Evidence for the Covalent Binding of SHAP, Heavy Chains of Inter-α-Trypsin Inhibitor, to Hyaluronan*

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We previously showed that serum-derived 85-kDa proteins (SHAPs), serum-derived hyaluronan associated proteins are firmly bound to hyaluronan (HA) synthesized by cultured fibroblasts. SHAPs were then identified to be the heavy chains of inter-α-trypsin inhibitor (ITI) (Huang, L., Yonedā, M., and Kimata, K. (1993) J. Biol. Chem. 268, 26725–26730). In this study, the SHAP-HA complex was isolated from pathological synovial fluid from human arthritis patients. The SHAP-HA complex was digested with thermolysin, followed by CsCl gradient centrifugation. The HA-containing fragments thus obtained were further digested with chondroitinase AC II and subjected to TSK gel high performance liquid chromatography (HPLC). Peptide-HA disaccharide-containing fractions (the SHAP-HA binding regions) were further purified by reverse phase HPLC. Major peaks were analyzed by protein sequencing and mass spectrometry (electrospray ionization mass spectrometry and collision induced dissociation-MS/MS). By comparison with the reported C-terminal sequences of the human ITI family, the peptides were found to correspond to tetrapeptides derived from the C termini of heavy chains 1 of and 2 of inter-α-trypsin inhibitor (HC1 and HC2), and heavy chain 3 of pre-α-trypsin inhibitor (HC3), respectively, and a heptapeptide from HC1. Mass spectrometric analyses suggested that the C-terminal Asp of each heavy chain was esterified to the C6-hydroxyl group of an internal N-acetylgalosamine of HA chain. This report is the first demonstration to give evidence to show the covalent binding of proteins to HA.

Hyaluronan (HA),† has been found as a ubiquitous compo-

⩠This work was supported partly by Special Coordination Funds of the Science and Technology Agency of the Japanese Government, by Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture, J (No. 06240247 to Y. O., No. 06608594 to M. Y.), by Grant-in-Aid for Encouragement of Young Scientists from Japan Society for the Promotion of Science (JSPS) (No. 93123 to M.Z.), and by a special research fund from Seikagaku Corp. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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†The abbreviations used are: HA, hyaluronan; SHAP, serum-derived hyaluronan associated protein; PAGE, polyacrylamide gel electrophoresis; IITI, inter-α-trypsin inhibitor; UTI, urinary trypsin inhibitor; HC1, heavy chain 1 of inter-α-trypsin inhibitor; HC2, heavy chain 2 of inter-α-trypsin inhibitor; HC3, heavy chain 3 of pre-α-trypsin inhibitor; HPLC, high performance liquid chromatography; ESI, electrospray ionization; MS, mass spectrometry; CID-MS/MS, collision induced dissociation MS/MS spectra.

26657
EXPERIMENTAL PROCEDURES

Materials—Streptomyces hyaluronidase was obtained from Seikagaku Corp., Tokyo, Japan; protease-free chondroitinase AC II (from Arthrobacter aurescens) was a gift from Dr. K. Yoshida of Seikagaku Corp.; thermolysin (three times crystallized), e-amino caproic acid, N-ethylmaleimide, and phenylmethylsulfonyl fluoride were purchased from Nagalai Tesque, Kyoto, Japan; rabbit immunoglobulins to human ITI, and goat immunoglobulins to rabbit immunoglobulins were from Dako Japan, Kyoto, Japan; DNase I was from Sigma; micro-BCA protein assay reagent was from Pierce; all reagents for protein sequence analysis were from Applied Biosystems, Chiba, Japan.

