Molecular Heterogeneity of the SHAP-Hyaluronan Complex

ISOLATION AND CHARACTERIZATION OF THE COMPLEX IN SYNOVIAL FLUID FROM PATIENTS WITH RHEUMATOID ARTHRITIS*

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We previously found that a coherent complex of SHAPs (serum-derived hyaluronan-associated proteins), the heavy chains of inter-α-trypsin inhibitor family molecules, with hyaluronan (HA) is accumulated in synovial fluid of patients with rheumatoid arthritis, and the complex is circulated in patient plasma at high concentrations. How the SHAP-HA complex participates in this disease is unknown. To address this question, it is essential to clarify the structural features of this macromolecule. The SHAP-HA complex purified from synovial fluid of the patients by three sequential CsCl isopycnic centrifugations was heterogeneous in density, and the fractions with different densities had distinct SHAP-to-HA ratios. Agarose gel electrophoresis and column chromatography revealed that there was no apparent difference in the size distribution of HA to which SHAPs were bound between the fractions with different densities. The SHAP-HA complex in the higher density fraction had fewer SHAP molecules per HA chain. Therefore, the difference between the fractions with different densities was due to a heterogeneous population of the SHAP-HA complex, namely the different number of SHAP molecules bound to an HA chain. Based on the SHAP and HA contents of the purified preparations, we estimated that an HA chain with a molecular weight of 2 × 10⁶ has as many as five covalently bound SHAPs, which could give a proteinaceous multivalency to HA. Furthermore, we also found that the SHAP-HA complex tends to form aggregates, judging from the migration and elution profiles in agarose gel electrophoresis and gel filtration, respectively. The multivalent feature of the SHAP-HA complex was also confirmed by the negative staining electron micrographic images of the purified fractions. Taken together, those structural characteristics may underlie the aggregate-forming and extracellular matrix-stabilizing ability of the SHAP-HA complex.

Hyaluronan (HA), a high molecular weight linear glycosaminoglycan composed of repeating disaccharide units of glucuronyl-N-acetylglucosamine, distributes ubiquitously in most mammalian connective tissues and the body fluids (1–3). The association with various hyaluronan-binding proteins (HABPs) including proteoglycans makes HA tremendously divergent in physiological function. For example, link proteins and aggrecan, major HABPs in cartilage, are implicated in the characteristic functions of this tissue such as elasticity by forming large macromolecular aggregates with HA in the extracellular matrix. Such extracellular matrices containing HA as a major component, which, hereafter, we call HA-rich matrices, play important roles in regulating cellular behavior in a variety of physiological and pathological processes via cell surface HA receptors, such as CD44 and RHAMM (4–6). Among a variety of HABPs that have been reported to date, SHAPs are the first and so far the only proteins covalently bound to HA (9, 10). The SHAP-HA complex was originally discovered from the HA-rich matrix of cultured mouse dermal fibroblasts, and SHAP was found to be derived from the serum supplemented to the culture media (the serum-derived hyaluronan-associated protein) (9, 11). SHAPs correspond to the heavy chains of plasma inter-α-trypsin inhibitor (ITI) family molecules and are bound to HA via a unique ester bond (10, 11). The ITI family molecules are synthesized by hepatocytes and secreted into blood at high concentrations (0.15–0.5 mg/ml plasma) (12). The heavy chains (HC1, HC2, and HC3) of these molecules are derived from three different genes, and either one or two of them are covalently bound to the light chain, bikunin, to form the ITI family members such as ITI, pre-α-trypsin inhibitor (ITI), and inter-α-trypsin-like inhibitor (13). Bikunin carries at the serine residue at position 10 an O-glycosidically linked chondroitin sulfate chain, to which the heavy chains are linked via ester bonds of

* The abbreviations used are: HA, hyaluronan; SHAPs, serum-derived hyaluronan-associated proteins; HC, heavy chain; ITI, inter-α-trypsin inhibitor; Pol, pre-α-trypsin inhibitor; HABP, hyaluronan-binding protein; CB, Coomassie Brilliant Blue; ELISA, enzyme-linked immunosorbent assay; PMFP, phenylmethylsulfonyl fluoride; HRP, horseradish peroxidase; BSA, bovine serum albumin; TM, The 3,3′,5,5′-tetramethylbenzidine.

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exactly the same type as that in a SHAP-HA complex (10, 14). During the formation of the SHAP-HA complex, HA is substituted for the chondroitin sulfate chain of bikunin, accompanied by the release of bikunin. Plasma also includes an enzymatic activity catalyzing the transfer of the heavy chains from the chondroitin sulfate to HA (11). Therefore, one can assume that the SHAP-HA complex is formed wherever and whenever plasma encounters HA.

The SHAP-HA complex has been found in the HA-rich matrix of expanded cumulus oophorus (15) and in the sera and synovial fluids of patients suffering from rheumatoid arthritis (10, 16). Previous studies (15, 17) suggested that the ITI family molecules, ITI and PoI, stabilized the HA-rich matrix of cultured cells and cumulus oophorus. Recently, the in vivo implications of the SHAP-HA complex in the extracellular matrix have become evident from our study on bikunin-knockout mice that are unable to form the complex (18). In the mice, the cumulus oophorus, an investing structure unique to the oocyte of higher mammals, had a defect in forming the extracellular HA-rich matrix, and the ovulated oocyte was unviviparized, leading to severe female infertility (18). However, it is yet unclear how the SHAP-HA complex contributes to the stabilization of an HA-rich matrix. Details on its molecular structure, particularly the number of SHAP molecules bound to an HA chain, would help us to understand the molecular interactions in the HA-rich matrix, not only between the SHAP-HA complexes but also between the SHAP-HA complex and other matrix components such as TSG-6 (19). Here for the first time, we have characterized a structural feature of the SHAP-HA complex isolated from pathological synovial fluid, and we provide evidence for the multiple binding of SHAPs to an HA chain.

