Evidence for a Two-Step Mechanism Involved in the Formation of Covalent HC·TSG-6 Complexes†

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Received January 18, 2006; Revised Manuscript Received March 29, 2006

ABSTRACT: IαI and TSG-6 interact to form a covalent bond between the *C*-terminal Asp α-carbon of an IαI heavy chain (HC) and an unknown component of TSG-6. This event disrupts the protein−glycosaminoglycan−protein (PGP) cross-link and dissociates IαI. In simple terms the interaction involves 5 components: (i) the IαI HCs, (ii) bikunin, (iii) chondroitin sulfate chain, (iv) TSG-6, and (v) divalent cations. To understand the molecular mechanism of complex formation, the effect of these were separately examined. The data show that although the mature covalent cross-link between the HCs and TSG-6 only involves the *C*-terminal Asp residue, the native fold of both IαI and TSG-6 was essential for the reaction to occur. Similarly, complex formation was prevented if the chondroitin sulfate chain was cleaved, releasing bikunin but maintaining the HC1 and HC2 PGP cross-links. In contrast, releasing the majority of the bikunin protein moiety by limited proteolysis did not prevent complex formation. An analysis of the divalent-cation requirements revealed two distinct interactions between IαI and TSG-6: (i) a noncovalent manganese, magnesium, or calcium-independent interaction between TSG-6 and the chondroitin sulfate chain (K_d 180 nM) and (ii) a covalent manganese, magnesium, or calcium-dependent interaction generating HC1·TSG-6, HC2·TSG-6, and high molecular weight (HMW) IαI. Significantly, both free TSG-6 and HC·TSG-6 complexes were able to bind the chondroitin sulfate chain suggesting that the sites on TSG-6 were distinct. On the basis of these findings, we propose a two-step reaction mechanism involving two putative binding sites. Initially, a cation-independent interaction between TSG-6 and the chondroitin sulfate chain is formed at site 1. Subsequently, a cation-dependent transesterification occurs, generating the covalent HC·TSG-6 cross-link at another site, site 2.

The bikunin proteins are composed of bikunin and one or two distinct but homologous heavy chains (HCs)[1] called HC1, HC2, and HC3. The components are intracellularly assembled forming heterodimers (PαI and HC2·bikunin) (the "·" represents a covalent linkage) or a heterotrimer (IαI, composed of HC1, HC2, and bikunin) (*1*, *2*). The interchain cross-links connecting the subunits are esters formed between the α-carbon of the HC *C*-terminal Asp and C-6 of an internal *N*-acetylgalactosamine of a chondroitin sulfate (CS) chain originating from Ser-10 of bikunin (*1*, *3*, *4*). This cross-link is called a protein−glycosaminoglycan−protein (PGP) cross-link (*3*, *4*). The CS chain is approximately 15 disaccharides long and is mainly unsulfated because only every fourth disaccharide carries sulfate in the form of chondroitin-4-sulfate (*5*). The bikunin subunit exhibits weak inhibitory activity against a broad spectrum of serine proteases (*6*). The bikunin proteins are found in plasma and in the extracellular matrix (ECM) (*7*).

In the ECM, the bikunin proteins interact specifically with tumor necrosis factor stimulated gene-6 protein (TSG-6). TSG-6 is a 35 kDa glycoprotein expressed during inflammation and inflammation-like conditions (*8*). The mRNA was originally identified following tumor necrosis factor (TNF) stimulation of human diploid FS-4 fibroblasts (*9*, *10*). TSG-6 is comprised of an *N*-terminal link module with affinity for hyaluronan (HA) and a *C*-terminal CUB domain of unknown function (*11*). In addition to HA, TSG-6 also interacts with other glycosaminoglycans (GAGs), including heparin, heparan sulfate, and CS (*12*, *13*). The interaction between IαI and TSG-6 is unique in that a covalent bond is formed between the two proteins (*14*). Recently, we demonstrated that two covalent complexes are formed, including HC1·TSG-6 and HC2·TSG-6 (*15*). Complex formation involves a transesterification, which cleaves the PGP cross-link and

† This work was supported by grants from the Danish Natural Science Research Council and the Novo Nordic foundation.

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[1] Abbreviations: ConABC, chondroitinase ABC; CS, chondroitin sulfate; DTT, dithiothreitol; ECM, extracellular matrix; FT, flow through; GAG, glycosaminoglycan; HA, hyaluronan, HC1, heavy chain 1; HC2, heavy chain 2; HCs, heavy chains; HMW, high molecular weight; IαI, inter-α-inhibitor; PAGE, polyacrylamide gel electrophoresis; PαI, pre-α-inhibitor; PGP cross-link, protein−glycosaminoglycan−protein cross-link; TSG-6, tumor necrosis factor stimulated gene-6 protein; "·", covalent linkage.

10.1021/bi060106s CCC: $33.50
Published on Web 05/23/2006

generates a new ester bond between the *C*-terminal Asp of the HCs and an unknown component of TSG-6 (*15*). The covalent complexes between IαI and TSG-6 have been identified in synovial fluid (*16*) and in the cumulus cell−oocyte complex formed during mammalian ovulation (*17*). TSG-6 transfers HCs from IαI to HA to generate HC·HA complexes (*18*−*20*). This reaction requires specific GAG structures including HA or chondroitin-0-sulfate (*19*). In contrast, TSG-6 does not transfer the HCs to dermatan-4-sulfate or chondroitin-6-sulfate (*19*). In addition, TSG-6 has the capacity to transfer HCs from the CS chain of one IαI molecule to the CS chain of another IαI molecule to generate high molecular weight (HMW) IαI (*15*). Both HC1·TSG-6 and HC2·TSG-6 are intermediates in these reactions (*20*). The physiological relevance of these interactions has been emphasized in animal models where the bikunin or the TSG-6 gene has been knocked out. Both bikunin and TSG-6 deficient mice were unable to assemble the ECM surrounding the oocyte and caused infertility (*18*, *21*).

