The Transfer of Heavy Chains from Bikunin Proteins to Hyaluronan Requires Both TSG-6 and HC2

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Tumor necrosis factor-stimulated gene-6 protein (TSG-6) is involved in the transfer of heavy chains (HCs) from inter-α-inhibitor (IαI), pre-α-inhibitor, and as shown here HC2-bikunin to hyaluronan through the formation of covalent HC:TSG-6 intermediates. In contrast to IαI and HC2-bikunin, pre-α-inhibitor does not form a covalent complex in vitro using purified proteins but needs the presence of another factor (Rugg, M. S., Willis, A. C., Mukhopadhyay, D., Hashall, V. C., Fries, E., Fülöp, C., Milner, C. M., and Day, A. J. (2005) J. Biol. Chem. 280, 25674–25686). In the present study we purified the required component from human plasma and identified it as HC2. Proteins containing HC2 including IαI, HC2-bikunin, and free HC2 promoted the formation of HC3-TSG-6 and subsequently HC3-hyaluronan complexes. HC1 or HC3 did not possess this activity. The presented data reveal that both HC2 and TSG-6 are required for the transesterification reactions to occur.

Tumor necrosis factor-stimulated gene-6 protein (TSG-6) is a multifunctional protein involved in inflammation and tissue remodeling (1). The 35-kDa protein is composed of a link and a CUB domain. The link domain mediates binding to glycosaminoglycans via two distinct binding sites (2, 3). The CUB domain in TSG-6 is less studied, but it has been shown recently that the domain mediates interaction with fibronectin (4). The bikunin proteins inter-α-inhibitor (IαI), pre-α-inhibitor (PαI), and HC2-bikunin contain the serine proteinase inhibitor bikunin, which is a member of the 12 Kunitz-BPTI inhibitor family (MEROPS data base) (5, 6). Bikunin carries a single chondroitin sulfate (CS) chain originating from Ser-10 (7) composed of both unsulfated chondroitin and chondroitin 4-sulfate (8, 9). Furthermore the bikunin proteins contain different heavy chains (HCs) selected from a group of five proteins (10), but only three of the five homologous HCs have been identified in complex with bikunin. IαI contains HC1 and HC2, PαI contains HC3, and HC2-bikunin contains HC2 (11, 12). The HCs are covalently attached to the CS chain originating from bikunin (7, 11). This unique cross-link has been designated a protein-glycosaminoglycan-protein cross-link (7).

TSG-6 and IαI HCs spontaneously form covalent complexes in vitro (13). The formation of these complexes involves a two-step process in which TSG-6 and the CS chain of bikunin initially interact in a divalent cation-independent way (14). Subsequently the HC:TSG-6 complexes are formed during a divalent cation-dependent transesterification reaction (14). These HC:TSG-6 complexes are intermediates in a process in which the HCs are transferred to hyaluronan (HA) (15).

Purified PαI and TSG-6 do not form a covalent complex (15), although HC3-HA complexes exist in vivo (16). However, the reaction will take place in the presence of serum (15) suggesting that the formation of HC3-HA requires another component (15). In the present study we purified this component from human blood and identified it as HC2. Both HC1 and HC3 failed to promote the TSG-6 transfer activity, and the data show that both HC2 and TSG-6 are required for transfer activity to be expressed.

