TSG-6 Modulates the Interaction between Hyaluronan and Cell Surface CD44

Received for publication, December 5, 2003, and in revised form, March 31, 2004
Published, JBC Papers in Press, April 1, 2004, DOI 10.1074/jbc.M313319200

Jayne Lesley‡, István Gáš, David J. Mahoney‡, Martin R. Cordell‡, Marilyn S. Rugg¶, Robert Hyman‡, Anthony J. Day¶¶, and Katalin Mikecz§**

From the ‡Molecular and Cell Biology Laboratory, The Salk Institute, San Diego, California 92186, §Departments of Orthopedic Surgery and Biochemistry, Rush University Medical Center, Chicago, Illinois 60612, ¶¶Medical Research Council Immunochemistry Unit, Department of Biochemistry, University of Oxford, Oxford OX1 3QU, United Kingdom

Interactions between CD44 and hyaluronan are implicated in the primary adhesion of lymphocytes to endothelium at inflammatory locations. Here we show that preincubation of hyaluronan with full-length recombinant TSG-6 or its Link module domain (Link_TSG6) enhances or induces the binding of hyaluronan to cell surface CD44 on constitutive and inducible cell backgrounds, respectively. These effects are blocked by CD44-specific antibodies and are absent in CD44-negative cells. Enhancement of CD44-mediated interactions of lymphoid cells with hyaluronan by TSG-6 proteins was seen under conditions of flow at shear forces that occur in post-capillary venules. Increases in the number of rolling cells were observed on substrates comprising TSG-6-hyaluronan complexes as compared with a substrate containing hyaluronan alone. In ligand competition experiments, cell surface-bound TSG-6-hyaluronan complexes were more potent than hyaluronan alone in inhibiting cell adhesion to immobilized hyaluronan. Link_TSG6 mutants with impaired hyaluronan binding function had a reduced ability to modulate ligand binding by cell surface CD44. However, some mutants that exhibited close to wild-type hyaluronan binding were found to have either reduced or increased activity, suggesting that some amino acid residues outside of the hyaluronan binding site might be involved in protein self-association, potentially leading to the formation of cross-linked hyaluronan fibers. In turn, cross-linked hyaluronan could increase the binding avidity of CD44 by inducing receptor clustering. The ability of TSG-6 to modulate the interaction of hyaluronan with CD44 has important implications for CD44-mediated cell activity at sites of inflammation, where TSG-6 is expressed.

Hyaluronan (HA), a high M₉ glycosaminoglycan composed of repeating disaccharides of glucuronic acid and N-acetylglucosamine, is present in the extracellular compartment of most tissues and is a major component of cartilage and synovial fluid. The synthesis of HA is often up-regulated in response to inflammation, tissue damage, or invasion by tumor cells or pathogens (1–3). CD44, a major cell surface receptor for HA, is present on a wide variety of cell types. For instance, most hematopoietic cells express CD44, but their HA binding function is tightly regulated so that they do not constitutively bind HA (4, 5). On T cells and B cells, HA binding can be activated in subsets of cells (5), where activating stimuli include phorbol esters and certain CD44-specific antibodies (6–8). CD44 has been shown to participate in the migration of leukocytes to inflammatory sites (9–12), where there is often an increase in CD44+ cells due to both the immigration of leukocytes to inflammatory sites and to a greater degree of CD44 expression on resident cells (1). CD44 expression is increased on many cell types in inflamed arthritic joints (11, 13), in which the synthesis and degradation of HA are also enhanced (14, 15).

TSG-6 (the secreted product of tumor necrosis factor-α-stimulated gene-6) is an HA-binding protein, the expression of which is very tightly regulated (for reviews, see Refs. 16–18). There is little or no constitutive expression of TSG-6 in adult tissues, but the protein is synthesized by fibroblasts, chondrocytes, monocytes, and vascular endothelial, and smooth muscle cells in response to stimulation with pro-inflammatory mediators or certain growth factors. TSG-6 protein has been detected at high levels in the synovial fluids of patients with arthritis and has been localized in articular cartilage and synovium from individuals with osteoarthritis and rheumatoid arthritis but is absent in normal joint tissues (16, 19). Although exogenous TSG-6 has been shown to have anti-inflammatory activity in certain in vivo model systems (20–24), the function of endogenously produced TSG-6 in the inflammatory milieu is unknown. It has been suggested that TSG-6 might modulate cellular interactions with HA at sites of inflammation (25).