In the present study, we have characterized the requirements for the covalent complex formation between TSG-6 and IαI. The 3D structures of both TSG-6 and IαI heavy chains were essential for complex formation to occur. In contrast, the bikunin protein moiety did not participate in the reaction. However, if bikunin was dissociated by cleaving the CS chain, then complex formation was prevented. Both IαI and free CS-substituted bikunin interacted noncovalently with TSG-6 in a divalent-cation-independent way as opposed to the covalent interaction that required magnesium, manganese, or calcium ions. The dissociation constant of the noncovalent interaction was determined to be 140−180 nM, and the interaction was mediated by the CS chain. On the basis of these findings, we propose a two-step reaction mechanism for the formation of covalent HC·TSG-6 complexes. Initially, TSG-6 interacts in a divalent-cation-independent way with the CS chain of IαI. Subsequently, HC·TSG-6 complexes are formed during a divalent-cation-dependent process.

MATERIALS AND METHODS

Materials. Chondroitinase ABC (ConABC) (EC 4.2.2.4), endoprotinase Glu-C (SPase V8) (3.4.21.19), zinc chloride, magnesium chloride, manganese chloride, calcium chloride (99.99+%, contains less than 0.04 ppm Mg and app. 0.05 ppm Mn), and horseradish peroxidase-conjugated goat anti-rabbit IgG were obtained from Sigma-Aldrich. Copper (II) sulfate was from Riedel-de Haën. ECL western blotting detection reagents, fast desalting PC 3.2/10 column, and cyanogens bromide activated sepharose were obtained from GE Healthcare. PVDF membrane (Immobilon-P) was from Millipore. Antisera against TSG-6, HC1, HC2, and bikunin were produced as previously described (*3*, *4*, *10*). IαI was purified from human plasma (*1*) obtained from Statens Serum Institut, Denmark. Human TSG-6 was expressed in insect cells and purified as previously described (*14*).

Purification of IαI-Derived Bikunin. IαI was treated with NaOH (*3*), and the dissociated protein was dialyzed against 20 mM Tris-HCl at pH 7.4 (buffer A) and applied to a Mono Q column (GE Healthcare, HR 5/5) connected to an FPLC system (GE Healthcare). The bound proteins were eluted with a linear gradient from 0 to 1 M NaCl in buffer A. Bikunin was detected by immunoblotting. Fractions containing glycosylated and unglycosylated bikunin were pooled separately and further purified by reverse phase HPLC using an Aquapore RP-300 column (Brownlee 4.6 mm × 22 cm) connected to an Äkta Explorer (GE Healthcare).

SDS−PAGE and Immunoblotting. Samples were boiled in an SDS sample buffer containing 10−50 mM dithiothreitol (DTT) and subjected to SDS−PAGE in 5−15% gradient gels (10 cm × 10 cm × 0.15 cm) using a glycine/2-amino-2-methyl-1,3-propandiol/HCl system (*22*). The gels were (i) stained for protein using Coomassie blue, (ii) stained for trypsin inhibitory activity using a counter staining technique (*1*, *23*), or (iii) transferred to PVDF membranes (*24*) and processed for immunoblotting using chemiluminescence.

Limited ConABC Digestion of IαI Followed by Analyses of Complex Formation. ConABC was dissolved in 40 mM Tris-HCl and 40 mM CH_3COONa at pH 8.0. A fixed concentration of IαI (0.5 μg) was titrated at 37 °C for 3 h using 0.0001−0.2 units of ConABC. Subsequently, the samples were divided equally, and one-half was placed at 0 °C, and the other half was incubated with 0.1 μg of TSG-6 at 37 °C for 15 min. All samples were finally analyzed by reduced SDS−PAGE and immunoblotting. The TSG-6-containing samples were analyzed using anti-TSG-6 antiserum as the primary antibody, and the other samples were analyzed using primary antibodies directed against HC1, HC2, and bikunin. The immunoblots were developed in a Typhoon scanner (GE Healthcare) and quantified using ImageQuant analysis software (GE Healthcare).

Limited SPase V8 Digestion of IαI to Remove the Bulk of Bikunin Followed by Analyses of Complex Formation. Approximately 5 μg of IαI was treated with SPaseV8 for 70 min at 25 °C using enzyme/substrate ratios of 1:100, 1:25, and 1:5 (w/w). The samples were divided, and one-half of the samples were boiled immediately to inactivate SPase V8. TSG-6 was added to the other half of the samples using a 1:1 IαI/TSG-6 ratio (w/w). The TSG-6-containing samples were incubated at 25 °C for additional 5 min, and afterward, these samples were also boiled. The TSG-6-containing samples were analyzed by reduced SDS−PAGE followed by immunoblotting. The immunoblots were developed, and the bands quantified as described above. The other samples were subjected to unreduced SDS−PAGE, and subsequently, bikunin inhibitory activity was visualized by the trypsin inhibitor counter staining procedure.

