Isolation and characterization of cDNAs encoding the heavy chain of human inter- α -trypsin inhibitor (I α TI): Unambiguous evidence for multipolypeptide chain structure of I α TI

 $(\alpha_1$ -microglobulin/calcium-binding domain/thiol-proteinase inhibitor)

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ABSTRACT Human inter- α -trypsin inhibitor (I α TI) is a plasma glycoprotein of M_r 180,000, which has been described as a single polypeptide chain. Recently, however, we proposed that I α TI might be composed of a heavy (H) chain (M_r = 95,000) and a light (L) chain ($M_r = 40,000$) synthesized by two separate mRNAs. In the present study we have characterized cDNAs for the H chain of I α TI. These cDNAs collectively covered two sequences (977 and 1450 base pairs in length) with single open reading frames. The deduced amino acid sequences were highly homologous to each other and well matched with partial amino acid sequences obtained from purified serum IaTI. RNA blot analyses of liver RNAs with H- or L-chain cDNAs as probes clearly identified two distinct mRNAs of 3.3 and 1.3 kilobases, which corresponded to H or L chain, respectively. Poly(A)⁺ RNAs hybrid-selected with H-chain cDNAs coded for polypeptide chains of M_r 90,000–95,000. These results unambiguously establish that IaTI is made of multipolypeptides, possibly including one H and two L chains. The H chain contains potential calcium-binding sites and also regions homologous to the proposed reactive site for thiolproteinase inhibitors. These data indicate that $I\alpha TI$ is a complex, multifunctional protein. mRNAs for both the H and L chains were found only in liver.

Inter- α -trypsin inhibitor (I α TI) is a serine protease inhibitor of M_r 180,000 present in human plasma. Polypeptides of lower M_r that immunologically cross-react with I α TI also have inhibitory activity and are present in normal plasma, urine, and bronchial mucus. These are called I α TI derivatives (1). Since I α TI shows a M_r of 180,000 on NaDodSO₄/PAGE, even in the presence of reducing agents, it has long been thought to be a single polypeptide chain. Recently, however, we have reported data to suggest that I α TI is synthesized by two separate mRNAs that code for the heavy (H) chain and the light (L) chain, respectively (2). The L chain gives rise to the I α TI derivatives (2). More recently, cDNAs for the L chain were isolated, and the L chain was found to contain amino acid sequences for two proteins: α_1 -microglobulin $(\alpha_1 m)$, an acute phase reactant, and a M_r 30,000 derivative of human IaTI (HI-30) (3, 4). HI-30 contains two homologous domains with an inhibitory site for elastase as well as an inhibitory site for trypsin and chymotrypsin (5, 6). The amino acid sequence of the H chain has not been determined, and no biological function has been reported for it.

In this paper, we report the isolation and nucleotide sequencing of partial cDNAs for the H chain.^{||} The H chain contains potential calcium-binding sites as well as sequences highly homologous to those at the reactive site of thiol-proteinase inhibitors. These data clearly indicate that $I\alpha TI$ is a multifunctional protein comprised of multipolypeptide chains that are synthesized in liver by two distinct mRNA species.

MATERIALS AND METHODS

Reagents. Various rabbit anti-human $I\alpha TI$ antisera were prepared as described (7). These included anti-I α TI antiserum containing both anti-H and anti-L chain antibodies, anti-L chain antiserum and anti-H chain antiserum (2). Biotinylated antirabbit IgG antibodies and avidin: biotinylated horseradish peroxidase complex were from Vector Laboratories (Burlingame, CA). Various baboon tissues were kindly provided to K. Kurachi by the Regional Primate Center (University of Washington, Seattle). T4 DNA polymerase and its Klenow fragment, dNTP, and ddNTP were from Boehringer Mannheim. Restriction enzymes and M13mp18 and M13mp19 phage DNA were from Bethesda Research Laboratories. Sma I-cut, dephosphorylated M13mp8 DNA, M13 sequencing primer, deoxyadenosine 5'-[α -³⁵S]thiotriphosphate (400 Ci/mmol; 1 Ci = 37 GBq), and [³²P]dCTP (3000 Ci/mmol) were from Amersham. GeneScreenPlus nylon membranes were from New England Nuclear. All other chemicals were of analytical grade.

Screening and Characterization of Human Liver cDNAs. About 1.6×10^5 recombinant phage plaques of a normal human liver cDNA library constructed in λ gt11 (provided by R. A. Lazzarini, National Institutes of Health) were screened with an anti-I α TI antiserum by a modification of the Young and Davis procedure (8). Positive phage clones were plaquepurified, and liquid phage stocks were prepared according to standard protocols (9). Then positive clones were immunoscreened for the expression of either the L or the H chain of I α TI. These clones were also screened with L-chain cDNA, designated pHuLITI1 (3), as a probe. Radiolabeled cDNA probes were prepared by nick-translation with [³²P]dCTP.