The HA binding domains of TSG-6 and CD44 both contain a “Link module,” a structural unit of about 100 amino acids that is present in many other members of the hyaladherin family including the proteoglycan aggrecan and cartilage link protein (17, 18, 26, 27). Although the HA-interaction surfaces in CD44 and TSG-6 appear to be similar (27–30), there are clearly

---

* This work was supported by National Institutes of Health Grants AI31613 (to R. H.) and AR45652 (to K. M.) and Arthritis Research Campaign Grants D0562 and M0625 (to A. J. D.). Flow cytometry facilities at the Salk Institute were supported by NCI, National Institutes of Health Core Grant CA14195 and the H. N. and Frances C. Berger Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

** The on-line version of this article (available at http://www.jbc.org) contains Supplemental Figs. S1 and S2.

† To whom correspondence may be addressed: MRC Immunochemistry Unit, Dept. of Biochemistry, University of Oxford, South Park Rd., Oxford OX1 3QU, UK. Tel.: 44-1865-275349; Fax: 44-1865-275729; E-mail: tony.day@bioch.ox.ac.uk.

‡‡ To whom correspondence may be addressed: Dept. of Orthopedic Surgery, Rush University Medical Center, 1735 W. Harrison St., 712 Cohn, Chicago, IL 60612. Tel.: 312-942-5767; Fax: 312-942-8828; E-mail: Katalin_Mikecz@rsh.net.

---

1 The abbreviations used are: HA, hyaluronan; ΔCY, cytoplasmic deletion mutant; FL-HA, fluorescein-conjugated HA; mAb, monoclonal antibody; NMR, nuclear magnetic resonance; TSG-6, tumor necrosis factor-stimulated gene-6; WT, wild type; MFI, mean fluorescence intensity; MES, 2-(N-morpholino)ethanesulfonic acid; DMEM, Dulbecco’s modified Eagle’s medium.
significant differences in the molecular details of HA binding in these proteins (29). For example, recent structural studies show that in the case of CD44, N- and C-terminal sequences flanking the Link module are required to form a folded and functionally active domain (27). Although TSG-6 binds HA with high affinity (28), the binding between CD44 and HA is much weaker (91, 32) and seems to depend on multivalent interactions (25). Therefore, extensive occupancy of HA with TSG-6 might be expected to inhibit its interaction with CD44 by reducing the number of sites available for binding.

In this study we have asked how the presence of TSG-6 influences the HA binding function of CD44+ cells. We have found that recombiant full-length human TSG-6 as well as its isolated Link module domain (Link_TSG6) are potent modulators of HA binding to CD44 on lymphoid cell lines. This finding has important implications for the regulation of CD44-mediated leukocyte migration to sites of inflammation.

EXPERIMENTAL PROCEDURES

Cell Lines—AKR1 is a CD44-negative mouse T lymphoma (isolated from an AKR/J mouse) that does not bind HA. AKR1/CD44+ is a transfectant of AKR1 with cDNA encoding the CD44.1 allele. AKR1/ CD44R41A is a transfectant of AKR1 that expresses a mutant CD44 molecule whose truncated cytoplasmic domain contains only the six most membrane-proximal amino acids and which binds soluble HA poorly without induction (36–38). EL4 is a T lymphoma that expresses CD44 at a level similar to AKR1/CD44+. HA binding to both AKR1/CD444/CY and EL4 cells can be induced by the CD44-specific mAb IRAWBE14 (5, 36, 39–41). AKR1/CD44R41A is a transfectant of AKR1 that expresses a mutant CD44 with an Arg→Ala substitution at a critical position (Arg-41) in the HA-binding site of CD44 (27, 30, 31). The AKR1/CD44R41A transfectant does not bind HA at all, and in contrast to AKR1/CD444/CY or EL4 cells, HA binding to this mutant cannot be induced by HA (27, 30, 36–38). All of the CD44+ cell lines used in this study express the hematoepoietic (H) or standard isoform of CD44 that contains none of the variant exon products (5, 42).