Reduction and Carboxymethylation of IαI and TSG-6 Followed by an Analysis of Complex Formation. IαI and TSG-6 were denatured in 6 M guanadinium hydrochloride containing 5 mM DTT and carboxymethylated using a final concentration of 15 mM iodoacetamide. The samples were desalted on a desalting column (Fast Desalting column PC 3.2/10) connected to a SMART system (GE Healthcare) equilibrated in 50 mM Tris-HCl and 50 mM NaCl at pH 8.0 containing 1 mM $MgCl_2$. Native and carboxymethylated protein samples were incubated at a 1:1 ratio (w/w) for 2 h at 37 °C and analyzed by reduced SDS−PAGE.

Complex Formation between IαI and TSG-6 in the Presence of Divalent Cations. Divalent cations were removed from the IαI preparation by adding EDTA to a final concentration of 2 mM. An equimolar amount of TSG-6 was added, and the buffer was exchanged using a desalting column (Fast Desalting column PC 3.2/10) connected to a

SMART system (GE Healthcare) equilibrated in 10 mM Tris-HCl at pH 7.6. The effect of various divalent cations on complex formation was then analyzed by adding $MgCl_2$, $ZnCl_2$, $CaCl_2$, $MnCl_2$, or $Cu(SO_4)$ to a final concentration of 1 mM. The samples were incubated for 2 h at 37 °C, boiled, and subjected to SDS−PAGE.

Analysis of the IαI and TSG-6 Divalent-Cation Interaction. Divalent cations were removed from the IαI or TSG-6 preparations as described above using a 10 mM Tris-HCl at pH 8.0. Five IαI samples were prepared containing 1 mM $MgCl_2$, $ZnCl_2$, $CaCl_2$, $MnCl_2$, or $Cu(SO_4)$. The samples were incubated for 20 min at 23 °C and desalted. Afterward, the 5 IαI samples were incubated using the desalted TSG-6 preparation for 2 h at 37 °C. Finally, the samples were analyzed by SDS−PAGE. A similar approach was used to determine if TSG-6 was able to coordinate the divalent cations involved in covalent complex formation.

Complex Formation between IαI and TSG-6 in a Divalent-Cation-Depleted Environment. IαI and TSG-6 were incubated at a 2:1 molar ratio in 20 mM Tris-HCl and 100 mM NaCl at pH 7.4 containing 2 mM in EDTA for 30 min at 37 °C. Parallel a sample without the addition of EDTA was incubated as controls.

Surface Plasmon Resonance Analysis. The kinetics of the interaction between IαI or bikunin and TSG-6 were determined by surface plasmon resonance analysis using Biacore model 3000 (Biacore). TSG-6 was immobilized on a CM5 sensor chip as previously described (*25*) at a density of 140 fmol/mm^2. The binding analysis was performed at the indicated concentrations using 10 mM Hepes, 150 mM NaCl, and 0.005% Tween 20 at pH 7.4 containing 3 mM EDTA. The kinetics of the interactions were evaluated using proprietary software (BIAevaluation 3.1). The concentrations of purified IαI and bikunin used in the experiments were determined by absorbance at 280 nm and the theoretical extinction coefficient.

Analysis of IαI/TSG-6 Complexes by Anti-Bikunin Immuno Adsorption. Protein G purified bikunin antiserum was immobilized on cyanogen bromide-activated Sepharose 4B, and a 0.8 mL column was packed. The column was equilibrated in 25 mM Tris-HCl, 0.137 M NaCl, and 2 mM $MgCl_2$ at pH 7.4 (buffer A). IαI and TSG-6 were incubated in a molar ratio of 1:1 in buffer A for 1 h at 37 °C and applied to the column. The column was washed with buffer A, and the bound proteins were eluted using 0.2, 0.5, and 1.0 M NaCl in 25 mM Tris and 2 mM $MgCl_2$ at pH 7.4 and finally with 0.1 M glycine at pH 2.7. Fractions were collected and analyzed by SDS−PAGE and immunoblotting using anti-TSG-6 or anti-bikunin as primary antibodies. As a control, TSG-6 in buffer A was applied to the column. The sample was eluted and analyzed as described above using an anti-TSG-6 antibody.

RESULTS

Intact CS Chain Is Required for the Formation of Covalent IαI·TSG-6 Complexes. The complexes formed between IαI and TSG-6 consist of HC1·TSG-6 and HC2·TSG-6 (*15*). Because neither the bikunin nor the backbone glycosidic bonds of the CS chain are components of the covalent complexes, we considered their role during complex formation. IαI was digested with increasing amounts of ConABC and, subsequently, incubated with TSG-6. The degradation pattern was evaluated following SDS−PAGE and western blotting using IαI and TSG-6 specific antibodies (Figure 1A and B). At low ConABC concentrations, bikunin is released from the HCs, whereas a more extensive digestion completely dissociates IαI (*5*). The amount of the different IαI degradation products were quantified and compared to the amount of HC·TSG-6 complex formation (Figure 1C). The results revealed that complex formation was prevented if the CS chain was cleaved, releasing bikunin from the IαI molecule. Furthermore, the free HCs were unable to form complexes. Apparently, the residual saccharides left on the HC1·HC2 complex or the free HCs have lost structural determinants important for complex formation. The data demonstrate that the continuity of the intact CS chain is essential for the formation of HC1·TSG-6 and HC2·TSG-6.

