TSG-6 Protein Binding to Glycosaminoglycans

Formation of Stable Complexes with Hyaluronan and Binding to Chondroitin Sulfates

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TSG-6 protein, up-regulated in inflammatory lesions and in the ovary during ovulation, shows anti-inflammatory activity and plays an essential role in female fertility. Studies in murine models of acute inflammation and experimental arthritis demonstrated that TSG-6 has a strong anti-inflammatory and chondroprotective effect. TSG-6 protein is composed of the N-terminal link module that binds hyaluronan and a C-terminal CUB domain, present in a variety of proteins. Interactions between the isolated link module and hyaluronan have been studied extensively, but little is known about the binding of full-length TSG-6 protein to hyaluronan and other glycosaminoglycans. We show that TSG-6 protein and hyaluronan, in a temperature-dependent fashion, form a stable complex that is resistant to dissociating agents. The formation of such stable complexes may underlie the activities of TSG-6 protein in inflammation and fertility, e.g. the TSG-6-dependent cross-linking of hyaluronan in the cumulus-oocyte complex during ovulation. Because adhesion to hyaluronan is involved in cell trafficking in inflammatory processes, we also studied the effect of TSG-6 on cell adhesion. TSG-6 binding to immobilized hyaluronan did not interfere with subsequent adhesion of lymphoid cells. In addition to immobilized hyaluronan, full-length TSG-6 also binds free hyaluronan and all chondroitin sulfate isoforms under physiological conditions. These interactions may contribute to the localization of TSG-6 in cartilage and to its chondroprotective and anti-inflammatory effects in models of arthritis.

TNFα-stimulated gene 6 (TSG-6) encodes a glycoprotein of ~35 kDa that is referred to as TSG-6 protein or TNF-inducible protein 6 (TNFIP6) (1–3). Expression of TSG-6 is up-regulated in various forms of arthritis (4). We and others (1, 5–10) have shown that TSG-6 protein exerts anti-inflammatory actions in murine models of acute inflammation and autoimmune arthritis. More recently, it has become clear that TSG-6 also plays an essential role in female fertility (1, 11). The protein consists of two domains, the N-terminal link module and the C-terminal CUB domain. The link module has long been recognized as a hyaluronan (HA)-binding domain (12, 13). The presence of the link module is a characteristic feature of members of the family of HA-binding proteins known as hyaladherins (14, 15). Although the link module is shared by all hyaladherins, TSG-6 is the only known member of this protein family containing a CUB domain. The CUB domain is present in a variety of proteins with diverse functions (16, 17). The CUB domain of spermadhesins, a family of proteins involved in fertilization, has been characterized as a glycosaminoglycan (GAG)- and oligosaccharide-binding module (17).

The study of the interactions between proteins and GAGs has been hampered by limitations of the assays used for their analysis. Adsorptive binding of GAGs to microtiter plates is not sufficiently stable to permit the application of stringent binding and washing conditions and, in particular, the use of dissociating agents. Adsorptive binding of GAG-binding proteins to the surface of microtiter plates, due to the differential hydrophobicity of the protein surface, may result in binding of these proteins in a preferred orientation. This may affect, and in extreme cases prevent, their interaction with GAGs. Another method used to investigate GAG-protein interactions, precipitation of GAG-protein complexes by cetyl pyridinium chloride (3, 18), may result in the nonspecific coprecipitation of some proteins and does not provide quantitative data.

These technical limitations may also have affected our understanding of the interactions of TSG-6 with HA and other GAGs. In addition, virtually all detailed studies addressing the binding of TSG-6 to HA have been carried out with the isolated link module, i.e. the N-terminal domain of TSG-6, and not with the full-length protein (19–23). However, the CUB domain, known as a GAG-binding domain from studies with other proteins, may also affect the binding of TSG-6 to GAGs (17, 24, 25). Therefore, the binding of full-length, glycosylated TSG-6 protein to GAGs deserves additional study.