Experimental Procedures

Materials—Magnesium chloride, HA sodium salt from human umbilical cord, and horse radish peroxidase-conjugated goat anti-rabbit IgG were obtained from Sigma-Aldrich. Chondroitinase ABC (EC 4.2.2.20) was either from Sigma-Aldrich or from Seikagaku. CNBr-activated Sepharose, ECL Western blotting reagents, and Mono Q 4.6/100 PE column were obtained from GE Healthcare. Mass spectrometry grade trypsin was from Promega. StageTips (C18) were from Proxeon Biosystems A/S. Antisera against TSG-6, HC1, HC3, and bikunin were produced as described before (11, 17, 18). With some modifications IαI, PαI, and HC2-bikunin were purified from human plasma also as described before (12). The plasma was obtained from Statens Serum Institut, Copenhagen, Denmark. CS-substituted bikunin was purified from NaOH-dissociated IαI as described recently (14). Human TSG-6 was expressed in insect cells and purified as described before (19). Protein concentrations were determined spectrophotometrically at 280 nm using theoretical extinction coefficients calculated by General Protein/Mass Analysis for Windows (GPMAW) software (Lighthouse Data).
**HC2 Promotes HC3-TSG-6 Complex Formation**

**Purification of HA Oligosaccharides**—HA (10 mg) was digested with 0.5 units of chondroitinase ABC for 1 h at 37 °C. The enzyme was inactivated by boiling and removed by reverse phase high pressure liquid chromatography (Aquapore RP-300) using water as the mobile phase. The flow-through was collected and applied to an anion exchange column (Mono Q 4.6/100 PE column, GE Healthcare) equilibrated in 20 mM Tris-HCl, pH 7.4 (buffer A). To separate the HA oligosaccharides the column was developed using a linear gradient from 0 to 0.4 M NaCl in buffer A. Relevant fractions were pooled, concentrated, and desalted by size exclusion chromatography (Superdex Peptide 10/300 GL (GE Healthcare) equilibrated in water) (22). The masses of the collected oligosaccharides were determined by MALDI time-of-flight mass spectrometry (Bruker Ultraflex, Bruker Daltonics, Bremen, Germany). The instrument was operated in linear and positive polarity mode using sinapinic acid (20 g/liter) as matrix. The concentration was determined by measuring the absorbance at 232 nm using 5500 cm⁻¹ M⁻¹ as the extinction coefficient (23).

**Purification of Plasma Component Required for HC3-TSG-6 Complex Formation**—Human plasma (3 ml) was diluted 3-fold in 20 mM Tris-HCl, pH 7.4 containing 20 mM EDTA. The sample was applied to a 5-ml HiTrap Q column (GE Healthcare) equilibrated in 20 mM Tris-HCl, 10 mM EDTA, pH 7.4 (buffer A) and operated at a flow rate of 2 ml/min. The column was developed using a 50-min 0–0.5 M NaCl gradient in buffer A. The eluate was monitored at 280 nm, and five fractions of 20 ml were collected. The collected fractions were dialyzed against 20 mM Tris-HCl, 140 mM NaCl, pH 7.4 and assayed for the ability to transfer HC3. A fixed amount of protein (2.5 μg) from each fraction was incubated with 0.3 μg of TSG-6 and 0.3 μg of Pol in the presence of 1 mM MgCl₂. Complex formation was monitored by reducing SDS-PAGE and immunoblotting using an anti-TSG-6 antibody. The fractions containing the HC3 transfer activity were pooled comprising a total volume 40 ml. The sample was concentrated (Centriprep YM-10, Amicon, Millipore) 20-fold, and 10% of the concentrate was subsequently applied to a size exclusion column (Superose 6, GE Healthcare) equilibrated in 20 mM Tris-HCl, pH 7.4 (buffer A). The column was eluted using a flow rate of 0.5 ml/min, and fractions of 1 ml were collected and assayed for HC3 transfer activity as described above. Active fractions were subjected to reducing SDS-PAGE and stained using Coomassie Blue before proteins were identified by peptide mass fingerprinting.

**Formation of HC-TSG-6 Complexes**—Bikunin proteins and TSG-6 were incubated in 50 mM Tris-HCl, 100 mM NaCl, pH 7.5 containing 1 mM MgCl₂ for 1 h at 37 °C at a 1:1 molar ratio. The complex formation was visualized directly by reducing SDS-PAGE and immunoblotting using an anti-TSG-6 antibody. Alternatively the complex-containing sample was subsequently treated with chondroitinase ABC for 2 h at 37 °C before the resulting products were visualized by reducing SDS-PAGE and immunoblotting using anti-HC1, anti-HC1 and -2, anti-HC3, and anti-TSG-6 antibodies.

