Characterization of Complexes Formed between TSG-6 and Inter-α-inhibitor That Act as Intermediates in the Covalent Transfer of Heavy Chains onto Hyaluronan*

Marilyn S. Rugg‡, Antony C. Willis‡, Durba Mukhopadhyay§, Vincent C. Hascall§, Erik Fries¶, Csaba Fülöp§, Caroline M. Milner‡, and Anthony J. Day¶

Characterization of Complexes Formed between TSG-6 and Inter-α-inhibitor That Act as Intermediates in the Covalent Transfer of Heavy Chains onto Hyaluronan*

The high molecular mass glycosaminoglycan hyaluronan (HA) can become modified by the covalent attachment of heavy chains (HCs) derived from the serum protein inter-α-inhibitor (IαI), which is transferred first onto TSG-6 and then onto HA. The formation of HC-HA is likely to play an important role in the stabilization of HA-rich extracellular matrices in the context of inflammatory disease (e.g. arthritis) and ovulation. Here, we have characterized the complexes formed in vitro between purified human IαI and recombinant human TSG-6 (an inflammation-associated protein implicated previously in this process) and show that these complexes (i.e. TSG-6-HC1 and TSG-6-HC2) act as intermediates in the formation of HC-HA. This is likely to involve two transesterification reactions in which an ester bond linking an HC to chondroitin sulfate in intact IαI is transferred first onto TSG-6 and then onto HA. The formation of TSG-6-HC1 and TSG-6-HC2 complexes was accompanied by the production of bikunin-HC2 and bikunin-HC1 by-products, respectively, which were observed to break down, releasing free bikunin and HCs. Both TSG-6-HC formation and the subsequent HC transfer are metal ion-dependent processes; these reactions have a requirement for either Mg2+ or Mn2+ and are inhibited by Co2+. TSG-6, which is released upon the transfer of HCs from TSG-6 onto HA, was shown to combine with IαI to form new TSG-6-HC complexes and thus be recycled. The finding that TSG-6 acts as cofactor and catalyst in the production of HC-HA complexes has important implications for our understanding of inflammatory and inflammation-like processes.

* This work was supported by the Medical Research Council and Arthritis Research Campaign Grants 16119 and 16539. 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 1754 solely to indicate this fact.

† To whom correspondence should be addressed. Tel.: 44-1865-275-349; Fax: 44-1865-275-729; E-mail: tony.day@bioch.ox.ac.uk

‡ The abbreviations used are: HA, hyaluronan; HC, heavy chain; IαI, inter-α-inhibitor; COC, cumulus-oocyte complex; CS, chondroitin sulfate; PrI, pre-α-inhibitor; Tricine, N[2-hydroxy-1,1-bis(hydroxymethyl)methyl]glycine; MES, 4-morpholineethanesulfonic acid; CAPS, 3-(cyclohexylamino)propanesulfonic acid; p-HA, polymeric hyaluronan; HA14, hyaluronan 14-mer.

Received for publication, February 4, 2005
Published, JBC Papers in Press, April 19, 2005, DOI 10.1074/jbc.M501332200

Printed in U.S.A.
flammmation-associated HA-binding protein composed mainly of contiguous Link and CUB modules (23–25) and appears to have an essential role in the transfer of HCs from Iol onto HA (7, 26, 27). Most importantly, Fülöp et al. (7) showed that Tsg-6"ΔC2" female mice are infertile due to their inability to form the HA-rich extracellular matrix that is essential for cumulus expansion, a phenotype that correlates with the total absence of HC-HA complexes in the oocytes of these animals; the administration of murine TSG-6, either as a recombinant protein or as a transgene, rescues the fertility of Tsg-6 null mice. In this study, HC3 (a component of P0I), in addition to HC1 and HC2, was missing from the cumulus matrix, indicating that TSG-6 is also necessary for the transfer of HC3 onto HA (7).

The expression of TSG-6 is up-regulated during COC expansion in the mouse and rat (27–33), where the protein has been shown to co-localize with HA and Iol in the cumulus matrix (30, 31). Western blot analyses revealed that TSG-6 is present as a free protein (~35 kDa) and as a species of ~120 kDa that is immunoreactive with both anti-TSG-6 and anti-Iol antibodies (30, 31). Characterization of this ~120-kDa band by mass spectrometry demonstrated that it contains TSG-6, HC1, and HC2, but not bikunin. On the basis of their molecular masses (i.e. TSG-6 is ~35 kDa, and each HC is ~80–85 kDa), the ~120-kDa band is thought likely to comprise a mixture of TSG-6-HC1 and TSG-6-HC2 complexes (31). These complexes are not sensitive to chondroitinase, indicating that the CS chain of Iol is not involved in their linkage, but they are cleaved by mild NaOH treatment, consistent with the presence of ester linkages. Therefore, the TSG-6-HC complexes may act as intermediates in the formation of HC-HA, as has been suggested (7). However, prior to the present study, this had not been investigated directly. Species of ~120 kDa can also be formed when human recombinant TSG-6 and human purified Iol are incubated together in vitro (27, 34, 35), and TSG-6-Iol complexes of this size have been detected in the synovial fluids of arthritis patients (36). However, the composition of the ~120-kDa species formed in vitro from human components, which was reported to contain TSG-6, HC2, and bikunin held together via a chondroitinase-sensitive linkage (34), indicates that it might represent a different type of complex (with TSG-6 replacing an HC on the CS chain) compared with that formed during ovulation in the mouse.

In this study, we have characterized the complexes formed in vitro between purified human Iol and recombinant human TSG-6 as TSG-6-HC1 and TSG-6-HC2 and have shown that they act as intermediates in the formation of HC-HA. This is accompanied by the production of bikunin-HC1 and bikunin-HC2 by-products, which break down to generate free bikunin and HCs. Both TSG-6-HC complex formation and subsequent HC transfer are metal ion-dependent processes, having a requirement for either Mg2+ or Mn2+. TSG-6, which is released upon the transfer of HCs from TSG-6 onto HA, was shown to combine with Iol to generate new TSG-6-HC complexes and thus acts as a true catalyst for the formation of HC-HA.

**EXPERIMENTAL PROCEDURES**

**Protein Expression and Purification**—Human full-length TSG-6 protein (Q allotype) was expressed in Drosophila Schneider-2 cells and purified to homogeneity as described (35), and the protein concentration was determined by amino acid analysis. Iol was purified from human serum (37), and its concentration was determined as described previously (38).

**Formation of TSG-6-Iol Complexes under Standard Conditions**—In the standard assay, recombinant full-length TSG-6 (80 µg/ml final concentration; 2.7 µM based on a molecular mass of 30 kDa) was incubated with Iol (320 µg/ml final concentration; 1.6 µM based on a molecular mass of 180 kDa) in 20 mM HEPES-HCl (pH 7.5), 150 mM NaCl, and 5 mM MgCl2 in a total volume of 25 µl for 2 h at 4 °C (i.e. on ice). The effects of protein concentration, temperature, ionic strength, pH, and metal ions on complex formation were investigated by varying these parameters individually while keeping all other conditions constant. Unless otherwise stated, 7.5 µl of each sample was analyzed on 10% (w/v) Tris/Tricine/SDS-polyacrylamide gels following reduction with 5% (v/v) β-mercaptoethanol in SDS protein sample buffer (5 min at 100 °C), and gels were stained with Coomassie Blue.