In the present study, we used covalently coupled HA to analyze the interactions between full-length recombinant TSG-6 protein and HA or chondroitin sulfate (CS). The use of coupled HA permits the use of a wide range of binding and washing conditions, thereby reducing or eliminating nonspecific interactions and facilitating the testing of the stability of protein-GAG complexes with the aid of dissociating agents. We show that both full-length TSG-6 and the TSG-6 link module, in a temperature-dependent fashion, form a stable complex with HA. We further demonstrate that TSG-6 binds to HA and all common forms of CS under physiological conditions. We also show that covalently coupled HA can be used to analyze the adhesion of cells to HA. The formation of a stable bond between TSG-6 and HA is likely to have important functional implications in inflammation and fertility.
**TSG-6 Binding to Glicosaminoglycans**

**EXPERIMENTAL PROCEDURES**

Reagents—Covalink-NH plates (Cov-NH) were purchased from Nunc, HA from rooster comb, and N-ethyl-N’-(3-dimethylaminopropyl) carbodiimide were purchased from Sigma, and N-hydroxysulfo succinimide was purchased from Pierce. Chondroitin 4,6-sulfate (C-4,6-S) and chondroitin 6-sulfate (C-6-S) were purchased from Sigma, and chondroitin 4-sulfate (C-4-S) was purchased from Calbiochem. According to information from the suppliers, C-4,6-S (Sigma C6737, lot 43K1125) contains 57% C-4-S and 43% C-6-S; C-6-S (Sigma C4384, lot 42K1434) contains 85% C-6-S and 15% C-4-S, and C-4-S (Calbiochem 230657, lot B27594) contains 80% C-4-S and 20% C-6-S. Dextran sulfate from *Leuconostoc* spp. with an average molecular weight of 10,000 was from Sigma (DE82). N-(3-dimethylaminopropyl)-N’-hydroxysulfosuccinimide (lauryl sulfobetaine (LSB), zwittergent), p-nitrophenyl phosphate, Trizma, and Tween 20 were purchased from Sigma. The biotinylated goat anti-rabbit IgG antibody was from Dako, and the streptavidin-alkaline phosphatase conjugate was purchased from Invitrogen. For the detection of TSG-6, a rabbit antibody raised against native recombinant TSG-6 was used.

**TSG-6 Link Module—** The link module of TSG-6 was expressed, using the baculovirus expression system, similar to the expression of full-length TSG-6. From a construct containing a full-length TSG-6 cDNA in pGEM-7Zl, an Sphl/HindIII fragment was isolated and digested with Hgal, and the Sphl/Hgal fragment (470 bp) was isolated. After filling in with M13 polymerase and digestion with BamHI, the fragment was ligated into the EcoRI/Smal-digested pBatPAX8 transfer vector. This construct encodes the link module of TSG-6 with a C-terminal extension that consists of the amino acid residues Tyr128 to Glu134 connecting the link module (lauryl sulfobetaine (LSB), zwittergent) p-nitrophenyl phosphate, Trizma, and Tween 20 were purchased from Sigma. The biotinylated goat anti-rabbit IgG antibody was from Dako, and the streptavidin-alkaline phosphatase conjugate was purchased from Invitrogen. For the detection of TSG-6, a rabbit antibody raised against native recombinant TSG-6 was used.

**TSG-6 Binding Assay—** Cov-NH and Cov-HA plates were blocked with 0.1% casein in TTBS. After 15 min to 2 h, the medium of BW 5147 cells were plated into wells of Cov-HA or Cov-NH plates after, the wells were washed three times with 2M NaCl and three times with TTBS, followed by incubation with a rabbit anti-TSG-6 immunoglobulin antibody (dilution 1:1000 in TTBS), a streptavidin-alkaline phosphatase conjugate (dilution 1:1000 in TTBS), and finally nitrophenyl phosphate, Trizma, and Tween 20 were purchased from Sigma. The biotinylated goat anti-rabbit IgG antibody was from Dako, and the streptavidin-alkaline phosphatase conjugate was purchased from Invitrogen. For the detection of TSG-6, a rabbit antibody raised against native recombinant TSG-6 was used.

