The Inflammation-associated Protein TSG-6 Cross-links Hyaluronan via Hyaluronan-induced TSG-6 Oligomers

Received for publication, April 5, 2011, and in revised form, May 5, 2011. Published, JBC Papers in Press, May 19, 2011, DOI 10.1074/jbc.M111.247395

Natalia S. Baranova, Erik Nilebäck, F. Michael Haller, David C. Briggs, Sofia Svedhem, Anthony J. Day, and Ralf P. Richter

From the Biosurfaces Unit, CIC biomaGUNE, Paseo Miramon 182, 20009 Donostia-San Sebastian, Spain, the Max-Planck-Institute for Metals Research, Stuttgart, Heisenbergstrasse 3, 70569 Stuttgart, Germany, the Department of Applied Physics, Chalmers University of Technology, 41296 Göteborg, Sweden, Q-Sense, Hägmpilsgatan 7, 42677 Västra Frölunda, Sweden, Hyalose, LLC, Oklahoma City, Oklahoma 73104, and the Wellcome Trust Centre for Cell Matrix Research, Faculty of Life Sciences, University of Manchester, Oxford Road, Manchester M13 9PT, United Kingdom

Tumor necrosis factor-stimulated gene-6 (TSG-6) is a hyaluronan (HA)-binding protein that plays important roles in inflammation and ovulation. TSG-6-mediated cross-linking of HA has been proposed as a functional mechanism (e.g. for regulating leukocyte adhesion), but direct evidence for cross-linking is lacking, and we know very little about its impact on HA ultrastructure. Here we used films of polymeric and oligomeric HA chains, end-grafted to a solid support, and a combination of surface-sensitive biophysical techniques to quantify the binding of TSG-6 into HA films and to correlate binding to morphological changes. We find that full-length TSG-6 binds with pronounced positive cooperativity and demonstrate that it can cross-link HA at physiologically relevant concentrations. Our data indicate that cooperative binding of full-length TSG-6 arises from HA-induced protein oligomerization and that the TSG-6 oligomers act as cross-linkers. In contrast, the HA-binding domain of TSG-6 (the Link module) alone binds without positive cooperativity and weaker than the full-length protein. Both the Link module and full-length TSG-6 condensed and rigidified HA films, and the degree of condensation scaled with the affinity between the TSG-6 constructs and HA. We propose that condensation is the result of protein-mediated HA cross-linking. Our findings firmly establish that TSG-6 is a potent HA cross-linking agent and might hence have important implications for the mechanistic understanding of the biological function of TSG-6 (e.g. in inflammation).

Hyaluronan (HA) is a structurally simple and linear polysaccharide. It is ubiquitous in the extracellular matrix of vertebrates and plays important roles in numerous physiological and pathological processes, such as inflammation, fertilization, embryogenesis, tumor development, osteoarthritis, and atherosclerosis (1, 2). HA is considered a “pericellular cue” (3) (i.e. it serves as a versatile scaffold within which other molecules are organized and regulated). A number of proteins, called hyaladherins (4), can bind to the flexible HA chains and engender self-assembly into large and hydrated multimolecular complexes (5–7).

The secreted product of tumor necrosis factor-stimulated gene-6 (TSG-6) (8, 9) is of particular importance for the formation and remodeling of HA-rich pericellular coats (10, 11) and extracellular matrices (12). There is little or no constitutive expression of TSG-6 in most adult tissues (with the exception of bone marrow (13) and epidermis (14)). Expression is elevated in response to stimulation with proinflammatory mediators or certain growth factors (8, 9, 15–18), and TSG-6 is detected in the context of many inflammatory diseases (19, 20) and in inflammation-like processes, such as ovulation (21, 22).

TSG-6 is composed mainly of two contiguous domains, a Link module and a CUB module (8, 17, 23). The Link module is conserved among members of the hyaladherin family (4) and is essential for binding to HA (23). Administration of recombinant human TSG-6 Link module (Link_TSG6) in vivo or in cell culture has been found to elicit biological responses comparable with those of full-length TSG-6 (13, 25–28), suggesting that Link_TSG6 is a useful model for the intact protein. Indeed, most of our current knowledge about the interaction between HA and TSG-6 comes from structural studies and in vitro binding assays on Link_TSG6. In contrast, only a few plate-based HA-binding assays have been reported for full-length TSG-6 (29–31).

Little is known about the function of the TSG-6 CUB module, although the fact that it is highly conserved between species suggests that it is important for at least some activities of TSG-6 (8, 31). A case in point is that the TSG-6-mediated covalent transfer of heavy chains of inter-α-inhibitor (IαI) onto HA requires the full-length protein (26, 32). Furthermore, we have recently identified that the CUB_C domain of TSG-6 (i.e. the α-inhibitor; SAV, streptavidin; oHA, oligomeric HA (9-mer); pHA, polymeric HA; OEG, oligoethylene glycol; SLB, supported lipid bilayer; QCM-D, quartz crystal microbalance with dissipation monitoring; RICM, reflection interference contrast microscopy; GuHCl, guanidine hydrochloride.

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CUB module together with the C-terminal region mediates the binding of TSG-6 to fibronectin (24).

Some of the functions of TSG-6 are clearly related to its ability to bind to HA. Moreover, TSG-6 and HA were reported to form stable complexes in solution that enhance or induce binding of HA to the cell surface receptor CD44 on lymphocyte cell lines (25). Based on this observation, it was proposed that either Link_TSG6 or full-length TSG-6 alone can cross-link HA and that cross-linking is functionally important in the regulation of inflammation (6, 25).

TSG-6 has a wide range of binding partners. Apart from HA, the protein interacts with many other glycosaminoglycans, such as chondroitin 4-sulfate, dermatan sulfate, and heparin/eparan sulfate; with the G1 domains of the proteoglycans aggrecan and versican; and with a number of other extracellular proteins, including Iol, pentaxin 3 (PTX3), and thrombospondin 1 (26–28, 33–37) (reviewed in Refs. 6, 8, and 9). Simultaneous binding of TSG-6 to HA and to either of these latter proteins has been suggested as alternative routes for HA cross-linking (6, 28).