**Effect of TSG-6 and Iol Protein Concentrations on TSG-6-Iol Complex Formation**—The amount of TSG-6 in the assay was varied from 1 to 8 µg (40–320 µg/ml final concentration) while keeping the amount of Iol constant at 8 µg (320 µg/ml final concentration). Alternatively, between 8 and 32 µg (320–1280 µg/ml final concentration) of Iol was used in the presence of 1 µg of TSG-6 (40 µg/ml final concentration). As a control, TSG-6 (1 µg) or Iol (8 µg) was incubated alone under standard assay conditions. These samples were analyzed by SDS-PAGE and by Western blotting using a rabbit anti-human polyclonal antibody raised against TSG-6 (39) as described (35).

**Effect of Temperature, pH, and Ionic Strength on TSG-6-Iol Complex Formation**—The amount of metal ions during formation of the TSG-6-Iol complex was tested by incubating TSG-6 and Iol with 1 mM EDTA in the absence or presence of 5 mM MgCl2, CaCl2, CoCl2, or MnCl2. Additionally, CaCl2 and CoCl2 were co-incubated with MgCl2 in the absence or presence of EDTA (i.e. 5 mM MgCl2 with 1 mM CaCl2 or ScCl2, 5 mM MgCl2 with 5 mM CaCl2 or CoCl2, and 1 mM Mgl2 and 1 mM EDTA with 5 mM CoCl2). Complex formation was also investigated at a range of MgCl2 concentrations (i.e. 0.1, 0.5, 1.0, 10.0, and 30.0 mM). The effect of metal ions on TSG-6-Iol complex formation was also examined using serum as the source of Iol. Mouse serum (20 µl, Rockland Immunocerehal) was incubated with 2 µg of human recombinant TSG-6 in the presence or absence of 2 mM EDTA and 5 mM CaCl2, MgCl2, or CoCl2 in a 25-µl reaction volume at 37 °C for 2 h. Samples (6 µl) were run on 4–20% precast gels (Invitrogen) and analyzed by Western blotting as described previously (31) using a rabbit anti-human polyclonal antibody raised against TSG-6 (39).

**Effect of Chondroitinase and NaOH Treatment on TSG-6-Iol Complex Stability**—TSG-6-Iol complexes were formed under standard conditions (i.e. 2 h at 4 °C; see above). Aliquots of the reaction mixture (7.5 µl) or of Iol incubated in the absence of TSG-6 were then diluted with an equal volume of water and incubated with either 1 µl of chondroitinase (10 million units; Seikagaku Corp.) or 1.5 µl of 1 M NaOH (0.1 M final concentration) was then added, followed by incubation for 2 h at 37 °C or for 10 min at room temperature, respectively. 1.5 µl of 1 M HCl was added to the NaOH-treated samples. To these (and a chondroitinase-only control (10 million units in 15 µl of H2O) and an untreated TSG-6-Iol complex (7.5 µl + 7.5 µl of H2O), both incubated for 2 h at 37 °C) were added 15 µl of 2X SDS protein sample buffer, followed by SDS-PAGE (with the whole sample loaded) or Western blotting (with one-third of the sample loaded) as described above.

**Characterization of TSG-6-Iol Complexes by N-terminal Sequencing**—Complements were formed under standard conditions and run on 10% (v/v) Tris/Tricine/SDS-polyacrylamide gels with or without chondroitinase (10 million units) or NaOH (0.1 M final concentration) treatment essentially as described above, except that the “untreated” sample was not incubated at 37 °C for 2 h, and in all cases, twice as much protein was loaded on per lane. The gels were electroblotted onto Hybond-P membranes (Amersham Biosciences) in 10 mM CAPS (pH 11) and 5% (v/v) methanol at 100 V for 2 h. The membranes were stained with Coomassie Blue for 75 s, destained in 50% (v/v) methanol for 10 min, and air-dried. Bands were excised (with reference to the standard control gel) and subjected to protein sequencing on an Applied Biosystems Procise 494A protein sequencer using standard “pulsed liquid for polyvinylidene difluoride-bound peptides” sequencing cycles. Bands that did not yield any visible sequence were excised from an identical gel and subjected to in-gel digestion with trypsin, followed by mass spectrometric analysis as described previously (31).

**Formation and Characterization of Iol-HA Complexes**—TSG-6-Iol
Formation of TSG-6-Iol and Iol-HA Complexes in Vitro

complexes were formed under standard conditions (2 h at 4 °C), except that HA was included in the reaction; 1 μg of medical grade low molecular mass polymeric HA (p-HA; ~120 kDa; Genzyme Corp.) or 1 μg of HA 14-mer (HA14; 2673 Da) prepared as described (40) was added to the assay, and samples were analyzed by Tris/Tricine/SDS-PAGE and protein sequencing as described above. For sequence analysis of the p-HA sample, three identical lanes were loaded (7.5 μl) for that at 0 V) and staining/destaining as described above. Equivalent bands (at the interface of the stacking and resolving gels) were excised from the three lanes and combined for sequencing.

Samples containing p-HA (or Iol alone) were also treated with Streptomyces hyaluronidase (Seikagaku Corp.) prior to SDS-PAGE analysis. The reaction mixture (7.5 μl) was diluted with an equal volume of water to which 1 μl of enzyme (10 milliunits) was added, followed by incubation at 37 °C for 2 h. Iol treated with NaOH as described above was included as a control.

Effect of Divalent Metal Ions on Iol-HA Complex Formation—TSG-6-Iol complexes were preformed under essentially standard conditions in the presence of 0.109 mM MgCl2 for 2 h at 4 °C, followed by the addition of ~1 mM EDTA and incubation for an additional 30 min. Divalent metal ions (MgCl2, MnCl2, CaCl2, and CoCl2) and 1 μg of HA14 were then added (20 μl of HEPES-HCl (pH 7.5), 150 mM NaCl, 0.1 μg of HA14, 1 mM EDTA, 5 mM Mg2+, and 40 μg/ml HA14 (final concentrations); 25 μl final volume) and incubated for 2 h at 4 °C. Control experiments were also performed in the absence of HA, EDTA, or metal ions. Alternatively, TSG-6-Iol complexes were preformed in the presence of 5.4 mM MgCl2 (5 mM final concentration), and then CoCl2 was added (1 or 5 mM final concentration) and incubated for 30 min at 4 °C before adding 1 μg of HA14 and incubating at 37 °C for 4 °C. In another experiment, TSG-6, Iol, and HA14 were incubated together for 2 h at 4 °C in the absence and presence of 5 mM metal ion (MgCl2, MnCl2, CaCl2, or CoCl2), but without the inclusion of EDTA; co-incubation of 5 mM MgCl2, MnCl2, or CaCl2 with 5 mM CoCl2 was also performed. Gel samples were prepared from 7.5-μl reaction volumes as described above.