EXPERIMENTAL PROCEDURES

Materials—Aminoacaproic acid, N-ethylmaleimide, phenylmethanesulfonyl fluoride (PMSF), and CsCl were purchased from Nacalai Tesque, Kyoto, Japan. Protease-free Streptomyces hyaluronidase, chondroitinase ABC, the HA-binding protein (HABP) derived from the N-terminal region of bovine aggrecan, the biotinylated HABP, mouse anti-human PG-M/versican monoclonal antibody, 2B1, and mouse anti-human decorin monoclonal antibody, 6B6, were from Seikagaku Corp., Tokyo, Japan. Rabbit anti-human ITI antibody (purified immunoglobulins) was from Dako, Glostrup, Denmark. Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG antibody (affinity-purified immunoglobulins) was from Jackson ImmunoResearch Laboratories, West Grove, PA. HRP-conjugated streptavidin was from Amersham Biosciences. HRP-conjugated protein A was from Organon Teknika Corp., West Chester, PA. Bovine serum albumin fraction V (protease-free grade) (BSA) was from Miles. Skim milk was from Difco. The 3,3′,5,5′-tetramethylbenzidine (TMB) solution and TMB stop solution were from Kirkegaard & Perry Laboratories. Micro-BCA Protein Assay Reagent kit was from Pierce. Western blot chemiluminescence reagent was from PerkinElmer Life Sciences. Hyperfilm ECL film and Sephareryl S-1000 were from Amersham Biosciences. Rabbit anti-human bikunin antibody (purified immunoglobulins) was prepared in this laboratory (18).

Purification of the SHAP-HA Complex from Pathological Synovial Fluid—The SHAP-HA complex was purified by a series of CsCl iso-osmolar centrifugations with increasing initial densities. Synovial fluid—fluid collected during an arthroscopic examination of patients with knee joint diseases, was washed and treated with solid CsCl. Then a density gradient was established by centrifugation at 40,000 rpm at 10°C for 2h with gentle mixing. The samples were added to 8% NaHCO3 (pH 9.5) at 4°C for 15 h and then centrifuged for 1 h at 20°C. The supernatant and washed pellets were combined and subjected to a second centrifugation with an initial density of 1.35 g/ml at 4°C for 2 h. The fractions were pooled for the measurement of unbound HA (free HA) and subjected to a third centrifugation with an initial density of 1.42 g/ml. The second gradient was partitioned into 27 fractions (1 ml/fraction) and the contents of HA, total glycosaminoglycan, and protein were measured by periodate/thiobarbituric acid reaction (20), carboxylase reaction, and micro-BCA assay, respectively.

Quantification of SHAP in the SHAP-HA Complex—Nunc Maxisorp microtiter plates were coated with HABP (2 μg/ml in 0.1 M sodium carbonate buffer (pH 8.25)) at 4°C for 15 h. The wells were washed twice with 200 μl of PBS-T, followed by blocking with 200 μl of 3% BSA in PBS-T at room temperature for 2 h. After three washes with 200 μl of PBS-T, 50 μl of each sample (500 ng HA/ml) in 1% BSA/PBS-T was added to the well, and the plates were incubated at 37°C for 2 h. After a wash, 25 μl of rabbit anti-human ITI antibody (diluted 1:2000 in 1% BSA/PBS-T) and 25 μl of HRP-conjugated goat anti-rabbit immunoglobulin antibody (diluted 1:1500 with 1% BSA/PBS-T) were added to each well and incubated at 37°C for 1 h. The wells were washed 3 times and then incubated with 50 μl each of TMB solution at 37°C for 20 min. The reaction was stopped by adding 50 μl of 1 M HCl, and the absorbance at 450/620 nm (A at 450 nm minus A at 620 nm) was measured on an immunoMini NI-2300 spectrophotometer. The assay was performed in triplicate.

Quantification of HA in the SHAP-HA Complex—Nunc Maxisorp microtiter plates were coated with 50 μl of each of rabbit anti-human ITI antibody (22 μg/ml in 20 mM NaHCO3 (pH 9.5)) at 4°C for 15 h and then blocked with 1% BSA in PBS-T for 60 min at 37°C. After three washes with PBS-T, 50 μl of each sample (300 ng HA/ml) in 1% BSA/PBS-T was applied to each well and incubated at 37°C for 60 min. The wells were washed and incubated with 50 μl of each of biotinylated-HABP (diluted 1:2000 with 1% BSA/PBS-T) at 37°C for 60 min. After a wash with PBS-T, 50 μl of HRP-streptavidin (1:500) was added to each well, and the plates were further incubated at 37°C for 60 min. Color development was achieved by incubating with 50 μl of TMB solution at 37°C for 5 min and then stopped with 50 μl of 1 M HCl. The absorbance at 450/620 nm (A at 450 nm minus A at 620 nm) was measured. The assay was performed in triplicate.

