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

(α_1-microglobulin/calcium-binding domain/thiol-proteinase inhibitor)

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ABSTRACT **Human inter-α-trypsin inhibitor (IαTI) is a plasma glycoprotein of M_r 180,000, which has been described as a single polypeptide chain. Recently, however, we proposed that IαTI 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 IαTI. 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 IαTI. 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 IαTI 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 thiol-proteinase inhibitors. These data indicate that IαTI is a complex, multifunctional protein. mRNAs for both the H and L chains were found only in liver.**

Inter-α-trypsin inhibitor (IαTI) is a serine protease inhibitor of M_r 180,000 present in human plasma. Polypeptides of lower M_r that immunologically cross-react with IαTI also have inhibitory activity and are present in normal plasma, urine, and bronchial mucus. These are called IαTI derivatives (1). Since IαTI shows a M_r of 180,000 on NaDodSO$_4$/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 IαTI 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 IαTI 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 (α_1m), an acute phase reactant, and a M_r 30,000 derivative of human IαTI (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 IαTI is a multifunctional protein comprised of multipolypeptide chains that are synthesized in liver by two distinct mRNA species.

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MATERIALS AND METHODS

Reagents. Various rabbit anti-human IαTI antisera were prepared as described (7). These included anti-IαTI antiserum containing both anti-H and anti-L chain antibodies, anti-L chain antiserum and anti-H chain antiserum (2). Biotinylated anti-rabbit 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-^{35}S]thiotriphosphate (400 Ci/mmol; 1 Ci = 37 GBq), and [^{32}P]dCTP (3000 Ci/mmol) were from Amersham. GeneScreen*Plus* 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 λgt11 (provided by R. A. Lazzarini, National Institutes of Health) were screened with an anti-IαTI antiserum by a modification of the Young and Davis procedure (8). Positive phage clones were plaque-purified, 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 IαTI. These clones were also screened with L-chain cDNA, designated pHuLITI1 (3), as a probe. Radiolabeled cDNA probes were prepared by nick-translation with [^{32}P]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 λgt11 clone was sonicated, and fragments of

Abbreviations: α_1m, α_1-microglobulin; IαTI, inter-α-trypsin inhibitor; HI-30, a M_r 30,000 derivative of human IαTI; H, heavy; L, light.

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‖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′-[α-^{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 IαTI 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 GeneScreen*Plus* membrane. Prehybridization, hybridization with radiolabeled cDNA probe(s), and washing were carried out as recommended for GeneScreen*Plus* by the manufacturer.

Amino Acid Sequences of IαTI and Its Peptides. Highly purified serum IαTI (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 IαTI peptides was also obtained by digestion of reduced and carboxymethylated IαTI 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 IαTI was kindly provided to us by K. Hochstrasser.

RESULTS

Human cDNAs for IαTI. In the initial screening of a normal human liver cDNA library for IαTI cDNA, 27 positive phage clones were identified with anti-IαTI 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 λHuHITI-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 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 IαTI. 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 IαTI showed that IαTI apparently does not inhibit papain, whereas human calpain I was weakly inhibited by IαTI. 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 λHuHITI-9 or -19. Immunoprecipitation of the *in vitro* translation products of human liver poly(A)$^+$ RNAs with anti-IαTI 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 IαTI, 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-IαTI 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 λHuHITI-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