Bikunin (Glu18-Phe145) Is Not Involved in the Formation of Covalent Complexes between IαI and TSG-6. To investigate the effect of bikunin on complex formation, the bulk of the protein was removed by limited SPaseV8 proteolysis of Glu_{18} (Figure 2A) (*3*, *6*, *26*). The dissociation of the proteolytically cleaved bikunin from the bulk of the IαI molecule was verified by size-exclusion chromatography (data not shown). A fixed amount of IαI was incubated with SPase V8 in ratios of 1:100, 1:25, and 1:5. The highest concentration completely dissociated bikunin and IαI (Figure 2B). Subsequent incubation of TSG-6 and the limited SPase V8 digests produced covalent complexes in all three samples, apparently identical to the complexes between intact IαI and TSG-6 (Figure 2C). The bands were quantified showing that the complexes in the three samples constituted 29, 29, and 26% of the total amount of TSG-6. The results suggest that the bikunin protein moiety is not important for the formation of HC1·TSG-6 and HC2·TSG-6. The small decrease in the ratio of complexes at the highest SPase V8 concentration is probably caused by adventitious proteolysis cleavages of the HCs and TSG-6.

The two forms of TSG-6 probably represent glycosylated and unglycosylated TSG-6 (Figure 2C) (*14*). Both of these forms are known to participate in covalent complex formation with IαI (*15*), but it is difficult to distinguish the two forms during SDS−PAGE possibly because the unglycosylated form is only present in small amounts.

Native Structures of the IαI and TSG-6 Protein Moieties Mediate Covalent Complex Formation. To investigate the relationship between the protein structures of IαI and TSG-6 and their effect on covalent complex formation, the proteins were reduced and carboxymethylated using denaturating conditions. Subsequently, immunoblotting with a TSG-6 specific antibody showed that the ability to form covalent complexes was abolished by the procedure (Figure 3). All of the involved polypeptides (HC1, HC2, bikunin, and TSG-6) contain disulfide bridges, and accordingly, the given treatment is likely to abolish the native protein structures. The results suggest that the tertiary structures of both the HCs and TSG-6 are important for HC1·TSG-6 and HC2·TSG-6 complex formation to occur. Moreover, because CS is unaffected by the denaturation protocol but is involved in the formation of the cross-link, the data suggest that a cooperative CS chain−protein interaction exists.

Formation of HC1·TSG-6 and HC2·TSG-6 Requires Magnesium, Calcium or Manganese Ions. The requirement

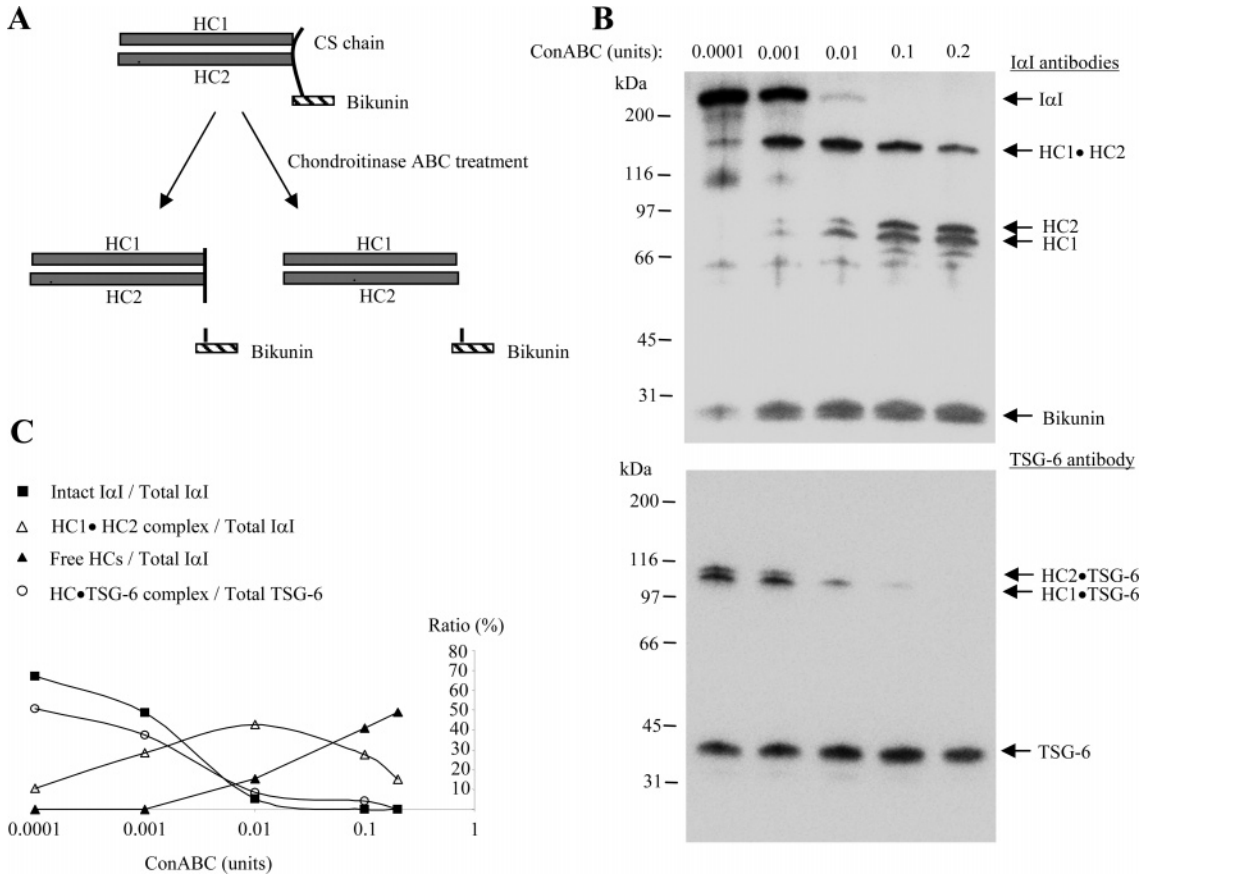

FIGURE 1: Covalent complex formation between IαI and TSG-6 requires the CS chain. A schematic illustrating the limited digestion of IαI with ConABC (Panel A). Immunoblot showing the limited digestion of a fixed amount of IαI with increasing amounts of ConABC (upper Panel B). After digestion, the samples were incubated with a fixed amount of TSG-6 (lower Panel B). The ratios between intact IαI/total IαI (■), between HC1·HC2 complex/total IαI (△), between free HC1 and HC2/total IαI (▲), and between HC·TSG-6 complex/total TSG-6 (○) are illustrated (Panel C). These results show that the covalent HC·TSG-6 complexes are formed only when CS is intact.