Immunoprecipitation of the SHAP-HA Complex—Anti-ITI antibody (110 μg) in 1.5 ml of PBS (pH 7.4) was mixed with an equal volume of PBS-equilibrated cellulose-protein A beads and incubated at room temperature for 2 h with gentle mixing. The beads were collected by centrifugation followed by five washes with three volumes each of PBS. The beads were resuspended in 200 μl of PBS-T and washed twice with PBS-T. The beads were mixed with the anti-ITI antibody-coated beads and incubated overnight at 4°C with gentle mixing. The beads were collected by centrifugation and washed 5 times with 3 volumes each of PBS. The supernatant and the first washing solution were pooled for the measurement of unbound free HA. The SHAP-HA complex bound on the beads was treated with 50 μM NaOH with 2 mM PMSF at room temperature for 3 h, washed twice with PBS-T containing 0.1% sodium deoxycholate, and then resuspended in 200 μl of PBS-T. The beads were washed once with 50 μM NaOH, and the two NaOH solutions were pooled. The amounts of unbound HA (free HA) and bound HA (in the form of the SHAP-HA complex) were measured by the carbazole reaction. After concentration in vacuo, HA was precipitated with ethanol, dried briefly, dissolved in 30 μl of TAE (40 μM Tris
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**Agarose Electrophoresis and Western Blotting of the SHAP-HA Complex and HA**—The SHAP-HA complex preparation (containing 30 μg of HA) was precipitated with ethanol, briefly dried, and then dissolved in 30 μl of TAE. If necessary, the precipitate of the preparation was dissolved in 30 μl of 0.2 M NaOH and incubated at room temperature for 4 h to completely break down the ester bond between SHAP and HA. The released HA was precipitated again with ethanol, dissolved in 30 μl of TAE buffer, and then subjected to 0.5% agarose gel electrophoresis (21). After electrophoresis, the gel was incubated in a freshly prepared solution (176 mg of L-ascorbic acid and 13.9 mg of FeSO₄ in 500 ml of TAE buffer) for 30 min to degrade the HA into low molecular weight HA. After two washes with TAE buffer for 20 min, the HA in the gel was blotted onto a Hybond N+ membrane by capillary action. HA was directly visualized by staining with 0.5% Alcian blue in 2% acetic acid for 15 min, followed by destaining with 2% acetic acid. Otherwise, the membrane was blocked with 20% skim milk in PBS-T overnight at room temperature with gentle shaking and incubated with biotinylated HABP (1 μg/ml) in PBS with 1% BSA at room temperature for 2 h and then with HPR-streptavidin (1:1000) in PBS with 1% BSA at room temperature for 1 h. The HA was finally visualized by enhanced chemiluminescence assay and exposed to Hyperfilm ECL.

**Gel Filtration of the SHAP-HA Complexes**—The SHAP-HA complex preparation was dialyzed against distilled water. An aliquot of the dialyzed preparation was treated with 0.2 M NaOH for 2 h at room temperature, followed by neutralization with HCl and subsequent addition of 10× PBS buffer to give the conventional PBS solution. For control, all additives were mixed first, and finally the aliquot was added. The sample was applied on to an analytical Sepharacryl S-1000 column (0.7 × 30 cm). The column was eluted with the conventional PBS solution at a flow rate of 12–15 ml/h, and 0.5-ml fractions were collected. 4 and 10 μl of each fraction were diluted with the PBS solution to make a total volume of 50 μl and then subjected to quantification of HA and SHAP, respectively.

**Quantification of HA by Inhibitory ELISA**—The inhibitory ELISA kit for HA was provided by Seikagaku Corp., Tokyo, Japan. Briefly, the microtiter plates were coated with HA-conjugated BSA and then blocked with BSA. To each well, the various concentrations of HA (standard) were added, together with a biotinylated HABP solution. After incubation, the plate was washed and further incubated with peroxidase-conjugated streptavidin. Finally, a color was developed, and the absorbance was measured as described above.

**Electron Microscopic Observation of the SHAP-HA Complex**—Negative staining of the SHAP-hyaluronan complex for electron micrographs and evaluation of the data were carried out as described previously (22). For negative staining, sample preparations (typical concentrations of about 10 μg/ml in Tris-buffered saline) were adsorbed to 400-mesh carbon-coated copper grids, washed briefly with water, and stained on two drops of freshly prepared 0.75% uranyl formate. The grids were rendered hydrophilic by glow discharge at low pressure in air. Specimens were observed in a JEOl JEM 1230 electron microscope operated at an 80-kV accelerating voltage. Images were recorded with a Gatan Multiscan 791 CCD camera. Molecular masses of globular protein domains from negatively stained images were estimated as described previously (22).

**RESULTS**

**Purification of the SHAP-HA Complex from Pathological Synovial Fluid**—The synovial fluid from rheumatoid arthritis patients was highly viscous and contained a variety of proteins at high concentrations. The SHAP-HA complex was isolated under dissociative conditions with 4 M guanidine HCl by three sequential CaCl₂ isopycnic centrifugations with initial densities of 1.35, 1.40, and 1.42 g/ml, respectively (Fig. 1A). After each centrifugation and subsequent fractionation, the density and uronate, HA, and protein contents of each fraction were measured. Fractions were numbered from the bottom to the top. After the first centrifugation, most of the proteins were recovered in the lower density fractions (ρ < 1.35 g/ml) (Fig. 1A). The higher density fractions (fractions 1–6) that contained less protein and more uronate were pooled and subjected to a second centrifugation. After the second centrifugation, we noticed that the relative content of protein to uronate in each fraction was not constant but tended to increase along with the decline in density (Fig. 1B). The fractions 3–9 of the second gradient were pooled and further purified by a third centrifugation. The fractions 5–15 of the third gradient that had densities between 1.5 and 1.37 g/ml contained most of the total HA, but the

