A Serum-derived Hyaluronan-associated Protein (SHAP) Is the Heavy Chain of the Inter α-Trypsin Inhibitor*

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We showed previously that hyaluronan (HA) synthesized by cultured fibroblasts firmly bound 85-kDa proteins. The proteins were derived from serum used for the culture and appeared to be covalently linked to HA (Yoneda, M., Suzuki, S., and Kimata, K. (1990) J. Biol. Chem. 265, 5247-5257). In these regards, we named this molecule SHAP (serum-derived HA-associated protein). Incubation of serum with exogenous HA under physiological conditions enabled us to prepare SHAP-HA complex without cell cultivation. The complex thus obtained from bovine or human serum was served for the characterization of SHAP. Digestion with HA-lyase and subsequent separation on SDS-polyacrylamide gel electrophoresis yielded two components, X and Y. Because of the block of their NH2 termini, peptides were obtained by the digestion of X and Y with V8 protease, separated on SDS-polyacrylamide gel electrophoresis and then subjected to the analysis. Peptides from X and Y showed a high degree of sequence similarity to the two heavy chains, HC2 and HC1, of human inter-α-trypsin inhibitor (ITI), respectively (over 80% with bovine SHAP and essentially 100% with human SHAP). Cross-reactivity with antibodies against ITI supported the findings. Direct digestion of the complex with V8 protease and the subsequent purification of the HA-resistant fragment complex were performed to identify the HA-binding domains. NH2-terminal sequences of the fragments suggested the participation of the COOH-terminal half of ITI with an amphipathic α helix structure in the HA binding.

It is generally accepted that hyaluronan (HA) plays modulatory roles in many fundamental biological processes, such as tissue morphogenesis, cell migration, and cell proliferation. HA is also involved in pathophysiological responses of tissues as in inflammation, angiogenesis, wound healing, and tumor invasion (1-5). In relation to such functional divergence of HA, the discovery of a growing number of HA-binding proteins suggests diverse but distinctive molecular features of the proteins. CD44 may be a typical example (6). Variant forms of CD44 generated by alternative splicing seem to have individual functions such as lymphocyte homing and tumor cell metastasis (7, 8).

We showed previously that HA extracted from the cell layer of cultured fibroblasts was complexed with an 85-kDa glycoprotein derived from the fetal calf serum used to supplement the culture medium (9). Subsequent studies have revealed that this protein appears to be covalently linked to HA and is immunologically and biochemically distinct from CD44 and other well characterized HA-binding proteins (10). Therefore, we have designated this protein as SHAP, serum-derived HA-associated proteins. SHAP appears to mediate the binding of HA to cell surfaces and may be one of the serum factors involved in HA metabolism in cultured fibroblasts (9).

Inter α-trypsin inhibitor (ITI) is a plasma protease inhibitor with as yet unknown physiological functions (11-14). Molecular biology has revealed that ITI is comprised of three genetically different peptides, a light chain and two heavy chains (HC1 and HC2) (15-18). Interestingly, the three peptides are covalently cross-linked by a unique linkage through chondroitin sulfate chains (19-23). The light chain has been named bikunin (17), because it contains two tandem repeats of Kunitz-type domains, and the trypsin inhibitor activity of ITI is localized within bikunin (11, 24). The ITI concentration in human serum is relatively high (0.45 mg/ml), but its serine protease inhibitory activity accounts for only about 5% of the total serum protease inhibitory activity (11). It has been proposed that ITI functions as a "protease shuttle" by complexing loosely with proteases and transferring them to other protease inhibitors for subsequent clearance of the second enzyme-inhibitor complex (25, 26).

In the present study, we have characterized the properties and structure of the SHAPs from both bovine and human serum. From sequence and homology data, we propose that SHAP corresponds to the two heavy chains (HC1 and HC2) of ITI. Possible mechanisms of ITI binding to HA as well as physiological roles of the two heavy chains in HA functions are discussed.