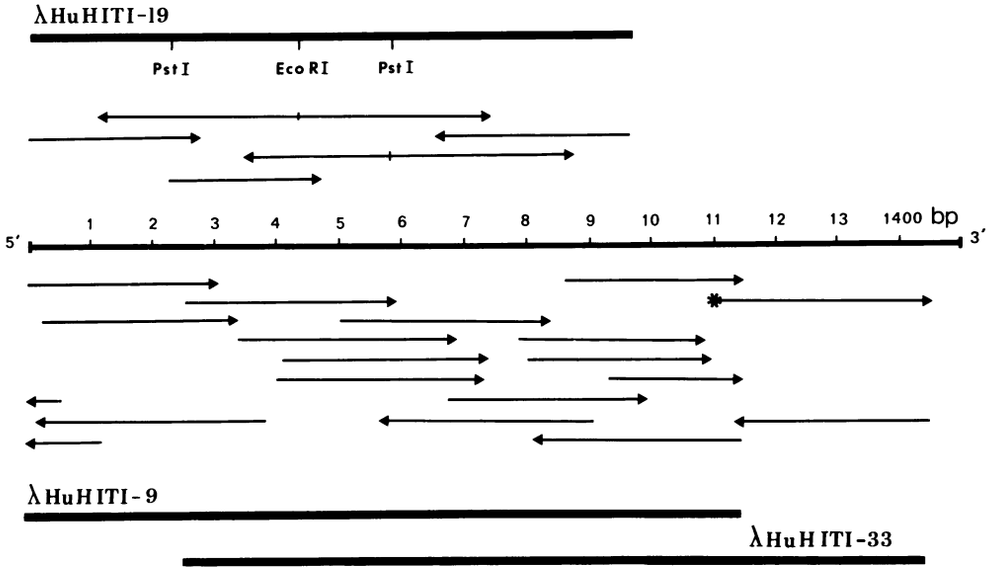
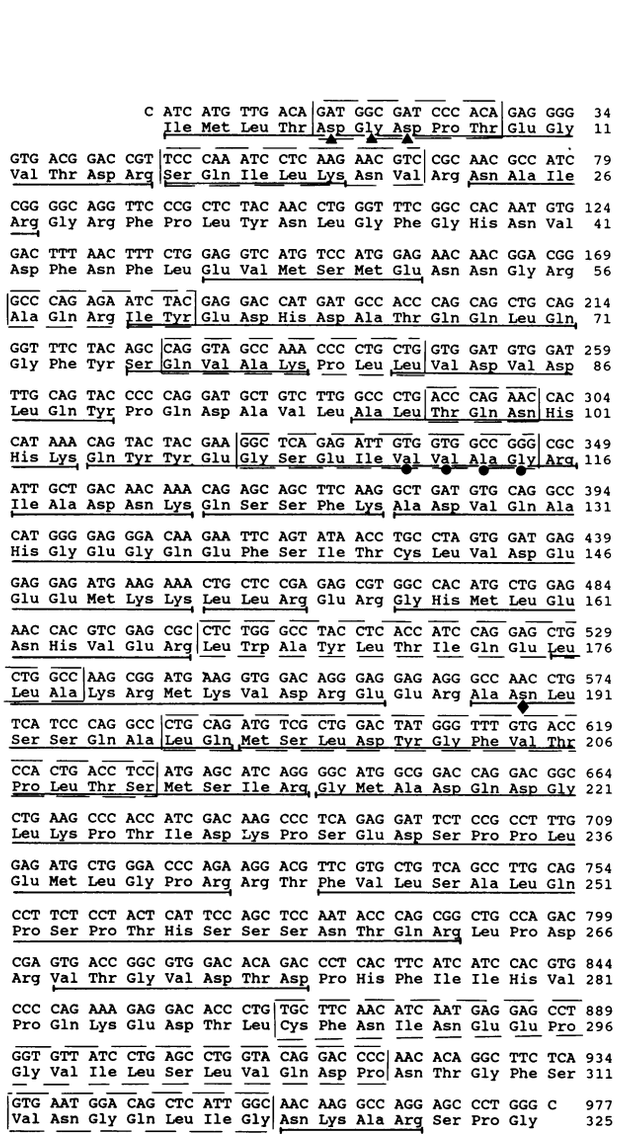


FIG. 1. Strategy for nucleotide sequencing of cDNAs λHuHITI-19 (*Upper*) and λHuHITI-9 and -33 (*Lower*). The arrows indicate the direction and extent of sequences determined for each cDNA fragment. The sequence with an asterisk at its 5′ end was primed with a specific, synthetic oligonucleotide. Most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