The effect of metal ions on the formation of Iol-HA complexes was also examined using serum as the source of Iol. Mouse serum (5 ml), 5 μg of high molecular mass HA (Healon GV, Pharmacia-Upjohn), and 250 ng of human recombinant TSG-6 were incubated for 24 h at 37 °C in the presence or absence of 2 ml EDTA and 5 ml CaCl2, MgCl2, or CoCl2 in 50 μl of phosphate-buffered saline. Aliquots of each reaction mixture (10 μl) were treated with 200 milliunits of Streptomyces hyaluronidase for 1 h at 37 °C. Hyaluronidase-digested and untreated samples were run on 4–20% precast gels and analyzed by Western blotting as described previously (31) using anti-Iol polyclonal antibody (Dako Corp.).

Requirement of TSG-6-Iol Complexes as Precursors in the Formation of Iol-HA—TSG-6-Iol complexes were preformed under essentially standard conditions (2 h at 4 °C) and then incubated at 4 °C for a range of times (0, 2, 4, and 22 h) before the addition of 1 μg of HA14, 1 μl of Tris/Tricine/SDS-PAGE, and 150 mM NaCl. Thus, at 37 °C, a band of ~80 kDa (corresponding to the running position of HC1) was also observed and is labeled I. Equivalent experiments done using the R allotype of TSG-6 (35) gave identical results (not shown).

FIG. 1. TSG-6-Iol complexes form rapidly at 4 and 37 °C. TSG-6 (Q allotype) and Iol were incubated together for various times at 4 °C (upper panel) or 37 °C (lower panel) and analyzed on Tris/Tricine/SDS-polyacrylamide gels stained with Coomassie Blue. Comparison of these reaction mixtures with TSG-6 or Iol proteins incubated alone for 2 h revealed that they contained three novel species: an ~130-kDa band (designated species A) and another ~120-kDa doublet, where species B and C are the upper and lower bands, respectively. At 37 °C, a band of ~80 kDa (corresponding to the running position of HC1) was also observed and is labeled I. Equivalent experiments done using the R allotype of TSG-6 (35) gave identical results (not shown).

as 30 s. Maximal amounts of the B/C doublet were formed after ~15 min at 37 °C and after ~60 min at 4 °C. At the higher temperature, an additional band of ~80 kDa (labeled I in Fig. 1) accumulated over time. This species ran at an identical position compared with HC1, and N-terminal sequencing revealed that it contained both HC1 and HC2 at an ~5:1 ratio (data not shown). Band I could therefore result from breakdown of the TSG-6-Iol complex or of other HC-containing species (see below). In this regard, an ~80-kDa band (albeit much fainter) and a band of the same apparent molecular mass as species A were also seen when Iol was incubated alone for 2 h at 37 °C, indicating that there was some breakdown of Iol at this temperature. Additionally, at 37 °C, a high molecular mass doublet (near the top of the gel) also accumulated over time during the incubation of TSG-6 with Iol, but significant amounts of this were not formed at 4 °C; this doublet was recently identified as a high molecular mass species of Iol containing additional HCs (41). Therefore, in subsequent experiments, incubations were generally carried out for 2 h at 4 °C to maximize Iol-TSG-6 complex formation while minimizing any production of high molecular Iol species or of degradative reactions.

Determining the Optimal Conditions for TSG-6-Iol Complex Formation—The effect of varying the protein concentration on the formation of the TSG-6-Iol complex (i.e. B/C doublet) was investigated as described under “Experimental Procedures.” Similar amounts of the B/C doublet were formed when 8 μg of Iol was incubated with between 2 and 8 μg of TSG-6, whereas less was seen under the other conditions tested (i.e. 1 μg of
TSG-6 in the presence of 8–32 μg of Iol (Supplemental Fig. S1). Assays conducted at different protein concentrations (e.g. 2 or 4 μg of TSG-6 with 16 μg of Iol) (data not shown) did not lead to significantly increased levels of the B/C doublet. Therefore, 2 μg of TSG-6 and 8 μg of Iol (i.e. the lowest concentrations that gave close to maximal complex formation) were used as the standard experimental conditions so as to minimize the amount of protein used in each assay.

From Fig. 2, it is clear that species A–C did not form when TSG-6 and Iol were incubated at pH 4.0 or 5.0 (under otherwise standard conditions). There also appeared to be somewhat less complex formation at pH 6.0 compared with pH 6.5–8.0, which all led to similar levels of the B/C doublet. Experiments at pH 7.5 in which the ionic strength was varied revealed that there was a reduction in the amount of TSG-6 complex formed between 100 and 300 mM NaCl (Fig. 2). From Fig. 2, it is clear that species A–C did not form when TSG-6 and Iol were incubated at pH 4.0 or 5.0 (under otherwise standard conditions). There also appeared to be somewhat less complex formation at pH 6.0 compared with pH 6.5–8.0, which all led to similar levels of the B/C doublet. Experiments at pH 7.5 in which the ionic strength was varied revealed that there was a reduction in the amount of TSG-6 complex formed between 100 and 300 mM NaCl (Fig. 2).

Preliminary experiments demonstrated that, in the absence of added metal ion, neither band A nor the B/C doublet was formed (Supplemental Fig. S2). However, these species were seen at similar levels when MgCl₂ was included in the reaction mixture at a wide range of concentrations (0.1–20 mM). MgCl₂ at the nominal concentration of 5 mM was chosen as the standard.

The above experiments therefore allowed us to determine the conditions under which optimal amounts of TSG-6-Iol complex could be formed. Under the standard conditions chosen (i.e. 80 μg/ml TSG-6 and 320 μg/ml Iol in 20 mM HEPES-HCl (pH 7.5), 150 mM NaCl, and 5 mM MgCl₂), which are close to physiological, Iol was at a concentration similar to that found in normal human serum (i.e. 400–500 μg/ml (42)).

**Effect of Divalent Metal Ions on TSG-6-Iol Complex Formation**—As described above, TSG-6-Iol complexes could be formed in the presence of MgCl₂, but did not form in the absence of added metal ion (Supplemental Fig. S2). Consistent with this, there was no complex formation in 0.1–50 mM EDTA (data not shown). Therefore, the effects of other divalent cations were investigated; TSG-6 and Iol were incubated under standard conditions with 5 mM M⁹⁺ (i.e. MgCl₂, CaCl₂, CoCl₂, or MnCl₂) and 1 mM EDTA, which was added to chelate any metal ion impurities. Fig. 3 shows that complex formation occurred with MgCl₂ or MnCl₂, but the B/C doublet was not seen in the presence of the other metal ions (e.g. Ca²⁺). It should be noted that experiments conducted in the absence of any EDTA also demonstrated that either Mg²⁺ or Mn²⁺ (but not Ca²⁺ or Co²⁺) ions could support complex formation (data not shown). Interestingly, co-incubation of 5 mM CoCl₂ with 1 mM MgCl₂, which was sufficient for optimal complex formation (Supplemental Fig. S2), was found to completely inhibit production of TSG-6-Iol (Fig. 3). Assays including both CaCl₂ (1 or 5 mM) and MgCl₂ (5 mM) showed that Ca²⁺ ions had no such inhibitory effect regardless of whether the experiments were done at 4 or 37 °C (data not shown).