EXPERIMENTAL PROCEDURES

Materials—Hyaluronan preparations with average molecular weights of 1.7 × 10⁶ to 9.5 × 10⁷ (measured by viscosity), protease-free Streptomyces hyaluronidase, and chondroitinase AC-II (Arthrobacter aurescens) were gifts of Seikagaku Corporation, Tokyo. Enzymes (Staphylococcus aureus V8, sequencing grade) was purchased from Boehringer Mannheim Biochemica, Mannheim, Germany. Polyvinylidene difluoride (PVDF) membrane (ProBlott) was kindly supplied by Applied Biosystems Japan. CAPS (3-cyclohexylamino)-1-propanesulfonic acid) was purchased from Sigma; bovine serum was from Hannai Eiken, Osaka, Japan; rabbit anti-human...
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inter-o,-stainin inhibitor antibody (IgG fraction) was from DAKO, Glostrup, Denmark. Human blood was kindly provided by volunteers in our laboratory.

Preparation of SHAP-HA Complex from Bovine and Human Serum—
The methods were essentially the same as the ones reported previously (9). Briefly, a 0.4 mg/ml HA solution in Hanks’s basal medium, 10 mM Tris-HCl, pH 7.4, 1:30 penicillin, 50 µg/ml streptomycin, 0.2% NaN3 was mixed with the same volume of human serum and incubated at 37°C for 24 h. Then, 1/10 volume of 4 M guanidine HCl in 0.2 M Tris-HCl, pH 8.0, protease inhibitors (10 mM EDTA, 10 mM N-ethylmaleimide, 5 mM phenylmethylsulfonyl fluoride, and 0.36 mM pepstatin) was added to the mixture and brought to a density of 1.4 g/ml by adding solid CsCl. A density gradient was established by ultracentrifugation at 40,000 rpm, 1°C for 48 h. The gradient was partitioned into 10 fractions, and the contents of HA and protein in each fraction were determined by the carbozole reaction and by measuring the absorption at 280 nm, respectively. The lower half of the gradient, which contained above 90% of total HA, was pooled and brought to a density of 1.45 g/ml by adding solid CsCl. Since the HA-binding reaction mixture contained a very high concentration of proteins, the first centrifugation was not usually enough to obtain the SHAP-HA complex. The second centrifugation and subsequent assays for HA and proteins in partitioned fractions were performed as described above. The SHAP-HA complex was also prepared from human serum as described above.

Purification of SHAP Components by SDS-PAGE—Polyacrylamide gel (7.5%) electrophoresis in the presence of 0.1% (w/v) SDS (27) was done under reducing conditions. Before electrophoresis, samples were precipitated with 3 volumes of 95% (v/v) ethanol containing 1.3% (w/v) potassium acetate at 0°C (28). The precipitates (about 400 µg of HA each) were dissolved in 500 µl of 50 mM sodium acetate, pH 5.0, containing 20 turbidity reducing units of protease-free Streptomyces hyaluronidase. The mixtures were incubated at 30°C for 2 h, followed by lyophilization. The samples were then dissolved in sample buffer containing 10% (v/v) mercaptoethanol, followed by heating at 100°C for 3 min. After electrophoresis, gels were stained with 0.1% (w/v) Coomassie Blue R-250, destained, and rinsed with cold water. The visualized SHAP bands were cut from the gels with a razor blade and soaked for 30 min in a solution of 12.5 mM Tris-HCl, pH 6.8, 0.1% (w/v) SDS, 1 mM EDTA with occasional swirling. The gel slices were subjected to the next step (29).

Peptide Mapping of SHAP Components by Limited Proteolysis in SDS-gel and Subsequent Gel Electrophoresis—Gels slices containing SHAP bands were placed in the sample wells of a 15% (w/v) SDS gel which contained 1 mM EDTA in both stacking and separating gels (29). Spaces around the slices in the sample wells were filled with 12.5 mM Tris-HCl, pH 6.8, containing 20% (w/v) glycerol. 10 µl of the same buffer containing various amounts of V4 protease and 1% (w/v) SDS was overlayed into each well and a second electrophoresis was run as usual (29). When the bromphenol blue dye reached to the bottom of the stacking gel, current was turned off and the gels left for 30 min to complete the digestion. Electrophoresis was then continued until the dye reached the front. Peptides derived from the SHAP components were detected by staining with Coomassie Blue R-250.