Hybrid Selection of RNAs and *in Vitro* **Translation.** *In vitro* translation of hybrid-selected $poly(A)^+$ RNAs of human liver and immunoprecipitation of the translation products were performed as described (2, 3).

Nucleotide Sequencing. cDNA inserts were subcloned into M13mp18 or M13mp19. Alternatively, the entire DNA of a recombinant $\lambda gt11$ clone was sonicated, and fragments of

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Abbreviations: $\alpha_1 m$, α_1 -microglobulin; I α TI, inter- α -trypsin inhibitor; HI-30, a M_r 30,000 derivative of human I α TI; H, heavy; L, light. [†]To whom requests should be addressed.

The sequences of the two domains reported in this paper are being deposited in the EMBL/GenBank data base (Bolt, Beranek, and Newman Laboratories, Cambridge, MA, and Eur. Mol. Biol. Lab., Heidelberg) (accession nos. J03012 and J03013).

500-900 base pairs in length were ligated to M13mp8 at the Sma I site according to a standard "shotgun" technique (10). DNA sequencing was performed by the dideoxy chain termination method with deoxyadenosine 5'- $[\alpha^{-35}S]$ thiotriphosphate and analyzed by polyacrylamide gel electrophoresis using a buffer gradient gel (11).

RNA Blot Analysis. RNA blot analysis of human liver $poly(A)^+$ RNAs with an I α TI L-chain cDNA probe has been described (3). Total RNAs were also prepared from various tissues of an adult male baboon by the guanidinium isothiocyanate procedure followed by centrifugation through a 5.7 M CsCl cushion (12). RNA preparations were electrophoresed in 1.5% agarose gels containing 6.7% formaldehyde in 20 mM phosphate buffer (pH 7.0). The agarose gels were then blotted onto a GeneScreenPlus membrane. Prehybridization, hybridization with radiolabeled cDNA probe(s), and washing were carried out as recommended for GeneScreenPlus by the manufacturer.

Amino Acid Sequences of $I\alpha TI$ and Its Peptides. Highly purified serum I α TI (13) was subjected to amino acid sequencing to determine its N-terminal end(s) at the protein sequencing facility of the University of Michigan. Recoveries for the first seven residues varied between 19% and 47%. In K. Hochstrasser's laboratory (Munich, F.R.G.), a set of IaTI peptides was also obtained by digestion of reduced and carboxymethylated IaTI with trypsin or Staphylococcus aureus V8 proteinase. Amino acid sequences were determined manually or by Edman degradation in a solid-phase sequencer. A library of peptide sequences for $I\alpha TI$ was kindly provided to us by K. Hochstrasser.

RESULTS

Human cDNAs for I α TI. In the initial screening of a normal human liver cDNA library for I α TI cDNA, 27 positive phage clones were identified with anti-IaTI antiserum and were plaque purified. Ten positive clones were detected with the anti-L chain antiserum, and 7 positive clones were identified with anti-H chain antiserum. None of these clones were detected with both anti-L chain and anti-H chain antisera. The radiolabeled L-chain cDNA probe (pHuLITI1) also hybridized to all clones that were detected with the anti-L chain antiserum (results not shown). Among the clones detected with the anti-H chain antiserum, several (designated λ HuHITI-9, -19, -33) were analyzed in further detail.

Nucleotide Sequence of cDNAs. Sequencing strategies for clones 9, 19, and 33 are shown in Fig. 1. The nucleotide





FIG. 1. Strategy for nucleotide sequencing of cDNAs λ Hu-HITI-19 (Upper) and λHuHITI-9 and -33 (Lower). The arrows indicate the direction and extent of sequences determined for each cDNA fragment. The sequence with an asterisk at its 5' end was primed with a specific, synthetic oligonucleotide. Most M13 subclones of clone λ Hu-HITI-9 were obtained by a shot-

gun technique.

sequence of clone 19 and the combined sequence of clones 9 and 33 are 977 and 1450 bases long, respectively, and contain a single open reading frame (Fig. 2). The amino acid sequences derived from the nucleotide sequences are in excellent agreement with the amino acid sequences obtained from various peptides derived from I α TI. So far the latter have confirmed about 60% of the amino acid sequence derived from the cDNAs (Fig. 2). No homology has been found between the amino acid sequences of the L(4) and H chains. A computer search of a protein data bank with the GenePro program (Riverside Scientific, Seattle) did not provide any evidence of significant homology to any other reported proteins.