**TSG-6-mediated HC to HA Transfer Assay**—The transfer experiment was performed as described above except that the proteins were incubated in a 25-fold molar excess of a 13-disaccharide long HA oligosaccharide (4940 Da). The transfer was observed by reducing SDS-PAGE. We chose one of the largest oligosaccharides that we were able to purify to homogeneity to obtain a clear band shift between free HCs and HC-HA.

**Assay to Determine Whether Free HC2 Promotes HC3-TSG-6 Complex Formation**—The role of free HC2 was analyzed by incubating purified HC2-bikunin (10 μg) and 0.01 unit of chondroitinase ABC at 37 °C overnight. A low concentration of enzyme was used to limit the chondroitinase ABC activity during subsequent complex formation. The digested sample was either used directly or heat-denatured, reduced, and S-carboxymethylated. Pol, TSG-6, and free HC2 were incubated as described above. Complex formation was evaluated by reducing SDS-PAGE and immunoblotting using anti-TSG-6 antibodies. Similar to this approach we also treated HC2-bikunin with NaOH, as described before (7), to generate free HC2. Afterward we lowered the pH by the addition of 600 mM Tris-HCl, pH 7.6 and incubated the dissociated HC2-bikunin with Pol and TSG-6. The final concentration of Tris in the sample and in the different controls for this experiment was 110 mM. Finally the complex formation was evaluated as described above.

**Assay to Determine Whether Free HC1 Promotes HC3-TSG-6 Complex Formation**—Iol (0.5 mg) was treated with 1.5 units of chondroitinase ABC for 3 h at 37 °C and applied to a Mono Q 4.6/100 PE column (GE Healthcare) equilibrated in 20 mM Tris-HCl, pH 7.4 (buffer A). Chondroitinase ABC did not bind to the column, and the dissociated Iol components were eluted using a linear gradient from 0 to 0.8 M NaCl in buffer A and a flow rate of 1 ml/min. The fractions were collected manually, and a fixed volume of each collected peak was analyzed by reducing SDS-PAGE and visualized by silver staining or immunoblotting using an anti-HC1 antibody. Silver-stained protein bands were identified by peptide mass fingerprinting. The presence of chondroitinase ABC in the flow-through was confirmed by incubating with Iol and analyzing the resulting reaction products by SDS-PAGE. An aliquot of each fraction was incubated with Pol and TSG-6 as described above. HC3-TSG-6 complex formation was visualized by reducing SDS-PAGE and immunoblotting using an anti-TSG-6 antibody.

**Assay to Determine Whether HC2 Is Required for the Transfer of HC from HC3-TSG-6 Complex to HA**—Purified Iol (3 mg) was immobilized on 2 ml of settled cyanogen bromide-activated Sepharose (GE Healthcare) according to the manufacturer’s suggestions. The Iol-Sepharose resin (50 μl), 4 μg of TSG-6, and 12 μg of Pol were then incubated for 2 h at 37 °C in 200 μl of 20 mM Tris-HCl, 137 mM NaCl, pH 7.4 containing 2 mM MgCl₂. The Iol-Sepharose was collected by gentle centrifugation, and the supernatant was removed. The supernatant (30 μl) containing HC3-TSG-6 was incubated with either HA oligosaccharides (2 μg) or with HA oligosaccharides (2 μg) and HC2-bikunin (1.2 μg) for 1 h at 37 °C. Finally the samples were...
subjected to reducing SDS-PAGE and immunoblotting using anti-TSG-6 and anti-HC3 antibodies.