Preparation and Characterization of Full-length Human TSG-6 and Link_TSG6—Full-length human TSG-6 (allelic variant TSG-6Q with Glu at amino acid position 144 in the pre-protein) was expressed in Drosophila Schneider 2 cells and purified by ion exchange chromatography and reverse-phase high performance liquid chromatography as described previously (43).

The Link module corresponds to residues 36–133 in the TSG-6 pre-protein (25). Cloning and overexpression of this module (Link_TSG6) in Escherichia coli has been reported previously (44, 45). Link_TSG6 mutants H4K, E6K, Y12F, K13E, F70V, and Y78F were asdescribed in Getting et al. (24) and Mahoney et al. (29). The additional H29K mutant was made in an identical manner and was analyzed by electrospray ionization mass spectrometry and one-dimensional NMR spectroscopy as before (29); H29K had an experimental mass within 0.8 Da of its theoretical mass and gave a one-dimensional NMR spectrum essentially identical to wild-type (WT) protein (data not shown). The affinity of the interaction between H29K and an octasaccharide of HA (HA3) was determined by isothermal titration calorimetry on a Microcal VP-ITC instrument at 25 °C in 5 mM Na-MES, pH 6.0, exactly as described for the other mutants (24). In this regard, HA3 has been shown to bind to Link_TSG6 with high affinity (46).

Analysis of HA Binding to Cell Surface CD44—HA binding to cells was determined by flow cytometry using HA (from rooster comb, protein A purified, Fluka, Buchs, Switzerland) and separated by filtration through Centricon filters (Millipore, Billerica, MA) of various molecular weight cut-off as described previously (35).

Determination of the enhancing/inducing activity of mutant Link_TSG6 proteins relative to the activity of WT Link_TSG6 was performed as follows. WT and mutant proteins preincubated with FL-HA at high concentrations (at a w/w ratio of 5:1) were diluted to 0.5 μg/ml FL-HA and assayed for binding to AKR1/CD44+ cells by flow cytometry. The MFI of FL-HA binding in the presence of each mutant protein was expressed as the percentage of FL-HA bound in the presence of WT Link_TSG6.

Flow Chamber Assays—The influence of TSG-6Q or Link_TSG6 (WT and mutant) proteins on HA-dependent cell rolling and adhesion was determined using a parallel flow chamber (GlycoTech, Rockville, MD) as described previously (32, 37). To allow for TSG-6 complex formation, HA was preincubated with TSG-6 proteins (or with diluent only) in 50 mM HEPES, pH 6.0, for 30 min at room temperature, as described for the FL-HA binding experiments. The volume was adjusted to 10 ml with phosphate-buffered saline. This substrate (containing HA or protein-HA complexes) was placed as a single spot in the center of a 60-mm plastic Petri dish, dried onto the surface overnight, washed extensively with phosphate-buffered saline, and blocked with DMEM containing 1% ultra-pure bovine serum albumin (Sigma). The flow chamber was placed over the bottom of the dish with the flow path covering the substrate-coated area. The chamber was perfused with a programmable syringe pump (Harvard Apparatus, Holliston, MA). Cells were accumulated at a shear force of 0.2 dyn/cm² followed by exposure to increasing levels of fluid shear (0.5, 1, 2, 4, and 8 dyn/cm², each for 2 min). Cell movement was recorded by streamline acquisition of images using a digital camera (RS Photometrics, Trenton, NJ) attached to a Nikon Diaphot inverted phase contrast microscope. Images were collected and analyzed using a Metaview image processing system (Universal Imaging). The number of rolling cells was determined by counting the cells that moved on the substrate parallel with the fluid flow (32, 37) at each level of shear force between 0.2 and 8 dyn/cm². To assess cell adhesion, the number of immobile cells on the substrate-coated area was determined at the end of each 2-min flow period. Cells in at least three fields of microscopic view were counted at each shear force, and the average number of rolling or adherent cells was calculated. The enhancement of HA-dependent cell rolling or firm adhesion by TSG-6 proteins was expressed as the ratio of the number of rolling or adherent cells on the protein/HA substrate to the number of rolling or adherent cells on the HA substrate. To determine the efficiency of Link_TSG6 mutants (relative to WT) to support cell rolling, WT and mutant proteins were preincubated with HA in a similar manner before immobilization in the flow chamber. The activity of substrates containing Link_TSG6 mutants was expressed as the percentage of the activity of WT Link_TSG6.

