The TSG-6 and IαI Interaction Promotes a Transesterification Cleaving the Protein-Glycosaminoglycan-Protein (PGP) Cross-link*

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During co-incubation of human inter-α-inhibitor (IαI) and human tumor necrosis factor-stimulated gene 6 protein (TSG-6) SDS-stable interactions are formed between the two proteins. We have analyzed the products of this reaction and characterized the mechanism of complex formation. Following the incubation seven new bands not previously identified were apparent in SDS-PAGE. Three of these bands did not contain TSG-6, including heavy chain (HC)1, bikunin, and free bikunin. In addition high molecular weight complexes composed of the same components as IαI, including HC1, HC2, and bikunin, were formed. The formation of these complexes was prevented by the addition of hyaluronan. The cross-links stabilizing these complexes displaying properties similar to the protein-glycosaminoglycan-protein (PGP) cross-link. The TSG-6-containing SDS-stable complexes were composed of HC1-TSG-6 or HC2-TSG-6 exclusively. Both glycosylated and non-glycosylated TSG-6 participated in the complex formation. The HC-TSG-6 cross-links were different from the PGP cross-link and were determined to be ester bonds between the α-carbonyl of the C-terminal Asp of the heavy chain and most likely a hydroxyl group containing the TSG-6 residue. The mechanism involved cleaving the PGP cross-link of IαI during a transesterification reaction. A TSG-6 hydroxyl group reacts with the ester bond between the α-carbonyl of the C-terminal Asp residues of HC1 or HC2 and carbon-6 of an internal N-acetylgalactosamine of the chondroitin-4-sulfate chain. An intermediate is formed resulting in a partitioning of the reaction between HC(1 or 2)/TSG-6 complexes and transfer of HC(1 or 2) to the chondroitin via competing pathways.

The bikunin proteins comprise a family of multichain serine protease inhibitors composed of free bikunin and bikunin in complex with one or two homologous heavy chains (HC) (1, 2).

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The on-line version of this article (available at http://www.jbc.org) contains supplemental Table S1.

TSG-6 is a secreted protein of ∼35 kDa produced during inflammation and inflammation-like processes (13). The mRNA encoding for the protein was originally identified following tumor necrosis factor treatment of human diploid FS-4 fibroblasts (14, 15). Like the members of the bikunin proteins, TSG-6 is thought to play a vital role in the ECM in general and particularly during cumulus cell-oocyte complex stabilization (13, 16). The mammalian ovulation is accompanied by the permeabilization of the blood/follicle barrier, allowing ingress of blood proteins (17). During this process hyaluronan (HA), IαI, and TSG-6 interact and stabilize the expansion of the cumulus cell-oocyte complex. The importance of these interactions has been emphasized in animal models where the bikunin or the TSG-6 genes have been knocked out. Both the bikunin- and TSG-6-deficient mice were unable to assemble the ECM surrounding the oocyte causing infertility (11, 16).

The interactions between TSG-6 and IαI have been studied in vitro. After a brief incubation of purified human IαI and human TSG-6 an apparent covalent complex between components of IαI and TSG-6 is formed (18, 19). It has been suggested that this complex is composed of HC2, bikunin, and TSG-6 (18).

HC2, heavy chain 2; IαI, inter-α-inhibitor; HC3, heavy chain 3; CS, chondroitin-4-sulfate; ECM, extracellular matrix; HMW, High molecular weight; TIC, trypsin inhibitor counter staining; TBS-T, Tris-buffered saline with Tween 20; LC-MS/MS, Liquid chromatography tandem mass spectrometry.
In addition the TSG-6 and I complex formation has been underscored in vivo by the biological importance of the cross-linking (20, 21).