Electrotransfer of the Digested Peptides to PVDF Membrane and the Peptide Sequencing—Peptides were transferred to PVDF membranes as described previously (30). After the second electrophoresis, the gel was rinsed in an electroblotting buffer of 10 mM CAPS, pH 11, 10% (v/v) methanol for 10 min with swirling. Electrotransfer of peptides to PVDF membrane was carried out in the above buffer at 40 V and 4°C overnight. The membrane was stained with 0.1% (w/v) Coomassie Blue R-250, 1% (w/v) acetic acid, 40% (v/v) methanol within 1 min, destained in 50% (v/v) methanol by changing the solution several times within 10 min, and finally rinsed with distilled water. The bands were excised from the membranes for sequencing. Sequencing of the peptides on PVDF membrane was done with a model 473A peptide Sequencer with BLOT program (Applied Biosystem, Inc., Foster City, CA). Homology searches used DNASIS software and a gene/protein sequence database characterization. The NIH-PFAM 0.01 protein homology search program (Hinrich Software Engineering Co., Tokyo).

Immunological Comparison between SHAP and ITI Subunits—Human ITI was partially purified from human serum as described by Salier et al. (29). D(-)-dextrorotidase AC-II digestion was used to remove the N-linked glycosylation (9). The presence of protease inhibitors (9) (0.1 unit of the enzyme for about 3 µg of ITI, 37°C for 2 h). When treated with Streptomyces hyaluronidase, the same conditions were used as those for SHAP. Digested samples were lyophilized and subjected to electrophoresis on 7.5% (w/v) gel. Proteins were transferred to PVDF membranes as described above. For immunostaining, each membrane was treated sequentially with 5% (w/v) skim milk, with rabbit anti-human ITI IgG (1:2000 dilution), and with protein A- peroxidase (1:3000 dilution). The color was developed using DAB-H2O2 solution.

Separation of the HA-binding Region from Human SHAP-HA Complex—Human SHAP-HA complex in 50 mM phosphate-buffered saline, pH 7.8, was digested with protease V4 at room temperature for 6-8 h with occasional swirling. The protease inhibitor phenylmethanesulfonyl fluoride (2 mM at final concentration) was added to inactivate the enzyme. The digest was brought to a density of 1.45 g/ml by adding CaCl2 as described above, and the sample was centrifuged at 70,000 rpm, 10°C, for 36 h. The gradient was divided into five fractions, and each fraction was assayed for HA and protein contents as described above. The lower two-fifths contained 80% of the total HA. The complex of HA and HA-binding region was precipitated by adding 2 volumes of 95% (v/v) ethanol, 1.3% (w/v) potassium acetate.

RESULTS

Isolation of SHAP from Bovine and Human Serum—We showed previously that direct incubation of serum with HA forms a SHAP-HA complex from the serum (9). In the present study, the method has been modified to give a maximal yield and to avoid nonspecific interactions between HA and proteins. Associative conditions were used for the purification steps instead of the dissociative conditions with high concentrations of detergents and guanidine HCl used previously (9). Formation of the SHAP-HA complex was dependent upon the incubation time and temperature, and the maximum was reached after the incubation at 37°C for 24 h (Fig. 1). The addition of protease inhibitors did not affect the yield except for EDTA, which inhibited formation of the complex (Fig. 1). Identical results were obtained when HA was incubated with human serum (data not shown). SDS-PAGE analysis of the SHAP-HA complex before and after treatment with Streptomyces hyaluronidase showed that bovine serum SHAP consists of two components, X and Y, having molecular masses of 85 and 80-kDa, respectively.

FIG. 1. Effects of incubation temperatures and times on the formation of the SHAP-HA complex, and inhibition of the formation by EDTA. The Hanks’s medium containing 0.4 mg/ml HA was incubated with the same volume of bovine serum and then incubated at 37°C (O) or 4°C (O) for the various times indicated. The SHAP-HA complex thus formed was purified under associative conditions and quantitated as described under “Experimental Procedures.” 10 mM EDTA was added to one sample prior to incubation at 37°C for 24 h (X). Quantification was done by densitometric determination of the relative staining intensities of SHAP bands after staining with Coomassie Blue R-250.
tively, as described previously (9). The molecular mass values of SHAP components from human serum were slightly lower than the bovine ones.

**Peptide Mapping and Sequencing of Bovine and Human Serum SHAPs**—In a preliminary study, the X and Y components of the bovine SHAP-HA complex were directly transferred from the SDS-gel to PVDF membrane for amino acid sequencing. However, the NH$_2$ termini were blocked. Therefore, protease V$_d$ digestion was used to create new NH$_2$ termini. The digestion was done in a second gel as described under “Experimental Procedures.” Bovine SHAP gave about 10 peptide bands from the X component and 6 peptide bands from the Y component in the mass range of 40 to 16 kDa, which could be distinctly detected after Coomassie Blue staining. The mapping patterns of the X and Y components were distinctly different (data not shown).