For competition experiments, cells were pretreated in suspension with HA or HA complexes with TSG-6 proteins before the rolling assays. First, HA and TSG-6 proteins (or diluent) were preincubated (1 μg/ml HA, 5 μg/ml protein) as described above. The mixture was then diluted with 1 ml of DMEM and incubated with the cells (4 × 10⁵/ml for 1 min) at 37 °C. The final volume was adjusted to 2 ml with supernatant of each cell population and incubated with unlabeled HA at pH 6.0. The cells were then washed extensively with DMEM and plated together on each well of a 24-well plate with adherent cell monolayers (32). Cell adhesion was determined as described above.
shown to reside within its Link module (24, 28, 29, 47); therefore, this well characterized domain (Link_TSG6) was used first to examine the effects of TSG-6 on the interaction of HA with cell surface CD44. Because HA_{20} is the minimum length of HA that can accommodate 2 Link_TSG6 molecules with optimal affinity\(^2\) (indicating that each Link module occupies \(-10\) sugar units), a 5:1 (w/w) ratio of Link_TSG6 (M, \(-19.9\)) to HA would be expected to saturate the majority of protein-binding sites on an HA chain. Therefore, this approximately equimolar ratio of Link_TSG6 to HA (when expressed as 10-mer eq) was used for initial titration experiments.

Link_TSG6 was tested for its ability to modify the binding of FL-HA to cell surface CD44 in three ways as follows. 1) Link_TSG6 (\(-1.5 \mu\text{M} \), final concentration) and FL-HA (3 \(\mu\text{g} / \text{ml} \), \(-1.5 \mu\text{M} \), 10-mer eq, final concentration) were diluted separately and added to the cell suspension. 2) Link_TSG6 (\(-20 \mu\text{M} \), final concentration) was preincubated for 30 min at room temperature with FL-HA (\(-20 \mu\text{M} \), 10-mer eq, final concentration) before dilution of the mixture and addition to cells. 3) Link_TSG6 was preincubated with CD44+ cells (up to 20 \(\mu\text{g} / \text{ml} \), final concentration for 30 min) before FL-HA was added.

Fig. 1A shows the results obtained with AKR1/CD44+ cells that express the active form of CD44 (i.e. bind HA constitutively). When Link_TSG6 and FL-HA were added to the cells according to protocol 1 (diluted before mixing) there was no difference in the FL-HA binding to CD44+ cells (filled circles) compared with that seen in the absence of protein (open triangles). Unexpectedly, HA binding was enhanced when FL-HA was preincubated with Link_TSG6 before dilution and incubation with the cells (protocol 2, open circles in Fig. 1A). Preincubation of AKR1/CD44+ cells with Link_TSG6 followed by the addition of FL-HA (according to protocol 3 above), had no effect on FL-HA binding to the cells (results not shown). We found, however, that once the FL-HA-Link_TSG6 complex was formed by preincubation at high concentrations, the complex remained active after overnight incubation at 4°C even though it had been diluted in cell-labeling buffer (data not shown).

For the experiments shown in Fig. 1A the ratio of Link_TSG6 to FL-HA was constant. The influence of Link_TSG6 present at a fixed concentration throughout the titration of FL-HA was also examined, and the ratio of Link_TSG6 to HA increased as the concentration of FL-HA was reduced (Fig. 1B). These results are expressed as “enhancement ratio” i.e. MFI of FL-HA bound in the presence of Link_TSG6 relative to MFI of FL-HA bound alone (in Fig. 1B (y axis) a ratio greater than 1.0 indicates enhancement, whereas a ratio of less than 1.0 represents inhibition of FL-HA binding). Without preincubation, there was no enhancement of FL-HA binding in the presence of Link_TSG6 at either 2 \(\mu\text{g} / \text{ml} \), open triangles or 10 \(\mu\text{g} / \text{ml} \), open circles. However, enhanced binding was observed (filled circles) after preincubation of Link_TSG6 (10 \(\mu\text{g} / \text{ml} \)) with FL-HA when FL-HA was present at concentrations greater than 0.3 \(\mu\text{g} / \text{ml} \). At very low concentrations of FL-HA (<0.3 \(\mu\text{g} / \text{ml} \)) and a high molar excess of Link_TSG6 (\(>30:1\), protein:HA 10-mer eq), there was inhibition of FL-HA binding regardless whether or not Link_TSG6 and HA were preincubated (Fig. 1B), although preincubation resulted in greater inhibition of binding than did the direct addition of Link_TSG6 and FL-HA. Binding of FL-HA to the cells from the preincubation mixture containing 10 \(\mu\text{g} / \text{ml} \) Link_TSG6 was uniformly prevented at all concentrations of FL-HA by the CD44-specific mAb KM81 (Fig. 1B) that inhibits HA binding to mouse CD44 (40).