**RESULTS**

**Coupling of HA to Cov-NH—** In order to evaluate the covalent binding of HA to Cov-NH, we coupled increasing amounts of biotinylated HA (HA-bio) to Cov-NH. Significant amounts of HA-bio were coupled if HA-bio concentrations of 100–500 µg/ml were used (Fig. 1A). Because the 5-fold increase in the HA-bio concentration from 100 to 500 µg/ml resulted in only a modest increase in the amount of bound HA-bio, 100 µg/ml HA, equivalent to a total of 10 µg of HA per well, was used for the covalent coupling of HA to Cov-NH in all experiments reported here. HA-bio coupled to Cov-NH proved to be resistant to treatment with 6 M guanidine HCl (Fig. 1B). In contrast, a significant part of HA-bio coupled covalently to Cov-NH was susceptible to treatment with hyaluronidase from *Streptomyces hyalurolyticus*, with ~70% of the bound HA-bio removed by treatment with 1 unit/ml hyaluronidase for 1 h (Fig. 1C). Treatment with up to 100 units/ml hyaluronidase did not result in further degradation of the immobilized HA-bio (data not shown).

**Binding of TSG-6 to Immobilized HA—** Next, we determined the binding of recombinant TSG-6 to Cov-HA. Purified recombinant TSG-6 bound to Cov-HA in a concentration-dependent fashion (Fig. 2A). Binding of TSG-6 to immobilized HA at 37 °C was clearly detectable at a TSG-6 protein concentration of 2 nM and increased up to 50 nM without indication of reaching saturation of binding. Even at a concentration of 50 nM TSG-6, there was very little HA-independent binding of TSG-6 to Cov-NH (Fig. 2A). Fig. 2B shows the kinetics of TSG-6 binding to immobilized HA at a concentration of 5 nM TSG-6 at 37 °C. TSG-6 binding was detectable within 5 min and increased steadily over a 2-h period. Little additional binding could be detected thereafter (data not shown). Although TSG-6 bound to immobilized HA much faster at the physiological temperature of 37 °C, binding was detected at 4 °C (Fig. 2C). In order to determine whether TSG-6 binds not only to immobilized HA, but also to HA in solution, we conducted competition experiments by adding increasing amounts of HA to the wells of a Cov-HA plate together with recombinant TSG-6. Fig. 2D shows that free HA efficiently competed, in a concentration-dependent fashion, with immobilized HA for the binding of 10 nM TSG-6.

**TSG-6 Forms a Stable Bond with HA in a Temperature-dependent Fashion—** In order to determine the stability of the bond between TSG-6 and immobilized HA, we incubated TSG-6 with immobilized HA at 37 °C for 2 h, removed unbound TSG-6 by washing with TTBS, and subjected bond TSG-6 to various dissociating agents for 15 min. Fig. 3A shows that most of the bound TSG-6 proved to be resistant to treatment with 6 M guanidine HCl containing 8% LSB, reducing SDS-PAGE sample buffer, or 25 mM NaOH. These findings show that much of the bound TSG-6 formed a stable complex with immobilized HA. About the same percentage of HA-bound TSG-6 that was resistant to treatment with 6 M guanidine HCl containing 8% LSB also resisted treatment with reducing SDS-PAGE sample buffer or 25 mM NaOH. The latter findings suggest that the same fraction of TSG-6 that resisted washing with guanidine HCl also resisted the other stringent washing conditions and is likely to represent the fraction of TSG-6 that formed a stable complex with HA (Fig. 3A). Treatment of TSG-6 bound to Cov-HA with guanidine HCl for up to 60 min did not affect the amount of TSG-6 that remained bound to Cov-HA (data not shown).