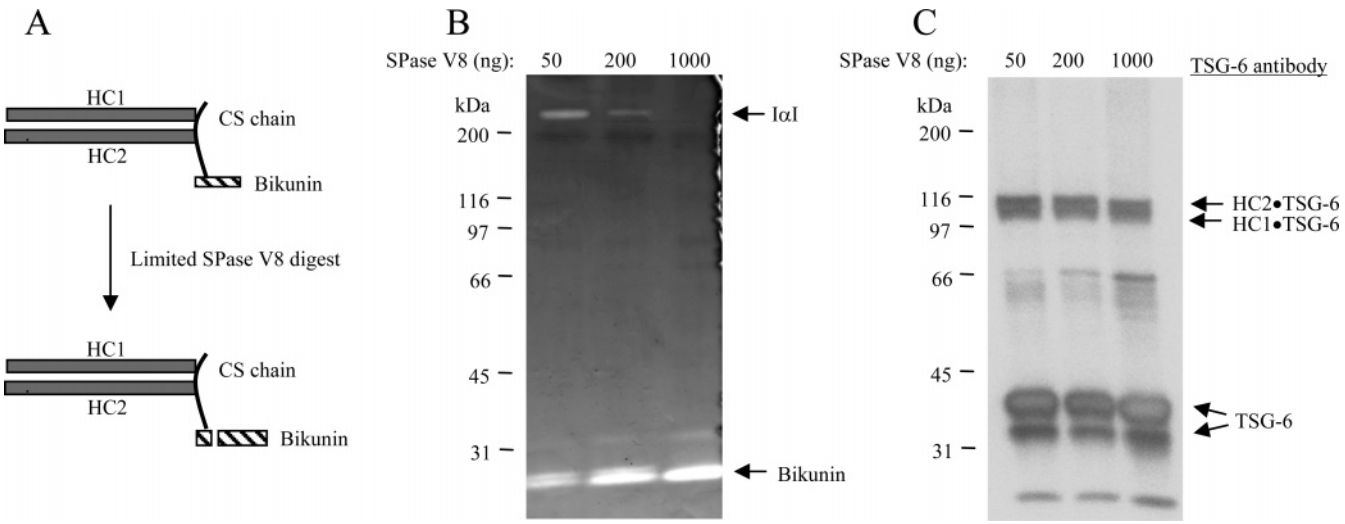

FIGURE 2: Bikunin is apparently not required for the covalent complex formation. A schematic illustrating the limited digest of IαI with SPase V8 (Panel A). SPase V8 preferentially cleaves Glu_{18}-Val_{19} in bikunin, releasing the inhibitory domains of bikunin. Trypsin inhibitor counter stained gel showing a digestion of a fixed amount of IαI with increasing amounts of SPase V8 (Panel B). Immunoblot of SPase V8 digestion of IαI and subsequent TSG-6 incubation (Panel C). These results demonstrate that the bulk of bikunin is not required for the formation of HC1·TSG-6 and HC2·TSG-6.

for divalent cations was evaluated by preparing divalent-cation-free IαI and TSG-6 preparations. These were subsequently incubated in the presence of various divalent cations. We found that magnesium, manganese, and calcium ions facilitated the formation of covalent HC1·TSG-6 and HC2·TSG-6 complexes, whereas zinc and copper did not (Figure 4). In addition, a similar approach was used to investigate whether IαI or TSG-6 are able to bind the divalent cations required for the formation of covalent complexes (data not shown). These results demonstrated that neither TSG-6 nor IαI were able to independently coordinate the divalent cations required for covalent complex formation.

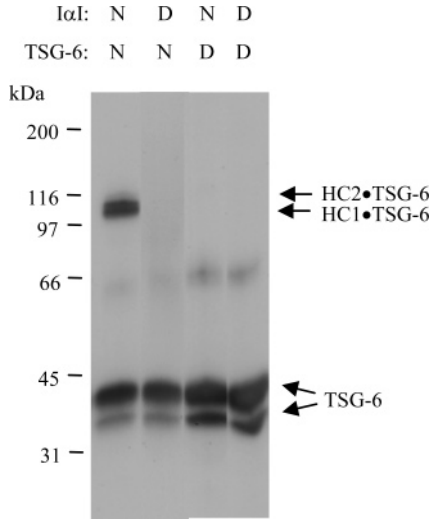

FIGURE 3: Native structures of both IαI and TSG-6 are required for the formation of HC·TSG-6 complexes. IαI and TSG-6 were separately reduced and carboxymethylated under denaturating conditions. Subsequently, the buffer was exchanged, and afterward, the capability of these pretreated proteins to participate in covalent complex formation was tested. N represents the native protein, and D represents the denaturated proteins. These results demonstrate that the native structure of both IαI and TSG-6 are necessary for the formation of covalent complexes.

