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Inter-α-Trypsin Inhibitor and Its Related Proteins in Syrian Hamster Urine and Plasma[1]

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Urinary excretion of trypsin inhibitor increased after injection of a carcinogen, *N*-nitrosobis(2-oxopropyl)amine, into Syrian hamsters. Two inhibitors were purified to apparent homogeneity from urine collected during the course of the carcinogenesis experiment. Their complete amino acid sequences were determined by Edman degradation of the intact proteins and partially degraded fragments. One corresponded to a hamster liver cDNA clone that hybridized with human bikunin probe [Ide *et al.* (1994) *Biochim. Biophys. Acta* 1209, 286-292], except that the protein sequence lacked C-terminal serine and the other was trypstatin, the C-terminal half of the bikunin molecule. Three proteins containing covalently linked bikunin were also identified in pooled blood plasma. They were all dissociated into heavy and light chains by treatment with chondroitinase ABC or 50 mM NaOH, but not by heating at 100°C in the presence of sodium dodecyl sulfate and dithiothreitol. N-terminal amino acid sequence analyses of the native chains and partially degraded fragments thereof revealed that these proteins are (i) human-type inter-α-trypsin inhibitor, consisting of heavy chains 1 and 2 and bikunin, (ii) bovine-type inter-α-trypsin inhibitor, consisting of heavy chains 2 and 3 and bikunin, and (iii) pre-α-trypsin inhibitor, consisting of heavy chain 3 and bikunin. Heterodimer of bikunin/heavy chain 1 or bikunin/heavy chain 2 was not detected. These results suggest that the composition, and hence function, of the inter-α-trypsin inhibitor family differs considerably from species to species.

Key words: bikunin, inter-α-trypsin inhibitor, pancreatic carcinogenesis, trypstatin, hamster.

Human inter-α-trypsin inhibitor (ITI) is a glycoprotein with a molecular mass of about 220 kDa, which was first reported as protein π (*1*) and later identified as a trypsin inhibitor (*2*). ITI migrates as a single band in SDS-PAGE in the presence of reagents that cleave disulfide bonds. This led earlier workers to conclude that ITI is a glycoprotein composed of a single polypeptide chain (*3*). Studies in the past several years (*4-14*), however, have revealed that (i) ITI is composed of three different polypeptide chains, *i.e.*, HC1, HC2, and light chain; (ii) these chains are synthesized in the liver from distinct mRNA precursors; (iii) these chains are covalently linked through an unusual structure, *i.e.*, ester linkage between the C-terminal carboxyl group of HC and C6 hydroxyl group of an internal GalNAc of the chondroitin sulfate chain which is attached to the light chain; (iv) the protein previously referred to as ITI contains three other related proteins (*i.e.*, PTI, consisting of HC3 and light chain, HC2/bikunin or inter-α-like inhibitor consisting of HC2 and light chain, and HC1/bikunin consisting of HC1 and light chain); and (v) the proteinase inhibitory activity resides in the light chain. Recently, Salier and associates (*15, 16*) showed that the mouse also contains three genes homologous to the respective human heavy chains, suggesting that the general occurrence of ITI gene family in mammals. However, it is also known that bovine ITI differs from its human counterpart in that it consists of HC2 and HC3 (instead of HC1 and HC2) and light chain (*14, 17*). Thus, further work is needed to generalize the above conclusion derived from human ITI and its relatives.

Although free heavy chains without light chain have not been demonstrated in physiological fluids, free light chain was found in urine and plasma as early as in 1955 as a protein with trypsin inhibitory activity (*18*). This protein has since been referred to as UTI, mingin, acid-stable trypsin inhibitor, and HI-30 according to its investigated properties (*19, 20*). It shows an apparent molecular mass of 60-80 kDa on gel filtration and 40-50 kDa on SDS-PAGE, while the amino acid sequence yields values of 15-16 kDa (*20*). This discrepancy, which caused considerable confusion regarding the molecular identity of "urinary trypsin inhibitor," is due mainly to the presence of chondroitin sulfate and *N*-glycan chains in the N-terminal region (Ser^{10} and Asn^{45}, respectively). It is now well established that HI-30 consists of two tandemly arranged domains of Kunitz-type trypsin inhibitor, and it has been renamed bikunin (*4*). Mild proteolysis of human bikunin releases

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Abbreviations: BOP, *N*-nitrosobis(2-oxopropyl)amine; HC1, heavy chain 1; HC2, heavy chain 2; HC3, heavy chain 3; HI-30, 30-kDa human inhibitor; ITI, inter-α-trypsin inhibitor; PTI, pre-α-trypsin inhibitor; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TIC, trypsin-inhibiting capacity; UTI, urinary trypsin inhibitor.