In this study we show that the interaction between I and TSG-6 leads to the formation of seven distinct reaction products not previously characterized. These include two high molecular weight (HMW) I species, HC1-TSG-6, HC2-TSG-6, HC1-bikunin, HC2-bikunin complexes, and free bikunin. The data suggest that the PGP ester bond between the a-carbonyl of the C-terminal Asp and carbon-6 of an internal N-acetylgalactosamine in the CS chain is cleaved during a transesterification reaction. An intermediate is formed, and a partitioning occurs between HC(1 or 2) and TSG-6 at molar ratios ranging from 1:0.1 to 1:8 for 60 min at 37 °C. An intermediate is formed, and a partitioning occurs between HC(1 or 2) and TSG-6 at molar ratios ranging from 1:0.1 to 1:8 for 60 min at 37 °C.

**Experimental Procedures**

**Materials**—Bovine testis hyaluronidase (EC 3.2.1.35), Proteus vulgaris chondroitinase ABC (EC 4.2.2.4), thermolysin (EC 3.4.24.27), hyaluronic acid sodium salt from human umbilical cord, 1,10-phenanthroline, and sodium chloride were obtained from Sigma. Bikunin and HC samples were obtained from Millipore. Biodyne B membrane (50 kDa) was from Millipore. Proteinase K was from Roche Molecular Biochemicals. Rabbit IgG (Sigma) was prepared for SDS-PAGE. Before SDS-PAGE the sample was filtered with a 0.45-μm Biodyne B membrane (27). The section containing the bands, including HC1-bikunin, HC2-bikunin, HC1-TSG-6, and HC2-TSG-6 complexes, were excised and the membrane pieces were treated with 100 μg/ml trypsin (10 μg/ml) and TSG-6 was more resistant to proteolysis than the HCs and conditions were established where the HCs were digested leaving TSG-6 intact. The reaction products were separated by a second SDS-PAGE analysis and electrophoretically transferred to an Immobilon-P membrane. After Coomassie Blue staining of the membrane, the band corresponding to TSG-6 was excised and analyzed by automated Edman degradation.

**Results**

**Incubation of I and TSG-6 Produce Seven New Protein Bands in SDS-PAGE**—The incubation of purified I and TSG-6 resulted in the appearance of seven protein bands when analyzed by reduced SDS-PAGE (Fig. 1). To aid in the description we used the following nomenclature: numbers refer to new bands that are covalently associated protein products of the reaction. These include HMW I (bands 1 and 2), HC2-TSG-6 (band 3), and HC1-TSG-6 (band 4). Letters refer to bands that appear to be the result of dissociation of one or more I components, including HC2-bikunin (band A), HC1-bikunin (band B), and free bikunin (band C).

**Bikunin-containing Complexes and Free Bikunin (Bands A–C) Are Generated during the Incubation of I and TSG-6**—The bikunin-containing bands formed during the incubation of I and TSG-6 were identified by non-reducing SDS-PAGE followed by TIC staining (Figs. 1 and 2). This technique enables the unambiguous identification of bikunin in contrast to Coomassie Blue staining (5). A fixed concentration of bikunin was incubated with increasing amounts of TSG-6. When equal amounts or a molar excess of TSG-6 was used (Fig. 2, lanes 7–9), a broad band about 30–45 kDa in size showed trypsin inhibitory activity (Fig. 2, band C). This band represents free bikunin carrying the CS chain (4, 8). Released bikunin does not migrate as a sharp band due to the heterogeneity of the attached CS chain. In addition to free bikunin two ~120-kDa fragments were detected for ~16 h at 37 °C. Following digestion, the trypsic peptides were extracted, filtered, and acidified prior to the mass spectrometry analysis.

