Identity of urinary trypsin inhibitor-binding protein with link protein

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Abstract

Urinary trypsin inhibitor (UTI), a Kunitz-type protease inhibitor, directly binds to some types of cells via cell-associated UTI-binding proteins (UTI-BPs). Here we report that
the 40-kDa protein (UTI-BP$_{40}$) was purified from cultured human chondrosarcoma cell line HCS-2/8 by UTI affinity chromatography. The purified UTI-BP$_{40}$ was digested with trypsin, and the amino acid sequence of the peptide fragments was determined. The sequence of six tryptic fragments of UTI-BP$_{40}$ was identical with subsequences present in human link protein (LP). Authentic bovine LP and UTI-BP$_{40}$ displayed identical electrophoretic and chromatographic behavior. The UTI-binding properties of UTI-BP$_{40}$ and LP were indistinguishable. The direct binding and competition studies strongly demonstrated that the NH$_2$-terminal fragment is the UTI binding part of the LP molecule, that the COOH-terminal UTI fragment (HI-8) failed to bind the NH$_2$-terminal subdomain of the LP molecule, and that the LP and UTI-BP$_{40}$ exhibited significant hyaluronic acid binding. These results provide a demonstration that the UTI-BP$_{40}$ is identical to LP and that the NH$_2$-terminal domain part of UTI is involved in interaction with the NH$_2$-terminal fragment within the LP, which is bound to hyaluronic acid into the extracellular matrix.

**Introduction**

Urinary trypsin inhibitor (UTI) is a Kunitz-type protease inhibitor that is responsible for the inhibition of several proteases in serum and urine as well as in amniotic fluid (1). We (2-4) and others (5,6) have found that UTI can directly bind to neoplastic cells as well as to non-neoplastic cells via cell-associated UTI-binding proteins (UTI-BPs) or specific UTI receptors. We recently reported that one of the proteins of the UTI-BP family is a pericellular matrix-associated glycoprotein of ~40-kDa (UTI-BP$_{40}$) that is thought to be very similar to human link protein (LP) (4). Our previous finding
demonstrated that UTI may be able to bind hyaluronic acid via the LP molecule, since UTI fails to directly bind hyaluronic acid (7). Link protein is present on a wide variety of cells including skin, fibroblasts (8), chondrocytes (9), chondrosarcoma cells (10-13), synovial cells (14), aorta (15), trachea (16), and hepatocytes (17-19). Characteristics of the LP molecule have been studied by a number of different laboratories (20,21), and it has been shown to mediate the interaction between proteoglycans and hyaluronic acid (22,23), a characteristic that may allow it to demonstrate pericellular matrix formation and stabilization (hyaluronic acid-rich matrix formation) (17). Several studies have suggested that a proteoglycan tandem repeat (PTR), found in most of the hyaluronic acid-binding molecules including LP and aggrecan, acts as a functional site of interaction with hyaluronic acid (24-26).

The present study was undertaken to define more clearly that relationship between proteins of the UTI-BP family and the LP molecule in human chondrosarcoma cell line HCS-2/8. For this, we first purified proteins of the UTI-BP family from the HCS-2/8 cells in a large scale. Sequencing of tryptic fragments of the UTI-BP40, chromatographic and electrophoretic examination, and comparison of UTI-binding properties has revealed the identity of UTI-BP40 with LP. Second, we tested a variety of antibodies raised against LP and hyaluronic acid-binding region (HA-BR) of aggrecan proteoglycan to determine whether anti-LP and anti—HA-BR antibodies cross-react with proteins of the UTI-BP family. In addition, the domain-specific antibodies to LP synthetic peptides were used as probes for determining structural analyses of the LP molecule. Finally, we studied the binding and competition effect of UTI fragments or LP subdomains on the solid-phase binding in attempts to localize ligand sites in the UTI structure and binding part in the LP molecule.

Materials and methods

Cells and culture conditions
Human chondrosarcoma cell line HCS-2/8 (27,28) (a gift from Prof. Dr. M. Takigawa; Department of Biochemistry and Molecular Dentistry, Okayama University Dental School, Okayama, Japan) was grown and cultured as previously described (29-31). The cells were cultured in RPMI 1640 supplemented with 10 % fetal bovine serum (FBS), 25 mM HEPES buffer (GIBCO), 2.5 mM glucosamine, 3 mM glutamine (GIBCO), 0.03 mM sodium pyruvate (GIBCO), 2.5 mM sodium lactate, 5 mM glucose (Yoneyama Chemical Co., Tokyo), 100 U/ml penicillin, and 100 µg/ml streptomycin in an atmosphere of 5 % CO₂, 95 % air. For immunohistochemistry, ~5 × 10⁴ cells were seeded on chamber slides and cultured.