several fragments such as HI-14 and HI-8 (*20, 21*). HI-14 or des(1-21)bikunin lacks the N-terminal glycopeptide but still contains two Kunitz-type domains, whereas HI-8 or des(1-77)bikunin contains only domain 2. Kido *et al.* (*22*) isolated a Kunitz-type trypsin inhibitor, named trypstatin, from rat peritoneal mast cells. This protein had sequence homology with domain 2 of human and bovine bikunin (*22*) and was later suggested to have been derived from rat bikunin by limited proteolysis (*23*). However, the occurrence of trypstatin *in vivo* has not been demonstrated in other species.

Human bikunin mRNA encodes two tandemly arranged in-frame proteins, α_1-microglobulin and bikunin, separated by the tripeptide Val-Arg-Arg, whose cleavage releases α_1-microglobulin and bikunin for further processing (*5, 6*). Although the same gene structure has been conserved in fish (*24, 25*) and mammals (*6, 26-28*), suggesting an important common function for the tandem expression of these proteins, the functional relationship between these two proteins remains virtually unknown. Human bikunin inhibits trypsin, chymotrypsin, and neutrophil elastase, but these proteinases are more efficiently inhibited by other inhibitors in plasma (*20, 21*). Furthermore, the inhibitory spectrum of bovine bikunin is considerably different from that of its human homologue (*20, 21*), and the deduced amino acid sequence around the reactive site of other bikunins also suggests differences in the target proteinases among species (*26*). These results cast doubt on the general role of bikunin as a proteinase inhibitor. Since rat trypstatin was copurified with tryptase, it was proposed that it may regulate the mast cell proteolytic activity (*22*). However, rat trypstatin affects neither human tryptase nor human chymase (*29*), in striking contrast to the fact that it inhibits rat chymase and tryptase (*22*). Moreover, the expression of activities of mast cell proteinases is extremely variable among experimental animals and even among tissues (*30*). Bikunin occurs at 100- to 500-fold elevated concentrations in the urine of patients with various malignant tumors (*31-33*) as well as in lung cancer tissue extracts (*34*) and the tumor fluid of ovarian cancers (*35*). Bikunin was also shown to be a growth factor for endothelial cells (*36*). Immunohistochemical staining revealed that bikunin is widely distributed in almost all malignant tumors (*37*), whereas it was detected in only a few normal tissues such as renal proximal tubules, cerebral glial cells, and bronchial epithelial cells (*37*). These results suggest that bikunin plays role(s) in carcinogenesis, tumor infiltration, or metastasis. However, these studies were carried out with antibodies, raised against bikunin, that do not distinguish between bikunin itself and trypstatin. Thus, it remains to be determined whether the positive substance is bikunin itself, trypstatin, or its complexed form with heavy chain(s). Under these conditions, the behavior of heavy chains is virtually unknown.

No congenital deficiency of ITI family proteins has been reported to date, suggesting that the absence of this gene family constitutes a lethal deletion. However, the function of ITI heavy chains is poorly understood. ITI stabilizes the cumulus extracellular matrix and supports the process of ovulation, possibly due to a hyaluronan-binding capacity (*38*). This activity seems to be unrelated to the proteinase inhibition by bikunin. No other physiological function of ITI heavy chains is known, although it was recently pointed out that the deduced amino acid sequence contains regions similar to multicopper-binding domain and von Willebrand type A domain (*16*). Since the human subject cannot be used as experimental material, it is necessary to establish an appropriate animal model in order to get further insight into the physiological role of the ITI family. Recently Syrian hamster has proven particularly useful for the study of pancreatic carcinogenesis, since the tumor induced by BOP is biologically and morphologically very similar to those encountered in humans (*39, 40*). However, proteinase inhibitors of rodents show marked species differences not only quantitatively but also qualitatively (*41-43*). Thus, clarification of molecular properties and species differences of the ITI family is prerequisite to using the hamster as an experimental model. The present study was undertaken to ascertain the occurrence of ITI and its family members in the hamster, and to compare their features with those reported from other mammals.

MATERIALS AND METHODS

Materials—Porcine trypsin and *Proteus vulgaris* chondroitinase ABC were purchased from Biozyme and Seikagaku Kogyo, respectively. Lysyl endopeptidase and BOP were purchased from Wako Pure Chemical Industries Ltd and Nacalai Tesque, respectively. Other materials were purchased from the sources described previously (*44*). Anhydrotrypsin was prepared by β-elimination of phenylmethane sulfonyltrypsin, and coupled to Sepharose 4B essentially as described previously (*45*). Basal diet (F-2 pellet) and choline-deficient diet were purchased from Funabashi Farm and Clea Japan, respectively.