**Liquid Chromatography Tandem Mass Spectrometry**—The LC-MS/MS analyses were performed using a Micromass Q-TOF Ultima Global mass spectrometer (Micromass/Waters) connected to a LC-Packs 950 Ultima nano LC System (LC-Packings). A nano-spray ion source was used to hold the packed PicoFrit™ columns (New Objective) and apply capillary voltage through a Valco union. The PicoFrit™ columns (75-μm ID × 10 cm) were packed with Zorbax SB C18, 3.5-μm reverse phase column material (Agilent) using a high pressure column Loader (Proxeon). The column was developed at a flow rate of 200 nl/min and linear gradients from 0.02% heptafluorobutyric acid/0.5% acetic acid in water (Buffer A) to 0.02% heptafluorobutyric acid/0.5% acetic acid in 75% acetonitrile/24.5% water (Buffer B). After data acquisition, the individual MS/MS spectra acquired for each of the precursors within a single LC run were combined, smoothed, deisotoped, and centroided using the Micromass Masslynx data processing software and output as a single Mascot-searchable peak list. The peak list files were used to query the Swiss-Prot data base using the Mascot program (25).
The bikunin-containing bands (Figs. 1 and 2, bands A and B) were observed. The upper band (band A) was significantly more intense than the lower band (band B) (lanes 4–6). The identity of the bikunin-containing complexes were established by a combination of (i) the apparent molecular mass in SDS-PAGE, (ii) immunoblotting using HC1, HC2, or bikunin-specific antibodies (data not shown), and (iii) the dissociation of both complexes by CS-degrading enzymes (Fig. 3, lanes 4 and 5). Based on these results we conclude that band A is composed of HC2-bikunin and band B of HC1-bikunin. These data show that free bikunin, HC1-bikunin, and HC2-bikunin are generated during the reaction between Iol and TSG-6. We note that a significantly smaller amount of HC1-bikunin was consistently produced as compared with HC2-bikunin.

High Molecular Weight Bands (Bands 1 and 2) Are Generated during Incubation of Iol and TSG-6—Following the incubation of purified Iol and TSG-6, two high molecular weight protein bands appear in both reduced and non-reduced SDS-PAGE (Fig. 1–5, bands 1 and 2). TIC gel analysis revealed that both protein bands contain bikunin (Figs. 1 and 2). If Iol was incubated with a molar excess of TSG-6 only very small amounts of these high molecular weight proteins were apparent. We conclude that the high molecular weight proteins are made of the same components as Iol (bikunin, HC1, and HC2) (1). The slower than expected migration in SDS-PAGE is most likely caused by a change in the bikunin/HC1/HC2 stoichiometry as compared with Iol. The molecular weight of the faster migrating HMW Iol band was estimated by reduced SDS-PAGE to be 71 kDa higher than Iol (data not shown). An increase of 71 kDa is consistent with the high molecular weight components of Iol, HC1, and HC2.
bands 3 were subsequently subjected to LC-MS/MS (Fig. 3, bikunin dissociated and migrated further). HC1/H18528 HC2/TSG-6 remained at the same position in the gel, while TSG-6 as compared with HC1/TSG-6 (Table S1, Supplemental Material). We noted that significantly as described above for the HMW I stabilizing the HC(1 or 2)/H18528 B degradation (data not shown). These properties were exploited HC2/TSG-6 cross-links were readily migrating HMW I HMW I TSG-6 cross-links were readily stabilized by differences in glycosylations (18). To determine if both TSG-6 HMW I/TSG-6 complex was prepared as described and separated by non-reducing SDS-PAGE. The HCTSG-6 band was electroluted and digested with thermolysin. TSG-6 was more resistant to proteolysis than the HCs, and conditions were established where TSG-6 remained intact following thermolysin digestion. The HCs were degraded, and the digest was separated by reduced SDS-PAGE and electrotransferred to a polyvinylidene difluoride membrane. N-terminal protein sequence analysis of the TSG-6 band revealed two sequences: the sequence LAQGSQVESTPPPHVRVEN(D) (Asp was not detected), corresponding to the C terminus of HC2, and WGFKDGIFHNSIWLERAAGVYH, which matches the N-terminal sequence of TSG-6. All residues except Asp-702 of HC2 were detected during Edman degradation suggesting that the C-terminal Asp-702 is involved in the HC2/TSG-6 cross-link.