In contrast, binding of TSG-6 to immobilized HA at 4 °C did not result in the formation of a similarly stable bond. TSG-6
was incubated in Cov-HA at 4 °C overnight and washed with TTBS before an additional wash under various stringent conditions was carried out for 15 min at ambient temperature. Fig. 3B shows that TSG-6 bound to immobilized HA at 4 °C was not only sensitive to treatment with dissociating agents such as 6 M guanidine HCl containing 8% LSB, 4 M guanidine HCl, or SDS-PAGE sample buffer but even to incubation at pH 2.5. Even incubation with 2 M NaCl resulted in a partial loss of the bound TSG-6 (Fig. 3B). A significant amount of the TSG-6 bound at 4 °C could be removed by washing at pH 4.5 (Fig. 3C). 0.5 M guanidine HCl was sufficient to remove most of the bound TSG-6, and even 250 mM guanidine HCl removed a significant part of TSG-6 bound to immobilized HA at 4 °C (Fig. 3C). Whereas TSG-6 that bound to immobilized HA at 4 °C formed a complex that was resistant to washing with TTBS, it did not withstand washing under more stringent conditions. This is in contrast to TSG-6 that bound to Cov-HA at 37 °C, which formed a stable complex that withstood the most stringent washing conditions (Fig. 3A). This result demonstrates that TSG-6 binding to HA and the formation of a stable complex are separate and most likely sequential events. At low temperature, e.g., 4 °C, the formation of a complex that is sensitive to dissociating agents is the end point of the reaction. At physiological temperature this binding reaction is followed by the transition of the initial complex into a complex that is resistant to dissociating agents.

In order to investigate the roles of link module and CUB domain to the formation of the stable complex, we expressed and purified the isolated TSG-6 link module. A comparison of the stability of the complexes formed by full-length TSG-6 and TSG-6 link module at 37 °C showed that both form a complex stable to treatment with 6 M guanidine HCl containing 8% LSB at almost exactly the same ratio relative to total binding (Fig. 3D).

**Binding of TSG-6 to Chondroitin Sulfates**—Our first study addressing the binding of TSG-6 to GAGs, using coprecipitation of GAG-TSG-6 complexes with cetyl pyridinium chloride, concluded that TSG-6 binds to HA but not to C-4,6-S or any other GAGs, with the possible exception of heparan sulfate (3). A later analysis of the binding of the isolated link module of TSG-6 to GAGs, based on the adsorptive binding of GAGs or protein to microtiter plates, demonstrated that the isolated link module, at pH 5.8, bound to HA and C-4-S with similar affinities (22). This study determined that the link module of TSG-6 did not interact with C-6-S (22). Most significantly, binding of the TSG-6 link module to HA or C-4-S at more physiological pH was not demonstrated (22).

Because of these intriguing data and the physiological significance of the interaction of TSG-6 with different isoforms of CS, we examined the binding of full-length TSG-6 to CSs, at the physiological pH of 7.5, using an assay based on competition between C-4-S and immobilized HA for the binding of TSG-6. Fig. 4A shows that C-4-S in solution competed efficiently with immobilized HA for the binding of TSG-6, comparable with HA in solution (Fig. 2D). Fig. 4, B and C, shows the same experiment with C-6-S and C-4,6-S, again demonstrating efficient under "Experimental Procedures." A–C, the amount of HA-bio present in the wells was determined with the aid of a streptavidin-alkaline phosphatase conjugate. B, stability of immobilized HA to treatment with 6 M guanidine. Immobilized HA-bio (Cov-HA-bio) was treated with either TTBS or 6 M guanidine HCl for 15 min at ambient temperature. Cov-NH and Cov-HA served as controls for the specificity of the detection reagents. C, digestion of immobilized HA-bio with hyaluronidase (HAse) from *S. haialurolyticus*. Cov-HA-bio was incubated with increasing concentrations of hyaluronidase in PBS, as indicated, for 1 h at 37 °C. Each data point represents the mean of six wells ± S.E. OD, optical density, absorbance.