In-gel Digestion and Peptide Mass Fingerprinting Analysis—The in-gel digestion procedure was performed essentially as described before (13, 24). Following digestion, the tryptic peptides were purified on a C18 Stagetip (Proxeon Biosystems A/S), described before (13, 24). Following digestion, the tryptic peptides were purified on a C18 Stagetip (Proxeon Biosystems A/S), and eluted directly onto the MALDI target using 1 μl of matrix (α-cyano-4-hydroxycinnamic acid). The peptide mass fingerprints were recorded in a Micromass Q-Tof Ultima Global mass spectrometer (Waters). Mass spectra were acquired in positive ion mode (range, 800–3000 m/z). Micromass MassLynx data processing software was used to generate a single Mascot-searchable peak list to query the Swiss-Prot protein data base (25). The searches were performed using a peptide mass tolerance of 50 ppm or better and propionamide modification of cysteine residues and allowed one or no missed tryptic cleavage site. Only significant hits as defined by Mascot probability analysis were accepted.

RESULTS

Purification of the Plasma Component Required for HC3-TSG-6 Complex Formation—Human plasma was separated by anion exchange chromatography (supplemental Fig. S1A), and the eluting fractions were assayed for HC3-TSG-6 cross-linking activity by immunoblotting. The HC3-TSG-6-promoting activity was present in the two last fractions from the anion exchange column. These fractions contain proteins that elute from 300 to 500 mN NaCl. The two fractions were pooled and subjected to size exclusion chromatography (supplemental Fig. S1B), and the fractions were assayed for HC3-TSG-6 complex formation. Because the migration in SDS-PAGE of the three HC3 is distinct (11), HC3-TSG-6, HC2-TSG-6, and HC1-TSG-6 could conveniently be distinguished. Because all three HC3-TSG-6 complexes were present in the same sample, the HC3-TSG-6-promoting activity was apparently co-eluting with Iol (Fig. 1A). The active fractions were further analyzed by reducing SDS-PAGE and peptide mass fingerprinting (Fig. 1, B and C, and supplemental Table S1). The results suggested that the protein corresponding to Iol (band a) contained the promoting activity. Peptide mass fingerprinting analysis failed to detect bikunin in the band most likely due to ion suppression effects in the mass spectrometer of the bikunin-derived peptides. The presence of bikunin in the fraction (band a) was subsequently confirmed by reducing SDS-PAGE and immunoblotting using an antibikunin antibody verifying that the HC3-TSG-6-promoting band contains Iol.

Iol and HC2-Bikunin Promote the Formation of HC3-TSG-6 Complex—To determine whether purified Iol or purified HC2-bikunin were able to promote the formation of HC3-TSG-6 cross-links, Pol, TSG-6, and Iol or HC2-bikunin were incubated and analyzed by reducing SDS-PAGE and immunoblotting using an anti-TSG-6 antibody. Both Iol and HC2-bikunin promoted the formation of HC3-TSG-6 complexes (Fig. 2A).

To confirm the formation of HC3-TSG-6 by mass spectrometry Iol, Pol, and TSG-6 were incubated to form HC-TSG-6 complexes, and the reaction products were treated with chondroitinase ABC to dissociate co-migrating HC-bikunin proteins (13) and finally separated by reducing SDS-PAGE. The band of interest was excised and analyzed by peptide mass fingerprinting. These analyses confirmed the presence of HC3-TSG-6 (supplemental Table S2). Finally the chondroitinase ABC-treated sample and the chondroitinase ABC-treated Iol and Pol were analyzed by reducing SDS-PAGE and immunoblotting using antibodies directed toward the HCs and TSG-6 (Fig. 2B). The samples were treated with chondroitinase ABC to simplify the interpretation of the results. The treatment dissociated the HC-bikunin complexes, but the HC1-HC2 complexes remained associated (Fig. 2B, lanes 4, 6, 10, and 12). In addition, the high molecular weight (HMW) Iol forms are similarly not completely degraded (Fig. 2B, lanes 6 and 12). The immunoblot reveals that truncated versions of especially HC3 exist in our preparation (Fig. 2B, lanes 8 and 9). Most importantly the immunoblot shows that in addition to the anti-TSG-6 antibody (Fig. 2B, lane 3) the anti-HC3 antibody also detected HC3-TSG-6 (Fig. 2B, lane 9), confirming the presence of the complex. Taken together, the data demonstrate that
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HC3-TSG-6 has been formed in vitro, and it is apparent that Iol or HC2-bikunin are required for the HC3-TSG-6 complex formation to occur. These proteins most likely represent the serum component required for complex formation.