HA Inhibits the Formation of HMW Iol —The effect of HA on the formation of HMW Iol was investigated as described under “Experimental Procedures.” When Iol and TSG-6 were incubated in the presence of HA the formation of HMW Iol was abolished (Fig. 5, lane 3). In addition Iol, HC1-bikunin, HC2-bikunin, HC1-TSG-6, and HC2-TSG-6 were not observed (Fig. 5, lane 3). The formation of a HA-HC complex is the most likely explanation for this observation (16, 29). These complexes are large and will not migrate into the gel. However, the interaction between HA and the HCs are mediated by an ester (30), which is vulnerable to mild NaOH treatment. Significantly, we observed that mild NaOH treatment of complex between Iol, TSG-6, and HA produced free HCs (Fig. 5, lane 4). The result shows that incubating Iol and TSG-6 in the presence of HA generates HA-HC complexes and inhibits the formation of HMW Iol.

Both the Glycosylated and the Non-glycosylated Forms of TSG-6 Are Capable of Forming a Covalent Complex with the HCs. The Iol/TSG-6 complex was analyzed by reduced SDS-PAGE and transferred to a Biodyne B membrane. The protein bands representing the HC1-bikunin, HC2-bikunin, HC1/TSG-6, and HC2/TSG-6 complexes were excised and subjected to a mild NaOH treatment. The proteins were extracted and analyzed by reduced SDS-PAGE and immunoblotting using anti-TSG-6 polyclonal antibodies (lane 1). TSG-6 was analyzed alone for comparison (lane 2). The glycosylated and the non-glycosylated forms of TSG-6 were both able to participate in the covalent complex formation.

addition of one more HC as compared with Iol. The slower migrating HMW Iol band similarly displayed an increase in size as compared with the faster migrating HMW Iol band. This suggests that HMW Iol are formed in a process where additional HCs are cross-linked to the CS-chain. We designate these reaction products HMW Iol.

Characterization of TSG-6-containing Protein Complexes (Bands 3 and 4) Generated during Incubation of Iol with TSG-6 —The Iol/TSG-6 complexes are resistant to both hyaluronidase (Fig. 3, lane 5, bands 3 and 4) and chondroitinase ABC degradation (data not shown). These properties were exploited to separate the TSG-6-containing bands from the co-migrating bikunin-containing bands in SDS-PAGE (HC1-bikunin, band B; HC2-bikunin, band A). After the digestion, HC1/TSG-6 and HC2/TSG-6 remained at the same position in the gel, while HC1-bikunin and HC2-bikunin dissociated and migrated further into the gel (data not shown). The TSG-6-containing bands were subsequently subjected to LC-MS/MS (Fig. 3, lane 5, bands 3 and 4). The analyses revealed that band 3 contained HC2 and TSG-6, whereas band 4 contains HC1 and TSG-6 (Table S1, Supplemental Material). We noted that significantly smaller amounts of HC2-TSG-6 as compared with HC1-TSG-6 were generated consistently during complex formation.

Stability of the HMW Iol Cross-links—The nature of the HMW Iol cross-links (bands 1 and 2) were investigated by analyzing their stability toward enzymatic or chemical dissociation protocols (Figs. 3 and 4). The complexes were dissociated by hyaluronidase (Fig. 3), chondroitinase ABC (data not shown), or by mild NaOH treatment (Fig. 4). This behavior is analogous to previous results obtained during the analysis of the PGP cross-link in Iol and Pgp (5, 8) suggesting that PGP cross-links are responsible for the cross-linking of HMW Iol.

Stability of the HC(1 or 2)/TSG-6 Cross-links—The cross-link stabilizing the HC(1 or 2)/TSG-6 interaction was investigated as described above for the HMW Iol proteins, including (i) stability toward hyaluronidase (Fig. 3), (ii) chondroitinase ABC (data not shown), and (iii) mild NaOH treatment (Fig. 4). Significantly, the HC(1 or 2)/TSG-6 cross-links were readily disrupted following NaOH treatment but was resistant to CS-degrading enzymes (Figs. 3 and 4, bands 3 and 4). Because NaOH dissociated the complex but CS-degrading enzymes did not, we conclude that an ester bond independent of the CS chain mediates the interaction. In addition the interaction between Iol and TSG-6 produced HC1-bikunin and HC2-bikunin as described above. These are generated at the same time as the HC2-TSG-6 or HC1-TSG-6 complexes are formed (Fig. 1). Because HC1 is positioned closest to the non-reducing end of the CS (8) the formation of HC1-bikunin is impossible if cross-link formation involved cleaving the CS chain. Accordingly, the formation of HC2-TSG6 or HC1-TSG-6 does not involve cleavage of the CS chain.