Two highly homologous domains (domains 1 and 2) were found in the partial amino acid sequences of the H chain (Fig. 2). Their relative positions in the H chain are not known at the present time. The sequence Val-Val-Ala-Gly-Lys in domain 2 of the H chain (amino acids 145-149) was found to be highly homologous to the reactive site of human α -2-thiol-proteinase inhibitor (14). A similar sequence is also found in domain 1. A preliminary experiment to determine thiol-proteinase inhibitor activity of IaTI showed that IaTI apparently does not inhibit papain, whereas human calpain I was weakly inhibited by I α TI. As shown in Fig. 3, sequences that are highly homologous to a well-defined calcium-binding site sequence (15) were also found in both domains of the H chain.

Analysis of Poly(A)⁺ RNAs Hybrid Selected with cDNAs of λ HuHITI-9 or -19. Immunoprecipitation of the *in vitro* translation products of human liver $poly(A)^+$ RNAs with anti-IaTI antiserum have yielded two different polypeptide chains with M_r values of 90,000-95,000 and 40,000, which correspond to the H and L chains of I α TI, respectively (2). In the present study, hybrid-selection experiments also showed that the DNA of clone 19 or 9 specifically retained mRNA fractions that encoded polypeptide chains of M_r 90,000-95,000. These polypeptides were immunoprecipitated by an anti-I α TI antiserum, whereas no L chain was concurrently detected (results not shown).

RNA Blot Analysis of Human Liver Poly(A)⁺ RNAs. The radiolabeled cDNA insert of λ HuHITI-9 or -19 was used as a hybridization probe to analyze liver $poly(A)^+$ RNAs. With either probe, a single population of mRNAs of about 3.3 kilobases (kb) was found (Fig. 4), whereas a probe containing the 3' half of the L-chain cDNA detected mRNA species of 1.3 kb, which is in good agreement with previous data (3). The latter result was also obtained with a probe derived from the