Purification of the urinary trypsin inhibitor-binding proteins (UTI-BPs)

The UTI-BPs were purified by UTI-coupled Sepharose 4B and molecular sieve chromatography as described previously (4). Briefly, purified human UTI (50 mg) was coupled to CNBr-activated Sepharose 4B (15 g dry weight=50 ml bed volume; Pharmacia, Uppsala, Sweden) according to the manufacturer's recommendations. HCS-2/8 cells (~1 × 10⁸ cells) were lysed in 5 ml of extraction buffer (20 mM Tris-HCl, pH 7.4 containing 150 mM NaCl, 1 % (w/v) Triton X-100 and 1 µg/ml Streptomyces hyaluronidase [Seikagaku Kogyo, Tokyo]) and incubated at 23 °C for 30 min. The resulting extract was centrifuged (5000 ×g, 30 min, 4 °C) and the supernatant was dialyzed and mixed with bovine serum albumin (BSA)-Sepharose beads previously equilibrated in 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.2 % Triton X-100, 10 mM benzamidine, 1 mg/ml leupeptin, 1 mM phenylmethyl sulfonylfluoride, 0.1 mg/ml ovalbumin, and 0.1 unit/ml aprotinin (all reagents from Sigma) using end to end rocking for 16 h at 4 °C. Unbound materials were again mixed with UTI-Sepharose beads using end to end rocking for 16 h at 4 °C. The affinity gel was then washed 10 times with 20 mM Tris-HCl, pH 7.4 containing 150 mM NaCl and twice with H₂O. Eluted materials were dialyzed and concentrated by ultrafiltration (Amicon; Tokyo, Japan) for analysis on a molecular sieve chromatography with high performance liquid chromatography (HPLC) system (Kanto Kagaku, Tokyo). The column was equilibrated in 4M guanidine-HCl, 50 mM Tris-HCl, pH 7.4 at a flow rate of 0.5 ml/min. Fraction size was 0.5 ml.
Calibration of the gel filtration column was with high and low molecular weight standard (Cosmo Bio, Tokyo). Eluant was monitored at 280 nm. The eluted fractions were assayed for dot blot analysis using anti-LP, LP\textsubscript{pep-N}, LP\textsubscript{pep-C}, HABR-1, and HABR-2 (see below). The fractions containing Mr ~100 kDa UTI-BP (UTI-BP\textsubscript{100}) was separated from the mixture of UTI-BP\textsubscript{45} and UTI-BP\textsubscript{40}. The mixture of UTI-BP\textsubscript{45} and UTI-BP\textsubscript{40} were further separated on reverse phase HPLC. This sample was applied to a 4.6 × 250-mm Vydac C-18 column (Kanto Kagaku) (2). The C18 columns were packed for high performance and equilibrated with 5 % acetonitrile (AN)/0.1 % trifluoroacetic acid (TFA) before loading. The material was pumped directly onto the column. The column was eluted at 1.0 ml/min with a gradient from 5 % AN to 50 % AN over 90 min. Eluent was monitored at 214 nm and 280 nm. The fractions eluting between 26 % and 29 % AN (UTI-BP\textsubscript{45}) and between 32 % and 34 % AN (UTI-BP\textsubscript{40}) were pooled, dialyzed, and concentrated. The amount of protein in the soluble fraction was quantified in a Bradford assay (Bio-Rad) using BSA as a standard (32).

**Purification of bovine link protein and hyaluronic acid-binding region (HA-BR) within aggrecan**

The isolation of hyaluronic acid-binding protein (HA-BP) derived from bovine nasal cartilage has been described in detail elsewhere (22,33). HA-BP was purified by affinity chromatography on hyaluronic acid covalently coupled to Sepharose. A purified preparation of HA-BP was supplied by Chugai Pharmaceutical Co. Ltd., and Seikagaku Kogyo, Co. Ltd., Tokyo. Five mg of HABP was concentrated using a Centricon 10 ultrafiltration tube by centrifuging at 200 ×g at 4 °C and then further purified by gel filtration chromatography on a column of Sepharose CL-6B (2.5 × 175 cm) equilibrated in 4 M guanidine HCl, 50 mM Tris-HCl, pH 7.4, as described by Tang et al (34). The crude hyaluronic acid-binding region (HA-BR) and LP peaks were fractionated by HPLC gel filtration using a SW3000 column (Kanto Kagaku, Tokyo). Aliquots of each fraction were tested for their immunoreactivity by a specific dot blot assay and a HA-BR peak (>100-kDa polydisperse band by Western blot) and a LP peak (~40-kDa band)
were obtained. The LP purified from HA-BP does not contain HA-BR within aggrecan, which was confirmed by Western blot analysis with specific monoclonal antibodies raised against HA-BR within aggrecan (mAbs HABR-1 and HABR-2).

Preparations of polyclonal antibodies raised against UTI and its derivative as well as against LP and its synthetic peptides

Highly purified preparation of UTI was supplied by Mochida Pharmaceutical Co., Tokyo. Chondroitinase ABC (Sigma) was used for enzymatic deglycosylation. Briefly, 1 mg of the purified UTI was incubated with 1.0 µg of chondroitinase ABC for 24 h at 37 °C. The COOH-terminal fragment of UTI (HI-8; Mr 8 kDa) prepared by trypsin digestion was a gift of Dr. Dan Sugino (Nissin Food Products; Shiga, Japan). Polyclonal antibodies against UTI and HI-8 were prepared by intradermal injection of rabbits with 0.1 mg of purified proteins emulsified in Freund's adjuvant. The antiserum was specific for UTI and had a 50 % maximal binding at a dilution of 1/10,000 in an ELISA. The antisera to UTI and HI-8 were reactive with the 240-kDa IαI, the 120-kDa Pre-α-inhibitor (PαI), the 40-kDa UTI and the Mr 8-kDa HI-8 in ELISA or Western blotting assays. Affinity-purified IgG was prepared by mixing 3 ml of antiserum with 1 ml of UTI (or HI-8)-Sepharose overnight at 4 °C. Following washing, the IgG was eluted with 0.1 M glycine-HCl, pH 2.5. The pH of the eluted fractions was immediately raised, and the IgG was stored at -20 °C.