**FIG. 1.** Covalent coupling of HA-bio to Cov-NH. A, dependence of the amount of HA-bio coupled to Cov-NH on the concentration of HA-bio used in the coupling reaction. The coupling was carried out as described...
competition with HA for the binding of TSG-6. An overlay of the data from these competition experiments (Fig. 4D) shows that the competition of C-4-S and C-4,6-S for the binding of TSG-6 is very similar to the competition by free HA, suggesting similar affinities of TSG-6 for HA and C-4-S or C-4,6-S. About 5-fold higher concentrations of free C-6-S were needed to achieve a 50% decrease in the binding of TSG-6 to immobilized HA (Fig. 4D), suggesting that TSG-6 binds to C-6-S with a slightly lower affinity than to either HA, C-4-S, or C-4,6-S. Nevertheless, the data clearly show that TSG-6 binds to all isoforms of CS without regard to the position of the sulfate groups and at physiological pH.

In order to test the structural specificity of the binding of TSG-6, we employed a sulfated but otherwise unrelated polysaccharide, dextran sulfate. In contrast to either free HA or chondroitin sulfates, dextran sulfate had little effect on the binding of TSG-6 to immobilized HA (Fig. 4E).

The major advantage of the competitive assay system that we employed for the analysis of TSG-6 binding to CSs is that it does not require any modification of the GAG and that it analyzes binding to CS in solution. An additional advantage is that it can easily be used to analyze the interaction between TSG-6 and other GAGs like heparan sulfate, keratan sulfate, or dermatan sulfate, for which only limited TSG-6 binding data or no data at all are currently available.

Effects of pH, Ionic Strength, and Metal Ions on the Binding of TSG-6 to HA—It was reported earlier that, in contrast to other members of the hyaladherin family such as aggrecan or link protein, the link module of TSG-6 does not bind to HA at neutral pH (23). TSG-6 link module binding to HA peaked at pH 5.5 to 6.0, and little binding was observed above pH 6.5 (23). This unique pH dependence of HA binding by TSG-6 suggested that in tissues TSG-6 would bind to HA only under inflammatory conditions associated with a lower pH and that this characteristic might contribute to the dissociation of aggrecan-link protein-HA aggregates in cartilage during inflammation (23). However, several other studies have demonstrated that TSG-6 binds to HA in vivo under noninflammatory conditions (9, 28, 29) and that TSG-6 is chondroprotective in experimental arthritis, rather
than being involved in cartilage dissociation (6–9).

Therefore, we examined the pH dependence of binding of full-length TSG-6 to immobilized HA. Our data indicate that TSG-6 binds to immobilized HA after binding at 4 °C. TSG-6 was incubated in Cov-HA at 4 °C overnight and washed three times with TTBS, followed by incubation with dissociating agents, at high pH (25 mM NaOH), low pH, or high ionic strength (PBS containing 2 M NaCl), as indicated, for 15 min at ambient temperature. C, TSG-6 bound to immobilized HA at 4 °C can be eluted at low pH or with low concentrations of guanidine HCl. TSG-6 was bound to Cov-HA at 4 °C overnight, washed three times with TTBS, followed by incubation with buffers of decreasing pH or with low concentrations of guanidine HCl, as indicated, for 15 min at ambient temperature. 100 mM glycine-HCl was used for pH 2.5 and pH 3.5, and 100 mM MES buffer was used for pH 4.5 to pH 6.5. D, stability of the bond between the TSG-6 link module and immobilized HA, formed at 37 °C, to dissociating agents. 40 nM TSG-6 link module and 10 nM TSG-6 was incubated in Cov-HA at 37 °C for 2 h. The wells were then washed three times with TTBS, followed by incubation with 8 M guanidine HCl containing 8% LSB, for 15 min at ambient temperature. A higher concentration of TSG-6 link module versus full-length TSG-6 protein was used to compensate for the decreased detection of link module by this antibody. Immunoblotting of full-length TSG-6 and TSG-6 link module, using the same antibody, confirmed that about a 4-fold molar excess of link module versus TSG-6 was necessary to obtain comparable bands (data not shown). Each data point represents the mean of six wells ± S.E. OD, optical density, absorbance.