			с	ATC 11e	ATG Met	TTG Leu	ACA Thr	GAT Asp	GGC Gly	GAT Asp	CCC Pro	ACA Thr	GAG Glu	GGG Gly	34 11	
GTG	ACG	GAC	CGT	TCC	CAA	ATC	CTC	AAG	AAC	GTC	CGC	AAC	GCC	ATC	79	
Val	Thr	Asp	Arg	Ser	Gln	Ile	Leu	Lys	Asn	Val	Arg	Asn	Ala	Ile	26	
CGG	GGC	AGG	TTC	CCG	CTC	TAC	AAC	CTG	GGT	TTC	GGC	CAC	AAT	GTG	124	
Arg	Gly	Arg	Phe	Pro	Leu	Tyr	Asn	Leu	Gly	Phe	Gly	His	Asn	Val	41	
GAC	TTT	AAC	TTT	CTG	GAG	GTC	ATG	TCC	ATG	GAG	AAC	AAC	GGA	CGG	169	_
Asp	Phe	Asn	Phe	Leu	Glu	Val	Met	Ser	Met	Glu	Asn	Asn	Gly	Arg	56	
GCC	CAG	AGA	ATC	TAC	GAG	GAC	CAT	GAT	GCC	ACC	CAG	CAG	CTG	CAG	214	
Ala	Gln	Arg	Ile	Tyr	Glu	Asp	His	Asp	Ala	Thr	Gln	Gln	Leu	Gln	71	
GGT	TTC	TAC	AGC	CAG	GTA	GCC	AAA	CCC	CTG	CTG	GTG	GAT	GTG	GAT	259	,
Gly	Phe	Tyr	<u>Ser</u>	Gln	Val	Ala	Lys	Pro	Leu	Leu	Val	Asp	Val	Asp	86	
TTG	CAG	TAC	CCC	CAG	GAT	GCT	GTC	TTG	GCC	CTG	ACC	CAG	AAC	CAC	304	
Leu	Gln	Tyr	Pro	Gln	Asp	Ala	Val	Leu	Ala	Leu	Thr	Gln	Asn	His	101	
CAT	AAA	CAG	TAC	TAC	GAA	GGC	TCA	GAG	ATT	GTG	GTG	GCC	GGG	CGC	349	
His	Lys	Gln	Tyr	Tyr	Glu	Gly	Ser	Glu	Ile	Val	Val	Ala	G1y	Arg	116	
ATT	GCT	GAC	AAC	AAA	CAG	AGC	AGC	TTC	AAG	GCT	GAT	GTG	CAG	GCC	394	
Ile	Ala	Asp	Asn	Lys	Gln	Ser	Ser	Phe	Lys	Ala	Asp	Val	Gln	Ala	131	
CAT	GGG	GAG	GGA	CAA	GAA	TTC	AGT	ATA	ACC	TGC	CTA	GTG	GAT	GAG	439	
His	Gly	Glu	Gly	Gln	Glu	Phe	Ser	Ile	Thr	Cys	Leu	Val	Asp	Glu	146	
GAG	GAG	ATG	AAG	AAA	CTG	CTC	CGA	GAG	CGT	GGC	CAC	ATG	CTG	GAG	484	
Glu	Glu	Met	Lys	Lys	Leu	Leu	Arg	Glu	Arg	Gly	His	Met	Leu	Glu	161	
AAC	CAC	GTC	GAG	CGC	CTC	TGG	GCC	TAC	CTC	ACC	ATC	CAG	GAG	CTG	529	
Asn	His	Val	Glu	Arg	Leu	Trp	Ala	Tyr	Leu	Thr	Ile	Gln	Glu	Leu	176	
CTG	GCC	AAG	CGG	ATG	AAG	GTG	GAC	AGG	GAG	GAG	AGG	GCC	AAC	CTG	574	
Leu	Ala	Lys	Arg	Met	Lys	Val	Asp	Arg	Glu	Glu	Arg	Ala	Asn	Leu	191	
TCA	TCC	CAG	GCC	CTG	CAG	ATG	TCG	CTG	GAC	TAT	GGG	TTT	GTG	ACC	619	
Ser	Ser	Gln	Ala	Leu	Gln	Met	Ser	Leu	Asp	Tyr	Gly	Phe	Val	Thr	206	
CCA	CTG	ACC	TCC	ATG	AGC	ATC	AGG	GGC	ATG	GCG	GAC	CAG	GAC	GGC	664	
Pro	Leu	Thr	Ser	Met	Ser	Ile	Arq	Gly	Met	Ala	Asp	Gln	Asp	Gly	221	
CTG	AAG	CCC	ACC	ATC	GAC	AAG	CCC	TCA	GAG	GAT	TCT	CCG	CCT	TTG	709	
Leu	Lys	Pro	Thr	Ile	Asp	Lys	Pro	Ser	Glu	Asp	Ser	Pro	Pro	Leu	236	
GAG	ATG	CTG	GGA	CCC	AGA	AGG	ACG	TTC	GTG	CTG	TCA	GCC	TTG	CAG	754	
Glu	Met	Leu	Gly	Pro	Arg	Arg	Thr	Phe	Val	Leu	Ser	Ala	Leu	Gln	251	
CCT	TCT	CCT	ACT	CAT	TCC	AGC	TCC	AAT	ACC	CAG	CGG	CTG	CCA	GAC	799	
Pro	Ser	Pro	Thr	His	Ser	Ser	Ser	Asn	Thr	Gln	Arg	Leu	Pro	Asp	266	
CGA	GTG	ACC	GGC	GTG	GAC	ACA	GAC	CCT	CAC	TTC	ATC	ATC	CAC	GTG	844	
Arg	Val	Thr	Gly	Val	Asp	Thr	Asp	Pro	His	Phe	Ile	Ile	His	Val	281	
CCC Pro	CAG Gln	AAA Lys	GAG Glu	GAC Asp	ACC Thr	CTG Leu	TGC Cys	TTC Phe	AAC Asn	ATC Ile	AAT Asn	GAG Glu	GAG Glu	CCT Pro	889 296	
GGT	GTT	ATC	CTG	AGC	CTG	GTA	CAG	GAC	CCC	AAC	ACA	GGC	TTC	TCA	934	
Gly	Val	Ile	Leu	Ser	Leu	Val	Gln	Asp	Pro	Asn	Thr	Gly	Phe	Ser	311	
GTG Val	AAT Asn	GGA Gly	CAG Gln	CTC Leu	ATT Ile	GGC Gly	AAC Asn	AAG Lys	GCC Ala	AGG Arg	AGC Ser	CCT Pro	GGG Gly	с	977 325	

FIG. 2. Nucleotide and amino acid sequences of H-chain cDNAs. (*Left*) λ Hu-HITI-19 (domain 1). (*Right*) λ HuHITI-9 and -33 (domain 2). Underlining indicates the amino acid sequences that match with those obtained from I α TI polypeptides (K. Hochstrasser, personal communication). Boxed areas indicate sequences that are highly homologous. •, Potential reactive sites as thiol-proteinase inhibitors; \blacktriangle , Ca²⁺-binding sites; •, potential sites for asparagine-linked carbohydrates.