Fig. 2 shows examples of the raw flow cytometry data used in calculating the results for Figs. 1, 3, and 4. CD44-negative, untransfected AKR1 cells do not bind FL-HA (Fig. 2, panel A). Panels B and C in Fig. 2 show binding of FL-HA alone (0.5 \(\mu\text{g} / \text{ml} \)) to the constitutively binding AKR1/CD44+ cell line (B) and to the inducible cell line EL4 (C) that does not bind FL-HA unless the cells are activated. Although AKR1/CD44+ cells bind with a MFI of 18 (18-fold over the background fluores-
ence of unlabeled cells). FL-HA binding to EL4 cells overlapped the background. Panels D–F of Fig. 2 show binding when FL-HA was preincubated with Link_TSG6 (using protocol 2). Binding to AKR1/CD44+ cells (panel E) was increased as indicated by the shift of fluorescence histogram to the right. EL4 cells bound FL-HA complexed with Link_TSG6 (Link_TSG6/FL-HA, labeled as Link_TSG6/FL-HA; panel F) significantly above background. Binding to both cell lines was further enhanced when FL-HA was complexed with full-length TSG-6 (TSG-6Q/FL-HA, labeled as TSG-6Q/FL-HA; bottom panels). However, FL-HA binding could not be induced by TSG-6 proteins in untransfected (CD44-negative) AKR1 cells (panels D and G). This observation together with the inhibition of Link_TSG6/FL-HA binding to AKR1/CD44+ cells in the presence of the KM81 mAb (see Fig. 1B) confirmed that enhancement/induction of HA binding by TSG-6 was mediated through cell surface CD44.

Influence of Full-length TSG-6 and Link_TSG6 on FL-HA Binding to the Cell Surface CD44 of Constitutively Active and Inducible Cell Lines—The effects of preincubating FL-HA with Link_TSG6 or the full-length protein (TSG-6Q) on the binding of HA to “constitutively active” AKR1/CD44+ cells and “inducible” EL4 cells were examined. TSG-6Q, like Link_TSG6, was able to enhance FL-HA binding to AKR1/CD44+ cells (see Fig.
2). Surprisingly, these proteins also caused a large induction of FL-HA binding to EL4 cells that in the absence of TSG-6 or Link_TSG6 did not interact with FL-HA to any significant extent (see Fig. 2, right panels). Fig. 3 shows that at equimolar ratios to HA, the full-length protein (filled circles) was more effective than Link_TSG6 (open circles) in enhancing FL-HA binding by both types of cells. From these experiments it is also clear that the ratio of enhancement of FL-HA binding by either full-length TSG-6 or Link_TSG6 was greater for the inducible EL4 cells than the constitutive AKR1/CD44+ cells (compare y axis scales in Fig. 3, A and B).

EL4 cells and AKR1/CD44ΔCY (expressing a “tailless” deletion mutant of CD44 lacking all but the six most membrane-proximal amino acids of the cytoplasmic domain) do not constitutively interact with FL-HA but can be induced to bind by treatment with the CD44-specific mAb IRAWB14 (5, 36). Fig. 4 shows a titration of TSG-6Q and Link_TSG6 on EL4 and AKR1/CD44ΔCY cells and compares the enhancement of FL-HA binding with that induced by a constant amount of IRAWB14. In these experiments the ratio of TSG-6Q or Link_TSG6 in the preincubation mixture was 5:1 (w/w), whereas FL-HA was serially diluted from 4 to 0.05 μg/ml. HA binding to both EL4 (Fig. 4A) and AKR1/CD44ΔCY (Fig. 4B) cells was greatly enhanced by both TSG-6Q (filled circles) and Link_TSG6 (open circles), particularly at higher FL-HA concentrations (>1 μg/ml) where the effect of IRAWB14 (open squares) was more modest.