Preparation of Antibodies—Antibodies against purified trypstatin and a synthetic hexadecapeptide, SLPEGVVDGVEVYSTK (which corresponds to the N-terminal residue of hamster mature HC3, see Fig. 6) were raised in rabbits essentially as described previously (*46*). The peptide was synthesized with an automatic peptide synthesizer, PSSM-8 (Shimadzu), purified by reversed phase chromatography using a C_8 column under the same conditions as described below, and coupled to rabbit albumin prior to immunization. Antibody raised against this peptide reacted with HC3 but neither HC1 nor HC2 (see below).

Assay of Trypsin-Inhibiting Activity—Activity of trypsin was determined using benzoyl-L-arginine *p*-nitroanilide as a substrate as described (*44*). The trypsin-inhibiting activity was measured in terms of the decrease in amidolytic activity of trypsin after incubation with a sample for 15 min at 37°C (*44*). The active sites of trypsin were determined by titration with 4-methylumbelliferyl *p*-guanidinobenzoate (*47*). One unit of inhibitory activity was defined as the inhibition of 1 μg of trypsin under the above assay conditions.

N-Terminal Amino Acid Sequence Analysis—Sequence analysis of purified proteins and partially degraded fragments thereof was carried by Edman degradation using an Applied Biosystems model 477A gas phase sequencer, essentially as described previously (*46*).

Deglycosylation of ITI and PTI—Plasma ITI and PTI were treated with chondroitinase ABC in 50 mM Tris-HCl buffer, pH 8.0, for 24 h at 37°C or with 50 mM NaOH for 5 min at 25°C, essentially as described (*9*).

Partial Degradation of Proteins, Separation and Se-

quencing of Fragments—Isolated proteins were partially degraded by treatment with CNBr (*46*), acetic acid (*48*), or trypsin (*49*). The proteins were also treated with lysyl endopeptidase (1.5 μg) at 37°C overnight in 50 mM Tris-HCl buffer, pH 8.2. Treated samples were *S*-pyridylethylated and applied to a C_{18} (RP-300, Applied Biosystems) column (2.1×30 mm) which had been equilibrated with 0.1% trifluoroacetic acid in water. The isocractic conditions were maintained for 10 min with the same solvent, followed by a gradient to 70% acetonitrile in 0.1% trifluoroacetic acid for 110 min. The flow rate was 0.1 ml/min and the absorbances were measured at 220, 260, and 280 nm. The fraction which passed through the column was then applied to a second C_8 (RP-18, Applied Biosystems) column (2.1× 30 mm), which had been equilibrated with 0.1% trifluoroacetic acid in water. Elution was conducted with the same conditions. Major peaks obtained from the above two columns were subjected to amino acid sequence analysis.

Animal Experiments—Eight-week-old female Syrian hamsters weighing about 90 g at the start of the experiment were used. Two animals were housed in each glass metabolic cage (Sugiyamagen Iriki) and given free access to water and food. Urine samples were collected in 24-h aliquots in plastic vessels containing sodium azide. The samples were kept frozen until use. Pancreatic carcinoma was induced by BOP using the protocol of Mizumoto *et al.* (*39*). The hamsters were subjected to three cycles of the augmentation pressure, and at the end of the experiment (93rd day), blood was withdrawn into a plastic syringe containing 0.1 M EDTA (final concentration, 4 mM) by cardiac puncture under ether anesthesia. Plasma was separated by centrifugation at 3,000 rpm for 30 min at 4°C and stored at −80°C until use. Pancreases and livers were removed and embedded in paraffin. Sections of 5 μm were cut and stained with hematoxylin and eosin.

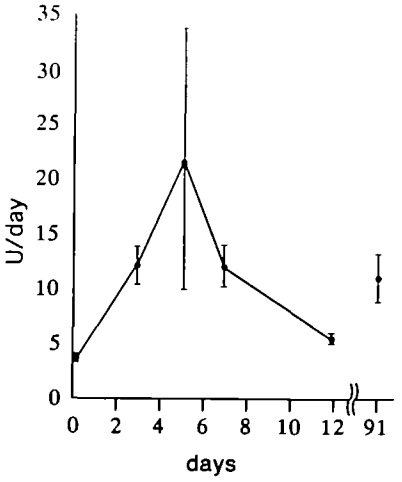

Fig. 1. **Change in urinary excretion of trypsin inhibitors after a subcutaneous injection of BOP.** TIC of the daily urine sample was determined as described in "MATERIALS AND METHODS." Two female hamsters were housed in one cage. Each point and bar represent the mean of four cages and the standard deviation, respectively. The difference between 5th day value and that of 3rd or 7th day is not statistically significant. The animals were killed at the 92nd day.