5' half of L-chain cDNA, which contains the sequence for $\alpha_1 m$ (see below).

Tissue Distribution of Expression for I α **TI Genes.** In RNA blot analyses, three cDNA probes were used. One is the insert from λ HuHITI-9, which detected the H chain. The other two probes of 591 and 501 base pairs were prepared from cDNA for L chain by Ava I digestion and correspond to the regions containing α_1 m and HI-30 domains, respectively (J.B., M.D.-M., R.S., J.P.M., and J.P.S., unpublished data). Of 13 different tissues obtained from a baboon, only liver was

GT	GGA	GGC	ACA	AAC	ATC	AAC	GAA	GCA	CTC	CTA	CGG	GCA	ATC	TTC	44
	Gly	Gly	Thr	Asn	Ile	Asn	Glu	Ala	Leu	Leu	Arg	Ala	Ile	Phe	14
ATT	TTG	AAT	GAA	GCC	AAT	AAC	TTG	GGA	CTG	TTA	GAC	CCC	AAC	TCC	89
Ile	Leu	Asn	Glu	Ala	Asn	Asn	Leu	Gly	Leu	Leu	Asp	Pro	Asn	Ser	29
GTC	TCG	CTG	ATC	ATT	TTG	GTT	TCT	GAT	GGA	GAT	CCA	ACA	GTG	GGC	134
Val	Ser	Leu	Ile	Ile	Leu	Val	Ser	Asp	Gly	Asp	Pro	Thr	Val	Gly	44
GAA	CTA	AAA	CTG	TCA	AAA	ATT	CAG	AAA	AAC	GTT	AAG	GAG	AAC	ATC	179
Glu	Leu	Lys	Leu	Ser	Lys	Ile	Gln	Lys	Asn	Val	Lys	Glu	Asn	Ile	59
C AA	GAC	AAT	ATC	TCC	TTG	TTC	AGT	TTG	GGC	ATG	GGA	TTT	GAT	GTG	224
Gln	Asp	Asn	Ile	Ser	Leu	Phe	Ser	Leu	Gly	Met	Gly	Phe	Asp	Val	74
GAC	TAT	GAT	TTT	TTG	AAG	AGA	CTG	TCC	AAT	GAA	AAC	CAT	GGA	ATT	269
Asp	Tyr	Asp	Phe	Leu	Lys	Arg	Leu	Ser	Asn	Glu	Asn	His	Gly	Ile	89
GCA	CAA	AGG	ATT	TAT	GGA	AAC	CAG	GAC	ACG	TCT	TCC	CAG	CTT	AAG	314
Ala	Gln	Arg	Ile	Tyr	Gly	Asn	Gln	Asp	Thr	Ser	Ser	Gln	Leu	Lys	104
AAA	TTC	TAC	AAC	CAG	GTC	TCC	ACT	CCA	TTG	CTC	CGG	AAT	GTT	CAG	359
Lys	Phe	Tyr	Asn	Gln	Val	Ser	Thr	Pro	Leu	Leu	Arg	Asn	Val	Gln	119
TTC	AAC	TAT	CCC	CAT	ACA	TCA	GTC	ACG	GAC	GTC	ACT	CAA	AAC	AAT	404
Phe	Asn	Tyr	Pro	His	Thr	Ser	Val	Thr	Asp	Val	Thr	Gln	Asn	Asn	134
TTC	CAT	AAC	TAC	TTT	GGA	GGC	TCA	GAG	ATT	GTG	GTG	GCA	GGA	AAA	449
Phe	His	Asn	Tyr	Phe	Gly	Gly	Ser	Glu	Ile	Val	Val	Ala	Gly	Lys	149
TTT	GAC	CCT	GCT	AAA	TTG	GAT	CAA	ATA	GAG	AGC	GTT	ATC	ACG	GCG	494
Phe	Asp	Pro	Ala	Lys	Leu	Asp	Gln	Ile	Glu	Ser	Val	Ile	Thr	Ala	164
ACT	TCG	GCT	AAC	ACG	CAG	TTA	GTC	TTG	GAG	ACC	CTG	GCC	CAG	ATG	539
Thr	Ser	Ala	Asn	Thr	Gln	Leu	Val	Leu	Glu	Thr	Leu	Ala	Gln	Met	179
GAC	GAC	TTG	CAG	GAT	TTT	CTA	TCG	AAA	GAC	AAG	CAT	GCA	GAT	CCC	584
Asp	Asp	Leu	Gln	Asp	Phe	Leu	Ser	Lys	Asp	Lys	His	Ala	Asp	Pro	194