Preparation of the SHAP-HA Complex from Pathological Synovial Fluid—The method was essentially the same as described previously (21, 22) with a slight modification. Briefly, to the pathological synovial fluid obtained from osteoarthritic patients (250 ml), the same volume of the extraction buffer was added. The extraction buffer contained 0.2 M Tris-HCl, pH 8.0, 8 M guanidine HCl, 10 mM EDTA, 10 mM amino caproic acid, 10 mM N-ethylmaleimide, and 2 mM phenylmethylsulfonyl fluoride. The extraction was performed at 4°C overnight with stirring, and the mixture was diluted and brought to a density of 1.35 g/ml with solid CsCl. A density gradient was established by centrifugation at 130,000 × g, 10°C, for 48 h (Hitachi, 70P-72). The gradient was partitioned into 16 fractions, and the contents of HA and proteins in each fraction were determined by carboxy disaccharide and micro-BCA method, respectively (21, 36). The lower one-fourth of the gradient which contained above 90% of the total HA was pooled. Furthermore, a second centrifugation with an initial density of 1.38 g/ml and subsequent assays for HA and proteins in partitioned fractions were performed as described above. The lower half of the gradient was pooled and subjected to a third centrifugation with an initial density of 1.45 g/ml, and subsequent assays for HA and proteins in partitioned fractions were as described above. The fractions from the bottom, nos. 4–10, which contained most of the total HA, were pooled and subjected to ethanol precipitation to collect the SHAP-HA complex (21).

Preparation of the SHAP-HA Binding Region—SHAP-HA complex (160 mg) was dissolved in 10 ml Tris-HCl, pH 7.5, containing 10 mM KCl and 3 mM MgCl₂ and treated with 300 units (100 μg) of DNase I at 37°C overnight with agitation. After CaCl₂ was added (the final concentration 2 mM), the mixture was treated with 100 μg of thermolysin at 37°C with agitation. After 6 h, another 100 μg of thermolysin were added, and further incubation for 3 h was performed. The same volume of 8 M guanidine HCl (extraction buffer) was added to the digested mixture. The density of the mixture was brought to 1.35 g/ml by adding solid CsCl, centrifugation at 130,000 × g for 48 h was performed. The HA component in the partitioned fractions was determined as described above. The HA-containing fractions were precipitated with ethanol and then subjected to digestion with chondroitinase AC II (67 milliunits/mg of HA) in 50 mM sodium acetate, pH 6.0, at 37°C overnight. The digested mixture was further subjected to TSK gel HPLC. Two TSK G3000PWXL (7.8 mm × 30 cm, Tosoh) columns were linearly connected for the better separation. The columns were eluted with 0.2 M NH₄HCO₃ at a flow rate of 0.5 ml/min. The absorbance was measured at 214 nm. The peptide-containing peak was eluted just before the main HA disaccharide peak. The first peak was collected and concentrated by lyophilization. The second HPLC using the same columns was carried out to completely remove a trace of the HA disaccharide product. The fractions containing the SHAP-HA binding region were collected and lyophilized.

Further Purification of the SHAP-HA Binding Region by Reverse Phase HPLC—The SHAP-HA binding region was further purified by reverse phase HPLC. The column (TSK ODS-120T, 4.6 mm × 25 cm, Tosoh) was equilibrated with 0.06% (w/v) trifluoroacetic acid in water. The peptides were eluted by an acetonitrile gradient 0 to 13% acetonitrile (37.7, w/v), typically at the concentration 2 mM, and the absorbance was measured at 214 nm.

Protein Sequence Analyses—Automated Edman degradation was carried out in an Applied Biosystems model 476A sequencer with on-line phenylthiohydantoin analysis using an Applied Biosystems model 476A sequencer. A potential difference of 3 kV was applied against the ground potential of the needle. Hot nitrogen gas was used as the counter gas to desolve sample droplets in the ion source. Argon gas was used at 0.13 Pa as the collision gas at 20 eV for CID-MS/MS spectra.

Other Analytical Methods—SDS-PAGE and immunoblotting were performed as indicated previously (21). Analysis of dehydro-disaccharides of hyaluronan, chondroitin, chondroitin 4-sulfate, and chondroitin 6-sulfate were performed as indicated elsewhere (37).

RESULTS

Isolation of SHAP-HA Complex from Human Synovial Fluid—Synovial fluid from knee joints of osteoarthritic patients was used as a source of the complex. Since the synovial fluid was highly viscous and contained a variety of proteins at high concentrations, dissociative conditions (in 4 M guanidine HCl) were used for purification. A series of three isopycnic centrifugations with initial densities of 1.35, 1.38, and 1.45 g/ml, respectively, were performed. The density and the contents of HA and proteins in each fraction were determined by centrifugation for 48 h (Hitachi, 70P-72). The gradient was partitioned into 16 fractions. Contents of HA and proteins in each fraction were determined by carboxy disaccharide and micro-BCA method, respectively.