RESULTS

Changes in Urinary TIC during Carcinogenesis—TIC of daily urine was assayed after a single subcutaneous injection of BOP (65 mg/kg body weight). As shown in Fig. 1, TIC increased severalfold, and decreased gradually. In contrast, no significant change was observed with control (saline-injected) animals (data not shown). Twelve days after the initial injection of BOP, the hamsters received a daily injection of ethionine (450 mg/kg) for four days followed a single injection of methionine (700 mg/kg) while being maintained on choline-deficient diet (*39*). During this augmentation pressure cycle, the animals lost appetite, which resulted in a loss of body weight, in accordance with the previous report (*39*). The daily urine volume also varied greatly from 2 to 13 ml per capita, during this period, making it practically impossible to follow the change in TIC. Accordingly, the urine was pooled during the carcinogenesis experiment and used for isolation of trypsin inhibitors.

Histological Findings of Liver and Pancreas—All hamsters were killed 92 days after the initial injection of BOP, and livers and pancreases were removed and examined histopathologically. Cholangiocarcinoma was detected in all 30 livers examined, but no hepatocellular carcinoma was found in any animals. On the other hand, 25 out of 30 animals (83%) developed pancreatic ductal carcinoma.

Purification of Two Trypsin Inhibitors from Urine—Pooled urine (2.5 liters, about 15 TIC units/ml) collected from BOP-injected hamsters was dialyzed against 50 mM Tris-HCl, pH 7.5, containing 0.15 M NaCl (Buffer A) and applied to an anhydrotrypsin-Sepharose 4B column (4×6 cm) which had been equilibrated with the same buffer. The column was washed with 2 liters of 50 mM Tris-HCl, pH 7.5, containing 0.5 M NaCl, then eluted with 1 liter of 50 mM glycine-HCl, pH 2.2, containing 0.5 M NaCl. Fractions were collected in tubes containing 1 M Tris-HCl, pH 8.6, and assayed for TIC. The active fractions were dialyzed against distilled water, freeze-dried, and dissolved in Buffer A prior to application to a Superose 12 column (1× 30 cm, Pharmacia), which had been equilibrated with Buffer A. The column was developed with the same buffer at a flow rate of 0.2 ml/min, and 0.5-ml fractions were collected. Two active peaks corresponding to 30- (peak A)

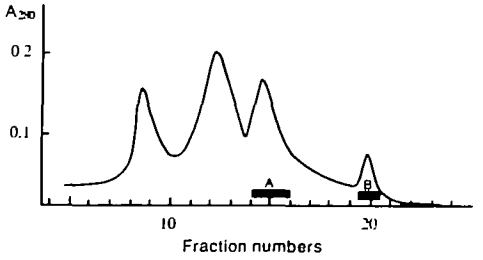

Fig. 2. **Elution profile of urinary proteins from a column (1× 30 cm) of Superose 12.** Fractions eluted from the anhydrotrypsin-Sepharose 4B column were pooled and applied to the Superose column as described in the text. The column was washed with 10 ml of Buffer A, then 0.5 ml fractions were collected and assayed for trypsin-inhibitory activity. The flow rate was 0.2 ml/min and the effluent was monitored at 280 nm. Fractions containing inhibitory activity are marked by horizontal bars A and B.

and 10-kDa protein (peak B) in this column were obtained (Fig. 2). Peak A material was desalted on a Sephadex G-25 column, freeze-dried, and redissolved in 10 ml of 0.1% trifluoroacetic acid. A 0.5-ml aliquot was subjected to reversed phase chromatography (SMART system, Pharmacia) using a C_8 column (2.1×30 mm) equilibrated with 0.1% trifluoroacetic acid. Elution was begun with a linear gradient of acetonitrile from 0 to 70% over 50 min at a flow rate of 0.2 ml/min. This chromatographic fractionation procedure was repeated with the remaining 19 aliquots. Peak B material was subjected to the same fractionation. From each column, one major and several minor fractions were eluted. The major fractions obtained from Peaks A and B materials from the above Superose 12 columns gave Preparations A (yield, 15 mg) and B (yield, 7 mg), respectively, both of which were homogeneous as judged from SDS-PAGE (data not shown) and showed trypsin-inhibitory activity even after the reversed phase chromatography. Approximately 60% of the initial TIC was recovered in Preparations A and B, suggesting that these two proteins are mainly responsible for the trypsin-inhibitory activity in the hamster urine under the conditions studied.