GAT	TTC	ACC	AGG	AAA	CTG	TGG	GCC	TAT	CTA	ACC	ATC	AAC	CAA	CTG	629
Asp	Phe	Thr	Arg	Lys	Leu	Trp	Ala	Tyr	Leu	Thr	Ile	Asn	Gln	Leu	209
CTA	GCT	GAA	CGA	AGC	CTG	GCT	CCT	ACA	GCT	GCC	GCC	AAG	AGA	AGA	674
Leu	Ala	Glu	Arg	Ser	Leu	Ala	Pro	Thr	Ala	Ala	Ala	Lys	Arq	Arg	224
ATT	ACA	AGA	TCG	ATC	CTG	CAG	ATG	TCT	CTA	GAC	CAC	CAC	ATT	GTG	719
Ile	Thr	Arg	Ser	Ile	Leu	Gln	Met	Ser	Leu	Asp	His	His	Ile	Val	239
ACT	CCG	CTG	ACC	TCG	CTG	GTG	ATC	GAG	AAC	GAG	GCT	GGG	GAT	GAG	764
Thr	Pro	Leu	Thr	Ser	Leu	Val	Ile	Glu	Asn	Glu	Ala	Gly	Asp	Glu	254
CGC	ATG	CTG	GCG	GAT	GCC	CCA	CCG	CAG	GAT	CCC	TCC	TGC	TGC	TCA	809
Arg	Met	Leu	Ala	Asp	Ala	Pro	Pro	Gln	Asp	Pro	Ser	Cys	Cys	Ser	269
GGG	GCC	CTG	TAT	TAC	GGC	AGC	AAA	GTG	GTT	CCA	GAT	TCC	ACC	CCG	854
Gly	Ala	Leu	Tyr	Tyr	Gly	Ser	Lys	Val	Val	Pro	Asp	Ser	Thr	Pro	284
TCT	TGG	GCC	AAT	CCT	TCA	GCA	ACG	CCC	GTG	ATC	TCC	ATG	CTG	GCA	899
Ser	Trp	Ala	Asn	Pro	Ser	Ala	Thr	Pro	Val	Ile	Ser	Met	Leu	Ala	299
CAA	GGA	TCT	CAG	GTG	CTA	GAG	TCC	ACG	CCA	CCC	CCA	CAT	GTG	ATG	944
Gln	Gly	Ser	Gln	Val	Leu	Glu	Ser	Thr	Pro	Pro	Pro	His	Val	Met	314
AGA	GTT	GAA	AAT	GAC	CCA	CAT	TCC	ATC	ATT	TAT	CTA	CCA	AAA	AGC	989
Arg	Val	Glu	Asn	Asp	Pro	His	Ser	Ile	Ile	Tyr	Leu	Pro	Lys	Ser	329
CAA	AAG	AAC	ATT	TGT	TTC	AAT	ATT	GAC	TCA	GAA	ССТ	GGA	AAA	ATC	1034
CTC	GAC	CTG	GCT	TCT	GAC	CCA	GAA	TCA	GGA	ATT	GTA	GTC	AAC	GGT	1079
CAG	CTT	GTT	GGT	GCC	AAG	AAG	ccc	AAC	AAT	GGA	AAA	CTA	AGC	ACC	1124
Gln	Leu	Val	Gly	Ala	Lys	Lys	Pro	Asn	Asn	Gly	Lys	Leu	Ser	Thr	374
TAT	TTT	GGA	AAA		GGA	TTT	TAT	TTC	CAA	Agt	GAA	GAC	ATA	AAA	1169
Tyr	Phe	Gly	Lys	Leu	Gly	Phe	Tyr	Phe	Gln	Ser	Glu	Asp	Ile	Lys	389
Ile	Glu	Ile	Ser	Thr	Glu	Thr	Ile	Thr	Leu	Ser	His	Gly	Ser	Ser	404
Thr	Phe	Ser	Leu	Ser	Trp	Ser	GAC Asp	ACG Thr	GCT Ala	CAA Gln	GTC Val	ACG Thr	AAT Asn	CAG Gln	1259 419
AGG	GTG	CAG	ATC	TCA	GTG	AAG	AAA	GAA	AAA	GTG	GTA	ACT	ATC	ACC	1304
Arg	Val	Gln	Ile	Ser	Val	Lys	Lys	Glu	Lys	Val	Val	Thr	Ile	Thr	434
CTG	GAT	AAA	GAG	ATG	TCC	TTT	TCT	GTT	TTA	CTT	CAT	CGT	GTT	TGG	1349
Leu	Asp	Lys	Glu	Met	Ser	Phe	Ser	Val	Leu	Leu	His	Arg	Val	Trp	449
AAG	AAG	CAT	CCC	GTC	AAT	GTT	GAC	TTT	CTG	GGA	ATC	TAC	ATA	CCC	1394
Lys	Lys	His	Pro	Val	Asn	Val	Asp	Phe	Leu	Gly	Ile	Tyr	Ile	Pro	464
CCT	ACA	AAC	AAG	TTC	TCA	CCT	AAA	GCC	CAC	GGA	CTA	ATA	GGC	CAG	1439
Pro	Thr	Asn	Lys	Phe	Ser	Pro	Lys	Ala	His	Gly	Leu	Ile	Gly	Gln	479
TTC Phe	ATG Met	CAG Gln	GA												1450 482