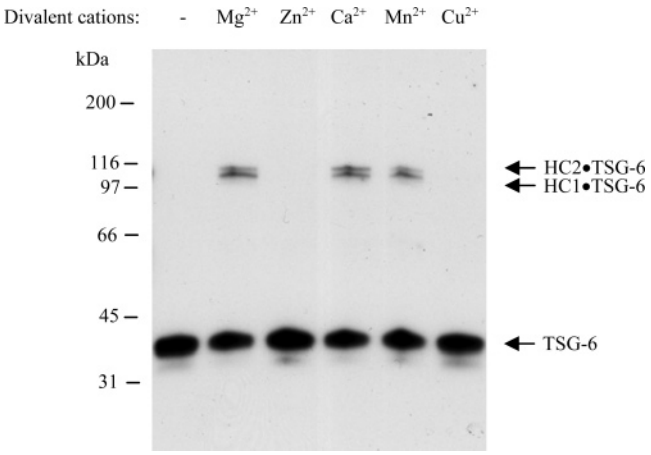

FIGURE 4: Formation of covalent complexes between IαI and TSG-6 requires magnesium, calcium, or manganese. IαI and TSG-6 were mixed in the presence of EDTA. Subsequently, EDTA and contaminating metal ions were removed. Afterward, the sample was divided and left to incubate without the addition of divalent cations or in the presence of the indicated divalent cations. Finally, all of the samples were subjected to reduced SDS−PAGE and subsequent immunoblotting with anti-TSG-6 antibody. These results demonstrate that the formation of HC1·TSG-6 and HC2·TSG-6 is a divalent-cation (magnesium, calcium, or manganese)-dependent process.

Formation of HMW IαI Depends on the Presence of Divalent Cations. We have previously shown that incubating IαI and TSG-6 in addition to HC1·TSG-6 and HC2·TSG-6 generates HMW IαI (*15*). To determine if the formation of the HMW IαI is a divalent-cation-dependent process, IαI and TSG-6 were incubated in the presence of EDTA and the products analyzed by SDS−PAGE (data not shown). The result shows that the formation of the HMW IαI, like the formation of HC·TSG-6 complexes, depends on divalent cations.

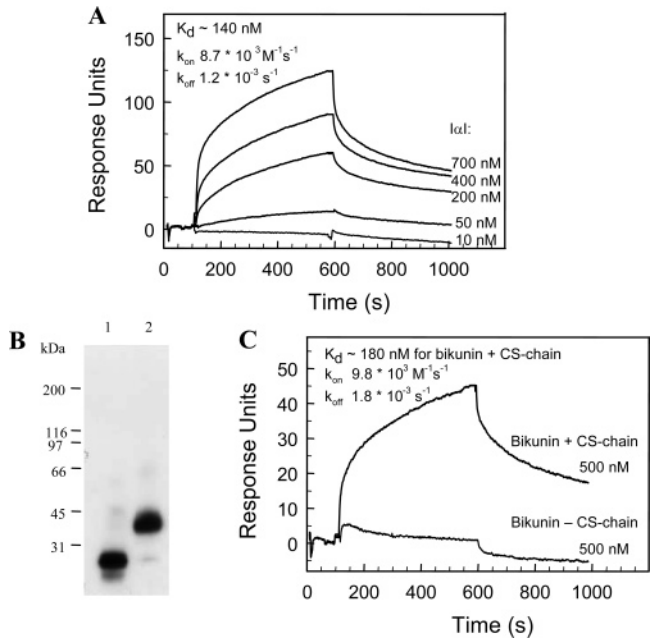

FIGURE 5: Surface plasmon resonance studies demonstrate an interaction between TSG-6 and CS chain of IαI. IαI was applied at the indicated concentrations to a flow cell containing immobilized TSG-6 (Panel A). The experiment was performed at physiological ionic strength in the presence of 3 mM EDTA to avoid covalent complex formation. The calculated K_d value is based on all sensorgrams using a 1:1 model of binding. We conclude that IαI and TSG-6 interact noncovalently by a divalent-cation-independent mechanism with a dissociation constant of 140 nM. Reduced SDS−PAGE showing purified bikunin without the CS-chain (Panel B, lane 1) and purified bikunin containing the CS-chain (Panel B, lane 2). The samples were transferred to a PVDF membrane for immunoblot analysis using a primary antibody directed against bikunin. The immunoblot shows that bikunin with and without the CS-chain have been purified. The two different bikunin forms were applied at the indicated concentrations to a flow cell containing immobilized TSG-6 (Panel C). The conditions were identical to the conditions used for IαI. The dissociation constant for bikunin with the CS-chain is 180 nM. Bikunin without the CS-chain does not interact with TSG-6. We conclude that the CS-chain mediates the divalent-cation-independent interaction between bikunin and TSG-6.

CS chain and TSG-6 Interact in a Noncovalently Divalent-Cation-Independent Way. As previously mentioned, our data show that the formation of HC1·TSG-6 and HC2·TSG-6 requires the CS chain of IαI, although the covalent complexes do not contain CS. It indicates that the CS chain participates in noncovalent interactions. By using a divalent-cation-depleted buffer, covalent complex formation between TSG-6 and IαI was prevented, and the existence of a noncovalent interaction was investigated by surface plasmon resonance spectroscopy. TSG-6 was immobilized on the sensor chip, and IαI was applied as a ligand using physiological conditions, except for the addition of 3 mM EDTA. The data demonstrated that IαI and TSG-6 also interacted in a divalent-cation-depleted environment with a dissociation constant (K_d) of 140 nM (Figure 5A). To determine if TSG-6 interacted with the CS-chain of IαI, we prepared bikunin with and without the CS chain (Figure 5B), and the interaction was studied as described above. The analysis showed that only CS-substituted bikunin interacted with TSG-6 (Figure 5C). The K_d value was determined to be 180 nM or similar to the IαI−TSG-6 dissociation constant (140

nM). In comparison, the K_d value between heparin and different heparin binding proteins is 60−80 nM for type I collagen (*27*), 120 nM for extracellular superoxide dismutase (*28*), 109−121 nM for P-selectin (*29*), and 12 nM for apolipoprotein E (*30*). These data indicate that the interaction between TSG-6 and the CS-chain is of relatively low affinity. We conclude that TSG-6 interacts with the CS-chain of IαI in a noncovalent divalent-cation-independent way.