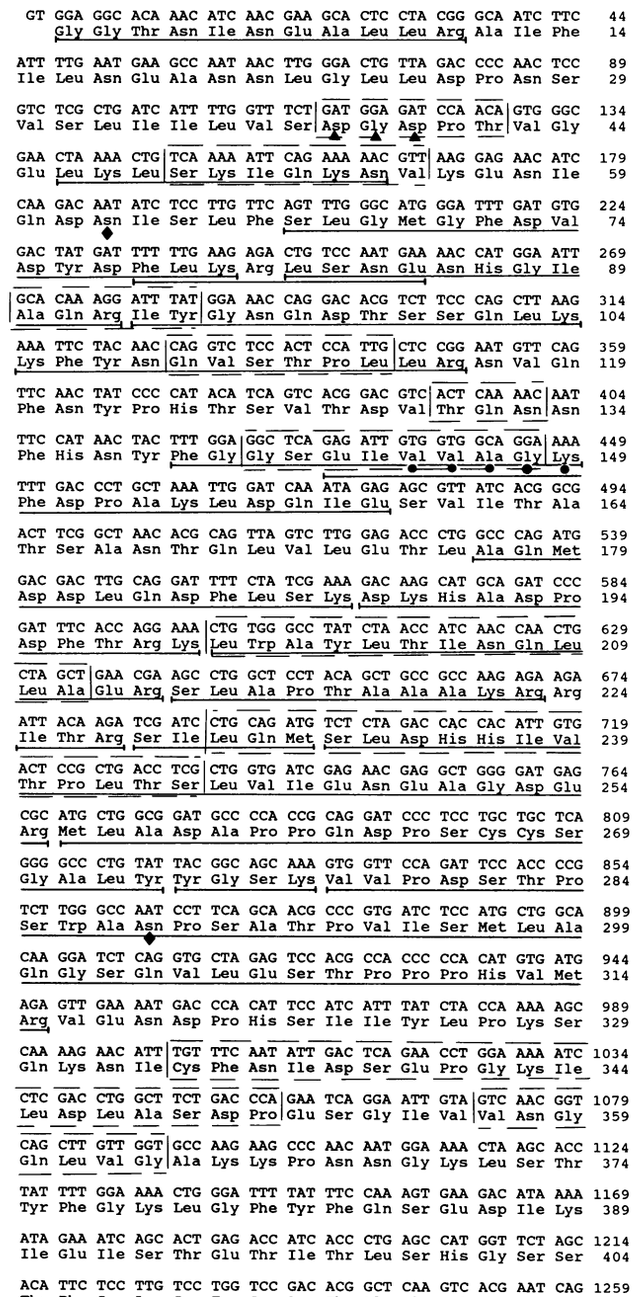


FIG. 2. Nucleotide and amino acid sequences of H-chain cDNAs. (*Left*) λHuHITI-19 (domain 1). (*Right*) λHuHITI-9 and -33 (domain 2). Underlining indicates the amino acid sequences that match with those obtained from IαTI polypeptides (K. Hochstrasser, personal communication). Boxed areas indicate sequences that are highly homologous. ●, Potential reactive sites as thiol-proteinase inhibitors; ▲, Ca^{2+}-binding sites; ◆, potential sites for asparagine-linked carbohydrates.

5′ half of L-chain cDNA, which contains the sequence for α_1m (see below).

Tissue Distribution of Expression for IαTI Genes. In RNA blot analyses, three cDNA probes were used. One is the insert from λHuHITI-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_1m 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 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 B-lymphoblastoid cell line.

DISCUSSION

In the present study, we have isolated and sequenced cDNA clones coding for IαTI. Amino acid sequences derived from cDNA clones λHuHITI-9, -19, and -33, which were among

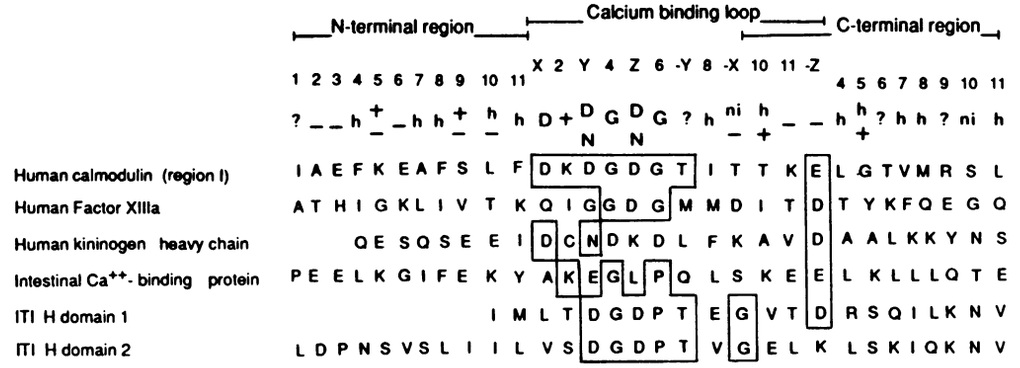


FIG. 3. Comparison of the potential Ca^{2+}-binding site sequences in domains 1 and 2 of the H chain with those found in other proteins. X, Y, Z, -Y, -X, and -Z refer to the vertices of the Ca^{2+} coordinating octahedron. Hydrophobic (h), negative (−), positive (+), nonionic (ni), and weakly conserved (?) 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 IαTI. In RNA blot analyses, a mRNA band of 3.3 kb was detected when the cDNA insert of λHuHITI-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-IαTI antiserum in a cell-free translation assay employing mRNAs isolated in a hybrid-select experiment using an H-chain cDNA obtained from either λHuHITI-9 or -19.