Experiments conducted with mouse serum as the source of Iol showed that TSG-6-Iol formed without any requirement for additional metal ions (Supplemental Fig. S3). When EDTA (2 mM) was included in the reaction mixture, complex formation was completely inhibited, which is consistent with the results obtained with purified components (see above). Addition of 5 mM MgCl₂ to these assays rescued the formation of TSG-6-Iol as expected. Interestingly, 5 mM CaCl₂ or CoCl₂ in the presence of 2 mM EDTA also led to complex formation (Supplemental Fig. S3). The likely explanation for these results is that the addition of Ca²⁺ or Co²⁺ releases sufficient Mg²⁺ from the EDTA (or serum proteins) to allow the reaction to proceed; this is consistent with the log K₁ values for the binding of these metal ions to EDTA.

**Characterization of the TSG-6-Iol Complex**—As described above, the incubation of TSG-6 and Iol under standard conditions led to the formation of three major species, i.e. the B/C doublet of ~120 kDa (band 2), which was immunoreactive with anti-TSG-6 antibody, and the ~130-kDa species A (band 1) (Fig. 4). These species were characterized by N-terminal sequencing (Supplemental Fig. S4). This analysis revealed that...
species A (band 1) contained the three protein chains of I/H9251 (i.e. bikunin, HC1, and HC2) (Table I). However, no TSG-6 was detected, consistent with its lack of immunoreactivity with anti-TSG-6 antibody. Band 2, which was cut into upper and lower portions corresponding to species B and C, respectively, contained TSG-6 in addition to both HCs (but no bikunin). As shown in Table I, the upper band contained TSG-6 and HC2 in similar amounts (as based on the initial sequencing yields), with about half as much HC1 present. The converse was observed for the lower band, which gave equivalent initial yields of TSG-6 and HC1, but less HC2. Therefore, from this analysis and considering the molecular masses of TSG-6 and the three chains of Iol (i.e. bikunin, HC1, and HC2) (Table I). However, no TSG-6 was detected, consistent with its lack of immunoreactivity with anti-TSG-6 antibody. Band 2, which was cut into upper and lower portions corresponding to species B and C, respectively, contained TSG-6 in addition to both HCs (but no bikunin). As shown in Table I, the upper band contained TSG-6 and HC2 in similar amounts (as based on the initial sequencing yields), with about half as much HC1 present. The converse was observed for the lower band, which gave equivalent initial yields of TSG-6 and HC1, but less HC2. Therefore, from this analysis and considering the molecular masses of TSG-6 and the three chains of Iol, it seems likely that species B and C correspond to complexes of TSG-6 with HC2 and HC1, respectively, whereas species A comprises a mixture of bikunin linked to one or the other of the HCs; given the proximity of species B and C (Supplemental Fig. S4), it is perhaps not surprising that both HC1 and HC2 were detected in these bands.

**Effect of Chondroitinase and NaOH on TSG-6/Iol Complex Stability**—The TSG-6/Iol complexes were formed under standard conditions and then treated with either chondroitinase ABC lyase (Ch'ase) or NaOH. These samples (along with untreated complex, Iol, and chondroitinase ABC lyase controls) were analyzed by SDS-PAGE (upper panel) or Western blotting with anti-TSG-6 antibody (lower panel). Numbered bands (bracketed) indicate species that were characterized by protein sequencing following electroblotting of an essentially equivalent gel onto a Hybond-P membrane (Supplemental Fig. S4). The chondroitinase enzyme (species 10, which was positively identified by mass spectrometry (59 peptides) (data not shown), but did not give any detectable N-terminal sequence) ran at a position between species 4 and 5 (i.e. the TSG-6/HC2 and TSG-6/HC1 complexes, respectively).

**Fig. 4.** TSG-6/Iol complexes are degraded by NaOH, but are insensitive to chondroitinase treatment. TSG-6/Iol complexes were formed under standard conditions and then treated with either chondroitinase ABC lyase (Ch'ase) or NaOH. These samples (along with untreated complex, Iol, and chondroitinase ABC lyase controls) were analyzed by SDS-PAGE (upper panel) or Western blotting with anti-TSG-6 antibody (lower panel). Numbered bands (bracketed) indicate species that were characterized by protein sequencing following electroblotting of an essentially equivalent gel onto a Hybond-P membrane (Supplemental Fig. S4). The chondroitinase enzyme (species 10, which was positively identified by mass spectrometry (59 peptides) (data not shown), but did not give any detectable N-terminal sequence) ran at a position between species 4 and 5 (i.e. the TSG-6/HC2 and TSG-6/HC1 complexes, respectively).
as the more widely separated bands 4 and 5 (with chondroitinase running between them). N-terminal sequence analysis of these species (Table I) showed that bands 4 and 5 correspond to complexes of TSG-6 with HC2 and HC1, respectively (i.e. these are equivalent to the upper and lower bands of species 2); in bands 4 and 5, there was only a relatively small amount of the alternative HC detected (i.e. 9 and 22%, respectively), presumably due to the slightly better separation of these bands (Supplemental Fig. S4). The reason underlying this improved separation is not clear, but it could result simply from the removal of higher molecular mass species (i.e. intact Iα1 and species A) or the presence of the chondroitinase enzyme itself, altering the mobility of species B and C, rather than necessarily being due to removal of chondroitin sulfate from the TSG-6-HC complexes.

**Model of TSG-6-HC Complex Formation**—The protein sequence analysis of the TSG-6-Iα1 reaction products and characterization of the species formed following treatment with chondroitinase or NaOH described above have allowed us to generate a model for TSG-6-Iα1 complex formation. As shown in Fig. 5, incubation of TSG-6 and Iα1 in the presence of metal ions (Mg²⁺ or Mn²⁺) leads to the formation of either a TSG-6-HC1 or TSG-6-HC2 complex (i.e. species C or B, respectively). This is likely to occur via a transesterification reaction in which the ester bond linking one or the other HC to CS is transferred onto TSG-6. Consistent with this, the TSG-6-HC complexes are degraded by mild NaOH treatment (to yield free HCs), whereas they are refractory to digestion with chondroitin ABC lyase. Via this mechanism, formation of TSG-6-HC1 would leave HC2 still attached to bikunin via the CS chain (i.e. bikunin-HC2) as a by-product, whereas if the TSG-6-HC2 complex were formed, then bikunin-HC1 would be left over. In this regard, species A (band 1 in Fig. 4) is likely to represent a mixture of the bikunin-HC1 and bikunin-HC2 by-products because sequencing revealed that this species contained a similar amount of bikunin (2.19 pmol) compared with the combined HCs (2.35 pmol) and that it was susceptible to cleavage with chondroitinase. In this model, there would be no cleavage of the CS chain upon transfer of an HC onto TSG-6 and consequently no CS "stub" left attached to either of the TSG-6-HC complexes. Overall, this model is similar to that proposed recently by Sanggaard et al. (41).