Antibodies against LP (pAb LP) purified from bovine cartilage were prepared in a similar manner. In addition, to generate anti-LP peptide antibodies, two synthetic oligopeptide sequences, 112VFLKGGSDSDAS123 (NH2-terminal fragment of LP) and 231TVPGVRNYGFWDKDKS246 (COOH-terminal fragment of LP), corresponding to the NH2-terminal domain and the COOH-terminal domain of human LP molecule, respectively, were selected. We searched for possible antigenic amino acid sequences on the LP molecule according to their predicted secondary structures and hydrophobicity. Each peptide was chosen based on their theoretical antigenic index and for specificity to the molecules. Antisera against LP synthetic oligopeptides were
obtained from rabbits immunized four times with 0.2 mg peptide conjugated with keyhole limpet hemocyanin together with Freund's adjuvant. Titration of antisera was performed by an ELISA, with peptides used for immunization as antigen. When the antibody titer reached a plateau, blood was totally collected, and the serum was separated. Polyclonal antibodies against NH$_2$-terminal fragment of LP (anti-LP$_{pep-N}$) and against COOH-terminal fragment of LP (anti-LP$_{pep-C}$) were prepared in a similar manner using the elute from protein A-Sepharose (Hitrap; Pharmacia, Uppsala, Sweden).

**Production of monoclonal antibodies raised against UTI and hyaluronic acid-binding region within aggrecan**

Male Balb/c mice were immunized at 14-day intervals by intraperitoneal injection of 20 µg affinity purified UTI. Three days after the last booster, spleen cells ($1 \times 10^8$) were fused with the mouse myeloma cell line NS-1 and seeded according to standard procedures (35). The antibodies were designated 2A6, 5C12, 4D1, and 8H11. 8H11 showed the strongest reactivity for UTI and reacted with the NH$_2$-terminal domain of UTI. mAb 8H11 was isolated from ascites fluid by chromatography on a protein A-Sepharose column and used for ELISA.

Monoclonal antibodies raised against HA-BR within aggrecan were prepared in a similar manner. Two antibodies were selected and designated HABR-1 and HABR-2. These mAbs were found to react with HA-BR of aggrecan, but not with LP. A list (antibody specificities and characterization) of the various mAbs and pAbs used in this study is shown in Table 1. A purified preparation of each antibody was biotinylated according to the method of Guesdon et al. (36), using N-hydroxsuccinimidyl biotinamidocaproate (Sigma) using the manufacturer's suggested procedures.

**Purification of UTI-BP$_{40}$ tryptic fragments**

Electrophoretically homogeneous UTI-BP$_{40}$ in PBS, pH 7.3, was treated with bovine pancreatic trypsin for 3 h at 37 °C at a 1 : 100 enzyme : substrate molar ratio. Peptides
were separated by 17% SDS-polyacrylamide gel electrophoresis under nonreducing conditions. The resulting gel was stained with Coomassie blue and electrophoretically blotted onto polyvinylidene difluoride (PVDF; Bio-Rad) membrane. In a parallel experiment, tryptic fragments were analyzed by reverse phase HPLC. Sequencing of the peptides was by Edman's degradation using an automated sequencer (Applied Biosystems model 477A) with on-line phenylthiohydantoin-derivative detection.

**Trypsin treatment of TLP-HA and TLP—HA-BR complexes**

Two major protein fractions have been isolated from a tryptic digest of the bovine proteoglycan complex (37-39): one of them, HA-BR, derives from the proteoglycan subunit and is located at its NH2-terminus, whereas the other (TLP) is a common trypsin fragment from LP; their homogeneity was assessed by SDS-PAGE. TLP differs from native LP by the removal of a short amino-terminal peptide from the native LP. Tryptic digestion of TLP-HA and TLP—HA-BR complexes was carried out according to previously reported experiments (24). The 22-kDa fragment, isolated from a tryptic digest of TLP-HA complex, corresponds to the COOH-terminal region of bovine TLP (termed as LP-C in this study). On the other hand, the 20-kDa fragment arising from TLP—HA-BR digest is the NH2-terminal fragment of TLP (termed as LP-N).