The C-terminal Asp of HC2 Is Involved in the HC2-TSG-6 Cross-link —The Iol/TSG-6 complex was prepared as described and separated by non-reducing SDS-PAGE. The HCTSG-6 band was electroluted and digested with thermolysin. TSG-6 was more resistant to proteolysis than the HCs, and conditions were established where TSG-6 remained intact following thermolysin digestion. The HCs were degraded, and the digest was separated by reduced SDS-PAGE and electrotransferred to a polyvinylidene difluoride membrane. N-terminal protein sequence analysis of the TSG-6 band revealed two sequences: the sequence LAQGSQVESTPPPHVRVEN(D) (Asp was not detected), corresponding to the C terminus of HC2, and WGFKDGIFHNSIWLERAAGVYH, which matches the N-terminal sequence of TSG-6. All residues except Asp-702 of HC2 were detected during Edman degradation suggesting that the C-terminal Asp-702 is involved in the HC2/TSG-6 cross-link.

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DISCUSSION

When IaI and TSG-6 interact apparent covalent interactions between TSG-6 and components of IaI are formed (18, 31, 32). The mechanism of the reaction and the identity of the products are not clear (18, 20). In the present study we have analyzed the polypeptide compositions of the reaction products, the chemical properties of the cross-links formed, and the reaction mechanism.

Characterization of the Reaction Products—Immunoblotting using TSG-6 antibody demonstrated that two TSG-6-containing complexes were formed during the incubation. In addition we show that HC1-bikunin, HC2-bikunin, free bikunin, and HMW IaI were produced (Fig. 7). In contrast to a previous study (18) we specifically dissociated the HC1-bikunin and HC2-bikunin complexes before SDS-PAGE. The IaI-TSG-6 complexes were resistant to both hyaluronidase and chondroitinase ABC, whereas HC1-bikunin and HC2-bikunin were readily dissociated. This prevented co-migration of HC1 or 2-bikunin and the two TSG-6-containing complexes. Subsequently, LC-MS/MS analyses of the two TSG-6-containing bands then revealed that they only were composed of HC1-TSG-6 or HC2-TSG-6 (Table S1, Supplementary Material). Bikunin is not involved in the TSG-6-containing complexes as suggested previously (18). The conflicting results reported previously (18, 20) have led to the suggestion that different complexes between IaI and TSG-6 exist in vivo (20). Although we are not able to explain the lack of HC1 or chondroitinase ABC sensitivity observed in a previous study (18), our data support that co-incubation of IaI and TSG-6 produce only HC1-TSG-6 and HC2-TSG-6 complexes.

In addition to the TSG-6-containing complexes the reaction produced other covalent protein complexes that we designate HMW IaI. These bands did not contain TSG-6 and were shown to be composed of the same components as IaI (Table S1, Supplementary Material, and Fig. 7). The bands behaved like IaI in terms of trypsin inhibitory activity and dissociation by both chemical and enzymatic procedures. They are likely the result of HC transfer from one IaI molecule to another during the reaction. Both the production of HC(1 or 2)-TSG-6 complexes and HMW IaI thus consume HCs and the detection of HC1-bikunin, HC2-bikunin, and free bikunin suggests that IaI served as a HC donor. In support of this we showed that more HC1-TSG-6 than HC2-TSG-6 was produced during the reaction (Fig. 3). Significantly, this was correlated with the formation of less HC1-bikunin than HC2-bikunin (Fig. 2). It is possible that the difference in the amount produced is caused by steric hindrance as HC2 is positioned closest to the reducing end of the CS (8). This could produce different environments for the two HCs and affect their ability to participate in the reaction.

In the present study we did not attempt to remove trace amounts of divalent cations from our protein preparations or buffer. Thus, we cannot out rule that the TSG-6-IaI complex formation is a metal ion-dependent process.