**TSG-6 Mediates the Transfer of HCs onto HA**—Recently, it was found that covalent Iα1-HA complexes do not form in the presence of metal ions (Mg²⁺ and Mn²⁺) and that it was susceptible to cleavage with chondroitinase. In this model, there would be no cleavage of the CS chain upon transfer of an HC onto TSG-6 and consequently no CS “stub” left attached to either of the TSG-6-HC complexes. Overall, this model is similar to that proposed recently by Sanggaard et al. (41).

Table I

| Sequence       | Initial yield | Protein |
|----------------|---------------|---------|
| Band 1 (species A) | AVLPQEEE      | Bikunin |
|                | 2.19          | HC2     |
|                | 1.58          | HC1     |
|                | 0.77          | HC1     |
| Band 2, upper (species B) | SLPGSEE       | TSG-6   |
|                | 0.85          | HC2     |
|                | 0.70          | TSG-6   |
|                | 0.39          | HC1     |
| Band 2, lower (species C) | SATORSGKS     | HC1     |
|                | 1.43          | HC1     |
|                | 1.42          | TSG-6   |
|                | 0.80          | HC2     |
| Band 3         | No visible sequence |        |
| Band 4         | WQFDG11F      | TSG-6   |
|                | 2.13          | HC2     |
|                | 1.72          | HC2     |
|                | 0.15          | HC1     |
| Band 5         | SATORSGKS     | HC1     |
|                | 1.29          | HC1     |
|                | 1.26          | TSG-6   |
|                | 0.29          | HC2     |
| Band 6         | SLPGSEE       | HC2     |
|                | 3.73          | HC2     |
|                | 0.41          | HC1     |
| Band 7         | SATORSGKS     | HC1     |
|                | 4.01          | HC1     |
|                | 0.17          | HC2     |
| Band 8         | SLPGSEE       | HC2     |
|                | 5.22          | HC2     |
|                | 0.42          | HC1     |
| Band 9         | SATORSGKS     | HC1     |
|                | 6.07          | HC1     |
|                | 0.24          | HC2     |
| Band 10        | No visible sequence |        |
| Band 11        | SATORSGKS     | HC1     |
|                | 0.91          | HC1     |
|                | 0.83          | HC2     |
|                | 4.03          | HC1     |
| Band 12        | SLPGESEE      | HC2     |
|                | 0.16          | HC2     |
sequencing showed to contain mainly HC1, but also a small amount of HC2 (Table I). Mass spectrometric analysis confirmed that both species were present, perhaps with more HC2 than indicated by sequence analysis (36 and 12 peptides of HC1 and HC2 identified, respectively) (data not shown). Given the different mobilities of HC1 and HC2 upon SDS-PAGE, it is surprising that both HC/H18528 complex should run at identical positions. However, when a longer HA oligomer (i.e. HA32-mer) was used, this also gave rise to a single novel species of HC/H11011 at 90 kDa (i.e. a higher apparent molecular mass than that of either HC1 or HC2). Mass spectrometry of this band identified 36 and 15 peptides of HC1 and HC2, respectively (data not shown), whereas amino acid sequencing indicated that there was much more HC1 (93%) than HC2 (7%). Importantly, the finding that the size of the HA oligomer used has a significant effect on the apparent molecular mass of the released HCs is consistent with the formation of stable complexes between the HCs and HA. It therefore seems likely that both HC1 and HC2 can become covalently linked to HA oligosaccharides and that this assay can be used to visualize the TSG-6-mediated transfer of HCs onto HA. In this regard, co-incubation of different w/w ratios of HA14 and p-HA with a TSG-6/I/H9251 mixture revealed that both HC1 and HC2 are likely to be as good substrates for HC transfer, i.e. the high molecular mass preparation did not lead to preferential transfer of HCs onto HA.

Fig. 5. Model of TSG-6/Iol complex formation. Iol consists of three protein chains (HC1, HC2, and bikunin) linked by a GS glycosaminoglycan. Both HCs contain von Willebrand factor A domains (blue). TSG-6 is composed mainly of contiguous Link and CUB modules (cyan and purple, respectively). TSG-6 and Iol can react in the presence of Mg2+ or Mn2+ ions to form one of two complexes (either TSG-6-HC1 or TSG-6-HC2), where bikunin-HC2 and bikunin-HC1, respectively, are by-products of this reaction. The position of the linkage between TSG-6 and the HCs (probably an ester bond) is shown between the C terminus of the HC (41) and the Link module of TSG-6 (see “Discussion”).

Fig. 6. Formation of HC-HA complexes in vitro from purified components. The left panel shows an SDS-polyacrylamide gel on which TSG-6 and Iol were incubated under standard assay conditions, but with the additional inclusion of low molecular mass p-HA or a defined oligosaccharide (HA14). The novel bands (i.e. bands 11 and 12) were identified by N-terminal sequencing and/or mass spectrometry (Table I) as a high molecular mass HC-HA complex (containing both HCs) and an HC-HA14 complex, respectively. Species II (∼160 kDa), which formed in the presence of HA14, was shown by mass spectrometry to contain both HC1 and HC2 (19 and 22 peptides, respectively) and is therefore likely to represent a complex in which both HCs are attached to the same HA oligomer. The right panel shows the results from SDS-PAGE analysis of TSG-6/Iol/p-HA reaction mixtures or an Iol control following treatment with (+) and without (−) Streptomyces hyaluronidase (H’ase). Iol degraded with NaOH was used to show the positions of HC1 and HC2.

Fig. 7. HC transfer assay containing different ratios of p-HA and HA14. TSG-6 and Iol were incubated with 1 µg of p-HA under standard conditions with variable amounts of competing HA14 (0–10 µg). When equal quantities of p-HA and HA14 were used, both a high molecular mass HC-HA complex and an HC-HA14 complex were formed in approximately equal amounts (i.e. based on intensity of Coomassie Blue staining). When p-HA was present in a w/w 10-fold excess over HA14, only the HC-HA complex was seen, and conversely, when the oligosaccharide was in excess, only the HC-HA14 complex was generated. This indicates that p-HA and HA14 are likely to be similarly efficient substrates for HC transfer, i.e. the high molecular mass preparation did not lead to preferential transfer of HCs onto HA.

Fig. 5. Model of TSG-6/Iol complex formation. Iol consists of three protein chains (HC1, HC2, and bikunin) linked by a GS glycosaminoglycan. Both HCs contain von Willebrand factor A domains (blue). TSG-6 is composed mainly of contiguous Link and CUB modules (cyan and purple, respectively). TSG-6 and Iol can react in the presence of Mg2+ or Mn2+ ions to form one of two complexes (either TSG-6-HC1 or TSG-6-HC2), where bikunin-HC2 and bikunin-HC1, respectively, are by-products of this reaction. The position of the linkage between TSG-6 and the HCs (probably an ester bond) is shown between the C terminus of the HC (41) and the Link module of TSG-6 (see “Discussion”).