In a previous paper (2), we proposed that IαTI 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-IαTI 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 IαTI 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 α_1m, 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 IαTI 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 IαTI has been cleaved between α_1m and HI-30 by an unidentified protease, which results in the loss of α_1m moieties from IαTI. This is further supported by the fact that IαTI isolated from serum

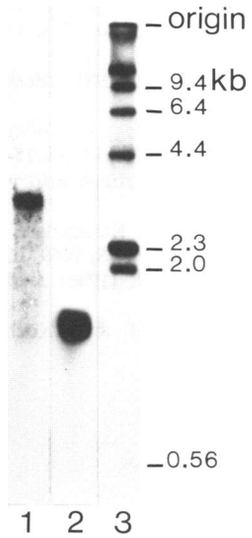


FIG. 4. RNA blot analysis of poly(A)$^+$ RNAs from human liver. Each lane was loaded with 5 μg of mRNA. Anode is at the bottom. Lanes: 1, hybridization with H-chain-specific probe (cDNAs λHuHITI-9 or -19); 2, hybridization with L-chain-specific probe (pHuLITI1; ref. 3); 3, size markers (in kb) (*Hin*dIII-restricted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

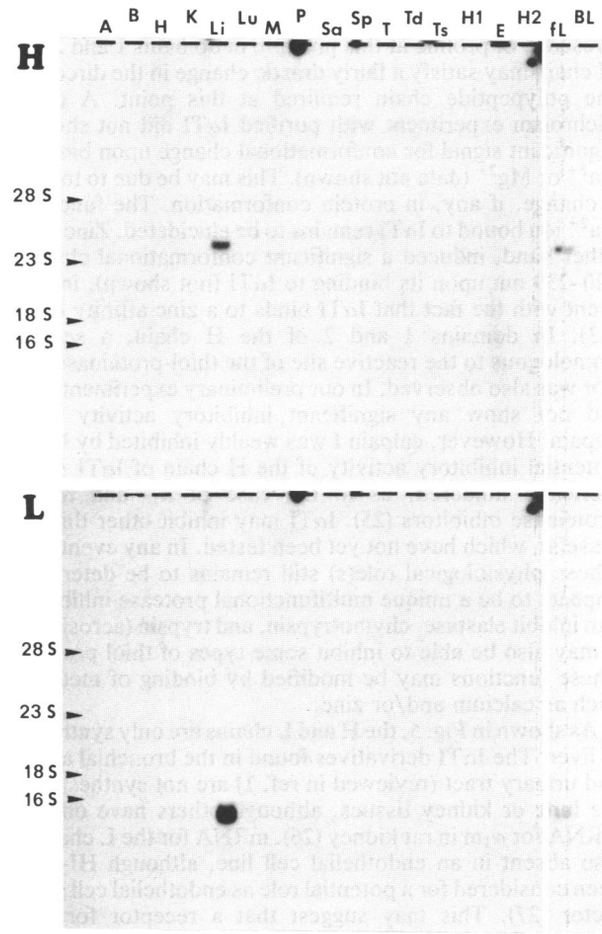


FIG. 5. RNA blot analysis of total RNAs from baboon tissues and human cell lines. Each lane was loaded with 20 μg of total RNA. Anode is at the bottom. (*Upper*) H-chain probe λHuHITI-9 was used. (*Lower*) Two L-chain probes containing the α_1m or HI-30 sequence gave identical results. A, adrenal; B, brain; H, heart; K, kidney; Li, liver; Lu, lung; M, muscle; P, pancreas; Sa, salivary gland; Sp, spleen; T, testis; Td, thyroid; Ts, thymus; H1 and H2, human hepatoma cell lines; E, human umbilical cord endothelium cell line; fL, human fetal liver (18 weeks old); BL, human B-lymphoblastoid cell line. Marker RNAs are indicated on the left.

is not immunoprecipitated by an anti-α_1m antiserum (J.P.S., unpublished data). Serum IαTI 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 NaDodSO$_4$/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 IαTI 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 IαTI 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 IαTI remains to be elucidated. Zinc, on the other hand, induced a significant conformational change at 220–230 nm upon its binding to IαTI (not shown), in agreement with the fact that IαTI 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, IαTI did not show any significant inhibitory activity toward papain. However, calpain I was weakly inhibited by IαTI. A potential inhibitory activity of the H chain of IαTI may be sterically hindered, as in the case of α_1- and α_2-thiol-proteinase inhibitors (25). IαTI may inhibit other thiol protease(s), which have not yet been tested. In any event, IαTI, 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 IαTI 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_1m 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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