found to express H or L chain (Fig. 5). Low level expression was also found in liver of a human fetus at the 18th week of gestation but was not detected in two human hepatoma cell lines, an umbilical cord endothelium cell line, or a Blymphoblastoid cell line.

DISCUSSION

In the present study, we have isolated and sequenced cDNA clones coding for I α TI. Amino acid sequences derived from cDNA clones λ HuHITI-9, -19, and -33, which were among

	,N-terminal region	Calcium binding loop C-terminal region								
	1 2 3 4 5 6 7 8 9 10 11 X 3	2 Y 4 Z 6 -Y 8 -X 10 11 -Z 4 5 6 7 8 9 10 11								
	?h ⁺ _hh ⁺ h D	$\begin{array}{c} + \begin{array}{c} D \\ N \end{array} \begin{array}{c} G \end{array} \begin{array}{c} D \\ O \end{array} \begin{array}{c} G \end{array} \begin{array}{c} h \\ n \end{array} \begin{array}{c} n \end{array} \begin{array}{c} ni \\ - \end{array} \begin{array}{c} h \\ - \end{array} \begin{array}{c} h \\ - \end{array} \begin{array}{c} h \\ + \end{array} \begin{array}{c} h \\ + \end{array} \begin{array}{c} h \\ n \end{array} \begin{array}{c} ni \\ + \end{array} \begin{array}{c} ni \\ ni \end{array} \begin{array}{c} h \\ ni \end{array}$								
Human calmodulin (region I)	IAEFKEAFSLFD	KDGDGT ITTKELGTVMRSL								
Human Factor XIIIa	ATHIGKLIV T K Q	IGGDGMMDITDTYKFQEGQ								
Human kininogen heavy chain	QESQSEEID	CNDKDL FKAV DAALKKYNS								
Intestinal Ca ⁺⁺ - binding protein	PEELKGIFEKYA	KEGLPO LSKEELKLLLOTE								
ITI H domain 1	IML	TDGDPTEGVTDRSQILKNV								
ITI H domain 2	LDPNSVSLIILV	SDGDPT VGELK LSKIQKNV								

FIG. 3. Comparison of the potential Ca^{2+} -binding site sequences in domains 1 and 2 of the H chain with those found in other proteins. X, Y, Z, -Y, -X, and -Z refer to the vertices of the Ca^{2+} coordinating octahedron. Hydrophobic (h), negative (-), positive (+), nonionic (ni), and weakly conserved (?) residues are indicated. The one-letter code for amino acids is used. The model of Ca^{2+} -binding site is obtained from ref. 15. The amino acid sequences of the first four proteins are from refs. 16–19, respectively. Homologies between proteins or with the model are boxed.

those detected with an anti-H chain antiserum, unambiguously matched the partial amino acid sequences obtained from various peptides of I α TI. In RNA blot analyses, a mRNA band of 3.3 kb was detected when the cDNA insert of λ Hu-HITI-9 or -19 was used as a probe. The size of this mRNA is in good agreement with the translation product (M_r 90,000–95,000) identified with an anti-I α TI antiserum in a cell-free translation assay employing mRNAs isolated in a hybrid-select experiment using an H-chain cDNA obtained from either λ HuHITI-9 or -19.