The separated peptides in the gel were transferred to a PVDF membrane and stained with Coomassie Blue R-250. Three major peptides (40, 20, and 18 kDa) derived from the X component, designated Bx1, Bx2, and Bx3, respectively, and one major peptide (19 kDa) from the Y component, designated By1, were chosen for sequencing. Bx1 and Bx3 had the same NH$_2$-terminal sequences, whereas that of Bx2 was different (Fig. 2). Thus, Bx3 appears to be a further digestion product of Bx1. Computer searches for sequence similarity in these peptides with other proteins revealed that peptides derived from the bovine X component (Bx1/Bx3 and Bx2) and the one from the Y component (By1) are highly homologous with the reported sequences for the two heavy chains of human inter-α-trypsin inhibitor (ITI) (HC2, 85 kDa and HC1, 78 kDa for X and Y, respectively).

SHAP from human serum was also subjected to the same processes. In this case, two major peptide bands on the PVDF membrane, designated Hx and Hy, which were derived from the X and Y components, respectively, were analyzed. Sequence search showed that the Hx1 and Hy1 sequences were identified as sequences in HC2 and HC1 of human ITI, respectively.

**Immunological Comparisons between SHAP and ITI**—We then isolated ITI from human serum by established methods (31) and further investigated the relationship between SHAP and ITI. Human SHAP-HA complex and intact ITI, either before or after the treatment with Streptomyces hyaluronidase or chondroitinase AC-II, were subjected to SDS-PAGE and, subsequently, to Western blotting with anti-human ITI IgG (Fig. 3). Although the antibodies were not equally reactive to the subunit chains, the result clearly showed that the X and Y components of human SHAP are recognized by the anti-human ITI IgG and are similar in mobilities and staining intensities with the two heavy chains, HC2 and HC1, derived from intact ITI by chondroitinase AC-II digestion. ITI, whether or not treated with *Streptomyces* hyaluronidase, gave similar results.

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**Table: Peptides (kDa) and Sequences**

| Peptides (kDa) | Homologous parts of ITI | Sequences comparison | % Homology |
|---------------|------------------------|---------------------|------------|
| Bovine        |                        |                     |            |
| Bx1 (40)      |                        | LEVFMGYVHFPAPENMPG  | 80         |
| Bx2 (20)      |                        | LEVFMGYVHFPAPENMPG  | 89         |
| Bx3 (18)      |                        | LEVFMGNYVHFPAPENMP  | 82         |
| Hx1 (19)      |                        | AKFINTINDGSLALTYY   | 44         |
| HC1 (1175-203)|                        | ASPFLPCILAQATIKSSFGKGVFLPFS |          |
| Human         |                        |                     |            |
| Hx1 (35)      |                        | VQYTLSSXYGQDSITSMAT | 100        |
| Hx1 (67-101)  |                        | VQYTLSSXYGQDSITSMAT |            |
| Hy1 (35)      |                        | IYKVRKGVQWHEFIDEQFGQ (kl) | 97            |
| Hy1 (103-137) |                        | IYKVRKGVQWHEFIDEQFGQ (kl) |            |

* The positions of amino acid residues

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**Fig. 2.** NH$_2$-terminal amino acid sequences of V$_d$ digestion products of bovine and human SHAPs and their high similarities with the heavy chains of ITI. Four peptides (Bx1, Bx2, Bx3, and By1) derived from bovine SHAP (see Fig. 2) and two peptides, Hx1 and Hy1 derived from the X and Y components of human SHAP, respectively, by V$_d$-protease digestion (approximately 100 pmol of each) were subjected to NH$_2$-terminal amino acid sequence analyses as described under “Experimental Procedures.” Sequence residues with a high reliability are shown. Underdetermined amino acid residues are represented as X. The corresponding amino acid sequences and positions of the homologous parts of the human ITI components are shown below the determined sequences for comparison. The apparent molecular sizes of bovine and human SHAP peptides are shown in parentheses. Percents of identical amino acid residues in each comparison are also shown on the right. The peptide from the bovine Y component had about 40% homology with the heavy chain, HC1 of human ITI, which was low, compared with the homologies of peptides derived from the bovine X component with the other heavy chain, HC2 of human ITI (over 80%). This may be due to the recent suggestion (23) that bovine ITI may consist of three subunits corresponding to human bikunin, HC2, and HC3 which remains to be sequenced.