Iol and HC2-Bikunin Promote the TSG-6-mediated Transfer of HC3 from Pol to HA—The transfer of HCs from bikunin proteins to HA is mediated by two sequential transesterifications, and the HC-TSG-6 complexes are intermediates in this process (15). Because Iol and HC2-bikunin promoted HC3-TSG-6 complex formation we investigated whether HC3 was transferred to HA. Pol, TSG-6, and HA oligosaccharides were incubated in the presence of Iol and subjected to reducing SDS-PAGE. When this sample (Fig. 3A, lane 7) is compared with the incubation of Iol, TSG-6, and HA it is apparent that a new band appears (Fig. 3A, lane 7, band a). The interactions between HA and HCs are mediated by an alkaline-sensitive ester, and the migration of the new band was sensitive to mild NaOH treatment (Fig. 3A, lane 8) supporting that the band represents HA-HC3. Indeed this was confirmed by peptide mass fingerprinting (Fig. 3B and supplemental Table S3). Furthermore we found that HC2-bikunin similarly to Iol promoted the TSG-6-mediated transfer of HC3 from Pol to HA (supplemental Table S4). Taken together these experiments demonstrate that HC2-bikunin and Iol promote the TSG-6-mediated transfer of HC3 from Pol to HA.

TSG-6 Generates HMW Pol—HMW proteins, containing bikunin and more than two HCs, are produced if bikunin proteins are incubated with TSG-6 (Fig. 3A, lanes 4 and 5) (13). However, when an excess of HA is added, HA is used as HC acceptor rather than the bikunin CS chain, and the formation of HMW proteins is abolished (Fig. 3A, lanes 6 and 7). The HMW proteins previously have been designated HMW Iol (13), but when/ if Iol, Pol, and TSG-6 are incubated together (Fig. 3A, lane 5), the term HMW bikunin proteins is more appropriate. This became apparent because immunoblotting, using an anti-HC3 antibody, revealed that HC3 was present both in the protein band corresponding to bikunin containing two HCs and in the protein band containing three HCs (data not shown) demonstrating that the TSG-6-mediated transfer of HCs also generates HMW Pol. The level of HMW Pol was, as expected, significantly reduced when Iol, Pol, and TSG-6 were incubated in the presence of HA.

TSG-6 Transfers HC2 from Purified HC2-Bikunin to HA—We have shown that HC2-bikunin and Iol promote the TSG-6-mediated transfer activity. These analyses indicated that although HC2-bikunin and Pol are structurally similar, containing bikunin and one HC, they are functionally different. Therefore we tested whether purified HC2-bikunin inter-
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| Identification  | a | b | c | d | e | f |
|-----------------|---|---|---|---|---|---|
|                | HC2 and TSG-6 | HC2 | HC3 | HC2 | HC2 | HC2 |

**FIGURE 4. TSG-6 transfers HC2, derived from HC2-bikunin, to HA.** A, TSG-6 was incubated without bikunin proteins (lane 1), with IαI (lane 2), with Pol (lane 3), or with HC2-bikunin (lane 4). Subsequently the samples were analyzed by immunoblotting using anti-TSG-6 antibody. This analysis demonstrates that HC2-bikunin and IαI interact covalently with TSG-6. B, TSG-6 was incubated with bikunin proteins and HA as indicated above the gel. The samples were separated by reducing SDS-PAGE and stained using Coomassie Blue. The HC2-bikunin preparation contained small amounts of intact and truncated free HC2 as indicated by the asterisk (*). C, the labeled bands were subjected to peptide mass fingerprinting (supplemental Table S5). Taken together, the mass spectrometry analysis, the migration of the proteins, and the sensitivity to NaOH treatment strongly indicate that band b corresponds to HC2-HA. The result shows that, in contrast to HC3 from Pol, TSG-6 does transfer HC2 from HC2-bikunin to HA.