700 triple stage quadrupole mass spectrometer equipped with an electrospray ion source (Analytica of Branford). A potential difference of 3 kV was applied against the ground potential of the needle. Hot nitrogen gas was used as the counter gas to desolve sample droplets in the ion source.

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SDS-PAGE and immunoblotting of the SHAP-HA complex thus prepared from human synovial fluid were performed to confirm that the complex was identical with the one prepared from the incubation mixture of human serum with HA as described previously (22) (Fig. 2). Proteins in the preparation from the synovial fluid were retained in the starting gels of SDS-PAGE, indicating that the complex in the synovial fluid preparation remained undissociated under both dissociative conditions of 4 M guanidine HCl during the centrifugation and 1% (w/v) SDS during the PAGE (Fig. 2A). This was also the case with the SHAP-HA complex prepared from the incubation mixture of human serum with HA as described previously (22).

Treatment of both complex preparations with HA-degrading enzymes, such as protease-free Streptomyces hyaluronidase or chondroitinase AC II released the proteins that corresponded to HC1 and HC2 of ITI (two bands with the higher mobility and the lower mobility, respectively), judging from both the immunoreactivities and the mobilities of proteins in the gels (Fig. 2,
Figure 2. Characterization of the SHAP-HA complex isolated from human pathological synovial fluid and comparison with the SHAP-HA complex prepared from the incubation mixture of human serum and hyaluronan. Aliquots (50 μg HA) of SHAP-HA complex isolated from the pathological synovial fluid were precipitated with ethanol, and incubated without HA degrading enzymes (lane 1), with Streptomyces hyaluronidase at 60 °C for 2 h (lane 2), with chondroitinase AC II at 37 °C for 2 h (lane 3). The samples were electrophoresed on SDS gel (9% gel, under nonreducing conditions) and the gels were stained with Coomassie Blue (A). About 1/20 of the same set of the samples were electrotransferred to nitrocellulose membrane after SDS-PAGE, and immunoblotted with antibodies to human ITI. The immune complexes were visualized by enhanced chemiluminescence assay (B). Aliquots of SHAP-HA complex prepared from the incubation mixture of human serum with HA were treated, and immunoblotted the same way as described above (C). Since the antibodies used in this experiment were more reactive to HC2 of ITI than the other heavy chains, the observed differences between the Coomassie Blue staining and immunostaining patterns could be explained by such differences in the immunoreactivity.

Figure 3. A lkali treatment of SHAP-HA complexes from human pathological synovial fluid and from the incubation mixture of human serum with hyaluronan. SHAP-HA complex isolated from pathological human synovial fluid was treated with 0.02 M NaOH for 0, 30, and 60 min (A). The complex from the incubation mixture of human serum and hyaluronan was subjected to the same treatment for 60 min (B). The samples after neutralization with acetic acid were treated with (−) without (−) Streptomyces hyaluronidase and subjected to SDS-PAGE and subsequent immunoblotting the same way as described in Fig. 2.

Purified preparation from the synovial fluid was neither contaminated with ITI nor with P1. The slight difference in mobility of the stained bands between the two samples could be explained by the difference in size of the products between the two enzymes. It is known that major digestion products of HA with Streptomyces hyaluronidase are tetramers and hexamers while the chondroitinase AC II digestion of HA yields dimers. In addition, there were also some differences between the Coomassie Blue staining and immunostaining patterns (Fig. 2, A and B). These differences could be explained by the apparent differences in the immunoreactivity of the anti-human ITI antibodies between HC1 and HC2.