**Solid-phase binding and competition assays**

The solid-phase binding assay has been described in detail elsewhere (26). A 96-well microtiter plate wells were coated with 100 µl of HA-BR (5 µg/ml), LP (2 µg/ml), LP-N (1 µg/ml), LP-C (1 µg/ml), or with UTI-BP40 (1 µg/ml) in bicarbonate buffer, pH 9.5, at 4 °C overnight. 100 µl of biotinylated UTI (0-10 µg/ml) was added to each well and was incubated for 2 h at room temperature. In a case of competition assay, studies on the binding of biotinylated UTI (10 nM) to immobilized UTI-BP40 or LP were performed in the presence of unlabeled competitors (UTI, HI-8, UTI-BP40, LP, LP-N, LP-C, α1-antitrypsin [α1AT], α2-antiplasmin [α2AP], and plasminogen activator inhibitor type-1 [PAI-1; reagents from Cosmo Bio Co. Ltd.; Tokyo]) for 2 h at 23 °C.
In a parallel experiment, 96-well microtiter plate wells were coated with 50 µl (100 µg/ml) of hyaluronic acid conjugated to phosphatidylethanolamine dipalmitoyl (HA-PE; a gift from Sekagaku Kogyo) in PBS at 4 °C overnight as described previously (26). For studies of specificity, the same amount of chondroitin sulfate (CS)-PE or heparan sulfate (HS)-PE (a gift of Sekagaku Kogyo) as HA-PE was used. After the wells were blocked with TBS containing 1 % BSA (1 h, 23 °C), 50 µl of UTI-BP40 or LP (0.5 µg/ml) was added to some of the plate wells (2 h, 23 °C). 50 µl of biotinylated UTI (0.1 µmol/L) was added to each well in the absence or presence of CS (50 µl, 100 µg/ml) or HS (50 µl, 100 µg/ml), and was incubated for 2 h at 23 °C. Horseradish peroxidase-conjugated avidin was used as detection probe.

**SDS-PAGE and Western blot**
The cell extracts, purified proteins, or tryptic fragments were dissolved in a sample buffer. The sample (20 µg protein/lane for cell extracts and 0.1~0.5 µg protein/lane for purified proteins) was processed for electrophoresis, using a SDS polyacrylamide gel under nonreducing conditions. The resulting gel was electrophoretically blotted onto PVDF membrane, which was blocked in TBS containing 2 % BSA, and then immunoblotted. The blot was subsequently processed for biotin-avidin-peroxidase method (40). Bands were visualized with the ECL detection system (Amersham, Tokyo). The membranes were then placed between two transparencies and exposed to Kodak film. In all experiments, some strips were incubated with non-immune rabbit (or mouse) IgG as a negative control.

**Statistical analysis**
The data presented are the mean of triplicate determinations in one representative experiment unless stated otherwise. Data are presented as mean ± standard deviation (SD). All statistical analysis was performed using StatView for Macintosh. The Mann-Whitney U test was used for the comparisons between different groups. P less than 0.05 was considered significant.
Results

_Determination of antibody specificity_

Characterization of mAb 8H11: Clone 8H11 was produced by somatic cell fusion. The interaction of mAb 8H11 with UTI was evaluated by immunoblotting with UTI, chondroitinase ABC-treated UTI (deglycosylated UTI), HI-8, and with UTI reduced with 2-mercaptoethanol (2-ME) (Figure 1). MAb 8H11 reacts with UTI and deglycosylated UTI, but not with HI-8 or with UTI reduced with 2-ME (Figure 1; Right panel). This suggests that the epitope resides in the NH2-terminal domain of UTI (since it is missing in the HI-8) and is destroyed by reduction of the disulfide bonds. In contrast, Western blot analysis indicated that polyclonal antibodies raised against UTI (pAb UTI) recognizes a determinant present on a wide variety of UTI preparations (UTI, deglycosylated UTI, HI-8, and UTI reduced with 2-ME) (Figure 1; Left panel). The ELISA data also confirmed that the 8H11 determinant was sequested in the NH2-terminal structure of UTI (data not shown).

Characterization of mAbs HABR-1 and HABR-2 and pAbs raised against LP and LP synthetic peptides as well as polyclonal antibodies raised against UTI-BP: By procedures described previously (2,4), the UTI-BP was purified from human HCS-2/8 cell lysates by UTI-coupled Sepharose 4B. As shown in Figure 2, HCS-2/8 cell-derived UTI-BP is composed of three different molecular species, around 100-kDa (UTI-BP100), the 45-kDa (UTI-BP45) and the 40-kDa (UTI-BP40). PAb raised against UTI-BP (pAb UTI-BP) reacted with all member of UTI-BP family (UTI-BP100, UTI-BP45, and UTI-BP40) and purified LP molecule, as well as with both the NH2-terminal immunoglobulin-like domain of LP (LP-N; ~20-kDa) and the COOH-terminal PTR domain of LP (LP-C; ~22-kDa). After immunoabsorption of anti-UTI-BP antibodies with LP, remaining antibodies recognized the UTI-BP100 and UTI-BP45 in UTI-BPs but
not the UTI-BP<sub>40</sub> (Figure 3). This shows that the 40 kDa band does not contain more than LP. The interaction of anti-LP antibodies (pAb LP) with UTI-BPs was also evaluated by immunoblotting with UTI-BP<sub>100</sub>, UTI-BP<sub>45</sub> and UTI-BP<sub>40</sub>. pAb LP reacted with UTI-BP<sub>40</sub> and purified LP molecule, as well as with both LP-N and LP-C. However, pAb LP failed to react with UTI-BP<sub>100</sub> or UTI-BP<sub>45</sub>. PAb LP<sub>pep-N</sub>, in which the epitope presents on the NH<sub>2</sub>-terminal domain of LP, reacted with UTI-BP<sub>40</sub>, LP, and LP-N, but not with LP-C, UTI-BP<sub>100</sub>, or UTI-BP<sub>45</sub>, whereas PAb LP<sub>pep-C</sub>, in which the epitope resides in the COOH-terminal domain of LP, reacted with UTI-BP<sub>40</sub>, LP, and LP-C, but not with LP-N, UTI-BP<sub>100</sub>, or UTI-BP<sub>45</sub>. Western blot analysis thus demonstrated that pAbs raised against LP synthetic peptides exclusively recognized both their respective domains of LP and UTI-BP<sub>40</sub>. It is unlikely that the UTI-BP<sub>100</sub> and UTI-BP<sub>45</sub> have antigenically cross-reactivity with LP. These results suggest that the UTI-binding sites purified from HCS-2/8 cells may contain other binding proteins or UTI receptors rather than LP.