To obtain direct evidence for and to understand the mechanisms behind TSG-6 mediated cross-linking, it would be desirable to be able to study the interaction between TSG-6 and a well defined supramolecular assembly of HA. Here, we present a novel experimental approach that realizes this goal. The method is based on the immobilization of either polymeric or oligomeric HA via their reducing end to protein-repellent planar solid supports. With a combination of several surface-sensitive biophysical techniques, namely quartz crystal microbalance with dissipation monitoring (QCM-D), ellipsometry, and colloid probe reflection interference contrast microscopy (RICM), we have quantified the binding kinetics of both recombinant human full-length TSG-6 (rhTSG-6) and Link_TSG6 to HA and correlated the binding to morphological changes of HA films. Our results provide novel insights into the molecular mechanism of HA cross-linking by TSG-6 and the properties of the ensuing HA supramolecular complexes, which have far reaching implications for the potential effect of TSG-6 in vivo.

EXPERIMENTAL PROCEDURES

Protein and Hyaluronan Preparations—Wild type human TSG-6 Link module (Link_TSG6, 10.9 kDa (38)) was expressed in Escherichia coli as described previously (38, 39). Full-length recombinant human TSG-6 (rhTSG-6; 30.1 kDa (31)) was expressed in Drosophila Schneider 2 cells and purified as described previously (31). Lyophilized streptavidin (SAv; Sigma) was taken up in ultrapure water. Stock solutions of all proteins, typically at 1 mg/ml, were aliquoted and stored at −20 °C.

Lyophilized polymeric HA (pHA), biotinylated at its reducing end and with well defined molecular masses of 58 ± 3 kDa (Select-HA B50) and 1080 ± 56 kDa (Select-HA B1000) was purchased from Hyalose (Oklahoma City, OK) as well as non-biotinylated HA of 262 ± 13 kDa (Select-HA 250). For reconstitution, HA was taken up in ultrapure water at a stock concentration of 1 mg/ml, gently shaken overnight, aliquoted, and stored at −20 °C. HA oligomers (oHA) with θ-glucuronic acid at both termini and with a ~3-nm spacer and a biotin moiety at their reducing end, were prepared as described in the supplemental material.

A Hepes buffer (150 mM NaCl, 10 mM HEPES, pH 7.4, 3 mM NaN3, 2 mM CaCl2 in ultrapure water) was used throughout all measurements, and protein and HA solutions at their final concentrations were prepared in this buffer except where otherwise stated.

Preparation of Protein Repellent, Biotin-functionalized Surface Coatings—Supported lipid bilayers (SLBs) on silica or glass surfaces were formed by the method of vesicle spreading (40), using small unilamellar vesicles that contained dioleoylphosphatidylethanolamine-cap-biotin and dioleoylphosphatidylcholine (both from Avanti Polar Lipids, Alabaster, AL) at a molar ratio of 1:9. Gold surfaces were functionalized with a dense oligoethylene glycol (COE) monolayer by incubation in an ethanolic solution of a biotin-OEG disulfide (SS-OEG-biotin) and plain OEG disulfide (SS-OEG; both from Polypure, Oslo, Norway) at a molar ratio of 1:99 (see supplemental material for details).

Assembly of HA Films on SLBs and OEG Layers—HA films were prepared as described previously for SLBs (41). Briefly, a dense SAv monolayer was formed by exposure of 10 μg/ml SAv (30 min) to a biotinylated SLB or OEG layer. Biotinylated HA was then grafted to the SAv monolayer by incubation of 1–10 μg/ml HA solution (supplemental Figs. S1 and S2). Samples were kept wet at all times.

Tuning of HA concentration and incubation time allowed for the HA density on the surface to be controlled in a quantitative manner (supplemental Fig. S3). The grafting density was set to 65 ± 5 ng/cm2 for 58-kDa HA in QCM-D measurements and to ~35 ng/cm2 for 1080-kDa HA in RICM measurements (supplemental Fig. S3), corresponding to mean anchor distances of 13 and 77 nm between neighboring chains, respectively. For ellipsometric measurements, densities of 58-kDa HA from 20 to 75 ng/cm2 were used, corresponding to mean distances of 24 to 12 nm, respectively. The surface density of oHA ranged between 13 and 28 ng/cm2, corresponding to mean distances of 6.6 to 4.5 nm.

Quartz Crystal Microbalance with Dissipation Monitoring—QCM-D measures changes in resonance frequency, Δf, and dissipation, ΔD, of a sensor crystal upon interaction of (soft) matter (e.g. biomolecules) with its surface (42). The QCM-D response is sensitive to the mass (including coupled water) and the viscoelastic properties of the surface adlayer. Adsorption and interfacial processes on silica or gold-coated QCM-D sensors were monitored in situ with subsecond time resolution under continuous flow of sample solution (43) (see supplemental material for details).

In Situ Ellipsometry—Ellipsometry measures changes in the polarization of light upon reflection at a planar surface. We employed ellipsometry in situ on silica or gold-coated silicon wafers as substrates that were installed in a custom-built open cuvette with a continuously stirred sample solution to quantify adsorbed biomolecular masses in a time-resolved manner (43) (see supplemental material for details).

Quantification of Binding Constants—The binding constant, Kd, and the saturation limit, Γmax, for the binding of TSG-6 to HA films was obtained from the surface densities at equilib-
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**RESULTS**

**Design of HA Model Films to Study TSG-6-Hyaluronan Interactions**—To analyze the interaction of TSG-6 with HA, we designed surface-confined films that present HA of selected molecular weights in a well defined supramolecular assembly (Fig. 1). The construction of the films was monitored and controlled by QCM-D (supplemental Figs. S1 and S2).