Preparation of the SHAP-HA Binding Region from SHAP-HA Complex—Since DNA has a density similar to HA in the CsCl centrifugation, the possibility that the SHAP-HA complex preparation was contaminated with DNA was not excluded. If this were the case, the DNA might disturb the subsequent processes. Therefore, the preparation was first subjected to DNase I digestion. The SHAP-HA complex was then digested with thermolysin. It was also possible that the bound HA protects SHAP from proteolysis (21). Therefore, a high enzyme/substrate ratio (1:40 in weight) and a long incubation time (9 h) were used to ensure complete digestion. The HA-containing products were purified by CsCl isopycnic centrifugations. The products were further digested with chondroitinase AC II overnight. In our preliminary test Streptomyces hyaluronidase digestion yielded not only different sizes of HA oligosaccharides (tetramers and hexamers) but also the SHAP-HA binding regions with these HA oligosaccharides, which caused difficulties in subsequent separation and purification of the binding regions from free HA oligosaccharides and those from each other. The digestion with chondroitinase II, instead, yielded the SHAP-HA binding regions containing only unsaturated disaccharide of HA. Subsequent HPLC on linearly connected two TSK gel columns gave a successful resolution of the binding regions from the HA disaccharide (Fig. 4A, peak 1 for the binding regions and peak II...
prepared from the human synovial fluid. HPLC onto C18 reverse phase HPLC. Elution with an acetoni- performed by applying the peak 1 from the second TSK gel rechromatographed onto the same column to ensure the com- for the HA disaccharide plus salt). The binding regions were rechromatographed onto the same column (B). The peptide-containing fraction (peak 1) was collected and lyophilized for the next purification step.

for the HA disaccharide plus salt). The binding regions were rechromatographed onto the same column to ensure the complete separation from the HA disaccharide (Fig. 4B, peak 1 for the binding regions, peak 2 for the residual HA disaccharide, and peak 3 for buffer salt).

The chromatography of peak II in Fig. 4A on SAX 10 column (37) indicated that none of any disaccharide products derived from chondroitin, chondroitin 4-sulfate and chondroitin 6-sulfate other than the HA disaccharide was detected in this fraction, judging from the elution positions of the standard disaccharides (data not shown). The result further confirmed no contamination with ITI and Pain in the SHAP-HA complex prepared from the human synovial fluid.

Further purification of the SHAP-HA binding regions was performed by applying the peak 1 from the second TSK gel HPLC onto C18 reverse phase HPLC. Elution with an acetonitrile gradient as described in the experimental procedures yielded 12 major peaks detected by the absorption at 214 nm and numbered by the numerical order (Fig. 5).

Peptide Sequences of the SHAP-HA Binding Regions—Amino acid sequences of peptides, if any, in each peak of the reverse phase HPLC were determined by an automated amino acid sequencer. Peaks 1, 2, 8, 9, and 12 did not contain any detectable amino acid residues. The amino acid sequences determined for peaks 3, 4, 5, 6, 7, 10, and 11 are shown in Table I. The fourth cycle for the shorter peptides and the seventh cycle for the longer peptides gave no peak corresponding to any standard amino acid residue under the employed conditions. Although there were other possibilities, the result suggested a modification of the C-terminal amino acid residues of those peptides. The residues might be covalently linked to hyaluronan in a similar way to the binding of the heavy chains to the chondroitin sulfate chain of bikunin in ITI family molecules (30, 34). In comparison with the C-terminal sequences of the heavy chains of human ITI and Pain, the C-terminal amino acid residue of each peptide might be Asp. In such a case, the

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carboxylate ion at the ester linkage. However, the ion at m/z 489 in Fig. 7 again indicated a structure in which the peptide was linked to the C6-hydroxyl group of the N-acetylhexosamine. Daughter ions at m/z 659 for peak 6 (Fig. 6) and at m/z 632 (with m/z 614) for peak 7 (Fig. 7) indicated an elimination of the terminal dehydroxyhexulonic acid, which suggests that the sugar unit directly linked to the peptides is N-acetylhexosamine in both samples. Thus, the negative charge was found localized in an acidic side chains in the peptides, and the sugar side negative ions were not observed.

CID MS/MS analysis of (M$_2$H)$_2$ at m/z 764 in peak 6 suggested that the same linkage occurs between HC3 and the HAdisaccharide (data not shown). However, its limited sample amount prevented us from carrying out detailed structural studies.