The Link_TSG6/FL-HA Complex Has a High Avidity for Cell Surface CD44 and Allows Stable Binding of Low M<sub>r</sub> HA Fragments—The relative avidity of FL-HA and Link_TSG6/FL-HA complexes for CD44 on AKR1/CD44+ cells can be compared by their sensitivity to blocking by unlabeled HA in a kinetic competition assay described in an earlier study (34). Binding of FL-HA at a defined concentration (0.3 μg/ml) alone or preincubated with Link_TSG6 (at a w/w ratio of 5:1) was blocked with

**Fig. 3.** Comparison of the effects of Link_TSG6 and full-length TSG-6 on FL-HA binding to cells expressing constitutively active or inducible CD44. A, constitutive AKR1/CD44+ cells were labeled with FL-HA, preincubated at high concentration with whole TSG-6 (9:1 w/w ratio, filled circles) or with Link_TSG6 (3:1 w/w ratio, open circles) with both proteins at the same molar concentration (7.6 μM). After a 30-min preincubation, mixtures were adjusted to 3 μg/ml FL-HA, and a series of 2-fold dilutions were assayed for binding to the cells. B, the same protocol was applied for the labeling of EL4 cells that express inducible CD44. Binding is expressed as enhancement ratio, i.e. MFI of cells incubated with TSG-6/FL-HA divided by the MFI of cells incubated with FL-HA alone. Enhancement gives a ratio greater than 1.0. The results shown are from one of two experiments.

**Fig. 4.** Induction of FL-HA binding to inducible cell lines by Link_TSG6, TSG-6Q, and CD44-specific mAb IRAWB14. Inducible cell lines EL4 (A) and AKR1/CD44ΔCY (B) were used to compare the induction of FL-HA binding by TSG-6 and the inducing mAb IRAWB14. Each cell line was assayed three or four times, and the data shown here are from a typical experiment. FL-HA was preincubated with TSG-6Q (filled circles) or Link_TSG6 (open circles) at high concentration at w/w ratios of 5:1 and diluted as indicated on the x-axis before the addition to the cells. For IRAWB14 treatment (open squares), cells were incubated with the mAb at a fixed concentration throughout the dilution of FL-HA. Results are expressed as enhancement ratio, as defined in Fig. 3. Binding of FL-HA alone to non-induced cells was equal to unstained cells (MFI = 1.0) except at the highest FL-HA concentration (4 μg/ml), when it was ≥1.5. Binding of FL-HA from the Link_TSG6/FL-HA complex to both EL4 and AKR1/CD44ΔCY cells was inhibited by the KM81 mAb, i.e. at the highest concentration of the components (4 μg/ml FL-HA and 20 μg/ml Link_TSG6) in the preincubation mixture, the enhancement ratio was < 2.0 for both cell lines in the presence of KM81.
TSG-6 Modulates Hyaluronan Binding by CD44

TABLE I

| Effect studied | FL-HA alone | Link_TSG6-FL-HA |
|----------------|-------------|-----------------|
| Concentration of unlabeled HA (µg/ml) required to block 50% of FL-HA binding<sup>a</sup> | 4.2 | 17.1 |
| Time (min) required for a 50% decrease in FL-HA binding in the presence of unlabeled HA<sup>b</sup> | 18 | 100 |
| Binding of low M<sub>i</sub> FL-HA fragments (MFI)<sup>c</sup> | | |
| ~30-kDa FL-HA | 1.0 | 2.1 |
| ~50-kDa FL-HA | 1.5 | 4.6 |

<sup>a</sup> FL-HA (0.3 µg/ml) or Link_TSG6-FL-HA complex was added to the cells in the presence of increasing concentrations of unlabeled HA. The concentrations of unlabeled HA at which the binding of FL-HA or Link_TSG6-FL-HA was blocked by 50% are indicated.