MAbs HABR-1 and HABR-2 (data not shown here) react with UTI-BP<sub>100</sub> but not with UTI-BP<sub>45</sub> or UTI-BP<sub>40</sub>, suggesting that the UTI-BP<sub>100</sub> is comprised of HA-BR of aggrecan fragment.

**Amino acid sequence of UTI-BP<sub>40</sub> tryptic fragments**

The purified UTI-BP<sub>40</sub> was digested with trypsin, and the resultant peptides were purified by immunoblotting or reverse phase HPLC and identified by NH<sub>2</sub>-terminal sequencing (Figure 4). Aliquots of each blotting or each pool were analyzed by gas-phase sequencing. A comparison with data in GenBank (Accession NM 001884) showed that the six tryptic peptides were identical with subsequences found in human LP. In every case, the UTI-BP<sub>40</sub> fragments corresponded to those expected from cleavage of LP at tryptic sites. The molecular mass of the six tryptic fragments was equivalent to 32.2 % of the mass of LP (354 aa).

**Reverse phase HPLC and SDS-polyacrylamide gel electrophoresis of UTI-BP<sub>40</sub> and LP**
Authentic LP and UTI-BP$_{40}$ displayed equivalent retention times (64 min) on a C18 reverse phase HPLC column, determined by A$_{214}$ detection. Similar electrophoretic migration (40 kDa) of the two proteins was observed in an SDS-15% polyacrylamide gel under nonreducing conditions.

**Binding and competition assays**

Specific binding of biotinylated UTI to immobilized potential ligands (LP, LP subdomains, UTI-BP$_{40}$, and UTI-BP$_{100}$): Since the yield of the UTI-BP$_{45}$ were small, studies on the specific binding of biotinylated UTI to immobilized UTI-BP$_{45}$ could not be carried out in the present study. We have separated the NH$_2$-terminal and the COOH-terminal regions of LP by subfragmentation with trypsin to confirm which one of the subdomains of LP interacts with UTI. The separated subdomains have been used to investigate epitope mapping of several antibodies (Figure 2) and have been extensively used in binding (Figures 5 and 6) and competition assays. The LP-C showed a 22-kDa single monomeric band whereas the LP-N represented a 20-kDa intense band and had additional fast migrating weak bands that appeared to be degradation products (see Figure 2; WB[pAb LP]). The solid-phase binding assay was used to support a more extensive analysis of the LP binding site (Figure 5). The LP, LP-N, and UTI-BP$_{40}$ exhibited significant biotinylated UTI binding, whereas the LP-C and UTI-BP$_{100}$ showed no significant affinity for UTI, even if the concentration of biotinylated UTI was increased to 1 µmol/L. Although UTI binding to different ligands cannot be quantitatively compared by plate binding, our results indicate that the subdomain for UTI binding is the NH$_2$-terminal domain within LP molecule and that UTI shows no significant affinity for HA-BR within aggrecan (UTI-BP$_{100}$). Our results support the hypothesis that HA-BR itself has an ability to bind UTI via the LP molecule, since HA-BR is known to directly and specifically interact with LP. To assure that the applied proteins stuck to the microtiter plate wells, we performed an immunodetection assay by using respective antibodies. The significant signals of absorbance at A$_{450}$ were obtained from these ligands tested, compared with those from the BSA control (data not shown).
Effect of ligands on the binding of biotinylated UTI to immobilized LP or UTI-BP$_{40}$: In order to localize and characterize ligand and binding structures in UTI, involved in the binding to UTI-BP$_{40}$ or LP, studies on the binding of biotinylated UTI to immobilized UTI-BP$_{40}$ or LP were performed in the absence or presence of unlabeled competitors (Figure 6). The UTI-BP$_{40}$—coated microtiter plate wells were incubated with biotinylated UTI at a concentration of 10 nM in the absence ($\Delta$OD$_{450}$ = 0.73) or presence of each competitor for 2 h at 23 °C. The inhibition of specific binding obtained with competitors in excess was for UTI (1 $\mu$M) 94 %, UTI-BP$_{40}$ (1 $\mu$M) 51 %, LP (1 $\mu$M) 53 %, and for LP-N (1 $\mu$M) 48 %, whereas the quenching caused by HI-8 and LP-C was insignificant (less than 20 %). Unrelated proteins ($\alpha_1$-AT, $\alpha_2$-AP, and PAI-1) and BSA failed to inhibit biotinylated UTI binding to immobilized UTI-BP$_{40}$. In a parallel experiment, potent inhibition by UTI, UTI-BP$_{40}$, LP, and LP-N was also observed in LP-coated microtiter plate wells (data not shown).