Fig. 6. Formation of HC-HA complexes in vitro from purified components. The left panel shows an SDS-polyacrylamide gel on which TSG-6 and Iol were incubated under standard assay conditions, but with the additional inclusion of low molecular mass p-HA or a defined oligosaccharide (HA14). The novel bands (i.e. bands 11 and 12) were identified by N-terminal sequencing and/or mass spectrometry (Table I) as a high molecular mass HC-HA complex (containing both HCs) and an HC-HA14 complex, respectively. Species II (∼160 kDa), which formed in the presence of HA14, was shown by mass spectrometry to contain both HC1 and HC2 (19 and 22 peptides, respectively) and is therefore likely to represent a complex in which both HCs are attached to the same HA oligomer. The right panel shows the results from SDS-PAGE analysis of TSG-6/Iol/p-HA reaction mixtures or an Iol control following treatment with (+) and without (−) Streptomyces hyaluronidase (H’ase). Iol degraded with NaOH was used to show the positions of HC1 and HC2.

Fig. 7. HC transfer assay containing different ratios of p-HA and HA14. TSG-6 and Iol were incubated with 1 µg of p-HA under standard conditions with variable amounts of competing HA14 (0–10 µg). When equal quantities of p-HA and HA14 were used, both a high molecular mass HC-HA complex and an HC-HA14 complex were formed in approximately equal amounts (i.e. based on intensity of Coomassie Blue staining). When p-HA was present in a w/w 10-fold excess over HA14, only the HC-HA complex was seen, and conversely, when the oligosaccharide was in excess, only the HC-HA14 complex was generated. This indicates that p-HA and HA14 are likely to be similarly efficient substrates for HC transfer, i.e. the high molecular mass preparation did not lead to preferential transfer of HCs onto HA.
TSG-6-mediated Transfer of HCs onto HA Is Metal Ion-dependent—Preliminary experiments demonstrated that, when TSG-6, IaI, and HA14 were incubated in the presence of EDTA under otherwise standard conditions, neither TSG-6-HC (species B/C) nor HC-HA14 was formed (data not shown). Therefore, to test the metal ion dependence of HC transfer onto HA, it was necessary to preform TSG-6-HC complexes, which was done in the presence of 0.109 mM MgCl2, prior to the subsequent addition of 1 mM EDTA, followed by the addition of HA14 in the absence and presence of various metal ions (see “Experimental Procedures”). In control experiments in which HA14 was added to preformed TSG-6-HC complexes in the absence of EDTA (i.e. in reactions containing a final concentration of 0.1 mM MgCl2), a band corresponding to HC-HA14 was seen (Fig. 8, upper panel, lane 1), whereas in the presence of 1 mM EDTA, no HC-HA14 was formed (lane 3). This clearly shows that the formation of HC-HA14 complexes requires the presence of metal ions and is inhibited by EDTA. As shown in Fig. 8 (upper panel), when HA14 was added in the presence of MgCl2, MnCl2, or CaCl2, the HC-HA14 complex formed. In a separate experiment in which TSG-6, IaI, and HA14 were all incubated together under standard conditions with 5 mM MgCl2, MnCl2, CaCl2, or CoCl2 in the absence of EDTA, the HC-HA14 complex (and the TSG-6-HC complex) formed only in the presence of Mg2+ or Mn2+ ions (Fig. 8, lower right panel). This demonstrates that Mg2+ or Mn2+ can support both TSG-6-HC complex formation and HC transfer, whereas Ca2+ or Co2+ alone does not give rise to either of these products. Therefore, in the experiment shown in Fig. 8 (upper panel), it is likely that transfer (i.e. formation of HC-HA14) occurred in the presence of Ca2+ due to its displacement of Mg2+ ions from the EDTA rather than having a direct effect on the reaction; this is not surprising given the much larger log Kf for the binding of Ca2+ to EDTA compared with Mg2+. However, the possibility that Ca2+, although not supporting the formation of the TSG-6-HC complex (Fig. 3), could be involved in subsequent HC transfer cannot be entirely ruled out.

Addition of CoCl2 to preformed TSG-6-HC complexes inhibited the subsequent formation of HC-HA14 (Fig. 8, upper panel). Interestingly, significant inhibition of the transfer reaction by CoCl2 was seen even when MgCl2 was present at a 5-fold higher concentration (i.e. 1 mM CoCl2 and 5 mM MgCl2) (Fig. 8, lower left panel). Co-incubation of TSG-6, IaI, and HA14 with 5 mM MgCl2 or MnCl2 in the presence of an equimolar concentration of CoCl2 did not lead to the formation of HC-HA14 or TSG-6-HC complexes (data not shown). These data indicate that Co2+ ions are potent inhibitors of TSG-6-mediated transfer of IaI HCs onto HA, even in the presence of Mg2+, presumably due to their tighter binding to the metal ion center involved in this reaction.

The requirement for divalent metal ions during HC transfer was also examined using mouse serum as the source of IaI and high molecular mass HA by Western blotting with anti-IaI antibody to visualize the species formed with or without treatment with Streptomyces hyaluronidase (Fig. 9). This showed that, in the absence of EDTA or additional divalent cations (i.e. with just the metal ions present in serum), the only species detected was either a high molecular mass smear corresponding to HC-HA complexes of polydispersed molecular masses (Fig. 9A) or a free HC (Fig. 9B) depending on whether the samples were untreated or hyaluronidase-digested, respec-
FIG. 9. Metal ion dependence of TSG-6-mediated transfer of HC onto HA using mouse serum as the source of Iol. Mouse serum, high molecular mass HA, and human recombinant TSG-6 were incubated at 37 °C in the presence or absence of 2 mM EDTA and 5 mM Ca\(^{2+}\), Mg\(^{2+}\), or Co\(^{3+}\). Untreated samples of these reaction mixtures (A) or those digested with Streptomyces hyaluronidase (H'ase) (B) were run on SDS-polyacrylamide gels and analyzed by Western blotting using a polyclonal antibody raised against Iol SDS-PAGE analysis of HC-HA\(_{14}\) complexes formed from preformed TSG-6-HC complexes that had been incubated for various times before the addition of HA. TSG-6-HC complexes were preformed under standard conditions (i.e. 2 h at 4 °C) and then incubated at 4 °C for 0, 2, 4, or 22 h before the addition of HA (labeled +). The TSG-6-HC complexes were visible in reactions containing HA (Fig. 10), was found to contain HC1 and HC2 at an intensity of species A (i.e. bikunin-HC by-products) was unaffected. In addition, in transfer experiments in which high concentrations of HA (e.g. 10 μg of p-HA or HA\(_{14}\)) were incubated with TSG-6 and Iol under otherwise standard conditions, very low amounts of TSG-6-HC complexes were visible. Again, the bikunin-HC complexes were present in normal amounts (Fig. 7). These data demonstrate that the TSG-6-HC complexes are consumed upon the formation of HC-HA, i.e. they are likely to act as intermediates in the transfer of HCs onto HA. To investigate this further and to determine the stability of the TSG-6-HC complexes, these were formed under standard conditions and then incubated for a range of times (i.e. 0, 2, 4, and 22 h) before the addition of HA (or water) and an additional incubation of 2 h. As shown in Fig. 10, in the absence of HA, there was no reduction in the amount of TSG-6-HC complex (B/C doublet) even after the maximal time of incubation (26 h in total); in fact, species B/C was more intense than at the former time points. This shows that the TSG-6-HC complexes are stable over relatively long time scales. However, a band of ~80 kDa (labeled I in Fig. 10) became more intense over time, and this may be the same species as the ~80-kDa band seen in reactions mixtures of TSG-6 and Iol incubated at 37 °C (Fig. 1, lower panel). Given the stability of the TSG-6-HC complexes, it seems probable that this species results from the breakdown of Iol and the bikunin-HC by-products. Upon sequencing, this band, which was also present in the HA-containing samples (Fig. 10), was found to contain HC1 and HC2 at an ~2:1 ratio (data not shown). The molecular mass of this band and the fact that it was formed in both the absence and presence of HA indicate that it is likely to correspond to free HCs, although it is not clear why HC1 and HC2 should migrate together; in both...
TSG-6 may be able to form new complexes with IHC onto HA led to the hypothesis that such molecules of TSG-6 may be released upon the transfer of HCs from TSG-6 to HA. TSG-6 was present at 0.27 or 0.054 μM for a total of 26 h (i.e. the 22-h time point in Fig. 10); the HC-HA14 complex was seen at all incubation times, along with a decrease in the intensity of the B/C doublet compared with reactions containing no oligosaccharide. This suggests that TSG-6 is a catalyst for HC transfer—The observation that TSG-6 can be recycled upon the transfer of HCs from TSG-6 to HA led to the hypothesis that such molecules of TSG-6 may be able to form new complexes with IHC and thus can be recycled. If this were the case, then it would be expected that suboptimal concentrations of TSG-6 should support, over time, the formation of significant amounts of HC-HA, given that neither IHC nor HA is limiting. To test this possibility, standard amounts of IHC (2.7 pmol of 1.8 μM) and HA14 (15 μM) were incubated with various concentrations of TSG-6 for 2, 4, or 22 h. Experiments conducted at 4 °C showed that, when TSG-6 was present at 0.27 or 0.054 μM (i.e. 10- and 50-fold lower concentrations than in the standard assay), very little, if any, HC-HA14 complex was formed after 2 or 4 h (data not shown); some transfer occurred with 0.27 μM TSG-6 (but not 0.054 μM) after 22 h.