In a first model system, pHA with a molecular mass of either 58 or 1080 kDa was immobilized via a biotin tag at its reducing end to solid supports that were previously functionalized with a protein-repellent coating and a dense monolayer of streptavidin (Fig. 1A). The grafting density can be controlled, and a highly hydrated film of partly stretched and entangled HA chains with up to several hundred nm in thickness, a so-called HA brush, is formed (41). Assuming a footprint of HA10 per TSG-6 protein (29, 46), each pHA chain of 58 and 1080 kDa can accommodate up to 30 and 571 TSG-6 molecules, respectively.

In a second approach, short oligomeric HA chains, comprising only nine monosaccharides (oHAs), were grafted to the surface (Fig. 1B). Grafting was again mediated by a biotin tag. A linker of about 3 nm in contour length (47) was placed between the biotin and the reducing end of HA to enhance the conformational freedom of the surface-bound oHA chains. Each oHA chain is 4.5 nm long and can interact with only one TSG-6 molecule at a time, and previous studies have shown that ~5 sugar monomers probably fill its HA-binding site (48).

Figures S1 and S2) and (ii) gold-supported OEG films exposing biotin groups (supplemental Fig. S2). The properties of the HA films were not affected by the choice of the primary surface layer (supplemental Fig. S4). SLBs were used for all studies related to Link_TSG6. OEG-covered surfaces were used for rhTSG-6 because they were found to exhibit better passivation against nonspecific binding of this protein (supplemental Fig. S5A).

**Binding of Full-length TSG-6 to HA Films**—To obtain quantitative insight into the kinetics of rhTSG-6 binding, we performed titration assays on oHA and pHA films using in situ ellipsometry (Fig. 2). The titration curves (Fig. 2C) exhibited a pronounced sigmoidal shape. The curves could be fitted well by the Hill equation (Equation 1) with exponents above 2, indicating that binding is cooperative. The exponents for pHA and oHA (2.7 ± 0.8 and 2.8 ± 0.5, respectively) coincided within experimental error (Table 1). The $K_{D}$ for pHA was similar to although slightly lower than for oHA (Table 1). On oHA, we found a stoichiometry of 2.7 ± 0.8 rhTSG-6 molecules per oHA chain (Table 1), indicating that more than one rhTSG-6 molecule can bind to oHA. The size of the proteins, the thickness of the passivation layer, and the length of oHA are drawn to scale; the thickness of the pHA films is compressed by up to 20-fold.

**Architecture of HA films.** A dense SAv monolayer was formed on a biotin-functionalized passivation layer. The passivation layer, either a silica-supported lipid bilayer or a gold-supported OEG layer (supplemental Figs. S1 and S2), was designed to inhibit nonspecific binding of TSG-6. HA chains were grafted through a biotin functionality at their reducing end to the SAv layer. A, pHA with molecular mass of 58 or 1080 kDa exposes up to several hundred binding sites for the HA-binding protein TSG-6 on each individual polymer chain. B, only one TSG-6 molecule can bind to oHA. The size of the proteins, the thickness of the passivation layer, and the length of oHA are drawn to scale; the thickness of the pHA films is compressed by up to 20-fold.
rhTSG-6 or on both. In fact, the supratetraoctiometric binding of rhTSG-6 to oHA provides strong indications that the binding of one rhTSG-6 molecule to HA is sufficient to induce the formation of protein dimers (or even larger oligomers). In this regard, we have observed previously that rhTSG-6 forms elongated end-to-end dimers in the presence of excess HA as well as larger species (49).

The dissociation of rhTSG-6 from HA films was generally slow. Only double exponentials (Equation 3) provided a good fit to the desorption curves upon rinsing of close-to-saturated HA films with buffer (Fig. 2, A and B, and Table 1). The fit did not reveal a significant fraction of irreversibly bound rhTSG-6 ($I_{\overline{m}} = 0$). The first dissociation rate constant was $\sim 4 \times 10^{-3}$ s$^{-1}$ for oHA and slightly smaller for pHA ($\sim 3 \times 10^{-3}$ s$^{-1}$). Notably, almost 50% of the protein on oHA but less than 10% on pHA desorbed with the faster dissociation rate. The second dissociation rate constant was at least 1 order of magnitude smaller than the first and slightly larger for pHA. One might be tempted to attribute the two apparent rate constants to two discrete unbinding events. The numbers are, however, also consistent with the presence of a spectrum of dissociation rates. The latter would be expected for the formation of a wide range of HA/TSG-6 complexes where the different interactions (e.g. HA-protein and protein-protein) probably have different stabilities.

Given the slow dissociation, not all rhTSG-6 could be unbound within experimentally accessible time scales. However, the remaining fraction could readily be eluted by an 8 M concentration of the dissociating agent guanidine hydrochloride (GuHCl) (Fig. 3). In contrast, the attachment of HA to our passivation layers via biotin and streptavidin was not disrupted by GuHCl (supplemental Fig. S7). rhTSG-6 hence does not form covalent complexes with HA, as had been suggested based on microtiter plate-based HA-binding assays (30).

**Full-length TSG-6 Oligomers Can Cross-link HA**—To test if rhTSG-6 that is bound to HA films can still bind additional HA, a "sandwich" assay by QCM-D was designed (Fig. 4). rhTSG-6 was first exposed to an oHA film. The total frequency shift for the oHA/rhTSG-6 film was $\sim 34$ Hz. The corresponding thickness of $\sim 6$ nm and the minor changes in dissipation are consistent with the formation of a rather dense layer of rhTSG-6; a control measurement on an HA-free surface did not show measurable binding of active rhTSG-6 (i.e. the small amount of nonspecifically bound rhTSG-6 was not able to bind to HA; supplemental Fig. S5B). This confirms that the rhTSG-6 protein was indeed immobilized via its binding to oHA and that rinsing in buffer induced a slow release of TSG-6 (between 62 and 67 min in Fig. 4A).