Amino Acid Sequence Analysis of the Urinary Trypsin Inhibitors—Amino-terminal sequence analysis of Preparation A identified only a single amino acid up to the 50th residue (Fig. 3). Yields of Ser and Asn in the 10th and 45th cycles, respectively, were very low. Fragments obtained by CNBr and lysyl endopeptidase treatments were also sequenced, and overlapping peptides were aligned to give a complete sequence of 144 amino acid residues (Fig. 3). Recently Ide *et al.* (*26*) reported the cDNA sequence which was obtained by screening hamster liver cDNA library with a cDNA probe encoding human bikunin, but no protein sequence data were presented in support of the cDNA sequence. The present amino acid sequence obtained by direct sequencing completely matches that deduced from the cDNA, except that the protein sequence lacks C-terminal serine. It is likely that hamster bikunin was cleaved prior to urinary excretion by a trypsin-like enzyme (since the penultimate residue is arginine) or by aminopeptidase. Human bikunin is known to have *O*- and *N*-glycans linked to Ser^{10} and Asn^{45}, respectively (*20*). Very low yields of the same amino acids at the same position in Preparation A suggest that the same positions are also glycosylated in the hamster counterpart. The N-terminal sequence analysis of Preparation B and its fragments identified 66 amino acids (Fig. 3), which were identical with the C-terminal half of bikunin (residues 78 to 144). These sequence data, together with the characteristic property that the trypsin-inhibiting activity was not lost in 0.1% trifluoroacetic acid, indicate that Preparations A and B are hamster bikunin and trypstatin, respectively.

Purification of ITI and Its Related Proteins from Hamster Plasma—Pooled hamster plasma (100 ml) containing, in final concentrations, 4 mM EDTA, 30 mM NaCl, and 10 mM Tris-HCl, pH 7.5, was applied to a column (2.5×8 cm) of Q-Sepharose Fast Flow (Pharmacia) which had been equilibrated with Buffer A. The column was washed with 300 ml of Buffer A and eluted with a linear gradient of NaCl in Buffer A up to the final concentration of 1 M. The fractions were assayed for TIC and immunoblotted using anti-trypstatin antibody. Fractions positive for both assays were eluted at about 0.3–0.4 M NaCl. They were combined and brought to half-saturation with solid ammonium sulfate. The precipitate was pelleted by centri-

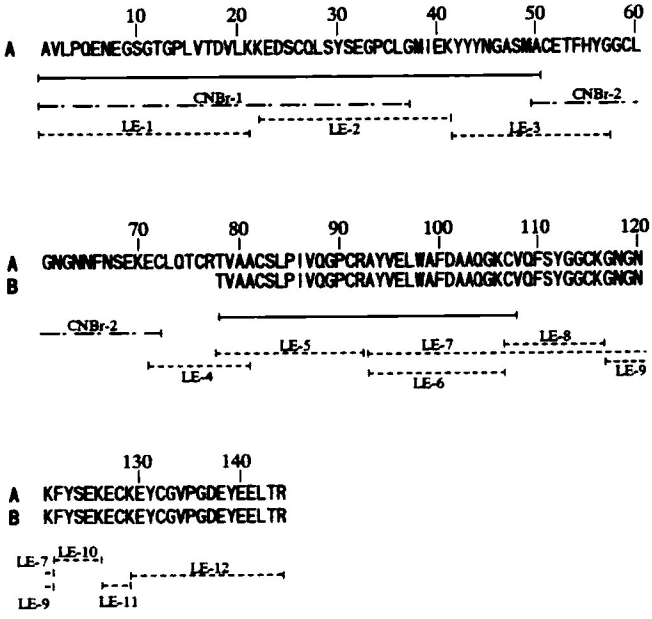

Fig. 3. **Amino acid sequences of hamster urinary trypsin inhibitors, Preparations A and B, and their fragments produced by CNBr or lysyl endopeptidase.** Solid line, native protein; CNBr, fragments obtained by CNBr treatment; LE, fragments obtained by lysyl endopeptidase treatment. The lysyl endopeptidase treatment of Preparation B gave eight fragments (LE-5 to LE-12), which were identical with those obtained from Preparation A.