![Image](318x215 to 562x737)

**Fig. 1. Isolation of the SHAP-HA complex from synovial fluid of patients with rheumatoid arthritis.** Guanidine HCl extract of pathological synovial fluid was subjected to three sequential CsCl isopycnic centrifugations under dissociative conditions. The initial densities in the three centrifugations were 1.35, 1.40, and 1.42 g/ml, respectively. The gradients were partitioned into 15 fractions after the first two centrifugations and 27 fractions after the third centrifugation. The density (Δ), the protein-to-HA ratio (○), and protein (■) in each fraction after the first, the second, and the third centrifugation are shown in A–C, respectively. C, the protein-to-HA ratio (×) of some fractions are also shown. A, the curve of protein concentration is truncated because the concentrations are over the scale. The maximal protein concentration was found to be 85.6 mg/ml in the top fraction (fraction 15).
protein-to-HA ratio still showed an increasing tendency, from 0.084 for fraction 5 to 0.148 for fraction 15 (Fig. 1C). Two-thirds each of the fractions 5–15 were pooled as the purified SHAP-HA complex. Comparing uronate and protein contents of the pooled fractions among the three centrifugations, we found that the relative amounts of protein to uronate decreased significantly between the first and second centrifugations but only a little between the second and third centrifugations (Table I). There was no further decrease when we performed the fourth centrifugation and compared the values with those for the third centrifugation (data not shown). Judging from these values and the distribution patterns of protein, uronate, and HA after each centrifugation, we concluded that the SHAP-HA complex in the starting synovial fluid of rheumatoid arthritis patients was mostly recovered in fractions 5–15 of the third gradient with the least contamination by other components. In this experiment, the final SHAP-HA complex preparation with 70 ml of synovial fluid (obtained from three rheumatoid arthritis patients) as starting material contained 3.08 mg of protein and 56 mg of HA.

Characterization of the Isolated SHAP-HA Complex—The purity of the SHAP-HA complex preparations was examined by CB staining and immunostaining after SDS-PAGE under reducing conditions. The pooled fraction after the second centrifugation without Streptomyces hyaluronidase digestion only gave the protein bands migrating faster than the SHAP bands (Fig. 2A, lane 1). The appearance of those bands such as the major one having molecular mass of 50 kDa was not altered by Streptomyces hyaluronidase digestion, and therefore, those protein bands were likely contaminants. The Streptomyces hyaluronidase digestion of the fraction gave two new bands having molecular masses of 75 and 85 kDa, which were stained with CB strongly and weakly, respectively (Fig. 2A, lane 2). They appeared to correspond to the SHAPs derived from HC1 and HC2 of ITI, respectively, according to the sensitivity to hyaluronidase digestion and the reported molecular sizes for human HC1 and HC2 (12, 23).

The fractions 5–14 on the third gradient, the representative fractions, did not give the protein bands migrating faster than the SHAP bands with or without the hyaluronidase digestion (Fig. 2B), which suggested that the third centrifugation was essential to purify the SHAP-HA complex from those contaminants. After Streptomyces hyaluronidase digestion, the fractions only gave the two bands having molecular masses of 75 and 85 kDa upon CB staining (Fig. 2B, lanes 1 and 3). Both of them were stained with the anti-human ITI antibody, confirming that they corresponded to ITI HCs (Fig. 2C). The results are consistent with our previous study showing that the SHAP-HA complex in human pathological synovial fluids was composed of both the HC1 and HC2 of the ITI (11). The CB staining pattern indicated that the synovial SHAP-HA complex involves more HC1 than HC2. Chondroitinase ABC digestion (Fig. 2B, lanes 2 and 4) did not yield any further bands except for the own enzyme bands (Fig. 2B, lane 6), which almost but not completely eliminated the possibility of contamination with chondroitin sulfate proteoglycans (Fig. 2B, lanes 2 and 4). This was also confirmed by no immunoreactivity on the membranes of chondroitinase ABC digested fraction 5 and 14 samples with antibodies against human PG-M/versican, human decorin, and human biguin (data not shown).

The Differences in the Density of the SHAP-HA Complex Fractions Are Due to Those in the Ratio of SHAP to HA—We noticed significant differences in the staining intensity of the SHAP bands between fractions 5 and 14 in Fig. 2, B and C, although we had applied aliquots containing the same amounts of HA. In both the CB- and immunostainings, fraction 14 of lower density (Fig. 2, B, lane 3, and C, lane 2, respectively) gave the more strongly stained bands than fraction 5 of higher density (Fig. 2, B, lane 1, and C, lane 1, respectively). Consistently, the protein-to-HA ratios of fractions 5–14 tended to increase with the decrease in density (Fig. 1C). The results suggested that, considering the high purity of these fractions, the SHAP protein contributed to the increasing relative content of protein in the low density fractions of the third gradient.

We then examined this possibility in detail by ELISA. The protein, uronate, and HA contents of fractions 5 and 14 are shown in Table II. For the ELISA of SHAP amounts, aliquots of these fractions containing the same amounts of HA were applied to HABP-coated wells for detection of the SHAP bound to HA with anti-ITI antibody (Fig. 3A). In the assay we first confirmed that the assay conditions gave a linear relationship between the HA amount and the SHAP absorbance. Then the applied amounts of HA (500 ng HA/ml) were chosen in a linear range to avoid any effect from the binding of HA (Fig. 3B). The result showed that SHAP was 1.40 times more abundant in fraction 14 than fraction 5 (Fig. 3C), which is very consistent with the observation that the protein-to-HA ratio of fraction 14 was 1.48 times higher than that of fraction 5 (0.124 versus 0.084, Table II) and confirmed that the two SHAP-HA complex preparations of different densities have distinct relative contents of SHAP to HA.