**FIGURE 5. Uncomplexed HC2 promotes the formation of HC3-TSG-6 complex.** TSG-6 or samples of TSG-6 incubated with Pol and/or different variants of HC2-bikunin, as indicated above the immunoblot, were subjected to reducing SDS-PAGE and immunoblotting with anti-TSG-6 antibody. The experiment shows that free HC2 promotes the complex formation between HC3 and TSG-6 and that the promoting effect requires the native fold of HC2. ChonABC, chondroitinase ABC.

The bikunin proteins HC2-bikunin, Pol, and IαI were incubated with TSG-6, and the complex formation was evaluated by reducing SDS-PAGE and immunoblotting using an anti-TSG-6 antibody (Fig. 4A). The analysis revealed that HC2 derived from purified HC2-bikunin reacted covalently with TSG-6. This was also confirmed by peptide mass fingerprinting of the complex from a Coomassie Blue-stained gel (Fig. 4, B and C, and supplemental Table S5). Like the complexes originating from IαI, this complex was dissociated by mild NaOH treatment and required divalent cations during the formation (data not shown). The result demonstrated that covalent complex formation did not depend on the presence of two HCs attached to the CS chain for complex formation with TSG-6 to proceed. Furthermore, we tested whether HC2 from purified HC2-bikunin was transferred to HA. TSG-6, HA oligosaccharides, and HC2-bikunin or Pol were incubated. The samples were analyzed by reducing SDS-PAGE, and protein bands of interest were identified by peptide mass fingerprinting (Fig. 4, B and C, and supplemental Table S5). The results revealed that HC2 derived from HC2-bikunin was transferred to HA (Fig. 4B, lane 7, band b) and that the HC2-HA cross-link was dissociated by mild NaOH treatment (Fig. 4B, lane 9). In addition, HC2 derived from HC2-bikunin was also transferred to the CS originating from bikunin in the absence of HA, generating HMW HC2-bikunin proteins (Fig. 4B, lane 5). In contrast and as expected, no transfer of HCs to either CS or HA was observed when Pol was incubated with TSG-6 + HA (Fig. 4B, lanes 4 and 6). These analyses demonstrate that Pol and HC2-bikunin are functionally different.

**Free HC2 Alone Promotes the Formation of HC3-TSG-6**—In simple terms IαI and HC2-bikunin are composed of bikunin, CS, and HCs. Bikunin is most likely not responsible for the HC3-TSG-6-promoting effect of IαI and HC2-bikunin because Pol contains bikunin. Indeed purified bikunin (containing the CS chain) was unable to promote the HC3-TSG-6 complex formation (data not shown). HC2 is the only other component shared by IαI and HC2-bikunin. To investigate the role of HC2 alone, HC2-bikunin was dissociated by chondroitinase ABC treatment. Free HC2 did not form a covalent complex with TSG-6 (Fig. 5, lane 4) (14), but significantly, free HC2 promoted...
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The observation that the native structure is important likely explains why free HC2 generated by NaOH treatment of HC2-bikunin failed to promote HC3·TSG-6 complex formation (supplemental Fig. S2). Alternatively in contrast to NaOH, chondroitinase ABC treatment is likely to leave a small CS fragment attached to the C-terminal Asp of the free HC2. It cannot be excluded that this CS fragment is important for the HC3·TSG-6-promoting activity.