Subsequent rapid incubation with pHA (262 kDa) resulted in a two-phase response (Fig. 4A). Initially, the frequency decreased rapidly, together with a pronounced increase in dissipation. Such a response is typical for the formation of a soft layer, as would be expected for the binding of pHA on top of the TSG-6-covered oHA film. For comparison, no pHA binding was observed on an rhTSG-6-free surface (supplemental Fig. S5C). Clearly, full-length TSG-6 dimers (or higher oligomers) that had already bound to oHA retained the ability to bind additional HA. In the second phase, both the frequency and the dissipation shift increased slowly, suggesting remodeling of the surface-bound film. The rate of frequency increase changed after removal of pHA from the solution phase, indicating that a supply of unbound HA must be involved in this process. A plausible explanation for the observed response would be that pHA captures some of the rhTSG-6 that is slowly released from the oHA film, and thanks to the cross-linking activity of rhTSG-6, a multilayer of pHA is formed on top of the oHA film (Fig. 4, B and C).

The cooperative binding of rhTSG-6 and its ability to cross-link HA have interesting implications with respect to the stability of rhTSG-6 oligomers in the absence of HA. If rhTSG-6 alone would form stable oligomers, their binding into the HA matrix would correspond to a multivalent interaction. Although multivalent binding would most likely exhibit an increased avidity, as compared with monovalent binding, it would not be cooperative. The observed cooperativity hence...
TABLE 1

| Binding parameters for the incorporation of Link_TSG6 and rhTSG-6 into oHA and pHA films, determined from titration curves as shown in Figs. 2 and 7 |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                 | $K_{n,s}$       | $k_{off}$       | $f^*$           | $k_{on}$         | $n$             | Stoichiometry at maximal binding$^*$ |
|                 | $10^{-6}$ M$^{-1}$ s$^{-1}$ | $10^{-3}$ s$^{-1}$ | $10^9$ M$^{-1}$ s$^{-1}$ | |
| Link_TSG6       | 6.1 ± 2.5       | 28 ± 3$^a$      | −86 ± 3%        | 4.6 ± 2.4       | 1.3 ± 0.3 per HA$_9$ |
| rhTSG-6         | 1.2 ± 0.4       | 43 ± 9%         | 0.052 ± 0.018   | 2.7 ± 0.8       | 2.7 ± 0.8 per HA$_9$ |
| Link_TSG6       | 5.5 ± 0.6       | 23 ± 8$^d$      | −79 ± 4%        | 4.2 ± 1.9       | 1 per 2.9 ± 0.3 HA$_9$ |
| rhTSG-6         | 0.79 ± 0.17     | 8 ± 1%          | 0.14 ± 0.07     | 2.8 ± 0.5       | 1 per 3.7 ± 1.0 HA$_2$ |

$^a$ $f = r/r_{total}$, where $r_{total} = r_{on} + r_{off}$, $r_{on}$ and $r_{off}$ are the binding and dissociation rates, respectively.

$^b$ Determined from $K_{n,s} = K_{off}/k_{on}$ for non-cooperative binding ($n = 1$).

$^c$ Determined from $K_{off}$ using Equation 1 and the surface density of immobilized HA.

$^d$ Determined from Equation 2.

$^e$ Determined from Equation 3 for desorption processes of 1-h duration and with $\Gamma_0$ set to 0.

Errors correspond to experimental uncertainties and variations across 2–3 measurements.

**FIGURE 3. Full-length TSG-6 can be fully eluted with dissociating agents.** A pHA (58 kDa) film that was previously close to saturated with rhTSG-6 and rinsed with Hepes buffer was exposed to 8 M GuHCl. All of the protein is covalently immobilized to HA. All processes, except the incubation with GuHCl, were monitored by ellipsometry. The HA film was stable in GuHCl (supplemental Fig. S7A). The measurement was repeated twice, and the displayed data are representative.

This implies that the oligomeric state of rhTSG-6 is induced (or stabilized) by HA.

With respect to cross-linking, the binding behavior of rhTSG-6 to oHA films merits detailed consideration. The average spacing between neighboring oHA strands, 4–7 nm, is comparable with the size of TSG-6; TSG-6 can be estimated to have a contour length, or

$$L = \frac{M}{\pi r^2 D}$$

where $M$ is the molecular mass of TSG-6, $r$ is the radius of the TSG-6 molecule, and $D$ is the hydrodynamic diameter of the TSG-6 molecule. By considering the conformations of TSG-6 in solution, we can estimate that the average size of TSG-6 is approximately 5–10 nm in length. The average spacing between neighboring oHA strands is therefore comparable with the size of TSG-6, and the binding of TSG-6 to oHA is consistent with this estimate.

The stoichiometry of the binding reaction can be estimated using the following equation:

$$K_{n,s} = \frac{k_{off}}{k_{on}}$$

where $K_{n,s}$ is the association constant for the binding of TSG-6 to oHA, $k_{off}$ is the dissociation rate constant, and $k_{on}$ is the association rate constant. The stoichiometry of the binding reaction can be estimated from the following equation:

$$n = \frac{k_{off}}{k_{on}}$$

where $n$ is the number of TSG-6 molecules bound per oHA chain.

Confined rhTSG-6 oligomers would mostly connect to only a single oHA chain. This is also consistent with the finding that immobilized TSG-6 is still able to bind pHA from the solution phase (Fig. 4A).

**Full-length TSG-6 Induces Strong Condensation of HA Films**—Next, we investigated how the influx of rhTSG-6 and the ensuing cross-linking affect the overall morphology of pHA films. Exposure of rhTSG-6 to a pHA (58 kDa) film induced a monotonous and strong decrease in the QCM-D frequency response (Fig. 5A). In contrast, the dissipation initially increased and then decreased. The decrease in particular is indicative of a rigification of the pHA film.