However, at 37 °C, a small amount of HC-HA14 complex was formed after 2 h even in the presence of the lowest concentration of TSG-6 (0.054 μM); and after 4 or 22 h, a significant amount of HC-HA14 was apparent (Fig. 11). Fig. 11 shows that, although there was a relatively constant amount of HC-HA14 generated between 2 and 22 h in reactions containing 2.7 μM TSG-6, when TSG-6 was present at lower concentrations, the HC-HA14 complex accumulated over time, with the concomitant disappearance of IHC. Importantly, in the samples containing 0.27 or 0.054 μM TSG-6, no HC-HA6 complexes (i.e. B/C doublet) were seen. It is clear, however, that TSG-6-HC complexes had been formed because species A, corresponding to the bikunin-HC by-products of this reaction (Fig. 5), were present. These data indicate that any HC-HA6 complexes made are converted into HC-HA14 and that the TSG-6 released is indeed recycled. If all of the IHC (45 pmol at 1.8 μM) was consumed, then a maximum of 45 pmol at HC-HA14 could be generated (i.e. one molecule of IHC is converted into one molecule of either HC1-HA14 or HC2-HA14), which would require 45 pmol of TSG-6-HC1/6-HC2 complexes to be formed and converted. Although not all of the IHC was consumed in any of these experiments, Fig. 11 shows that ~50% of the IHC disappeared after 22 h in the presence of 0.054 μM TSG-6 (1.3 pmol). This corresponds to the formation of >20 pmol of HC-HA14, which is >15-fold the amount of TSG-6 present.

Therefore, the above data show that TSG-6 can be recycled. Thus, it not only acts as an essential cofactor for HC transfer, but is also a true catalyst for this reaction, as illustrated in Fig. 12.

DISCUSSION

We have shown here that the incubation of human recombinant TSG-6 with purified human IHC led to the formation of TSG-6-IHC1 and TSG-6-IHC2 complexes with the generation of the corresponding bikunin-HC2 and bikunin-HC1 by-products in a pH-, salt strength-, and metal ion-dependent manner. Our characterization of the TSG-6-IHC complexes and bikunin-HC by-products and the mechanism of complex formation we have proposed (Fig. 5) (43) agree well with a recent study (41). Earlier work from Wisniewski et al. (34) suggested that the TSG-6-IHC complexes formed in vitro consist of TSG-6-bikunin-HC2 and that this complex is susceptible to degradation by chondroitinase, which does not agree with our data. Furthermore, Sanggaard et al. (41) demonstrated that, in the TSG-6-HC2 complex at least, TSG-6 is linked directly to the C-terminal aspartic acid of HC2, providing definitive evidence that TSG-6-HC2 complexes are covalent in nature, as was suspected (31). Given our previous finding that monoclonal antibody A38, which recognizes an epitope in the TSG-6 Link module (44), can inhibit the formation of the TSG-6-HC complexes (27), it seems likely that this domain is directly involved in complex formation and may be the site of covalent attachment (as shown in Fig. 5). However, the Link module alone is unable to form a covalent complex with IHC (45), indicating that other regions of TSG-6 are also necessary for this process (see below).

Sanggaard et al. (41) also observed and characterized high molecular mass species that are likely to correspond to IHC with...
out. The formation of bikunin in this way is likely to be of biological relevance because the inhibition of certain serine proteases occurs only when this inhibitor is in its free state (e.g. tissue kallikrein in the context of asthma).2

We have demonstrated here that TSG-6-HC complexes act as intermediates in the formation of HC-HA. It is likely that this also involves a transesterification reaction in which the ester bond linking a HC to TSG-6 is transferred onto HA; it has been shown previously that HCs become attached to HA via an ester bond between the carboxylates of their C-terminal aspartic acid residues and the C-6 hydroxyls of GlcNAc residues (3). In other words, the TSG-6-mediated formation of HC-HA complexes requires two sequential transesterification reactions involving the “same” ester bond at the C terminus of the HC. Given that, in our assays, the only protein components present were TSG-6 and Iol, it is probable that this transesterase activity is located in one or the other (or both) of these molecules. It is very unlikely that this activity could be due to a contaminating enzyme because we have used many different preparations of these proteins and have seen no variability in the formation of either TSG-6-HC or HC-HA. In this regard, our results indicate that TSG-6-HC complex formation and HC transfer onto HA are metal ion-dependent processes with a requirement for either Mg2+ or Mn2+ and are inhibited by cobalt. The similarity of their metal ion dependences (and inhibition) suggests that the same metal ion center may be involved in both reactions. Divalent metal ion-binding sites are likely to be present in the HCs because they each have a single von Willebrand factor A domain (Figs. 5 and 12) containing the metal ion-dependent adhesion site consensus sequence known to support the binding of Mg2+/Mn2+ in other proteins (46). Interestingly, TSG-6 is also likely to bind metal ions. Recent structural studies on complement C1s have revealed that its CUB1 module contains a Ca2+/Mg2+-binding site, which would also be expected to accommodate Mn2+, where the residues involved in metal ion coordination are also conserved in the CUB module of TSG-6 (47). Further work will be necessary to determine whether these metal ion-binding sites in Iol and TSG-6 are involved in the transesterification processes. It should be noted that, although our data clearly show that Ca2+ ions do not support the formation of TSG-6-HC complexes and are not a requirement for HC transfer, we cannot completely rule out their involvement in the second transesterification step.