Taken together, the analyses demonstrated linkages between

| Cycle | Starting sample | Peaks (pmol) |
|-------|----------------|--------------|
| 1     | V(326)         | VAGS (9, 10, 20, 12) | V(26) | V(34) | V(51) | V(40) | V(4.6) | V(13) |
| 2     | EDT (82, 44, 7) | ED (2.2, nq) | ED (11, 1.9) | DE (25, 4.3) | DE (19, 0.8) | T(2.1) | T(5.4) |
| 3     | NTG (70, 40, 42) | N(nq) | NTG (5.2, 1.7, nq) | TNG (9.3, nq, nq) | TN (28, 4, 1) | G(3.2) | G(9.0) |
| 4     | V(18)          | _          | _          | _          | _          | _          | _          | _          |
| 5     | D(9.2)         | _          | _          | _          | D(1.4) | D(4.3) |
| 6     | T(5.4)         | _          | _          | _          | T(1.7) | T(3.9) |
| 7     | _              | _          | _          | _          | _          | _          | _          |

* Sample before reverse phase HPLC.

Peptides A, B, C and D were determined by comparison of the sequence data for each peak (see Table I) with cDNA sequences of heavy chains of ITI and Pial from gene bank. The amino acids in brackets were predicted from cDNA sequences, and the presence was confirmed by mass spectrometry. Percentage of each peptide was calculated from the yields of amino acids in the sequence data.

### Table II

Peptide compositions of peaks obtained by reverse phase HPLC of SHAP-HA binding regions and molecular masses of the negative ion of each peptide and its expected HAdisaccharide-derivative

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| Peptides | A | B | C | D |
|----------|---|---|---|---|
| Peptides | HC2 | V-E-N-(D) | HC1 | V-D-T-(D) |
| HC3 | V-D-G-(D) | HC1 | V-T-G-V-D-T-(D) |
| Content (%) | 57 | 19 | 11 | 13 |

* The value was obtained by subtraction of 18 from the sum of molecular masses of each peptide and an unsaturated hyaluronan (ΔHA) disaccharide since H$_2$O would be removed for the formation of the covalent linkage.

**Fig. 6. Negative ion ESI CID-MS/MS of the Peak 6 sample.** Precursor ion: (M − H)$^-$ at m/z 835. The inset shows proposed fragmentations. Weak signals from m/z 650–900 are mostly due to instrument noise.

carboxylylate ion at the ester linkage. However, the ion at m/z 489 in Fig. 7 again indicated a structure in which the peptide was linked to the C6-hydroxyl group of the N-acetylhexosamine. Daughter ions at m/z 659 for peak 6 (Fig. 6) and at m/z 632 (with m/z 614) for peak 7 (Fig. 7) indicated an elimination of the terminal dehydroxyhexulonic acid, which suggests that the sugar unit directly linked to the peptides is N-acetylhexosamine in both samples. Thus, the negative charge was found localized in an acidic side chains in the peptides, and the sugar side negative ions were not observed.

CID MS/MS analysis of (M − H)$^-$ at m/z 764 in peak 6 suggested that the same linkage occurs between HC3 and the HA disaccharide (data not shown). However, its limited sample amount prevented us from carrying out detailed structural studies.

Taken together, the analyses demonstrated linkages be-
between the C-6 of the reducing terminal N-acetylhexosamine residue of HA disaccharide and a carboxyl group of the C-terminal aspartic acid of the peptides derived from the heavy chains, HC1, HC2, and HC3 of ITI and Puv.

**DISCUSSION**

Enghild et al. (30, 34) showed that three different heavy chains (HC1, HC2, and HC3) were covalently linked to bikunin by chondroitin sulfate chain in ITI and Puv. We have shown in this study that a similar linkage structure exists between SHAPs (HC1, HC2, and HC3) and HA. Mass spectrometric analyses revealed esterification of the carboxyl group of the C-terminal Asp with C-6 hydroxyl group of reducing terminal N-acetylhexosamine of the unsaturated HA disaccharide. Thus, we conclude that SHAPs (heavy chains of ITI and Puv) are covalently linked to HA by the esterification with the C6-hydroxyl groups of N-acetylglucosamine of HA via the C-terminal Asp.