In a previous paper (2), we proposed that $I\alpha TI$ may be composed of H- and L-polypeptide chains, which are synthesized by separate mRNAs in liver, based on the data obtained by immunoprecipitation of translation products by anti-IaTI antisera. Isolation and characterization of cDNAs for the H chain in the present study, in addition to the data reported for L-chain cDNA (3, 4), provide unambiguous evidence that $I\alpha TI$ is composed of H and L chains, which are synthesized separately in liver tissue. This is also in good agreement with the reported observation that $\alpha_1 m$, which is contained in the L chain, is an acute phase protein (20), whereas the H chain is not (1). Amino acid sequence analysis of highly purified serum I α TI in the present study gave two major N-terminal sequences. One sequence was Ser-Leu-Pro-Gly-Glu, which may correspond to the N-terminus of the H chain. The other sequence was Ala-Val-Leu-Pro-Gln-Glu, which corresponds to the N-terminal sequence of HI-30 (6). This clearly indicates that L chain of isolated IaTI has been



FIG. 4. RNA blot analysis of poly(A)⁺ RNAs from human liver. Each lane was loaded with 5 μ g of mRNA. Anode is at the bottom. Lanes: 1, hybridization with H-chain-specific probe (cDNAs λ HuHITI-9 or -19); 2, hybridization with L-chain-specific probe (pHuLITI1; ref. 3); 3, size markers (in kb) (*Hind*III-restricted λ DNA that was end-labeled by the Klenow filling-in reaction).

cleaved between $\alpha_1 m$ and HI-30 by an unidentified protease, which results in the loss of $\alpha_1 m$ moieties from I α TI. This is further supported by the fact that I α TI isolated from serum





is not immunoprecipitated by an anti- α_1 m antiserum (J.P.S., unpublished data). Serum I α TI of M_r 180,000, therefore, may be composed of one H chain $(M_r 95,000$ without carbohydrates) covalently linked to two HI-30 moieties of the L-chain. HI-30 has an apparent M_r of 44,000 in NaDodSO₄/ PAGE due to its very high carbohydrate content (21). The bonds linking the H chain and HI-30 are not susceptible to reducing agents, indicating that a bond(s) other than a disulfide bond(s), such as the isopeptide bond(s) found in fibrin or fibronectin (22) or the Lys-Lys bond(s) found in collagen (23), may be involved. The nature of the bonds still remains to be determined.

The H chain of I α TI clearly contains homologous domains as shown in Fig. 2. The relative positions of domains 1 and 2 in the H chain are not yet known. The possibility that the H chain is heterogeneous and each H chain may contain either of these domains cannot be excluded. In domains 1 and 2, we found two similar sequences, which are homologous to the sequence for a well-defined calcium-binding site. In this sequence, an aspartic acid and two asparagine/aspartic acid residues provide chelating ligands for calcium. Position 6 (mostly glycine) permits folding of the polypeptide chain to form a proper orientation of ligands to chelate the metal ion (15). Replacement of aspartic acid in position X with other amino acids has been reported (see Fig. 3). A glycine in position 6 is not an absolute requirement (24) and the presence of proline at this position in domains 1 and 2 of the H chain may satisfy a fairly drastic change in the direction of the polypeptide chain required at this point. A circular dichroism experiment with purified I α TI did not show any significant signal for conformational change upon binding of Ca^{2+} or Mg^{2+} (data not shown). This may be due to too small a change, if any, in protein conformation. The function of Ca^{2+} ion bound to I α TI remains to be elucidated. Zinc, on the other hand, induced a significant conformational change at 220–230 nm upon its binding to I α TI (not shown), in agreement with the fact that $I\alpha TI$ binds to a zinc affinity column (13). In domains 1 and 2 of the H chain, a sequence homologous to the reactive site of the thiol-proteinase inhibitor was also observed. In our preliminary experiments, $I\alpha TI$ did not show any significant inhibitory activity toward papain. However, calpain I was weakly inhibited by $I\alpha TI$. A potential inhibitory activity of the H chain of $I\alpha TI$ may be sterically hindered, as in the case of α_1 - and α_2 -thiolproteinase inhibitors (25). IaTI may inhibit other thiol protease(s), which have not yet been tested. In any event, $I\alpha TI$, whose physiological role(s) still remains to be determined, appears to be a unique multifunctional protease inhibitor. It can inhibit elastase, chymotrypsin, and trypsin (acrosin), and it may also be able to inhibit some types of thiol proteases. These functions may be modified by binding of metal ions such as calcium and/or zinc.

As shown in Fig. 5, the H and L chains are only synthesized in liver. The I α TI derivatives found in the bronchial airways and urinary tract (reviewed in ref. 1) are not synthesized by the lung or kidney tissues, although others have observed mRNA for α_1 m in rat kidney (26). mRNA for the L chain was also absent in an endothelial cell line, although HI-30 has been considered for a potential role as endothelial cell growth factor (27). This may suggest that a receptor for HI-30 molecule is present on the endothelial cell surface.

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