The identity of UTI-BP$_{40}$ with LP is directly provided by the following competition assays. First, studies on the binding of anti-UTI-BP antibodies to immobilized UTI-BP$_{40}$ were performed in the presence of LP (data not shown). We carried out an immunodetection assay by using biotinylated anti-rabbit IgG and avidin-peroxidase. This experiment showed that LP (1 $\mu$M) was able to give ~90 % inhibition of anti—UTI-BP antibodies binding to UTI-BP$_{40}$ bound to a plate. Second, UTI-BP$_{40}$ (1 $\mu$M) almost completely blocked anti-LP antibodies binding to LP bound to a plate (data not shown). Thus, the antibodies were each blocked to greater than 90 % by the antigens indicated.

*biotinylated UTI binding to LP or UTI-BP$_{40}$ anchored via hyaluronic acid*

We studied the interaction of LP or UTI-BP$_{40}$ with hyaluronic acid (HA), chondroitin sulfate (CS), or heparan sulfate (HS). Biotinylated UTI was added to the HA-PE—, CS-PE—, or HS-PE—coated wells preincubated with or without LP or UTI-BP$_{40}$ (Figure 7). The LP and UTI-BP$_{40}$ exhibited significant HA binding, whereas LP and UTI-BP$_{40}$ showed no significant affinity for CS or HS. These results indicated that there is no
significant difference in UTI binding activity between LP and UTI-BP$_{40}$ bound to immobilized HA. In addition, we added LP or UTI-BP$_{40}$ to the HA-PE-coated plate wells together with CS and HS to see if either could compete with LP or UTI-BP$_{40}$ binding. However, neither CS nor HS could compete with LP or UTI-BP$_{40}$ binding to HA. These results strongly indicated the specific interaction of both LP and UTI-BP$_{40}$ with HA, but not CS or HS. The direct binding studies strongly demonstrated UTI does not directly and specifically interact with HA, CS, or HS (data not shown). These results support the hypothesis that UTI has an ability to bind HA via the LP molecule or UTI-BP$_{40}$. We confirmed again that the applied proteins (LP or UTI-BP$_{40}$) stuck to HA-coated microtiter plate wells (data not shown).

**Discussion**

Extracellular proteolysis is required in inflammation and in tumor processes where cell migration and invasion occurs (41-45). A growing body of evidence demonstrated that UTI effectively inhibits tumor cell invasion and metastasis. Tumor cell-associated plasmin, but not urokinase activity, was efficiently inhibited by UTI (46-49). UTI interacts with a variety of cell types including neoplastic and non-neoplastic cells. The presence of UTI at the cell surface has been explained by the demonstration of UTI binding sites on the cell membranes. These UTI-binding protein (UTI-BP) and UTI receptor have been recently detected, but only a few have been isolated or extensively characterized (2-6,50). We initially reported the presence of proteins of the UTI-BP family on human choriocarcinoma SMT-cc1 cells and uterine fibroblasts (2,4). While the UTI-BP$_{40}$ was described as an UTI-binding site, it was able to bind hyaluronic acid as well and has been localized abundantly in cartilage and ovary in mice and rats (in submission). Therefore, in the present study we tried to isolate and characterize proteins of the UTI-BP family from human chondrosarcoma HCS-2/8 cells in a large scale, since
these cells expressed cartilage proteoglycans associated with hyaluronic acid to form proteoglycan aggregates (51,52).

Using the UTI-immunoaffinity beads, several proteins of the UTI-BP family were purified from HCS-2/8 cell extracts. The UTI-BP$_{40}$ was the major band consistently and specifically bound to UTI. The UTI-BP$_{100}$ and UTI-BP$_{45}$ were the minor bands directly or indirectly bound to UTI. The present study extended characterizations of UTI-BP$_{40}$. The purification of apparent homogeneity of UTI-BP$_{40}$ gave us access to partial amino acid sequence information. First, the amino acid sequences of tryptic fragments of UTI-BP$_{40}$ were identical with subsequences found in human LP. Second, UTI-BP$_{40}$ is identical with LP with respect to molecular weight and behavior on reverse phase-HPLC chromatography and on SDS-PAGE. Third, a number of domain-specific anti-LP antibodies cross-react with the UTI-BP$_{40}$. Fourth, authentic LP and UTI-BP$_{40}$ displayed similar UTI binding. The binding of biotinylated UTI by LP and UTI-BP$_{40}$ was specific in that unrelated proteins (α$_1$-antitrypsin, PAI-1, and α$_2$-antiplasmin) and BSA did not inhibit it. The IC$_{50}$ for unlabeled UTI was in the low nanomolar range. However, we could not explain why UTI-BP$_{40}$, LP, and LP-N only give ~50% inhibition of UTI binding to UTI-BP$_{40}$ bound to a plate. The NH$_2$-terminal LP fragment (Mr 20 kDa) showed substantial UTI binding activity, whereas the COOH-terminal LP fragment (22 kDa) did not have UTI binding ability, indicating that the subdomain for UTI binding is the NH$_2$-terminal domain within LP molecule. Furthermore, LP and UTI-BP$_{40}$ specifically bind hyaluronic acid. The common structural motif in LP for hyaluronic acid binding appears to be a PTR module within the COOH-terminal region of this protein. Thus, we have been able to identify the NH$_2$-terminal fragment within the LP molecule as a probable binding domain for UTI. Fifth, the COOH-terminal UTI fragment (HI-8) failed to bind the LP itself or the NH$_2$-terminal subdomain of the LP molecule. Sixth, UTI-BP and LP exist in association with hyaluronic acid into extracellular matrix of the cultured cells. Collectively, we conclude that UTI-BP$_{40}$ is LP, and that the NH$_2$-terminal domain part of UTI is involved in
interaction with the NH$_2$-terminal fragment within the LP, which is bound to hyaluronic acid into the extracellular matrix (Figure 8).