**Fig. 3. Stability of TSG-6-HA complexes formed at 37 or 4 °C.** A, stability of the bond between TSG-6 and immobilized HA, formed at 37 °C, to dissociating agents. 50 nM TSG-6 was incubated in Cov-HA at 37 °C for 2 h. The wells were then washed three times with TTBS, followed by incubation with dissociating agents, or 25 mM NaOH, as indicated, for 15 min at ambient temperature. B, TSG-6 fails to form a stable bond to immobilized HA after binding at 4 °C. TSG-6 was incubated in Cov-HA at 4 °C overnight and washed three times with TTBS, followed by incubation with dissociating agents, at high pH (25 mM NaOH), low pH, or high ionic strength (PBS containing 2 M NaCl), as indicated, for 15 min at ambient temperature. C, TSG-6 bound to immobilized HA at 4 °C can be eluted at low pH or with low concentrations of guanidine HCl. TSG-6 was bound to Cov-HA at 4 °C overnight, washed three times with TTBS, followed by incubation with buffers of decreasing pH or with low concentrations of guanidine HCl, as indicated, for 15 min at ambient temperature. 100 mM glycine-HCl was used for pH 2.5 and pH 3.5, and 100 mM MES buffer was used for pH 4.5 to pH 6.5. D, stability of the bond between the TSG-6 link module and immobilized HA, formed at 37 °C, to dissociating agents. 40 nM TSG-6 link module and 10 nM TSG-6 was incubated in Cov-HA at 37 °C for 2 h. The wells were then washed three times with TTBS, followed by incubation with 8 M guanidine HCl containing 8% LSB, for 15 min at ambient temperature. A higher concentration of TSG-6 link module versus full-length TSG-6 protein was used to compensate for the decreased detection of link module by this antibody. Immunoblotting of full-length TSG-6 and TSG-6 link module, using the same antibody, confirmed that about a 4-fold molar excess of link module versus TSG-6 was necessary to obtain comparable bands (data not shown). Each data point represents the mean of six wells ± S.E. OD, optical density, absorbance.

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**Fig. 4. Binding of BW 5147 Cells to Immobilized HA.** The thymoma cell line BW 5147 has been known to aggregate in the presence of HA and to constitutively express an active form of the cellular HA receptor CD44 (30). By using HA immobilized to Co-
FIG. 4. CS competes efficiently with immobilized HA for the binding of TSG-6. A, increasing concentrations of C-4-S were incubated with 10 nM TSG-6 in Cov-HA for 2 h at 37 °C. B, increasing concentrations of C-6-S were incubated with 10 nM TSG-6 in Cov-HA for 2 h at 37 °C. C, increasing concentrations of C-4,6-S were incubated with 10 nM TSG-6 in Cov-HA for 2 h at 37 °C. D, comparison of the inhibition of TSG-6 binding to immobilized HA by either free HA or CS isoforms. Data from different experiments were normalized so that the binding of TSG-6 to Cov-HA in the absence of competing GAG equals 1. E, failure of dextran sulfate to compete with immobilized HA for the binding of TSG-6. 10 nM TSG-6 was incubated in the absence or the presence of the indicated concentrations of free dextran sulfate in Cov-HA for 2 h at 37 °C. Each data point represents the mean of six wells ± S.E. OD, optical density, absorbance.
valink plates, we found that BW 5147 cells bound readily to immobilized HA but not to Cov-NH that was used as a control for nonspecific binding (Fig. 6). After cells bound to immobilized HA, they could not be removed by aspiration of the medium and repeated washing with PBS. Binding was fast and could be detected within 10 min, i.e. it seemed to be determined entirely by the sedimentation rate of the cells (data not shown). Most significantly, binding of 10 nM TSG-6 protein to the immobilized HA before the addition of BW 5147 cells did not affect subsequent cell adhesion (Fig. 6). BW 5147 continued to grow on immobilized HA with no significant change of their growth rate (data not shown).