The present study using ESI CID-MS/MS technique did not provide information to show which of the two carboxylate groups in the C-terminal Asp participated in the ester linkage formation. This is because the low energy collision-induced dissociation in the quadrupole instrument under our conditions failed to cleave C-C bonds in the aspartic acid. We assume tentatively that the α-carboxylate is the one which covalently bonds to the C6-hydroxyl group of the N-acetylhexosamine by an ester linkage, by analogy to the previous reported structure for the interchain linkage between the heavy chains and the light chain (bikunin core protein) via a chondroitin sulfate chain originating from the light chain in ITI or Puv (30, 34). This suggests that SHAP-HA complex may be formed simply by transferring the heavy chains from the chondroitin sulfate to HA (substitution reaction of HA for the chondroitin sulfate). However, the mechanism for the reaction has not been determined yet.

We noted that two fractions with different retention time in reverse phase HPLC contained peptides with the identical amino acid sequence (for example, peaks 4 and 6 in Tables I and II). Since there was no significant difference observed between those two fractions by peptide sequencing or by ESI CID-MS/MS, this could be due to difference of the anomeric configuration of hydroxyl group at the reducing end (α and β) of the HA disaccharide.

In mouse ovulation, preovulatory synthesis of hyaluronan within the cumulus mass plays an important role for cumulus expansion (8). Recently, Chen et al. (38, 39) reported that the cumulus extracellular matrix stabilizing factor in fetal bovine serum is a member of the ITI family, and that stabilizing ability is achieved through its direct binding to HA, which is sensitive to ionic strength and has a dissociation constant of 1.9 × 10^{-8} M at pH 7.2. Therefore, the properties of the interaction of cumulus extracellular matrix stabilizing factor with HA appear to be different from those for the formation of SHAP-HA complex.

The tight binding of ITI to HA in human pathological synovial fluid was firstly reported in 1965 (40). The present results show that the formation of a covalent linkage is involved in this binding. TSG-6, a 35-kDa glycoprotein of the proteoglycan tandem repeat HA-binding family, also exists in pathological synovial fluid of patients with arthritis (41). Recently, Wisniewski et al. (20) have shown that TSG-6 forms a covalently bound complex (120 kDa) with serum ITI. This TSG-6/ITI complex was formed rapidly even in the apparent absence of other proteins at 37 °C, but not at 4 °C. TSG-6 appeared to form a direct covalent bond to the chondroitin 4-sulfate chain of ITI for the stability of the TSG-ITI complex (20). In our study, we have not detected any peptides derived from TSG-6 in the SHAP-HA binding regions prepared from human pathological synovial fluid. Therefore, TSG-6 may not be involved in the formation of SHAP-HA complex in synovial fluid.

It is interesting to note that the formation of the SHAP-HA complex from HA and ITI or Puv is accompanied by the release of bikunin. Bikunin contains two tandem repeats of Kunitz-type domains, and the trypsin inhibitor activity of ITI is localized in this part (23, 42). Bikunin is also identified in urine as UTI (urinary trypsin inhibitor), which was shown to be a urine proteoglycan with molecular mass ranging from 40 to 45 kDa (43–45). Some tumor cells have UTI receptors (46). UTI and fragments derived from UTI by limited proteolysis efficiently inhibit tumor cell invasion and metastasis (46–49). Therefore, the formation of SHAP-HA complex might be related to some defense mechanism from proteolysis.

Acknowledgments—We are grateful to Drs. H. Habuchi and N. Sugiura in this laboratory for assistance in glycosaminoglycan analysis and HPLC techniques, respectively, and to Dr. S. Nagasawa of
the Pharmaceutical Department, Hokkaido University, and Dr. H. Kobayashi of Department of Obstetrics and Gynecology, Hamamatsu University School of Medicine, for providing us with authentic human ITI and UTI, respectively. M. Zhao wishes to thank Dr. C. Sun, Shanghai Institute of Biochemistry, for her continuous support.

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