Heterogeneous Structures of the SHAP-HA Complex—Several factors may cause the change in the SHAP-to-HA ratio of SHAP-HA complex preparations: the content of free HA, the number of SHAP molecules bound to an HA chain, and the length of the SHAP-bound HA chain. By using fractions 5 and 14, we examined which factor contributed most to the change.

First, we analyzed the content of SHAP-bound HA in the fractions by taking advantage of the ELISA strategy. The wells were coated with anti-human ITI antibody so that only the SHAP-HA complex could be captured on the wells. Biotinylated HABP was then used to detect the HA in the wells (Fig. 4A). Again, we first determined the range where the absorbance shows a linear relationship with the amount of HA applied (Fig. 4B). Then the samples with a proper amount of HA (300 ng/ml) were applied to give the absorbance in a linear range. Under such assay conditions, we did not detect any significant difference in the amount of HA between fraction 5 and fraction 14 (Fig. 4C). The results suggested that the two preparations had similar amounts of HA bound to SHAP and that the observed difference in the SHAP-to-HA ratio might be caused by the molecular heterogeneity of the SHAP-HA complex.

We next compared the length of the HA chain of the complex between fraction 5 and fraction 14 by agarose gel electrophoresis followed by either Alcian blue staining or b-HABP staining. When either fraction without any treatment was separated on agarose gel, a significant portion of HA was retained at the origin of the gel, and the rest migrated but formed a very broad band in the gel (Fig. 5, lanes 1 and 2). When both fractions were pretreated with alkali to cleave the ester bond between SHAP and HA, all HA migrated into the gels (Fig. 5, lanes 3 and 4). Judging from migration patterns in comparison with those of HA molecular weight markers, the molecular masses of the HA

| Centrifugation | Protein | Uronate | HA | Protein/ Uronate | Protein/ HA |
|---------------|---------|---------|----|-----------------|-------------|
| 1             | 0.616   | 1.712   | 0.360 |                 |             |
| 2             | 0.197   | 1.172   | 0.168 |                 |             |
| 3             | 0.140   | 0.695   | 1.296 | 0.201           | 0.108       |
chains of both fractions were about 1,800 kDa, and there was no significant difference in their size distribution between fraction 5 and fraction 14 (Fig. 5, lanes 3 and 4). We prepared the samples for electrophoresis from the fractions of the CsCl gradients in the dissociative 4 M guanidine HCl solution by either ethanol precipitation or dialysis against TAE buffer, and in both cases the same results as described above were obtained (data not shown).

We further examined the aggregation of the SHAP-HA complex by gel filtration using Sephacryl S-1000, the molecular sieving gel media that are so far known to have the largest molecular size exclusion limit. As shown in Fig. 6, the SHAP-HA preparations of both fractions 5 and 14 had similar elution patterns, with a peak at the elution position where the HA standard of mean molecular mass of 2,130 kDa was eluted. It seems that the free HA and the SHAP-HA complex were not separated under the chromatographic conditions because the elution pattern of SHAP in the complex superposed largely that of HA. It is of note that only about 65% of loaded HA in both fraction 5 and 14 were recovered after chromatography. However, when the samples were alkali-treated before chromatography, the yield was elevated to nearly 100% in both the fractions. The results suggest that almost the one-third of the SHAP-HA complex had been retained on the top of the column (Fig. 6, A and C) until the SHAPs were removed from HA by an alkaline treatment (Fig. 6, B and D). The HA involved in the SHAP-HA aggregation was not largely different in the chain size in fraction 5 and in fraction 14, although only a small population of the HA with smaller size increased in fraction 14 (Fig. 6, A and C).

For the further confirmation of the aggregate formation of the SHAP-HA complex and in relation to the mechanism of the aggregation, it is important to compare the SHAP-to-HA ratio between the material eluted from the column and the sample loaded to the gel filtration column. The ratios of the former

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**TABLE II**

| Fraction no. | Density (g/ml) | Protein (mg/ml) | Uronate (mg/ml) | HA (mg/ml) | Protein/Hydroxyapatite (HA) |
|--------------|---------------|----------------|-----------------|------------|----------------------------|
| 5            | 1.470         | 0.121          | 0.716           | 1.432      | 0.084                      |
| 14           | 1.380         | 0.142          | 0.617           | 1.141      | 0.124                      |

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**Fig. 2.** SDS-PAGE and immunoblot analysis of the isolated SHAP-HA complex. A, the pooled fractions after the second centrifugation. Each lane contains 20 μg of HA digested with lane 2 or without lane 1) Streptomyces hyaluronidase and is stained with Coomassie Brilliant Blue. B, fraction 5 (lanes 1 and 2) and fraction 14 (lanes 3 and 4) after the third centrifugation. Each lane contains 20 μg of HA, which was treated by Streptomyces hyaluronidase in lanes 1 and 3, and by chondroitinase ABC in lanes 2 and 4. The same amounts of hyaluronidase and chondroitinase were loaded in lanes 5 and 6, respectively. The proteins are stained by Coomassie Brilliant Blue. C, fractions 5 (lane 1) and 14 (lane 2) after the third centrifugation. Each lane contains 3 μg of HA digested by Streptomyces hyaluronidase. The released SHAP was detected with anti-ITI antibody.