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**Fig. 3.** Immunological comparison of SHAP components (X and Y) with heavy chains of ITI (HC1 and HC2) using rabbit anti-human ITI antibodies. Human SHAP-HA complex was incubated with *Streptomyces* hyaluronidase (lane 1). Human ITI was incubated with chondroitinase AC II (lane 2), with the hyaluronidase (lane 3), and without enzymes (lane 4) as described under “Experimental Procedures.” Alkphs with about 10-μg proteins were subjected to the SDS-PAGE on a 7.5% gel under reducing conditions, followed by electrotransfer to a PVDF membrane. Immunostaining with rabbit anti-human ITI IgG was done as described under “Experimental Procedures.” Arrows indicate expected migration positions of intact ITI (a), two HC (b, HC plus bikunin (c), HC2 (d), HC1 (e) and bikunin (f).
identical staining patterns, with bands of slow mobilities, indicating that ITI at approximately 240 kDa, unlike the SHAP-HA complex, is not sensitive to this enzyme. It was noted, however, that ITI in the solution tended to dissociate into subunit chains during the incubation even in the presence of protease inhibitors. The diffused bands at approximately 120 kDa in Fig. 3, lanes 3 and 4 may account for a complex with one light chain and the one heavy chain. This possibility could be explained by the disappearance of these bands after chondroitinase AC-II digestion of ITI and the concomitant appearance of the two major bands at 85 and 80 kDa and the one faint band at about 30 kDa, which correspond to the two heavy chains and the one light chain of ITI, respectively (Fig. 3, lane 2). The chondroitinase digest contained two narrow bands at approximately 180 kDa and near the origin (Fig. 3, lane 2). These could correspond to aggregates of the subunit chains in which the linkage formation by chondroitinase-resistant bonds is involved or to heterogeneity in ITI (19, 21).

When the above samples of human SHAP and ITI were stained with anti-bovine SHAP antibodies that were developed in our previous study (9), staining was too weak to be reproduced photographically, but appeared to be similar to that observed with anti-human ITI IgG described above. The difference might be due to a species difference in the antigenicity.

Bovine SHAP was not stained with anti-human ITI IgG (data not shown). This might also be due to some differences in animal species and/or immunological properties.

Characterization of the HA-binding Region of Human SHAP—Human SHAP-HA complex was treated with protease V. The peptide-HA complex thus produced was purified by the same methods as for intact SHAP-HA complex and analyzed on SDS-PAGE. Coomassie Blue-stained peptide bands (major Hp1 and Hp2) appeared around 35–40 kDa on the gel only after treatment with Streptomyces hyaluronidase. Anti-human ITI IgG reacted with these peptide bands (Fig. 4). The results indicate that they were bound to HA and were derived from the SHAP-HA complex. Hp1 and Hp2 (38 and 37 kDa, respectively) were transferred to PVDF membrane, and their NH2-terminal amino acid sequences were determined (Fig. 5). The peptides had the identical NH2-terminal sequences. The sequences corresponded to the sequence of the heavy chain 1 (HC1) of ITI from position 353. Considering their apparent molecular masses (the 37-kDa chain corresponds to approximately 340 amino acid residues), and the HA-binding domain in the HC1 chain must be localized between position 353 and near the COOH-terminal end, position 638. Consistent with this suggestion, a computer-assisted analysis (37) identified an amphipathic α-helical structure (residues 508–563) that may bind to HA.

DISCUSSION

In the present study we have suggested that the SHAPs correspond to the two heavy chains (HC1 and HC2) of ITI.

Full-length sets of cDNAs for three subunits, HC1, HC2, and the one light chain (bikunin) of human ITI, have been characterized recently (15–18). The light chain is responsible for all the serine protease inhibitory activity of ITI (11, 24). Some potential roles of the two heavy chains have been proposed based on their sequence similarities with other proteins, although there is no direct evidence. Calcium-binding sites and the proposed VWA domains (the triplicated type A domain of von Willebrand factor) were found in the sequences of HC1 and HC2 (15, 32). VWA domains are widespread in adhesive proteins and receptors such as cartilage matrix proteins and the integrin α subunit. The similarity between HC2 and VWA is ~23%, which is only slightly below the value that suggests a similar folding topology (32). Hence, this domain in the heavy chains of ITI may function in recognizing proteins which then make the complex with the inhibitor (32).