However, the conclusion from the set of immunoprecipitation experiments is that anti-LP antibodies can immunoprecipitated approximately 60% of UTI binding activity from the cell extracts (data not shown). Incomplete reduction after immunoprecipitation could be due to the presence of a heterogenous population of UTI binding proteins, since some fractions (UTI-BP$_{100}$ and UTI-BP$_{45}$) of which may be unable to react with the LP-related antibodies. It is likely that UTI can bind to components rather than members of the LP molecules. The UTI-BP$_{100}$ was identified immunologically as a HA-BR within aggrecan molecule. Therefore, UTI-BP$_{100}$ and aggrecan G1 domain share similar epitopes or that they are closely related if not identical molecules. It is unlikely that the HA-BR within aggrecan is another candidate for UTI-BP, since UTI does not directly bind to HA-BR. We have considered that the HA-BR may bind UTI via the LP molecule. The identity of UTI-BP$_{100}$ as aggrecan G1 domain should also be established by amino acid sequencing, tryptic maps, and specific binding and competition experiments.

In addition, the minor UTI-BP$_{45}$ was also specifically isolated. It is unlikely that UTI-BP$_{40}$ may represent a degradation product of UTI-BP$_{45}$, since anti-LP antibodies did not cross-react with UTI-BP$_{45}$. This may be a novel protein or may be a subunit of UTI-BP complex, each having the ability to bind UTI. Since the yield of UTI-BP$_{45}$ was small, studies on the specific binding of UTI to UTI-BP$_{45}$ could not be carried out in the present study. Whether the UTI-BP$_{100}$ represents the aggrecan G1 domain or the UTI-BP$_{45}$ is a new member of the UTI-BP family remains an open question.

The molecular weight of LP produced by HCS-2/8 cells is almost the same as that of UTI-BP$_{40}$ (Takigawa et al., 1999; unpublished data). LP is synthesized by the chondrosarcoma cells themselves and stabilizes the binding between proteoglycan subunits and hyaluronic acid (34,53,54). Since LP is found in the extracellular matrix, it is thought to be involved in the organization of an hyaluronic acid-rich matrix. We reported for the first time that LP directly binds UTI, which corresponds to a light chain.
of inter-α inhibitor. These data strongly demonstrate that a locally produced and expressed UTI-binding sites accumulate free UTI and/or inter-α inhibitor into the extracellular matrix of the chondrosarcoma cells.

It is possible that the UTI serves a number of different functions through the LP molecule. UTI could interact with LP anchored in hyaluronic acid-rich matrix on the surface of tumor cells. This may result in the effective inhibition or regulation of tumor cell-associated protease activity. Furthermore, our previous studies demonstrated the specific internalization of UTI by tumor cells (2,50,55). The process of UTI-BP- or UTI receptor-mediated endocytosis has been the subject of extensive study. Proteins of the UTI-BP family may be involved in the active endocytosis of UTI. Further research will reveal additional characteristics for the very interesting proteins of the UTI-BP family.

In summary, the present study characterizes the proteins of the UTI-BP family biochemically, immunologically and immunohistochemically, and identified UTI-BP40, which is identical with LP. Our results strongly support that UTI binding site is located on the NH2-terminal region of this molecule and the NH2-terminal domain part of UTI may be involved in interaction with the NH2-terminal fragment within the LP, which is bound to hyaluronic acid into the extracellular matrix via the COOH-terminal PTR domain part of the LP molecule. Several lines of evidence demonstrate that the UTI-BP100 is the aggrecan G1 domain, although a definitive answer can only be found after sequencing and cloning. Whether UTI-BP45 is a novel member of the expanding UTI-BP family also remains an open question.

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| Antibody | Characteristic Feature |
|----------|-----------------------|
| UTI      | urinary trypsin inhibitor |
| UTI-BP   | UTI-binding protein     |
| LP       | link protein           |
| HI-8     | COOH-terminal domain of UTI |
| HA-BR    | hyaluronic acid-binding region |
| SMT-cc1  | choriocarcinoma cell line |

1), see refs. 2 and 4.
Figure legend

Figure 1  Characterization of antibodies raised against UTI
SDS-PAGE followed by Western blot of purified UTI and its derivatives under nonreducing and reducing conditions. The samples (0.1 µg) were analyzed by 5-18 % gradient SDS-PAGE and transferred to PVDF membranes. Western blot analysis of the UTI and its derivatives were conducted for pAb raised against UTI (Left panel) and mAb 8H11 (Right panel) reactivities. Lane 1, UTI; lane 2, chondroitinase ABC-treated UTI; lane 3, HI-8; and lane 4, UTI treated with 2-mercaptoethanol (2-ME). The molecular masses (in kDa) and the positions of marker standards are indicated to the left.