To determine whether free HC1 promoted the HC3·TSG-6 complex formation 1ol was treated with chondroitinase ABC, and the resulting products were separated by anion exchange chromatography (Fig. 6A). The collected fractions were subsequently analyzed by SDS-PAGE, peptide mass fingerprinting, and immunoblotting toward HC1 (Fig. 6, B, C, and D, and supplemental Table S6). These analyses demonstrated that free HC1 has been purified. Finally we evaluated whether the free HC1 promoted HC3·TSG-6 complex formation (Fig. 7). Taken together the results show that free HC2, and not HC1, promotes the formation of HC3·TSG-6.

HC2 Is Involved in the Transfer of HC3 from HC3·TSG-6 Complexes to HA—The data presented so far provide evidence that HC2 and TSG-6 in collaboration generate the covalent HC3·TSG-6 complex. The role of HC2, during the transfer of HC3 from HC3·TSG-6 complexes to HA, was investigated by immobilizing 1ol on CNBr-activated Sepharose. Pol-TSG-6, and 1ol-Sepharose were then incubated, and the supernatant was analyzed. Because 1ol was immobilized HC1-TSG-6 and HC2-TSG-6 complexes remained associated with the Sepharose leaving only the generated HC3·TSG-6 complexes in solution (Fig. 8A, lane 3). HA oligosaccharides were then added, and the sample was incubated with or without HC2-bikunin.

FIGURE 6. Purification of free HC1. A, initially 1ol was digested with chondroitinase ABC and applied to a Mono Q 4.6/100 PE column (GE Healthcare). The column was developed, and fractions (1–4) were subsequently analyzed by reducing SDS-PAGE and visualized by silver staining. B, the labeled bands from the silver-stained gel were identified by peptide mass fingerprinting (supplemental Table S6). C, finally the starting material (SM) before separation on the Mono Q column and the collected peak fractions (P1–P4) were analyzed by reducing SDS-PAGE and immunoblotting using anti-HC1 as primary antibody. Taken together, the analyses reveal that free intact and truncated HC1 was purified (P1). HC2 co-eluted with bikunin and the HC1·HC2 complex (P2–P4) preventing the purification of HC2 using this method. mAU, milliabsorbance units.

the formation of HC3·TSG-6 (Fig. 5, lane 6). This also demonstrates that covalent complex formation between HC2 and TSG-6 is not part of the mechanism that results in HC3·TSG-6 complex formation. In addition, denaturation of the free HC2 abolished the HC3·TSG-6-promoting activity (Fig. 5, lane 7). The observation that the native structure is important likely explains why free HC2 generated by NaOH treatment of HC2-bikunin failed to promote HC3·TSG-6 complex formation Subsequently the samples were analyzed by immunoblotting using anti-TSG-6 antibody. The results revealed that the amount of HC3·TSG-6 remained unchanged in the presence of HA (Fig. 8A, lane 4). This indicates that TSG-6 alone is unable to transfer HCs from HC-TSG-6 to HA. The addition of both HC2-bikunin and HA to the supernatant significantly reduced the level of HC3·TSG-6, suggesting that HC3·HA was produced (Fig. 8A, lane 5). The formation of HC3·HA was also confirmed
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by immunoblotting using an anti-HC3 antibody (Fig. 8B, lane 5). Small amounts of HC3-HA were observed before the addition of HC2-bikunin (Fig. 8B, lane 4). This is likely to be explained by trace amounts of HC2, HC2-bikunin, or Iol released from the Sepharose. The formation of HC3-TSG-6 was not observed on the anti-HC3 immunoblot because it co-migrates with Iol and is present in a much smaller amount than Iol. The anti-HC3 immunoblot clearly demonstrated the formation of the previously mentioned HMW Iol (Fig. 8B, lanes 3–5). These data suggest that HC2 is required for both the first and the second transesterification.