We employed colloidal probe RICM to quantify the variations in film thickness upon the addition of rhTSG-6 (Fig. 5B). To extend the range of potential thicknesses, we used HA of larger molecular mass (1080 kDa) while maintaining the total mass of HA per surface area comparable with the previously described pHA films (supplemental Fig. S3). Titration of rhTSG6 initially resulted in a gradual decrease in film thickness. Significant film condensation was already observed at 0.16 $\mu$M rhTSG-6 in the bulk solution, and the film thickness decreased by more than 2-fold, from 400 to 180 nm, upon exposure to 0.33 $\mu$M rhTSG-6 protein. Based on the kinetic data that we had obtained by ellipsometry (Table 1), a bulk concentration of 0.33 $\mu$M rhTSG-6 would result in an average occupancy of one rhTSG-6 molecule per 44 HA$_2$ repeating units (one protein every HA$_8$; Fig. 5B, inset), which is equivalent to one protein every 44 nm of HA contour length, or ~65 TSG-6 molecules per HA chain.

How can this rather low occupancy induce such a strong condensation of the HA films? In the absence of TSG-6, the surface-grafted pHA films form a so-called polymer brush (41). Such a brush can be pictured as a strongly hydrated and highly dynamic meshwork of entangled polymer chains that are weakly stretched in the direction perpendicular to the surface (51, 52) (Fig. 6A). The water content in the 1080-kDa pHA films (51, 52) predicts that the size of the meshes (or "holes") in the pHA meshwork is comparable with the mean distance between the anchor points of neighboring HA chains (~80 nm for 1080-kDa
pHA films). The introduction of cross-linkers will force neighboring HA chains closer together and decrease the mesh size (Fig. 6B). As a result, the film’s thickness will decrease, whereas its rigidity will increase. Given that the films have a large mesh size to start with, a rather small amount of cross-linkers can already have an appreciable effect on the film thickness. The frequency increase upon subsequent rinsing with buffer indicates slow desorption of some rHTSG-6. The rapid addition of pHA (262 kDa) without biotin linker resulted in a two-phase response that is indicative of two overlapping processes. The initial decrease in frequency indicates binding of pHA to the oHA-bound rHTSG-6. The concomitant strong increase in dissipation is characteristic for the formation of a soft and highly hydrated film. The subsequent increase in frequency is probably a result of desorption of rHTSG-6 and/or migration of rHTSG-6 inside the pHA film. This process continues, albeit at a slower pace, after rinsing in buffer. The measurement was performed twice, and the data shown are representative.

FIGURE 4. Full-length TSG-6 cross-links HA. A, sandwich assay, monitored by QCM-D. The start and duration of the incubation with different samples and buffer are indicated (solid arrows and dashed arrows, respectively). A film of surface-bound oHA was incubated with rHTSG-6 until equilibrium, reaching a frequency shift of $\Delta f = -34$ Hz and a dissipation shift of $\Delta D = 1.1 \times 10^{-6}$, indicating the formation of a rather dense and rigid film of about 6 nm in thickness. The frequency increase upon subsequent rinsing with buffer indicates slow desorption of some rHTSG-6. The rapid addition of pHA (262 kDa) without biotin linker resulted in a two-phase response that is indicative of two overlapping processes. The initial decrease in frequency indicates binding of pHA to the oHA-bound rHTSG-6. The concomitant strong increase in dissipation is characteristic for the formation of a soft and highly hydrated film. The subsequent increase in frequency is probably a result of desorption of rHTSG-6 and/or migration of rHTSG-6 inside the pHA film. This process continues, albeit at a slower pace, after rinsing in buffer. The measurement was performed twice, and the data shown are representative. B, schematic illustration of the final sandwich structure. Our data indicate that HA-cross-linking is mediated by rHTSG-6 oligomers (here represented by the simplest possible oligomers (i.e. dimers)). The Link module and the CUB_C domain of TSG-6 are schematically indicated in light blue and green, respectively. C, for comparison, a molecular model of a TSG-6 dimer with two bound HA octasaccharides derived from small angle x-ray scattering data (49) is also shown; the Link module structure (blue) in its HA-bound conformation (50), docked HA oligomers (orange (48, 50)), and CUB module model (green (31)) are shown in space-filling representations, whereas the N- and C-terminal regions of TSG-6 (for which no structural data are available) are represented as protein backbone traces predicted from the scattering data (49).

FIGURE 5. Rigidification and condensation of HA films upon influx of full-length TSG-6. A, binding of rHTSG-6 to a pHA (58 kDa) film was monitored by QCM-D. The strong decrease in frequency upon exposure to rHTSG-6 is initially accompanied by an increase, followed by a decrease, in dissipation. The latter indicates rigidification of the HA film. B, variations in the thickness of a pHA (1080 kDa) film as a function of increasing rHTSG-6 concentration, quantified by colloidal probe RICM. The film retained its collapsed state upon rinsing in buffer (data not shown). The inset shows the thickness as a function of the occupancy of HA (calculated from B, using Equation 1 and the data for pHA in Table 1). Error bars, S.D. values for 10 independent measurements on the same surface.

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pHA films). The introduction of cross-linkers will force neighboring HA chains closer together and decrease the mesh size (Fig. 6B). As a result, the film’s thickness will decrease, whereas its rigidity will increase. Given that the films have a large mesh size to start with, a rather small amount of cross-linkers can already have an appreciable effect on the film thickness. The above considerations illustrate that cross-linking is an efficient route for the condensation of HA films and rationalize the increase in rigidity and the decrease in thickness that we have observed upon introduction of rHTSG-6 in pHA films (Fig. 5).

Maximal film collapse, to about 100 nm, was induced at a protein concentration of 0.7 $\mu$M in the bulk solution. This corresponds to an occupancy of one rHTSG-6 per about 10 HA$_2$ repeating units (HA$_{20}$) and a protein concentration inside the film of around 1.0 mM, or 30 mg/ml. Notably, the film thickness did not decrease further but instead slightly increased upon further increasing the rHTSG-6 bulk concentration to 2.3 $\mu$M. At this point, the rHTSG-6 concentration inside the HA film reached a value of ~2.3 mM, or 70 mg/ml (i.e. TSG-6 occupied a significant fraction (about 5%) of the total volume in the HA film). We propose that crowding of rHTSG-6 prevents further film condensation. The thickness of the maximally collapsed film did not change over a period of at least 1 h following removal (by rinsing) of the remaining rHTSG-6 in solution, con-
firms that release of rhTSG-6 is slow and that the protein-HA complexes are rather stable, as previously observed by ellipsometry (Fig. 2A).