Figure 2  Characterization of antibodies raised against UTI-BP and HA-BR as well as LP and its fragments
SDS-PAGE (15 % gel) of purified proteins (UTI-BP [1 µg/lane]; LP, LP-N and LP-C [0.2 µg/lane]) under nonreducing conditions. Western blot (WB) of the UTI-BP, LP and its subdomains using pAbs raised against UTI-BP (pAb UTI-BP), LP (pAb LP), LP-N (pAb LP pep-N), and LP-C (pAb LP pep-C) as well as mAb raised against HA-BR within aggrecan (mAb HABR-1). The molecular masses (in kDa) and the positions of marker standards are indicated to the left. The result of Western blot using mAb HABR-2 was same as that using mAb HABR-1.

Figure 3  Characterization of anti—UTI-BP antibodies immunoabsorbed with LP
12 % SDS-PAGE and Western blot of UTI-BP (1 µg/lane) using anti—UTI-BP antibodies (lane 1) and antibodies immunoabsorbed with LP (lane 2) under nonreducing conditions. The molecular masses (in kDa) and the positions of marker standards are indicated to the right.
Figure 4  Amino acid sequence of human link protein and tryptic fragment of UTI-BP<sub>40</sub>

The upper row, aa sequence of LP; the lower row, aa sequence of tryptic fragments of UTI-BP<sub>40</sub>. X, unidentified residue.

Figure 5  The solid-phase binding assay: Specific binding of biotinylated UTI to immobilized potential ligands

Data points represent average ± SD for estimates done in triplicate. □, LP-coated wells; ■, LP-N—coated wells; →, UTI-BP<sub>40</sub>—coated wells; ▲, UTI-BP<sub>100</sub>—coated wells; and ▼, LP-C—coated wells. Figure is representative of 4 independently conducted experiments. *, p<0.05

Figure 6  Effect of ligands on the binding of biotinylated UTI to immobilized UTI-BP<sub>40</sub>: Competition-inhibition of biotinylated UTI binding to immobilized UTI-BP<sub>40</sub> by UTI, HI-8, UTI-BP<sub>40</sub>, LP-N, LP-C, α<sub>1</sub>-antitrypsin (A1AT), α<sub>1</sub>-antiplasmin (A2AP), and plasminogen activator inhibitor type-1 (PAI-1). Specific binding observed in the presence of increasing concentrations (10<sup>-9</sup>-10<sup>-6</sup> mol/L) of UTI, HI-8, UTI-BP<sub>40</sub>, LP-N, and LP-C was expressed. Data points represent average ± SD for estimates done in triplicate. Figure is representative of 3 independently conducted experiments. *, p<0.05

Figure 7  Biotinylated UTI binding to LP or UTI-BP<sub>40</sub> anchored via hyaluronic acid

Biotinylated UTI (0.1 µg/ml) was added to the HA-PE—coated wells preincubated with LP (0.5 µg/ml; ■■■), UTI-BP<sub>40</sub> (0.5 µg/ml; □□□), or BSA (1.0 µg/ml; □□□) in the absence or presence of CS (100 µg/ml) and HS (100 µg/ml). Data points represent average ± SD for estimates done in triplicate. Figure is representative of 3 independently conducted experiments. *, p<0.05
Figure 8  Schematic representation of the hypothesized interaction of UTI with link protein and hyaluronic acid.

Link protein lacks a transmembrane domain and contains one Ig domain and two PTR loops. Binding to hyaluronic acid is mediated through sequences at the tip of the PTR loops. The Ig loop of the link protein may permit interaction with UTI. The NH$_2$-terminal domain of UTI is involved in interaction with the NH$_2$-terminal fragment within the LP, which is bound to hyaluronic acid into the extracellular matrix.
Figure 1
Western blot (pAb UTI)

Western blot (8H11)
Figure 2

SDS-PAGE

WB (mAb HABR-1)

WB (pAb LP)

 WB (pAb LP pep-N)

 WB (pAb LP pep-C)

 WB (pAb UTI-BP)
Figure 5

Concentrations of biotinylated UTI (mol/L)
Figure 6

Biotinylated UTI, % BOUND

Concentrations of competitors (mol/L)
Figure 7

Coating   Pretreatment

HA-PE     LP
          LP+CS+HS
          UTI-BP40
          UTI-BP40 +CS+HS
          BSA

CS-PE     LP
          UTI-BP40
          BSA

HS-PE     LP
          UTI-BP40
          BSA

OD$_{450}$

*
Figure 8

protease inhibitor domain

C-terminus (HI-8)

Link protein (LP) = UTI-BP40

UTI

N-terminus

LP-N

LP-C

N

C

PTR

Hyaluronic acid (HA)
Identity of urinary trypsin inhibitor-binding protein with link protein
Hiroshi Kobayashi, Yasuyuki Hirashima, Guang Wei Sun, Michio Fujie, Takashi Nishida,
Masaharu Takigawa and Toshihiko Terao

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