TSG-6·HC Complexes Apparently Interact with the CS chain. As described above, the CS-chain of free bikunin and IαI bind to TSG-6. To investigate whether the HC·TSG-6 complexes retained their ability to bind the CS chain, an anti-bikunin affinity column was prepared. First, TSG-6 was applied to the column, and as expected, no binding was detected (Figure 6A). Then, IαI was allowed to form complexes with TSG-6 in a divalent-cation-containing environment, and the products were applied. The column was washed by increasing the NaCl concentration and, finally, eluted by lowering the pH. The fractions were analyzed by immunoblotting using bikunin (Figure 6B) or TSG-6 antisera (Figure 6C). As expected, bikunin-containing proteins bound strongly to the column and eluted when the low pH buffer was applied (Figure 6B). Significantly, free TSG-6 and HC·TSG-6 complexes also bound to the column and eluted with approximately 0.5 M NaCl (Figure 6C). This interaction was only observed when bikunin, which included the CS chain, was bound to the column. These data indicate that the HC·TSG-6 interaction does not interfere with TSG-6 binding to the CS chain. Although an excess of HCs compared to TSG-6 is present during incubation, a part of TSG-6 does not interact covalently with the HCs (Figure 6C). However, the free TSG-6 still interacts noncovalently with the CS chain (Figure 6C). The results suggest that both forms of TSG-6, including free TSG-6 and HC·TSG-6 complexes, interact noncovalently with the CS chain of IαI. Furthermore, the results demonstrate that even though the K_d value for the interaction between TSG-6 and the CS-chain is relatively low, 200 mM NaCl does not disrupt the binding, emphasizing the physiological relevance of the interaction.

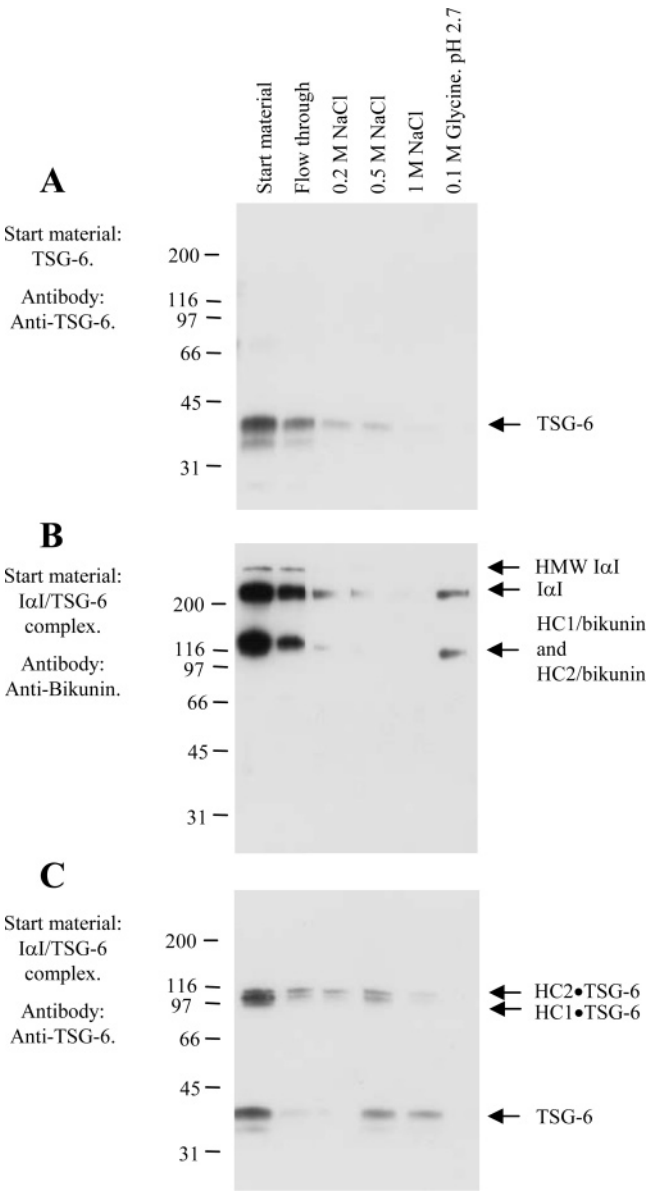

FIGURE 6: HC·TSG-6 complexes interact with the CS-chain of IαI. TSG-6 (Panel A) or IαI−TSG-6 complexes (Panels B and C) were applied to a bikunin antibody column, and the bound proteins were subsequently eluted stepwise as indicated above Panel A. The fractions corresponding to the different elution steps were analyzed by reduced SDS−PAGE. Subsequently, the proteins were transferred to a PVDF membrane and analyzed with anti-TSG-6 antibody as the primary antibody (Panels A and C) or with anti-bikunin antibody as the primary antibody (Panel B). The immunoblots show that both free TSG-6 and HC·TSG-6 complexes interact with IαI.