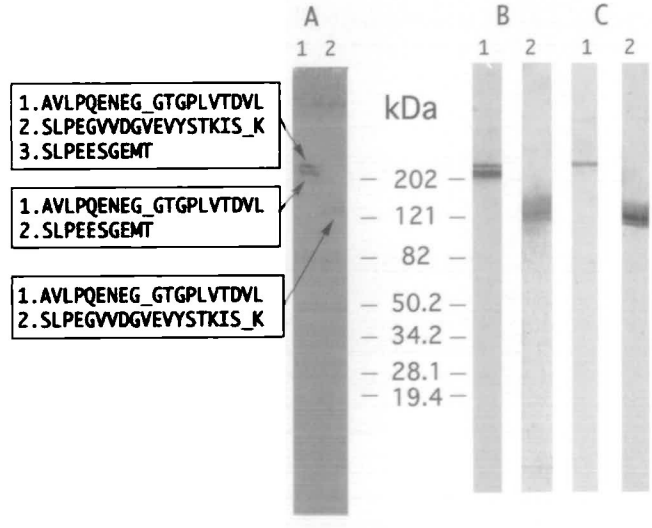

Fig. 4. **Electrophoresis and N-terminal sequence analysis of Preparations C and D isolated from hamster plasma.** (A) SDS-PAGE of Preparations C (lane 1) and D (lane 2) followed by Coomassie Blue staining. The N-terminal sequences of each band are presented in boxes at left. Underlined spaces indicate the failure to detect PTH amino acid; these residues were identified as cysteine by pyridylethylation prior to amino acid sequencing (see Fig. 6). (B) SDS-PAGE of Preparations C (lane 1) and D (lane 2) followed by immunoblotting with anti-trypstatin. (C) SDS-PAGE of Preparation C (lane 1) and D (lane 2) followed by immunoblotting with anti-hexadecapeptide. The positions of molecular mass markers under the same electrophoretic conditions are indicated in kDa.

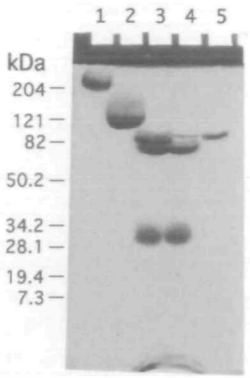

Fig. 5. **SDS-PAGE of Preparations C and D isolated from hamster plasma after treatment with chondroitinase ABC.** Lanes 1 and 2, Preparations C and D, respectively, before enzyme treatment; lanes 3 and 4, Preparations C and D, respectively after chondroitinase ABC treatment; lane 5, chondroitinase ABC. The gel was stained with Coomassie Blue.

fugation at 8,000 rpm for 20 min, dissolved in 6 ml of Buffer A containing 4 mM EDTA, and applied to a column (2.5×100 cm) of Superdex 200 which had been equilibrated with the same solvent. The active fractions were pooled and applied to a column (2.5×5 cm) of CM Affigel-Blue, washed with Buffer A, and eluted with 50 mM Tris-HCl, pH 7.5, containing 2 M NaCl. The flowthrough and eluted fractions contained 125- (Preparation C) and 225-kDa protein (Preparation D), respectively, on SDS-PAGE (Fig. 4A). Immunoblotting revealed that Preparation C reacted with anti-trypstatin (Fig. 4B, lane 2) and anti-hexadecapeptide (Fig. 4C, lane 2). Preparation D showed a doublet (Fig. 4A, lane 1), of which both bands reacted with anti-bikunin (Fig. 4B, lane 1), but the only slower-moving band reacted with anti-hexadecapeptide (Fig. 4C, lane 1). No dissociation of these bands were observed on SDS-PAGE after heating in the presence of 1% dithiothreitol (data not shown). When Preparations C and D were treated with chondroitinase ABC prior to SDS-PAGE, both proteins were cleaved into 82- and 30-kDa components (Fig. 5, lanes 3 and 4). Similar cleavage was also observed when these Preparations were treated with 50 mM NaOH at 37°C for 5 min (data not shown).

Amino-Terminal Sequencing of Preparations C and D—Preparation C (125-kDa band) on Immobilon (Fig. 4A, lane 2) was cut out of the sheet and subjected to N-terminal sequence analysis. This band gave two amino acid residues in approximately equal amounts in each cycle (Fig. 4A, lower left box). One sequence was identified as hamster bikunin by comparison with the sequence obtained with the experiment described above (Fig. 4). The other (sequence 2) is homologous with human and mouse HC3s (see Fig. 6). The sequence corresponding to HC1 or HC2 was not detected in this band. These results are consistent with the immunoblotting data (Fig. 4, B and C) and indicate that Preparation C is the hamster counterpart of human PTI. Each band of the 225-kDa doublet (Fig. 4A) was cut from the sheet and subjected to N-terminal sequence analysis. The faster-moving band gave two amino acid residues in each cycle (Fig. 4A, middle left box); one (sequence 1) is identical with the N-terminal region of urinary bikunin, while the other (sequence 2) is homologous with human and