<sup>b</sup> The time at which 50% of FL-HA (at 0.3 µg/ml alone or in complex with Link_TSG6 dissociated from the cell surface in the presence of 250 µg/ml unlabeled HA.

<sup>c</sup> Binding of low M<sub>i</sub> fragments of FL-HA to the cell surface (at 0.6 µg/ml alone or in complex with Link_TSG6, expressed as MFI.

serial dilutions of unlabeled HA. As can be seen from Table I, row 1, ~4× more unlabeled HA was required to block 50% of the binding of Link_TSG6-associated FL-HA to AKR1/CD44+ cells than for blocking the binding of FL-HA alone. This suggests that the Link_TSG6-FL-HA complex has a higher avidity for cell surface CD44 than does FL-HA. Significantly, unlabeled HA (at concentrations greater than 500 µg/ml) was able to block the binding of the Link_TSG6-FL-HA complex completely (data not shown). These data together with the findings that TSG-6-FL-HA complexes do not bind to constitutive or inducible cell lines in the presence of the blocking mAb KM81 (Fig. 1B and data not shown) nor bind to the AKR1/CD44Arg41Ala transfectant that expresses a mutant CD44 molecule unable to bind HA (see Supplemental Fig. S1) indicate that the HA-binding site of CD44 is responsible for interaction with the complex.

FL-HA bound to cell surface CD44 dissociates in the presence of excess unlabeled HA (34). Table I (row 2) shows that dissociation of the Link_TSG6-FL-HA complex was greatly retarded with FL-HA alone (a half-life of 100 and 18 min, respectively), suggesting a greater avidity of the complex for cell surface CD44.

The FL-HA used for analysis of binding to CD44+ cells is prepared from rooster comb HA with an average M<sub>i</sub> of ~2 × 10<sup>6</sup> (48). Stable binding of FL-HA to CD44+ cells is not observed with HA of molecular mass below 100 kDa (35). As shown in Table I, FL-HA fragments with estimated M<sub>i</sub> of ~30 (row 3) and ~50 kDa (row 4) bound poorly to AKR1/CD44+ cells on their own but exhibited significant binding (over 2-fold increase in MFI) if they were preincubated with Link_TSG6 at a 5:1 (w/w) ratio.

TSG-6 Enhances CD44-dependent Cell Rolling on an HA Substrate—CD44 mediates lymphocyte rolling on HA in parallel plate flow chamber experiments that mimic the conditions of blood flow in post-capillary venules (37, 49). As shown in Fig. 5A, when TSG-6Q (open circles) or Link_TSG6 (open triangles) was included in the HA substrate (each preincubated with HA at a 5:1 w/w ratio) there was a significant increase in the number of AKR1/CD44+ cells that rolled compared with the number of cells that rolled on immobilized HA alone (filled circles). Enhancement of HA-dependent rolling by TSG-6 proteins was seen even at a high shear stress (8 dyn/cm<sup>2</sup>), although the number of rolling cells decreased at increasing fluid shear on all substrates. Thus, although cell capture and rolling were increased on the substrates containing TSG-6-HA complexes at each level of shear force, the cells exhibited an overall trend of increased detachment at higher shear forces as they did on the substrate containing HA alone (Fig. 5A).

A small fraction of AKR1/CD44+ cells, particularly those with very high CD44 expression, adhere tightly to HA and do not roll under flow (37). As compared with HA alone, there was a modest enhancement of tight adhesion to the HA substrate containing TSG-6Q but no significant enhancement of adherence to the Link_TSG6-HA substrate (Fig. 5B). When HA was preincubated with bovine serum albumin or another HA-binding protein (HA-BP from cartilage), no enhancement of rolling or firm adhesion was observed.3

The AKR1/CD44ΔCY transfectant, which requires induction to bind FL-HA (36, 38), is capable of attaching to and rolling on immobilized HA (37), as is the EL4-inducible cell line. The number of AKR1/CD44ΔCY cells and EL4 cells that rolled on substrates containing TSG-6 proteins was enhanced relative to HA alone, although in these cases there was no significant enhancement of firm adhesion (Fig. 6).