**Binding of Link\_TSG6 to HA Films**—To compare the HA binding behavior of full-length TSG-6 with that of the Link module, Link\_TSG6 was titrated into both oHA and pHA (58 kDa) films, and the binding kinetics were quantified by ellipsometry (Fig. 7). The titration curves (Fig. 7C) could be fitted well with Equation 1 and a Hill coefficient of ~1, revealing dissociation constants of ~5 μM (Table 1). The Hill coefficient of unity and similar $K_D$ values for oHA and pHA indicate that interactions between adjacent binding sites along a given polymeric HA chain do not affect binding. Furthermore, Link\_TSG6 bound stoichiometrically to oHA at saturation (Table 1). All of these findings are consistent with a simple one-site model (i.e. in which all binding sites are identical (as also found in recent confocal fluorescence recovery after photobleaching analysis of the binding of Link\_TSG6 to pHA in solution phase (53))), in stark contrast to rhTSG-6.

Overall, the observed simple, non-cooperative binding and the micromolar affinities for Link\_TSG6 are consistent with earlier solution phase studies by calorimetry on HA oligosaccharides (29, 46, 50, 54) and by confocal fluorescence recovery after photobleaching on polymeric HA (53). However, it should be noted that these measurements were performed at different pH and ionic strength from those used here and that both parameters have been found to significantly affect the binding affinity (29, 34, 53, 54). A detailed quantitative comparison of the binding affinities is hence far from trivial.

For polymeric HA, we found a stoichiometry of 2.9 ± 0.3 HA disaccharides per Link\_TSG6 at saturation (Table 1). This number is similar to the minimum size of HA oligomer that binds with maximal affinity to Link\_TSG6 (HA₇ with d-glucuronic acid at both termini (50)), but it is smaller than the decameric footprint that has been reported based on calorimetric binding studies with HA oligosaccharides of different size (29). The desorption curves upon rinsing of close-to-saturated HA films with buffer could be fitted well by simple exponential fits (Equation 2 and Fig. 7, A and B). The dissociation rate constants (Table 1) for Link\_TSG6 were similar for oHA and pHA films but more than 5-fold larger than the fastest desorption rates observed for rhTSG-6. Most Link\_TSG6 could be readily dissociated within experimental time scales, although a proportion of the protein could not be removed by rinsing in Hepes buffer (i.e. ~10 and ~25% for the oHA and pHA films, respectively) (Fig. 7, A and B). Control measurements with Link\_TSG6 on HA-free surfaces (supplemental Fig. S6A) revealed minor non-specific binding that was comparable in magnitude with the irreversibly bound amounts on oHA films. It remains unclear if the significantly larger amount of irreversibly bound Link\_TSG6 protein in pHA films is specifically bound to HA. It might also reflect some tendency of the protein to aggregate at the high concentrations reached in the HA film. In this regard,
Link_TSG6 has been found to be stable at concentrations in solution phase up to at least 2 mM in the presence of a 1:1 molar ratio of HA$_8$ (50), although precipitation has been reported under some conditions in the presence of a larger HA oligomer (29). However, the protein fraction that remained bound upon rinsing in buffer could readily be eluted in 8 M GuHCl (Fig. 7A) (i.e. Link_TSG6, as rhTSG-6, did not engage in a covalent interaction with HA).

Condensation of HA Films by Link_TSG6—Next, we tested how Link_TSG6 affects the morphology of pHA films, as compared with rhTSG-6. Titration of Link_TSG6 at bulk concentrations ranging from 0.05 to 5 μM into pHA (58 kDa) films was monitored by QCM-D (Fig. 8A). A remarkable decrease in dissipation, concomitant with a decrease in frequency, occurred at concentrations above 3 μM, indicating rigidification of the film (43, 55, 56); in comparison, we had observed a similar decrease in dissipation already at a 8-fold lower concentration of rhTSG-6 (Fig. 5A). The QCM-D responses reversed upon gradually decreasing the Link_TSG6 concentration in solution. Compared with the responses at increasing protein concentrations, a hysteresis was observed (i.e. the process was only partly reversible).

Colloidal probe RICM revealed a gradual decrease in thickness upon titration of Link_TSG6 into pHA (1080 kDa) films (Fig. 8B), from 427 ± 4 nm in the absence of proteins to 66 ± 16 nm in the presence of 10 μM Link_TSG6. Stepwise elution of Link_TSG6 resulted in a gradual thickness increase. A hysteresis in thickness between adsorption and desorption processes (Fig. 8B) was consistent with QCM-D data (Fig. 8A) and the irreversibly bound protein fraction observed by ellipsometry (Fig. 7A).

In order to compare the potency of rhTSG-6 and Link_TSG6 to condense HA films, it is useful to consider the degree of condensation as a function of occupancy of HA chains with proteins rather than the bulk protein concentration (Fig. 8C). At low occupancies, full-length TSG-6 was considerably more potent in condensing HA than Link_TSG6. To reach a 2-fold decrease in thickness, for example, only one rhTSG-6 per 44 HA$_2$ repeating units (HA$_{44}$) but more than one Link_TSG6 per six HA$_2$ repeating units (HA$_{12}$) were required.

Within experimental error, the film thickness decreased linearly with the occupancy for Link_TSG6. At the maximal experimentally assessed protein uptake, one Link_TSG6 per 4.0 HA$_2$ repeating units (HA$_4$) (which is somewhat higher than the stoichiometry at saturation predicted from the Hill equation; Table 1), the film thickness had attained ~15% of its original value, and the concentration of Link_TSG6 inside the film was ~3.2 mM, or 35 mg/ml.