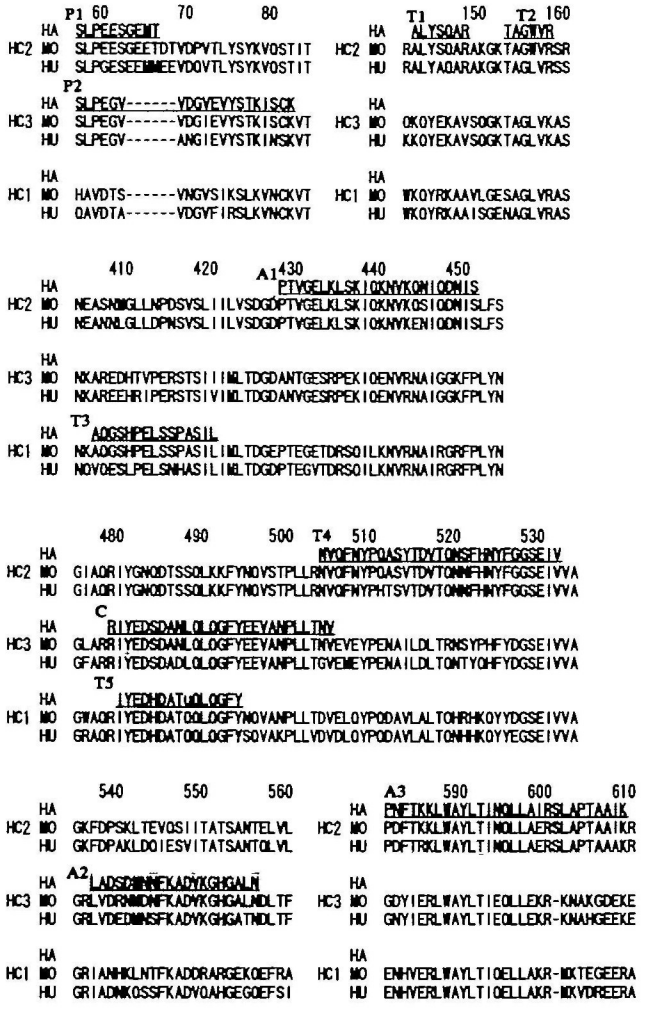

Fig. 6. **N-Terminal amino acid sequences of native chains and fragments obtained by treatment with CNBr and trypsin in comparison with HC1, HC2, and HC3 of mouse and human ITI (*16*).** Prefixes P, A, T, and C indicate the sequences of native protein, and fragments obtained by treatment with acetic acid, trypsin, and CNBr, respectively. Amino acid residues are numbered according to Chan *et al.* (*16*) and shaded when identical with those of the hamster proteins. HA, hamster; HU, human; MO, mouse.

mouse HC2s (see Fig. 6). The slower-moving band of Preparation D (225-kDa band) gave three amino acid residues in each cycle (Fig. 4A, upper left box), two of which could be assigned to the sequences identified in the band with faster mobility. The third sequence was obtained by subtracting the above two sequences and was found to be homologous with the human and mouse HC3s (see Fig. 6). The results suggest that the slower-moving band corresponds to bovine ITI consisting of HC2, HC3, and bikunin (*14, 17*).

Partial Degradation of Preparation D and N-Terminal Sequencing of Its Fragments—The above results indicate that the faster-moving band of Preparation D consists of HC2 and bikunin, which is not compatible with its molecular size, *i.e.*, 225 kDa. It has been reported that N-terminal blockage, especially of HC1, sometimes occurs during PAGE (*50*), and previous workers could only establish the N-terminal sequence of bikunin and HC2 by direct se-

quencing of human ITI (*50, 51*). Thus, it is possible that the faster-moving band of 225-kDa doublet is N-terminally blocked. To clarify this point, hamster ITI preparation was partially degraded and the internal sequences were determined. Owing to shortage of material, Preparation D was treated, without prior separation into two components, with chondroitinase ABC followed by partial degradation and sequencing as described above. A total of nine fractions obtained by the reversed phase chromatography were subjected to N-terminal sequencing, and the results are shown in Fig. 6, aligned with human and mouse heavy chains. Two peptides clearly indicate the presence of HC1 in Preparation D: the trypsin fragment T3 (corresponding to positions 406-420) is identical with mouse HC1 but different from the same regions of HC2 or HC3; and peptide T5 (positions 480-494) is identical with that of human and mouse HC1, but different from that of HC2 or HC3. The remaining seven peaks all could be aligned with either HC2 or HC3 (Fig. 6). These results, together with the direct N-terminal sequencing of Preparation D bands, indicate that hamster plasma contains two ITIs: one consisting of HC1, HC2, and bikunin (human type); the other consisting of HC2, HC3, and bikunin (bovine type). The former has a slightly higher mobility than the latter on SDS-PAGE.