The HC(1 or 2)-TSG-6 Cross-link—Several lines of evidence suggest that the cross-linking of IaI and TSG-6 involves a transamidation reaction in which the PGP cross-link is cleaved, including (i) the cross-linking reaction dissociates IaI producing free bikunin and HC(1 or 2)-bikunin (Fig. 2); (ii) HC-TSG-6 is not dissociated by CS-degrading enzymes suggesting that the cross-link does not contain [GlcUA-GalNAc] disaccharides (Fig. 3); (iii) the complexes are readily dissociated by mild NaOH treatment suggesting an ester bond (Fig. 4); and (iv) the HC2-TSG-6 cross-link was shown to be between the C-terminal Asp residue of HC2 and TSG-6. The most likely scenario is that during complex formation the ester bond between the α-carbon of the C-terminal Asp and the carbon-6 of an internal N-acetylgalactosamine is cleaved (Fig. 8A). A new ester bond is formed between the C-terminal Asp residues of the HCs and a TSG-6 residue containing a functional hydroxyl group, including Tyr, Ser, Thr, or a carbohydrate moiety (Fig. 8A). It has been suggested that the two TSG-6 bands observed following reduced SDS-PAGE, are glycosylated and non-glycosylated TSG-6 (18). The fact that both forms are involved in the covalent complex formation (Fig. 6) suggests that TSG-6 hydroxyl groups are not carbohydrate-derived.

The HMW IaI Cross-link—The cross-links stabilizing HMW IaI are different from the HC-TSG-6 cross-link. The HC-TSG-6 cross-link was resistant to CS-degrading enzymes, whereas HMW IaI readily dissociated and the HMW IaI cross-links are likely analogous to the PGP cross-link of IaI (Fig. 3). Recently it has been demonstrated that TSG-6 is able to transfer HCs from IaI to unsulfated chondroitin (29). We have previously shown that the bikunin CS is heterogeneously sulfated (8). The sulfate groups were mainly associated with disaccharides near the reducing end while the CS at the non-reducing end did not carry sulfate groups. It is possible that HMW IaI is formed by a similar process where HCs are transferred from the CS of one IaI molecule to the unsulfated CS region of another IaI molecule (Fig. 8B). Consequently, IaI molecules with more than two HCs are produced, accounting for the higher molecular weight (Fig. 7). HA is a better HC acceptor than chondroitin (29), and the fact that HA abolished the formation of HMW IaI (Fig. 5), provides additional evidence for this hypothesis.

Mechanism of Complex Formation—The cross-linking reaction did not cleave the CS chain. If cleavage of the CS chain was part of the mechanism HC1-bikunin would not have been de-
tected according to the position of the HCs on the CS (8). In addition CS-degrading enzymes were unable to dissociate the HC(1 or 2)/H18528TSG-6 complex (Fig. 3) implying that [GlcUA-Gal-NAc] disaccharides were not involved in the cross-link. This is in contrast to the HMW I/H9251I complex, which was readily cleaved by CS-degrading enzymes. Based on these findings the mechanism is likely to involve the formation of an intermediate that partitions to generate HC(1 or 2)/H18528TSG-6 complexes or transfer HCs to CS via competing pathways. In favor of this mechanism two different covalent products are produced (HC/H18528TSG-6 and HMW I/H9251I) and a progressively lower level of HMW I/H9251I was generated when a fixed concentration of I/H9251I was titrated with increasing amount of TSG-6 (Fig. 2).

Taken together the data suggest that the cross-linking of Iol and TSG-6 involves a transesterification where the PGP cross-link is cleaved. A new ester bond is formed between the C-terminal Asp-carbonyl of the HCs and a TSG-6-derived hydroxyl donor most likely a Tyr, Ser, or Thr residue or possibly a glycan moiety. In a competing reaction HCs are transferred to the CS chain of an Iol molecule by forming a new ester bond between C-terminal Asp-carbonyl of HCs and a carbon-6 of an internal N-acetylgalactosamine of the CS producing a new PGP cross-link (Fig. 8).

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