DISCUSSION

The transfer of the IαI HCs to HA can be divided into two events: (i) the formation of HC·TSG-6 complexes (*15*) and (ii) the transfer of the HCs from the HC·TSG-6 complexes to HA (*20*). In both steps, a transesterification is involved (*15*, *20*). According to a recent study, the transfer of the HCs from IαI to HA depends on Mn^{2+} or Mg^{2+} (*20*). In contrast to our results, it was concluded that Ca^{2+} was unable to facilitate HC·TSG-6 complex formation (*20*). The reason for this discrepancy is unclear because we used high grade calcium chloride in our study so as to diminish the possibility of Mn^{2+} or Mg^{2+} contamination. In accordance with our studies, it has also been reported that Ca^{2+} is essential for the TSG-6 mediated transfer of HCs from IαI to HA in synovial fluid (*31*). HMW IαI is formed during the IαI−TSG-6 interaction (*15*). These complexes were similarly dependent on divalent cations, supporting the hypothesis that HMW IαI is formed in a process similar to the formation of HC·HA.

A direct binding or coordination of divalent cations by the involved proteins has been suggested because both IαI and TSG-6 contain putative divalent-cation binding sites (*20*). However, neither IαI nor TSG-6 were able to independently coordinate divalent cations. The divalent cations might alternatively be coordinated in the interface between IαI and TSG-6 during the transition state of the process, generating HC·TSG-6 complexes. It has previously been reported that certain metal-ion-dependent adhesion sites only coordinate the divalent cations in the ligand bound state (*32*), and the coordination of the divalent cations in the IαI−TSG-6 interaction might resemble this.

Noncovalent interactions between IαI and the link module of TSG-6 exist (*33*), and it has been suggested that these are mediated via a protein−protein interaction between the

link module of TSG-6 and the protein moiety of bikunin (*12*). This finding was based on the observations that (i) TSG-6 does not interact with purified HCs and (ii) the interaction was not interrupted by the addition of chondroitin-4-sulfate. In contrast, we found that TSG-6 interacted directly with the CS chain and not with the protein moiety of bikunin. Using surface plasmon resonance, the K_d value of the interaction between TSG-6 and glycosylated bikunin was determined to be 180 nM, whereas no interaction was observed between deglycosylated bikunin and TSG-6. The K_d value for the interaction between IαI and TSG-6 in a divalent-cation-depleted environment was similar (140 nM). The lack of importance of the bikunin protein moiety was further emphasized by the ability of IαI molecules lacking the bulk of bikunin to form HC·TSG-6 complexes. Because the CS chain is necessary for the generation of HC1·TSG-6 and HC2·TSG-6, the mechanism of covalent complex formation is likely to be initiated by this noncovalent-cation-independent interaction with the CS chain. As previously mentioned, TSG-6 does not interact with free HCs (*12*), but the native form of the HCs are required for covalent complex formations. It indicates that the HCs guide the CS chain during the formation of HC1·TSG-6 and HC2·TSG-6.

We have shown that TSG-6 binds to bikunin via the CS chain, and recently, it was shown that the bikunin binding site on TSG-6 overlaps the HA binding surface (*12*). This suggests that the HA- and IαI CS-interacting grooves are similar. TSG-6 undergoes a conformational change during HA interaction (*34*), and it is reasonable to hypothesize that a similar conformational change takes place during the formation of the HC·TSG-6 complex. A HA heptasaccharide or larger is required to obtain maximum affinity between HA and TSG-6 (*34*). We have previously suggested that the IαI HCs are positioned relatively close to each other on the CS chain (*5*), and the CS segment between the HC1 and HC2 following limited ConABC digestion is likely to be too small to fit the TSG-6 binding groove. It explains why the HC1·HC2 complex did not participate in HC·TSG-6 complex formation.

The formation of HC·TSG-6 complexes did not abolish the ability of TSG-6 to interact with the CS chain of IαI. Hence, TSG-6 simultaneously interacts covalently and noncovalently with IαI: covalently via the HC·TSG-6 cross-link and noncovalently via the HC·TSG-6 interaction with the CS chain. The covalent cross-link between the HCs and TSG-6 is mediated by an ester bond between the α-carbon of the *C*-terminal Asp residue of the HCs and a TSG-6-derived hydroxyl donor, most likely a Tyr, Ser, or Thr residue (*15*). The fact that covalent complex formation does not disrupt the noncovalent interaction indicates that the involved residue from TSG-6 is not situated in the groove interacting with the CS chain in IαI. Interestingly, the link module of TSG-6 is able to interact with IαI, but the formation of covalent complexes requires full-length TSG-6 (*33*), indicating that the residue involved in the covalent complexes is positioned on the CUB domain of TSG-6. Because the groove in TSG-6, which interacts with the CS chain of IαI, is similar to the HA interacting groove, our results also suggest that HC·TSG-6 complexes interact with HA in vivo. Such an interaction could mediate the initial interaction required for the second transesterification generating HC·HA complexes.

The formation of the HC·TSG-6 complexes is likely to involve a two-step reaction mechanism. Initially, TSG-6 recognizes the CS chain of IαI in a divalent-cation-independent reaction. Subsequently, TSG-6 forms covalent complexes with the HCs in a divalent-cation-dependent manner. These interactions require native TSG-6 and native IαI but do not depend on the bikunin moiety. Furthermore, we conclude that the formation of HMW IαI also is a divalent-cation-dependent reaction. Finally, we show that TSG-6 remains associated with the CS chain after the formation of HC·TSG-6 complexes, indicating that the CS chain interacting site is distinct from the HC binding site on TSG-6.

ACKNOWLEDGMENT

We thank Drs. Steen Vang Petersen and Henrik Karring for helpful suggestions.

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BI060106S