**DISCUSSION**

Earlier studies have provided evidence for the binding of TSG-6 to HA (3) and for the binding of the isolated TSG-6 link module to HA and C-4-S under acidic but not under neutral conditions (22, 23). Further studies have provided a detailed analysis of the interaction between the TSG-6 link module and HA (19–21, 31). Most of these studies were carried out at 25 °C, whereas the interaction of TSG-6 with HA at 37 °C was not studied. In addition, the interactions between the glycosylated full-length TSG-6 protein and HA, and in particular between TSG-6 and other GAGs, have not been investigated in detail. We have devised an assay system for the investigation of the interactions of TSG-6, or other GAG-binding proteins, with GAGs that does not only permit the determination of binding but is also uniquely suitable for the analysis of the stability of TSG-6-HA complexes to treatment with dissociating and denaturing agents. Our data show that TSG-6 protein, or the isolated TSG-6 link module, in the absence of any other factors, forms a stable complex with immobilized HA in a temperature-dependent fashion. Stable complex formation by the TSG-6 link module indicates that the CUB domain is not essential for the formation of stable TSG-6-HA complexes. These results provide...
adherent cells in Cov-NH. The absorbance at 615 nm, provides a measure of the number of adherent cells. The concentration of the dye eluted from the cells, determined by PBS, the remaining adherent cells were stained with naphthol blue black. The concentration of the dye eluted from the cells, determined by the absorbance at 615 nm, provides a measure of the number of adherent cells. Each data point represents the mean of six wells ± S.E. Microscopic observation confirmed the virtually complete absence of adherent cells in Cov-NH. OD, optical density, absorbance.

the first evidence for the formation of a complex between HA and any hyaladherin that is stable to treatment with dissociating agents.

Although the temperature-dependent formation of stable TSG-6-HA complexes has not been reported earlier, it has been noticed that the interaction between the TSG-6 link module and HA shows unusual features at temperatures at and above 35 °C. Isothermal titration calorimetry, applied to the interaction of the TSG-6 link module with an HA octamer, revealed a linear correlation between the enthalpy change and the temperature in the range from 10 to 30 °C (20). However, the enthalpy change increased greatly between 35 and 40 °C. Based on additional NMR data, Kahmann et al. (20) suggested that ligand-induced protein refolding contributes to this enthalpy change. HA-induced refolding of the TSG-6 link module at 37 °C may provide a potential mechanism for the formation of stable TSG-6-HA complexes at this temperature, or it may at least contribute to it.

Earlier, inter-α-inhibitor (IαI), a normal plasma protein, was shown to form stable complexes with TSG-6 and HA (26, 29, 32–34). IαI consists of three polypeptide chains, two so-called heavy chains and bikunin, that are linked by a C-4-S chain, resulting in a protein-GAG-protein bridge (35–37). The two heavy chains of IαI are linked to the C-4-S chain by an unusual ester bond (36). These same chains, in a reaction dependent on TSG-6 (1, 33), can be transferred to HA, where they are again linked by an ester bond (34, 38). These ester bonds are sensitive to treatment with alkali (37–39). We addressed the possibility, however remote, of the formation of an ester bond between TSG-6 and immobilized HA by treating preformed TSG-6-HA complexes with 25 mM NaOH. This treatment did not release more TSG-6 from Cov-HA than other stringent washing conditions (Fig. 4A). This finding does not support the notion that TSG-6 forms an ester bond with HA.

We show here that full-length TSG-6 does not only bind to C-4-S under acidic conditions as has been reported earlier (22), but it binds to all common isoforms of CS under physiological conditions. This has important physiological implications. Sulfated chondroitin, forming side chains of aggrecan, is a significant structural component of cartilage. Aggrecan is a target of proteolytic attack during arthritis resulting in cartilage erosion. Data obtained in murine models of experimental arthritis consistently indicated that TSG-6 has a chondroprotective effect associated with its anti-inflammatory activity (6, 7, 40). This chondroprotective effect of TSG-6 in mice with antigen-induced arthritis includes the almost complete inhibition of aggrecan cleavage by stromelysin and aggrecanase (8). The presence of TSG-6 in cartilage has been demonstrated, and it is likely that the chondroprotective effect of TSG-6 is associated with its localization in cartilage (41). Our data suggest that besides binding to HA, TSG-6 binding to CS may also be responsible for its localization in cartilage and may directly contribute to its inhibition of aggrecan degradation in experimental models of arthritis.