It has been reported previously that Ca2+ ions are in fact essential for the coupling of Iol to HA and that Mg2+ ions are not involved (48), which is at odds with our findings. The reason for this is not clear, but may relate to the fact that the previous study was conducted in synovial fluid containing EDTA, where it is likely that the addition of Ca2+ ions may release sufficient Mg2+ from the EDTA or the various proteins present to allow the reaction to proceed. We also found that the formation of TSG-6-HC complexes in serum/EDTA could be rescued by the addition of CaCl2 (Supplemental Fig. S3), even though it is clear that Ca2+ does not support this reaction in assays containing just the purified proteins and no additional metal ions (Fig. 3).

A major issue relating to our proposed model of HC-HA formation is the question of where the energy that drives these reactions comes from. Although the generation of both TSG-6-HC and HC-HA complexes occurred rapidly even at 4 °C (e.g. HC-HA was visible after only 30 s) (data not shown), these

---

2. R. Forteza, S. Casalino-Matsuda, C. M. Milner, M. E. Monzon-Medina, and A. J. Day, manuscript in preparation.
processes are temperature-dependent because they did not occur efficiently at 4 °C when the amount of TSG-6 was limiting, unlike the situation at 37 °C (Fig. 11). However, although heat energy is required, the major driving force underlying the formation of a new ester bond must be derived from the fission of the existing covalent linkage. In other words, the energy of these processes is stored in the CS/HC linkages of the IaI molecule (15, 16), which are formed intracellularly during its biosynthesis (49). Consistent with this, we have found that incubating individual HC1 or HC2 (alone or together) with TSG-6 did not lead to either TSG-6/HC or HC-HA complex formation in the absence or presence of HA (data not shown).

The composition of TSG-6/IaI complexes (i.e. TSG-6/HC1 and TSG-6/HC2) made from human proteins in vitro and their sensitivity to NaOH, but not chondroitinase, indicate that they are likely to be the same as those characterized previously from murine COCs in vivo (31). Therefore, the same processes of cumulus matrix expansion may occur in both mice and humans, i.e. the cross-linking of the HA-rich COC matrix via the generation of HC-HA (7, 8, 27, 43). In this regard, the human recombinant TSG-6 used in this study can rescue the expansion of COCs from Tsg-6−/− mice (7). Furthermore, recent work has indicated that HC-HA is formed during cumulus expansion in the pig, where this may also be mediated by TSG-6 (50), making it possible that this is a mechanism common to eutherian (placental) mammals.

Given the rapidity of TSG-6/HC and HC-HA complex formation in our assay system, it seems likely that these complexes would form in vivo wherever TSG-6, IaI, and HA meet; TSG-6/HC can be formed under a wide range of pH (6.0–8.0) and salt strength (50–300 mm) conditions (Fig. 2), including acid pH values that are often associated with inflammation. For example, TSG-6/IaI complexes of ~120 kDa, which are likely to correspond to TSG-6/HC, and HC-HA have been detected in a variety of inflammatory processes (e.g. rheumatoid arthritis and osteoarthritis (4, 10, 11, 13, 36) and asthma) and inflammation-like settings (e.g. ovulation (reviewed in Ref. 43) and cervical ripening (39)). In this regard, TSG-6 expression (24) and HA synthesis (51) are up-regulated in response to inflammatory mediators, and IaI can ingress into tissue spaces from serum due to either vasodilation (10, 13) or regulated changes to specific blood-tissue barriers, such as in the ovarian follicle (52, 53). The stability of the TSG-6/HC complexes would further increase the likelihood of HC-HA being produced once an HA molecule is encountered. Consistent with this notion, it has been reported that the synthesis of HA is the rate-limiting step in the formation of the cumulus matrix (54). In other cases where there is a high concentration of HA (e.g. synovial fluid), it would be expected that any TSG-6/HC formed would be converted quickly into HC-HA, and thus, there would not be an accumulation of the TSG-6/HC complex. For example, in synovial fluids from arthritis patients, there is only a small amount of ~120-kDa complex detected relative to the amount of free TSG-6 (36).

Importantly, the finding that TSG-6 acts as a true catalyst in the transfer of HCs onto HA is likely to mean that low levels of TSG-6 expression could lead to a significant production of HC-HA over time, which may be of relevance to diseases that take many years to develop (e.g. atherosclerosis). Further research is clearly necessary to understand the biological functions of HC-HA, to identify the locations of TSG-6/HC-HA in physiological and pathological processes, and to determine the precise molecular details of their formation.

Acknowledgment—We thank Dr. Mike Kinter (Mass Spectrometry Laboratory for Protein Sequencing, Lerner Research Institute, Cleveland Clinic Foundation) for mass spectrometric analysis.
Mildner, A. M., Moon, J. B., Mott, J. E., Mutchler, V. T., Tomich, C. C., Watenpaugh, K. D., and Wiley, V. H. (1998) Structure 6, 923–935
47. Gregory, L. A., Thielens, N. M., Arlaud, G. J., Fontecilla-Camps, J. C., and Gaboriaud, C. (2003) J. Biol. Chem. 278, 32157–32164
48. Jessen, T. E., and Ødum, L. (2003) Osteoarthritis Cartilage 12, 142–148
49. Thogersen, I. B., and Enghild, J. J. (1995) J. Biol. Chem. 270, 18700–18709
50. Nagyova, E., Camaioni, A., Prochazka, R., and Salustri, A. (2004) Biol. Reprod. 71, 1838–1843
51. Butler, D. M., Vitti, G. F., Leizer, T., and Hamilton, J. A. (1988) Arthritis Rheum. 31, 1281–1289
52. Chen, L., Mao, S. J., and Larsen, W. J. (1992) J. Biol. Chem. 267, 12380–12386
53. Camaioni, A., Hascall, V. C., Yanagishita, M., and Salustri, A. (1993) J. Biol. Chem. 268, 20473–20481
54. Ødum, L., Jessen, T. E., and Andersen, C. Y. (2001) Zygote 9, 283–288
Characterization of Complexes Formed between TSG-6 and Inter-α-inhibitor That Act as Intermediates in the Covalent Transfer of Heavy Chains onto Hyaluronan
Marilyn S. Rugg, Antony C. Willis, Durba Mukhopadhyay, Vincent C. Hascall, Erik Fries, Csaba Fülöp, Caroline M. Milner and Anthony J. Day

J. Biol. Chem. 2005, 280:25674-25686.
doi: 10.1074/jbc.M501332200 originally published online April 19, 2005

Access the most updated version of this article at doi: 10.1074/jbc.M501332200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

Supplemental material:
http://www.jbc.org/content/suppl/2005/04/29/M501332200.DC1

This article cites 53 references, 29 of which can be accessed free at
http://www.jbc.org/content/280/27/25674.full.html#ref-list-1