DISCUSSION

The present results generally confirm the earlier report (*39*) that hamsters subjected to BOP initiation followed by three cycles of augmentation pressure developed, within 3 months, pancreatic ductal carcinoma that is histopathologically very similar to human pancreatic cancer. The present results also showed that cholangiocarcinoma was induced in virtually all hamsters, but no hepatocellular carcinoma was detected in any hamster examined. This is also in agreement with the previous results (*39*), except that the incidence of cholangiocarcinoma was higher than previous reported (40-46%). Bax *et al.* (*52*) reported that BOP alkylated DNA of almost all tissues in hamster and rat, but that the tumors induced were largely different between the two species. In rats, BOP caused tumors mainly in colon, liver, lungs, and urethra, whereas in hamsters BOP was a potent and selective carcinogen for liver and pancreas (*53*). Thus, DNA damage is not the main factor responsible for the difference in the pancreatic carcinogenesis between hamster and rat. Furthermore, the majority of liver neoplasms induced in the rat and hamster were hepatocellular carcinoma and cholangiocarcinoma, respectively (*39, 40, 53*). This also indicates that the difference in hepatic carcinogenesis in these species was not due to the degree of DNA damage. Thus, these results suggest that events in the promotion stage after BOP initiation may play an important role(s) in the neoplastic transformation of the duct cells in the hamster liver and pancreas. The hamster is unique among experimental animals in that its pancreas contains cells resembling hepatocytes, sometimes called pancreatic hepatocytes (*54*). These cells increase in number when the pancreas is injured by various stresses, such as the augmentation pressure used in the present experiment, *i.e.*, a choline-deficient diet followed by administration of ethionine and BOP. Although virtually nothing is known of the physiological function of pancreatic hepatocytes, it is possible that they catabolize BOP to a metabolite produced in liver parenchymal cells, and that this metabolite acts as a promoter for the development of ductal tumors. This may explain why the hamster, but not the rat, produces pancreatic ductal carcinoma as well as cholangiocarcinoma. However, further work is needed to substantiate this hypothesis.

In the present study, bikunin was isolated in good yields from urine of the animals undergoing carcinogenesis. This further indicates the similarity between the hamster model and human tumors, since patients with malignant tumors were reported to excrete this protein in greatly elevated concentrations (*33-35*). At present, however, the origin of urinary bikunin is unclear. Since mRNA encoding bikunin was detected only in the liver, it was proposed that bikunin was secreted from the liver and taken up by cancer tissues or excreted in the urine (*23*). Bikunin contains no signal peptide, and hence it may be complexed with heavy chain(s) and excreted as ITI or PTI followed by cleavage of the connecting carbohydrate chain. Virtually nothing is known, however, of this transport pathway or mechanism. Hamsters excreted trypstatin in amounts comparable to that of bikunin, whereas there is virtually no report for the occurrence of trypstatin in human urine. The biological significance of this difference is not clear.

Mammalian ITIs can be classified into two types, one consisting of HC1, HC2, and bikunin and the other consisting of HC2, HC3, and bikunin. Human and bovine plasmas contain only the former and the latter, respectively (*5, 13, 14*). The present paper reports for the first time the presence two types of ITI in the same species. However, in the present study, tumor-bearing hamsters were used, and it remains to be determined whether the healthy hamsters also contain the same types of ITI. Mammalian PTIs can be classified into three types: HC1/bikunin, HC2/bikunin, and HC3/bikunin. Human contains all the three types (*14*). In contrast, hamster and bovine contain only HC3/bikunin (the present result) and HC2/bikunin (*14, 17*), respectively, although the presence of other types in minute quantity cannot be ruled out. The biological significance of these species differences in the composition of ITI families is not yet clear. Recently Chan *et al.* (*16*) showed that mRNAs for HC2 and HC3, but not for HC1 or α_1-microglobulin/bikunin, were transcribed in mouse brain, while mRNAs for these proteins were all transcribed in mouse liver. This suggests that there is a functional difference between HCs and that HCs without bikunin may also play a role(s). Thus, the data so far available indicate that expression and function of proteins belonging to the ITI superfamily differ not only from species to species but also from organ to organ, and the hamster may provide a good model for solving these problems.

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