Several mechanisms might be considered for the rigidification and condensation of pHA films by Link_TSG6. In analogy with our arguments for rhTSG-6 (Fig. 6), cross-linking is one possible driving force. It would be probable that Link_TSG6 dimers (or larger oligomers), rather than monomers, act as cross-linkers. Only about six carbohydrate monomers are on average available per Link_TSG6 at saturation in pHA films (Table 1); if a monomer were sufficient for cross-linking, then the average footprint per HA chain would need to be an HA trisaccharide unless two Link_TSG6 molecules can simultaneously bind to the same part of the HA chain. Based on the structure of the HA binding site (48, 50), this is unlikely. Furthermore, it should be noted that previous studies on Link_TSG6 by analytical ultracentrifugation (46) and NMR spectroscopy (57) indicated that, in solution phase, this protein domain is monomeric in both the absence and presence of HA$_8$.

An alternative route toward condensation and rigidification of HA films would be via the condensation and/or rigidification of individual HA chains. Recent molecular modeling studies on Link_TSG6 suggest that HA chains bend locally in order to fit into the HA-binding site, as is the case for CD44 (58), and such bending might induce an apparent chain shortening. The bending is pronounced; however, it is unclear at present whether alone it could explain the more than 6-fold decrease in film thickness that we have observed. It has also been proposed that the dense coverage of an HA chain with many hyaladherins, like

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beads on a string, induces or stabilizes distinct HA conformations (5) that are more condensed and/or more rigid than the free HA chain. An increase in chain rigidity would decrease the entropically driven stretching of the individual chains in the HA brush in the direction perpendicular to the surface (51, 52) and might thereby induce rearrangement into a thinner but more densely packed film. The final film density and the linear relationship between film thickness and occupancy would be consistent with such a scenario. Based on the present experimental data, it appears difficult to exclude any of the above mechanisms, which might even act jointly.

**Link_TSG6 Induces Total Film Collapse at Low Ionic Strength**—Previous studies have shown that the affinity of Link_TSG6 increases strongly with decreasing ionic strength, with submicromolar affinities being reached at 5 mM NaCl, as a result of electrostatic repulsion between HA chains. Error bars, S.D. values for 10 independent measurements on the same surface.

The dramatic decrease in dissipation upon exposure to 1 μM Link_TSG6 in 5 mM NaCl indicates complete film collapse and rigidification. The film remains collapsed after rinsing in 5 mM NaCl (marked by an asterisk) but recovers partly in 150 mM NaCl. β, variations in the thickness of a pHA (1080 kDa) film as a function of the Link_TSG6 concentration in solution at 5 mM NaCl determined by colloidal probe RICM (Fig. 8). A more than 2-fold decrease in film thickness was already observed at Link_TSG6 concentrations of 0.1 μM, and close to complete collapse is attained at 1 μM. Data acquired in 150 mM NaCl under otherwise identical conditions is shown for comparison (C; from Fig. 7B). Note that the decrease in ionic strength induced a swelling of the protein-free HA film, from 450 ± 20 nm at physiological ionic strength to about 1 μm in 5 mM NaCl, as a result of electrostatic repulsion between HA chains. Error bars, S.D. values for 10 independent measurements on the same surface.

**DISCUSSION**

Using a novel experimental platform, based on films of end-grafted HA, in conjunction with a toolbox of surface-sensitive characterization techniques, we have investigated the interaction of TSG-6 with HA in an ultrastructural context. The experimental platform is interesting for several reasons. First, the morphology and quantity of immobilized HA is well controlled, and nonspecific binding to the underlying surface is low. These are prerequisites for detailed and quantitative binding studies. Second, the binding behavior on both oligomeric and polymeric HA can be interrogated on the same immobilization platform and directly compared. Third, with a toolbox of surface-sensitive characterization techniques, the amount and kinetics of protein binding can be correlated with changes in the physicochemical properties, such as the dimensions, mechanical properties, and morphology, of the resulting HA ultrastructures.

Key results of our study are the direct experimental evidence that full-length TSG-6 alone can cross-link HA (Fig. 4) and that the cross-linking induces condensation of HA at what are likely to be physiologically relevant concentrations (Fig. 5). Furthermore, our study provides novel and quantitative insights into the interaction between TSG-6 and HA. The cooperative binding of rhTSG-6 to both oHA and pHA (Fig. 2) and its supras-
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Toehiometric binding to oHA (Table 1) provide evidence for HA-induced protein oligomerization.

The HA binding behavior of Link_TSG6 at physiological ionic strength is distinctly different from that of rhTSG-6; binding of the former is both simpler and weaker. The lack of cooperative interaction might well explain the weaker binding of Link_TSG6. However, we cannot rule out the possibility that, in addition to the Link module, some other part of the TSG-6 protein makes a contribution to HA binding. This could occur in a number of ways, for instance via a direct interaction of a neighboring region of the protein with the bound HA (e.g. an extension of the Link module binding groove (48, 50)) or via an allosteric effect on the affinity of the Link module (e.g. stabilizing the Link module in its bound conformation (50, 57)).

Although less potent than rhTSG-6, Link_TSG6 retains the ability to condense and rigidify HA films. At present, the sizes of the HA-induced rhTSG-6 oligomers and the mechanism underlying Link_TSG6-mediated HA-film condensation and rigidification remain unclear. The observed Hill coefficients above 2 on oHA and pHA films and the maximal binding stoichiometry above 2 on oHA films (Table 1) indicate that rhTSG-6 must form oligomers that are larger than dimers. Small angle x-ray scattering data suggested the formation of rhTSG-6 dimers (Fig. 4C) as well as larger species in the presence of oHA octasaccharides in solution (49), and it is possible that a spectrum of oligomer sizes is also present in our HA films.