It is likely that the SHAPs may be the two heavy chains of ITI, but without the light chain. They could be involved in the formation of HA-rich extracellular matrix through their calcium-dependent HA-binding activity. In agreement with this possibility, preparations of SHAP did not show trypsin inhibitory activity (data not shown). Furthermore, a chelating reagent, EDTA, inhibited the HA binding activity (Fig. 1).

In 1965, a report described that ITI tightly bound to HA in human pathological synovial fluid (33). A recent study also suggested that ITI, when bound to HA, influences the degradation of HA (34). More recently, Chen et al. (35) have reported that the cumulus extracellular matrix stabilizing
factor in fetal bovine serum is a member of the ITI family. They proposed that extracellular matrix stabilizing factor is essential for the process of cumulus cell expansion through its stabilization of the predominant structural component, HA, in the expanded cumulus matrix. These reports are totally consistent with the possible functions of SHAP.

In the present study, we have localized the HA-binding domain to the COOH-terminal half of the HC1 chain of human ITI by NH2-terminal sequence analysis and apparent sizes of the V4 protease-resistant fragments (HP1 and HP2) that remained bound to HA (positions from 353 to the COOH-terminal end). Although resistant fragments derived from the HC2 chain were missed in the present experiment, the high similarity between amino acid sequences in the HC1 and HC2 chains (17, 18) suggests a similar localization of the HA binding activity in the HC2 chain.

There are several different proteins involved in HA binding (see the review by Toole (1)). Of them, CD44 (37–39), PG-M/vericain (hyaluronectin) (40–42), aggrecan (43, 44), and the link protein (45) share a homologous domain for HA binding. Recently, a novel type of HA receptor, unrelated in amino acid sequence to these HA-binding proteins, was shown to mediate tumor cell motility (46). The heavy chains of ITI showed no similarity in amino acid sequences to either of these two families of HA-binding proteins. Hence, SHAP may represent a third type of HA-binding protein.

The SHAP-HA complex could not be dissociated under any dissociative condition tested. Thus, the linkage appears to be covalent (9). Furthermore, the complex contains only the two heavy chains of ITI, not the light chain (Fig. 2). Thus, ITI may first interact with HA via its HA-binding domains in a manner similar to other HA-binding proteins. Then, through some unknown mechanism, the light chain (bikunin) is released, and covalent bonds are produced at the same time or later. Alternatively, HA might substitute for the light chain in the interaction between HA and ITI. In relation to these possibilities, it should be noted that the heavy chains of ITI as single chains or dimers are difficult to detect in normal blood while bikunin is present significantly (13, 21, 23). It is known that a chondroitin sulfate chain is involved in linking the three chains of ITI (19–23). Recent work by Enghild et al. (47) suggested that ITI has a covalent cross-linkage between the light and heavy chains through chondroitin 4-sulfate that originates from an O-glycosidic linkage to Ser-10 of the light chain. HC2 is esterified to C-6 of an internal galactosamine of the chondroitin sulfate via the α-carbon of the COOH-terminal Asp. They also showed that pre-α-trypsin inhibitor (PrtI) possesses identical chondroitin sulfate-mediated cross-linkages between the chondroitin sulfate chains (19, 20). Thus, it is possible that a covalent linkage is formed by the simple substitution of HA for the chondroitin sulfate of the light chain during the interaction between ITI and HA. We have tried to test the direct binding between ITI and HA by incubating intact ITI or dissociated ITI with HA under the same conditions as those used when preparing SHAP-HA complex. However, we have not been successful in detecting significant binding so far (data not shown). Hence, there may be some factors in serum involved, for example enzyme(s) mediating substitution reactions of HA for the chondroitin sulfate. In order to understand the functions of SHAP, it may be important at this stage to elucidate the linkage structure between HA and SHAP. Wang and Underhill (48) showed that HA could be nonenzymatically linked to protein through an alkali-sensitive bond. However, considering the above discussion, this may not be the case for the binding of HA to SHAP.

Over all, our present study suggests important involvements of ITI in HA functions. It may act as a carrier of HA in serum or a binding proteins between HA and other matrix protein, including those on cell surfaces in tissues to regulate the localization, synthesis, and degradation of HA which are essential to cells undergoing biological processes.

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