There is abundant evidence that CD44-HA interactions mediate adhesion of leukocytes and activated T cells and are involved in the rolling of these cells along vessel walls and in the extravasation of activated T cells (42–49). We therefore used the murine thymoma cell line BW 5147 to study the adhesion of cells to immobilized HA and the effect of TSG-6 binding on cell adhesion. BW 5147 cells express an active form of the HA receptor CD44 and aggregate in the presence of HA (30). Although BW 5147 cells bound readily to immobilized HA, but not to control wells free of HA, TSG-6 binding to the immobilized HA did not affect the firm adhesion of these cells to immobilized HA (Fig. 6). Therefore, it appears unlikely that TSG-6 causes its potent anti-inflammatory effect by preventing the firm adhesion of activated leukocytes to HA. Lesley et al. (50) reported recently that TSG-6 modulates the binding of HA to CD44. Preincubation of HA with an excess of TSG-6 resulted in a modest increase of the binding of fluorescein-labeled HA to cells and of the rolling of cells on immobilized HA, but it had little effect on the firm adhesion of these cells (50). This latter finding is in good agreement with our data, despite the fact that Lesley et al. (50) used different cell lines, higher TSG-6 concentrations, and a different assay system to determine cell adhesion.

The formation of a stable bond between TSG-6 and HA may significantly contribute to changes in the extracellular matrix that are associated with processes like ovulation and inflammation. Mammalian oocytes are surrounded by cumulus cells forming a compact cumulus cell-oocyte complex. During ovulation, this complex undergoes a dramatic expansion (51). This expansion of the ovariocytic follicle is caused by rapid HA synthesis, resulting in the formation of an HA-rich extracellular matrix. Studies in knock-out mice have shown that three genes, encoding TSG-6, pentraxin 3 (PTX3), and bikunin, one of the polypeptide chains of IαI, are essential for the structural integrity of the cumulus cell-oocyte complex during ovulation and that the disruption of any one of these genes impairs female fertility (1, 11, 52–54). It is assumed that the stability of the cumulus cell-oocyte complex matrix is achieved by cross-linking of HA. Only the heavy chains of IαI have so far been shown to form a stable bond with HA (34, 38), but the formation of stable complexes between TSG-6 and HA under physiological conditions may also contribute to the cross-linking of HA. Additional work is required to elucidate the details of this cross-link.

New and unusual HA structures that contain heavy chains of IαI have also been identified in inflammatory processes where they act as leukocyte receptors. These structures, currently referred to as “HA cables,” have been detected on the surface of
smooth muscle cells after infection with virus, treatment with poly(I-C), or as a result of so-called endoplasmic reticulum stress, and they are also found in inflammatory lesions of the colon in patients with Crohn’s disease and ulcerative colitis (55–57). Although the presence of Igl heavy chains in these structures suggests a role for TSG-6 in their formation, TSG-6 was not detected in the HA cables (58). However, TSG-6 was present in distinct filamentous structures on the surface of the same cells (58). The formation of a stable bond between TSG-6 and HA could contribute to the formation of stable TSG-6-HA structures that may persist when TSG-6 itself, after transient expression in response to proinflammatory cytokines, is no longer produced. We noticed earlier that the anti-inflammatory effect of recombinant TSG-6 administered to mice with collagen-induced arthritis persisted for at least 2 weeks after the last TSG-6 injection, although no TSG-6 was detectable in the sera of these mice (6). Formation of a stable bond between TSG-6 and HA may promote sequestration of TSG-6 in the extracellular matrix of HA-rich tissues, e.g. in cartilage, possibly providing an explanation for the unusual persistence of the anti-inflammatory effect of recombinant TSG-6.

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