**Fig. 3.** Comparison of the SHAP-HA complexes in fractions 5 and 14 after the third centrifugation by sandwich ELISA. A, schematic representation of the ELISA strategy. The SHAP-HA complex is immobilized via its HA moiety and detected via its SHAP moiety. B, the standard curve for the ELISA system showing a linear relationship between the HA amount and the absorbance within the indicated range. C, when the same amount of HA (500 ng/ml) was examined, about 1.4 times more SHAP was detected in the SHAP-HA complex preparation in fraction 5 than in fraction 14.
were lower than the one of the latter (Fig. 6, B and D, insets), indicating that the material lost during the gel filtration had the high SHAP-to-HA ratio, therefore suggesting that the aggregation may happen to the population of the SHAP-HA complex with the high SHAP-to-HA ratio.

Taken together, the SHAP-HA complex in physiological solutions is heterogeneous in structure and exists as aggregates at least in part, which raises the interesting question of how the SHAP-HA complexes interact with each other to form aggregates.

Number of SHAP Molecules Bound to an HA Chain—Because agarose gel electrophoresis of fraction 5 and fraction 14 revealed that the samples likely included free HA (Fig. 5, lanes 1 and 2), we tried to isolate the SHAP-HA complex by immunoprecipitation using anti-ITI antibody-coated beads as described under “Experimental Procedures” and analyzed the size of SHAP-bound HA. Hexuronate contents determined by the carbazole reaction indicated that the HA in the supernatant solution (free HA) and the precipitates (the SHAP-HA complex) accounted for 41.6 and 58.3% of the total HA, respectively, in fractions 5, and 41.0% and 59.0%, respectively, in fractions 14. The results suggested that both fractions contained the same amounts of free HA and SHAP-bound HA and were consistent with the ELISA data described above (Fig. 4). The immunoprecipitated SHAP-HA complexes were treated with alkali to release the HA, which was subsequently fractionated on agarose gel and detected by biotinylated HABP. The results confirmed that there was no significant difference in the molecular mass of the SHAP-bound HA between fraction 5 and fraction 14 although only a small population of smaller size increased in fraction 14 (data not shown).

The observations strongly argued for a difference in the number of SHAP molecules bound to an HA chain of the same size. More SHAP in the HA chain decreased the density of the SHAP-HA complex. Given that the average molecular masses of HA and SHAP were 1,800 and 80 kDa, respectively, the molecular ratio of SHAP to HA was calculated to be 3.2 and 4.8 in fraction 5 and fraction 14, respectively, according to the contents of HA, protein (Table II), and the percentage of free HA in the total HA population in the two preparations. We assume that the SHAP-HA complexes in fraction 5 and 14 are composed of 3 and 5 SHAP molecules per HA chain, respectively, as shown in Fig. 7. Although we have not analyzed the SHAP-HA complexes in all fractions, the present results suggest that a SHAP-HA complex of different density may have a different number of SHAP molecules per HA chain.

Electron Microscopic Observation of the SHAP-HA Complex Structure—Application of the negative staining made the complex clearly visible, revealing HA-strands (white strands) as well as SHAP globules (arrows and arrowheads) in the network (Fig. 8). The SHAP globules are rather homogeneous in size and match previously published dimensions of ITI heavy chains (diameter about 11 nm after rotary metal shadowing) (24). The observation of selected areas of HA networks with SHAP particles at higher magnification demonstrated that individual HA strands are resolved with heavy chains appearing either as single globules (arrowheads) or as globules with short
extended domains (arrows) binding to the HA (Fig. 8). This agrees with previous observations that the glycosaminoglycan-binding domains of ITI heavy chains (24) appear to be flexible and sometimes fold back to form a single globule only. In addition, it is of note that the lower density fractions show an image of the HA network with more globules, which is consistent with the above conclusion. Furthermore, the images of two globules coming together suggest the possible involvement of SHAPs in the aggregation of HA strands.

**DISCUSSION**

In the present study, we purified the SHAP-HA complex from pathological synovial fluids to a homogeneous level on SDS-PAGE by sequential CsCl isopycnic centrifugation. In fractions 5–15 of the third gradient, no band was detected at the positions corresponding to ITI (180 kDa), PoI (120 kDa), and the covalent ITI/TSG-6 complex (120 kDa) by Coomassie Blue staining and immunostaining. TSG-6 (tumor necrosis factor-stimulated gene 6), an inflammatory HABP of 35 kDa, has been shown to be present in synovial fluid of patients with rheumatoid arthritis and some other forms of arthritis but not in normal human joints (25). Interestingly, this molecule in the patient synovial fluid has been found to form a covalent complex with ITI molecules via the chondroitin sulfate chains (25). Because the chondroitinase ABC digestion did not alter the
staining patterns on SDS-PAGE, the SHAP-HA complex preparations we obtained were free of contamination by ITI family proteins and other proteoglycans. Although the present purification protocol was not designed to separate the SHAP-HA complex from free HA, the immunoprecipitation performed in this study enabled us to do so. As described under "Results," about 41% of the HA was free of a SHAP molecule.