Our observations that rhTSG-6 binds HA more strongly than Link_TSG6 (Table 1) and that both protein constructs can be fully released from HA by a dissociating agent (Figs. 3 and 7A) contrast with an earlier study (30) that had reported similar binding properties and non-dissociable HA-rhTSG-6 complexes for full-length TSG-6 and the TSG-6 Link module in plate binding assays with immobilized HA. These assays, however, were performed at a non-physiological ionic strength of 500 mM NaCl. This and the limited control on the immobilization of analytes in these plate binding assays might explain the discrepancies with our study.

Implications for the Function of TSG-6 in Vivo—For pHA films containing a concentration of ~1 mg/ml HA, we observed significant condensation already at 0.16 μM rhTSG-6, and maximal condensation was attained at 0.7 μM (Fig. 5). These concentrations are likely to be physiologically relevant. For example, HA is present in synovial fluid and umbilical cord at ~1 mg/ml (59) and is also found at high concentrations in other tissues, such as skin (~0.2 mg/ml (59)) and the expanded cumulus matrix surrounding the mature oocyte (~0.2–0.5 mg/ml (60)). Although it has been suggested that TSG-6 is present at up to ~0.6 μM in synovial fluids from patients with inflammatory arthritis (i.e. based on data from Western blots (18)), more recent ELISA analysis suggests a maximum of ~3 nM (61). However, given that the likely source of this protein is synovium and cartilage (62), its local concentration in certain regions of these tissues is likely to be much higher than this (e.g. in the HA-containing pericellular matrix of chondrocytes that are synthesizing TSG-6). Similarly, there is likely to be a high local concentration of TSG-6 in the HA-rich cumulus matrix formed during cumulus-oocyte complex expansion, prior to ovulation (21, 22). Thus, the effects that we observed in vitro may have physiological relevance. The TSG-6-mediated cross-linking has two major consequences, condensation and rigidification, and both might be functionally important.

The high affinity of TSG-6 for HA and the ensuing cross-linking might lead to the formation of rather dense and spatially confined HA matrices. For example, Simpson et al. (11) have shown that the coordinated expression of HA and TSG-6 during fibroblast to myofibroblast differentiation is necessary for the formation of a pericellular coat during normal wound healing and that this diminishes with age. Furthermore, it might be envisaged that a TSG-6-mediated contraction of the chondrocyte pericellular matrix during inflammation might serve to promote matrix remodeling that could contribute to its chondroprotective properties (53). Such dense matrices could not only locally sequester TSG-6 but might also enhance the retention of HA and matrix-associated molecules. Because the cross-links formed between TSG-6 and HA are reversible and the HA chains are flexible, it is likely that such matrices can dynamically adopt various shapes as a function of external cues. Upon uniaxial stretching, for example, highly elongated “fiber-like” assemblies with particular mechanical properties might form (6). Extracellular matrix rigidity has emerged as an important regulator of cellular behavior (63–65). Thus, TSG-6-induced modulation of the local mechanical properties of the extracellular space might directly affect the phenotype of adjacent cells.

Relevance of the Cross-linking of HA-rich Pericellular Coats for Leukocyte Homing—Lesley et al. (25) have shown that decoration of HA with either Link_TSG6 or rhTSG-6 promotes adhesion and rolling of CD44+ T-lymphocytes and suggested that cross-linked HA/TSG-6 complexes might be the adhesion-promoting agent (6). Our direct evidence for TSG-6-mediated cross-linking and condensation (i.e. the formation of dense HA networks) supports this hypothesis. Cross-linking probably increases the valence of interactions between HA and the cell surface. For example, rigidifying HA might reduce the entropic cost of cell surface receptor binding (45). It might also trigger a redistribution/clustering of cell surface receptors (66) or promote their conformational up-regulation (58). Interestingly, condensation occurs gradually with increasing rhTSG-6 concentration, from 0.1 to 0.7 μM (Fig. 5), and local cross-linking of HA in the endothelial matrix by TSG-6 might hence be a dynamic regulator of the inflammatory response (6, 25).

It should be noted that other hyaladherins or TSG-6-binding proteins (34, 35) might alter the HA cross-linking activity of TSG-6 in subtle ways that remain to be elucidated. A case in point is the catalytic action of TSG-6 in mediating covalent transfer of heavy chains from Ia1 onto HA, which leads to a mechanism of HA cross-linking different from that described here (6, 32, 67). In this regard, the heavy chain transfer activity of TSG-6 but not the formation of TSG-6/HA complexes leads to a promigratory phenotype of proximal tubular epithelial cells (10). Also, the cross-linking of HA in the cumulus-oocyte complex matrix was shown to involve at least two proteins, Ia1 and PTX3, in addition to TSG-6 (12, 35, 60, 68–70), in a way that remains only partly understood.

On the other hand, the binding of TSG-6 to other glycosaminoglycans (chondroitin 4-sulfate, heparin/heparan sulfate) and highly hydrated proteoglycans, such as aggrecan and versican.
(8, 27, 33, 34, 37) suggests that the cross-linking activity of TSG-6 might not be restricted to HA. In this regard, we showed previously that TSG-6 could promote the interaction of fibronectin with thrombospondin-1, probably by bridging between these two proteins via interactions mediated by its CUB_C domain and Link module, respectively (24). By simultaneously interacting with and cross-linking various components of pericellular and extracellular matrices, TSG-6 might play a central role as a matrix reorganizer. Our finding that the formation of the HA cross-linking entities (i.e. the TSG-6 oligomers) is induced by the very presence of HA might be of particular importance in the regulation of such reorganization processes.

The experimental approach that we have applied in this study can in the future be readily extended to study the cross-talk between HA, TSG-6, and their binding partners. Hence it is likely to provide novel insight into the mechanisms behind TSG-6-induced reorganization of pericellular and extracellular matrices.

Acknowledgments—We thank Douglas Dyer, Dr. David J. Mahoney, Dr. Caroline M. Milner, Marilyn S. Rugg, and Dacha Tungsoongnoen for assistance with production of recombinant proteins and Drs. Charles D. Blundell and Anja Bernecker for helpful discussions.

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