DISCUSSION

The present study confirms that TSG-6 alone is unable to transfer HC3 from Iol to HA in vitro (15). This observation has been puzzling in view of the fact that HC3-HA has been observed in vivo (16), and studies with TSG-6 knock-out mice showed that TSG-6 transfers HC1 and HC2 from Iol and HC3 from Iol to HA in vivo (26). Apparently another component is present in vivo that is required for the completion of the HC3 transfer (15). We show in this study that this component is HC2 and that TSG-6 in concert with HC2 is responsible for the TSG-6-mediated transfer of HC3 to HA.

The HCs exist in five variations (10, 27). These all contain a vault protein inter-α-trypsin domain and a von Willebrand type-A domain (10). Despite the sequence identity between the HCs, the present study demonstrates that significant functional differences exist.

Free HCs are not present in vivo, and although free HC2 promotes HC3-TSG-6 complex formation, the physiologically relevant proteins are HC2-bikunin and Iol. Therefore, the co-localization of bikunin proteins, TSG-6, and HA is likely to ensure transfer of all HCs to HA in vivo. The primary means of regulation is probably at the level of expression and the localization of the reactants. Bikunin proteins are constitutively expressed and present in blood (6) in contrast to the expression of both TSG-6 and HA (1, 28). The interaction between the bikunin proteins and TSG-6/HA is established when the bikunin proteins access the extracellular space, for instance during ovulation (29). If bikunin proteins and TSG-6 co-localize in the absence of HA or unsulfated chondroitin, which also acts as an HC acceptor (30), TSG-6 is likely to transfer the HCs to the CS chain of bikunin generating HMW Iol and HMW Iol.

HC2-bikunin is a consequence of the Iol and TSG-6 interaction forming HC3-TSG-6 complexes (13, 15). In the present study, we show that HC2-bikunin and TSG-6 generate a covalent complex. This demonstrates that the by-product of the first

FIGURE 7. HC1 fails to promote the formation of HC3-TSG-6 complex. TSG-6, Iol, and the peak fractions (P1–P4) derived from the anion exchange purification of HC1 (see Fig. 6) were incubated and analyzed by reducing SDS-PAGE. Various control samples were included as indicated. Bands of interest were visualized by immunoblotting using an anti-TSG-6 antibody. The immunoblot confirmed that the HC2-containing peaks (P2–P4) promote HC3-TSG-6 complex formation. In contrast, peak 1, which contains free HC1, did not promote the formation of HC3-TSG-6.

FIGURE 8. HC2 participates in the transfer of HC3 from HC3-TSG-6 to HA. Iol and TSG-6 were incubated in the presence of Iol coupled to CNBr-activated Sepharose. Subsequently, the sample was spun to deposit the Sepharose, and the supernatant was removed. The supernatant was either analyzed directly (lane 3) or initially incubated with HA (lane 4) or with HA and HC2-bikunin (lane 5). As a control TSG-6 was incubated with Iol (lane 1) or Iol (lane 2), respectively. All samples were subjected to immunoblotting either with anti-TSG-6 antibody (A) or with anti-HC3 antibody (B). The experiment shows that immobilized Iol promotes HC3-TSG-6 complex formation. Furthermore, the result demonstrates that HC2 is required for the subsequent transfer of HC3 from HC3-TSG-6 to HA.
reaction could be used for subsequent HC•TSG-6 complex for-
formation thereby releasing free bikunin. Previously it has been 
shown that free bikunin is generated during the Iα1/TSG-6 
interaction as a result of HC•bikunin breakdown (15, 31). Here 
we show that this alternative pathway also generates free 
bikunin as TSG-6 strips the CS chain of the HCs. This may 
impact the regulatory role of bikunin within the extracellular 
protease network (31, 32).

In summary, we report that HC2-bikunin forms a covalent 
complex with TSG-6 and that HC2 is the additional factor 
required for the HC3/TSG-6 complex formation. Our studies 
show that HC2 and TSG-6 in collaboration promote the trans-
fer of HCs from bikunin proteins to HA.

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