The molecular properties of the SHAP-HA complex preparations appear to be source-dependent. We also purified the SHAP-HA complex from another two batches of rheumatoid arthritis synovial fluids (one batch from three or four patients) by the same protocol (data not shown). In one sample, the protein-to-HA ratio was 0.39 after the first centrifugation but reduced to 0.09 after the third centrifugation. In the other sample, the ratio was 0.16 after the first centrifugation and 0.03 after the third. These values were significantly lower than those of the present sample (Table 1). A possible reason for the high HA content in these samples is that it could be a reflection of the disease state and, if so, is worthy of further study. It is also possible that the starting fluids might have been collected mistakenly from patients receiving intra-articular HA injections. In addition, some low molecular weight proteins were detected in those two preparations, suggesting the degradation of SHAP as a result of an inflammatory response, although the possibility of degradation during the sample handling should not be excluded.

Characterization of the purified SHAP-HA complex led to the clarification that an HA chain can form a covalent complex with multiple SHAP molecules. It has been shown that the heavy chains of ITI consist of a globular structure with a diameter of 11 ± 2 nm at their N-terminal halves, and a thin extension with a length of about 15 nm at their C-terminal halves, by which the heavy chains are bound to chondroitin sulfate (24). The projected length of an HA disaccharide is estimated to be 0.8–1.0 nm (26). Accordingly, the length of an HA chain with a molecular weight of $1.8 \times 10^6$ in its extended form is about 400 times the diameter of a globular head of SHAPs. Therefore, it is reasonable for such a long HA chain to have more than five SHAPs bound to it. In fact, HA rarely exhibits an extended conformation in solution. Instead, it tends to form random coils and a molecular network depending on its concentration and the presence of other components such as iron and HA-interacting molecules (26). The effect of SHAP binding on the conformation of HA chains remains to be established. However, as suggested from the electron micrographs (Fig. 8), the notion of multivalent SHAP binding is important in considering the formation of HA aggregates. In the present study, we did observe that the purified SHAP-HA complexes are prone to form aggregates that were not dissociated by electrophoretic separation in 0.5% agarose gel (Fig. 5) and by molecular sieving-based separation on Sephacryl S-1000 gels (Fig. 6). An early study also found the retention of SHAP-HA complex when synovial fluids or follicle fluids were subjected to agarose gel electrophoresis (27). Our results were in agreement with the observation and further indicated that the SHAP-HA complex itself was enough to form the aggregates, which implies an interesting role for SHAP in the regulation of HA aggregation. Such a role is apparently distinct from the previously described one, which showed that the formation of protein-HA complexes helps protect the cartilage proteoglycan-HA aggregates from disruption by oxygen-derived free radicals and hydrogen peroxide (28, 29).

It is well known that cells undergoing active proliferation, differentiation, and migration frequently form an extracellular HA-rich matrix, which influence significantly cellular behavior during many developmental and pathological processes (3). In early studies on the matrices of cultured fibroblasts, mesothelial cells and cumulus-oocyte complexes (15, 17), ITI and PoI were shown to be able to stabilize the HA-rich matrices, suggesting a role for the SHAP-HA complex in stabilizing the HA-rich cell coats. Recently, SHAP-HA complex-deficient mice were generated, and the physiological importance of the complex established as being essential for the expansion of the cumulus oophorus (18). In preovulatory follicles, the cumulus cells start to synthesize HA actively upon a gonadotropin surge. Simultaneously, gonadotropin stimulates the opening of the blood-follicle barriers to allow the influx of ITI family molecules, which then form SHAP-HA complexes with the locally synthesized HA. Such a reaction is essential for the formation of the cumulus HA-rich matrix, which plays a crucial role in successful fertilization (18). Similarly, the SHAP-HA complexes in pathological synovial fluids were formed between the local HA and the plasma-derived ITI (10, 16). It is plausible to speculate that the cumulus SHAP-HA complex is also multivalent, and such a molecular feature contributes to its central role in the organization and stabilization of the cumulus matrix.

Enzyme factor(s) may be directly involved in the formation of the SHAP-HA complex. The factor(s) has been described either in the plasma or at local sites including preovulatory follicles and joints and shows strict divalent cation dependence (11, 30, 31) but has not been cloned yet. Because the enzyme(s) and the ITI family molecules (one of substrates) are present abundantly in serum, the serum level of the SHAP-HA complex seems dependent on the serum HA level. Indeed, we have observed a positive correlation between the serum SHAP-HA level and the greatly elevated serum HA level in patients with rheumatoid arthritis (16), acute inflammatory hepatitis, and...
some cancers.\textsuperscript{2} Consistent with this, an increase in the concentration of ITI family molecule(s) with single heavy chains such as PAI has been described in the serum of a patient with rheumatoid arthritis (32). One may speculate that the transfer of one of the two heavy chains of ITI to HA caused this increase as reaction products. Similarly, Chawla et al. (33) reported a great increase in the urinary bikunin level and simultaneous decrease of ITI in cancer patients. As we previously reported (34), malignant cancer tissues frequently generated HA-rich matrices with a significant accumulation of the SHAP-HA complex, which was purified from pathological synovial fluids. Such a molecular feature may be important in regulating the formation of HA aggregates during the construction of an extracellular HA-rich matrix. In this sense, one can consider that the SHAP-HA complex may be an active form of HA. The control of the SHAP-to-HA ratio in a SHAP-HA complex may comprise many factors, such as HA-binding proteoglycans and link protein included in HA-rich matrices, the enzymatic factor(s) that catalyzes the transfer reaction between the ester bonds, the effusion of circulating ITI family molecules, and so on. A recent report (35) describing TSG-6 null mutation in mouse revealed important roles of TSG-6 in the cumulus SHAP-HA complex formation. All remain to be further investigated and would help us understand the formation and regulation of the HA-rich matrix and the related events and pathogenesis.

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REFERENCES

1. Balaz, E. A. (1965) in The Amino Sugar (Balazs, E. A., and Jeanloz, R. W., eds) Vol. IIA, pp. 401–460, Academic Press, New York
2. Sundblad, L. (1965) in The Amino Sugar (Balazs, E. A., and Jeanloz, R. W., eds) Vol. IIA, pp. 259–289, Academic Press, New York
3. Laurent, T. C., and Fraser, J. R. (1992) FASEB J. 6, 2397–2404
4. Toole, B. P. (1999) Curr. Opin. Cell Biol. 2, 839–844
5. Heinegard, D., Björnsson, S., Mørgelin, M., and Sommarin, Y. (1998) Wener-Gren Int. Ser. 72, 113–122
6. Underhill, C. (1992) J. Cell Sci. 103, 283–288
7. Masselis-Smith, A., Belch, A. R., Munt, M. J., Turley, E. A., and Pilarski, L. M. (1996) Blood 5, 1891–1899
8. Jackson, R. L., Busch, S. J., and Cardin, A. D. (1991) Physiol. Rev. 71, 481–539
9. Yoneda, M., Suzuki, S., and Kimata, K. (1990) J. Biol. Chem. 265, 5247–5257
10. Zhao, M., Yoneda, M., Ohashi, Y., Kuroko, S., Iwata, H., Ohnuki, Y., and Kimata, K. (1995) J. Biol. Chem. 270, 26657–26663
11. Huang, L., Yoneda, M., and Kimata, K. (1993) J. Biol. Chem. 268, 6630–6635
12. Mizon, C., Balduyck, M., Albani, D., Michalski, C., Burnouf, T., and Mizon, J. (1996) J. Immunol. Methods 190, 61–70
13. Salier, J. P. (1990) Trends Biochem. Sci. 15, 435–439
14. Englund, J. J., Sulvesen, G., Høfta, S. A., Thogersen, I. B., Rutherfurd, S., and Pizzo, S. V. (1991) J. Biol. Chem. 266, 747–751
15. Chen, L., Mao, S. J. T., McLean, L. R., Powers, R. W., and Larsen, W. J. (1994) J. Biol. Chem. 269, 28282–28287
16. Kida, D., Yoneda, M., Miyaura, S., Ishimaru, T., Yoshida, Y., Ito, T., Ishiguro, N., Iwata, H., and Kimata, K. (1989) J. Rheumatol. 26, 1230–1238
17. Blom, A., Porth, H., and Fries, E. (1995) J. Biol. Chem. 270, 9988–9971
18. Zhao, M., Yoneda, M., Zhao, M., Yingsung, W., Yoshida, N., Kitagawa, Y., Kamawara, K., Suzuiki, T., and Kimata, K. (2001) J. Biol. Chem. 276, 7693–7696
19. Makhopadhyay, D., Hascall, V. C., Day, A. J., Salustri, A., and Fulep, C. (2001) Arch. Biochem. Biophys. 394, 173–181
20. Yoneda, M., Yamagata, M., Suzuki, S., and Kimata, K. (1998) J. Cell Sci. 111, 265–273
21. Lee, H. G., and Cowman, M. K. (1994) Anal. Biochem. 219, 278–287
22. Engell, J., and Furtthmayr, H. (1987) Methods Enzymol. 145, 3–78
23. Elmore, T., Daveau, M., and Salier, J. P. (1992) Biochim. Biophys. Acta 110, 1019–1024
24. Blom, A. M., Mørgelin, M., Oyen, M., Jarvet, J., and Fries, E. (1999) J. Biol. Chem. 274, 298–304
25. Wisniewski, H. G., Burgess, W. H., Oppenheim, J. D., and Vilcek, J. (1994) Biochemistry 33, 7423–7430
26. Cowman, M. K., Liu, J., Li, M., Hittner, D. M., and Kim, J. S. (1998) Wener-Gren Int. Ser. 72, 17–24
27. Jessen, T. E., Odum, L., and Johnsen, A. H. (1994) Biol. Chem. Hoppe-Seyer 375, 521–526
28. Hovda, K., Poulsen, S., and Brocks, P. (1988) Ann. Rheum. Dis. 47, 377–385
29. Greenland, R. A., and Mey, W. W. (1980) Arthritis Rheum. 23, 455–463
30. Chen, L., Zhang, H., Powers, R. W., Russell, P. T., and Larsen, W. J. (1996) J. Biol. Chem. 271, 19409–19414
31. Odum, L., Andersen, C. Y., and Jessen, T. E. (2002) Reproduction 124, 249–257
32. Odum, L., Hansen-Nord, G., and Byrjalsen, T. (1986) Clin. Chim. Acta 162, 189–194
33. Chawla, R. K., Rausch, D. J., Miller, F. W., Vogler, W. R., and Lawson, D. H. (1984) Cancer Res. 44, 2718–2723
34. Yoneda, M., Zhao, M., Watanabe, H., Yamada, Y., Huang, L., Nagasawa, S., Nishimura, H., Shimomura, T., Isoai, Z., and Kimata, K. (2000) in New Frontiers in Medical Sciences: Redefining Hyaluronan (Abatangelo, G., and Weigel, P. H., eds) pp. 21–50, Elsevier Science Publishers B.V., Amsterdam
35. Falop, C., Szanto, S., Makhopadhyay, D., Bardos, T., Kamath, R. V., Rugg, M. S., Day, A. J., Salustri, A., Hascall, V. C., Glant, T. T., and Mikecz, K. (2003) Development 130, 2253–2261