Biding of TSG-6-HA Complexes to CD44 Antagonizes Cell Adhesion to Immobilized HA—HA, bound from solution to constitutive CD44+ cells, can inhibit the rolling and firm adhesion interactions of the cells with immobilized HA in a concentration-dependent manner.4 Therefore, it might be expected that HA, bound to the surface of AKR1/CD44+ cells in complex with full-length TSG-6 or Link_TSG6, would be more efficient than HA alone in blocking the rolling and adhesion interactions of the cells with the immobilized HA substrate. As shown in Fig. 7, cells pretreated with HA, TSG-6Q-HA, or Link_TSG6-HA exhibited reduced rolling on and adhesion to immobilized HA. The protein-HA complexes (bound to cells at w/w ratios of 5:1 to HA) had greater inhibitory effects on these interactions than HA alone. Both TSG-6Q-HA and Link_TSG6-HA as well as HA alone reduced firm adhesion more efficiently than rolling (compare Figs. 7, A and B).

Activity of Mutant Link_TSG6 Proteins—The HA binding properties of several mutant Link_TSG6 proteins with single amino acid substitutions are shown in Table II. The H29K mutant generated here was shown by NMR spectroscopy to have a WT fold, as is the case for the other mutants that were analyzed previously (24, 29). Isothermal titration calorimetry experiments with the H29K mutant revealed that the alteration of His at amino acid 29 to Lys has no effect on the interaction of Link_TSG6 with HA (Table II). H4K has a WT fold, as is the case for the other mutants that were analyzed previously (24, 29). Isothermal titration calorimetry experiments with the H29K mutant revealed that the alteration of His at amino acid 29 to Lys has no effect on the interaction of Link_TSG6 with HA (Table II). H4K has a slightly reduced affinity for HA, whereas Y12F, K13E, F70V, and Y78F have significantly lower HA binding activities (be-

<sup>3</sup> I. Gál, A. J. Day, and K. Mikecz, unpublished data.

<sup>4</sup> I. Gál and K. Mikecz, unpublished observations.
tion 6 may allow the formation of an additional ionic interaction between HA and the protein (26).

The effects of Link_TSG6 mutants on the interactions between HA and CD44 on AKR1/CD44+ cells (i.e. binding of FL-HA from solution and rolling on immobilized HA) were compared with that of the WT protein (Table II). It can be seen from these results that mutants with a significant reduction in HA binding activity (i.e. Y12F, K13E, F70V, Y78F) were also deficient in their ability to enhance the HA binding function of CD44. This supports the conclusion, from the data above, that enhancing/inducing activity depends on the interaction of Link_TSG6 with HA. However, the H4K mutant, which only has a slightly reduced HA binding affinity, exhibits significantly reduced ability to modulate CD44 function. In addition, H29K, which is on a face of the Link module opposite from the HA-binding site (Fig. 8), exhibited slightly increased enhancement of both FL-HA binding and cell rolling (160 and 115% of WT activity, respectively; Table II) while having WT binding to HA8. Clearly, the residues involved in TSG-6-mediated enhancement of CD44 HA binding activity (i.e. His-4, Tyr-12, Lys-13, His-29, Phe-70, and Tyr-78) are widely spaced on the Link module. The results indicate that a large surface area that extends beyond the structural boundaries of the HA-binding site of the TSG-6 Link module is involved in mediating enhancement of HA binding to CD44.

**Fig. 5.** Rolling and adhesion interactions of constitutive AKR1/CD44+ cells under flow with immobilized substrates containing HA or TSG-6-HA complexes. Rolling (A) and firm adhesion (B) of the cells to HA-containing substrates were assayed in a parallel flow chamber as described under "Experimental Procedures." For the experiments shown the substrates were prepared using 1 μg of HA (filled circles) or 1 μg of HA preincubated with 5 μg of either TSG-6Q (open circles) or WT Link_TSG6 (open triangles) at high concentrations and spotted onto the plastic bottom of the chamber in a 10-μl final volume. This was based on the results of flow cytometry assays where preincubation of TSG-6Q or Link_TSG6 at 5:1 w/w protein:HA ratios gave rise to significant enhancement of FL-HA binding (Fig. 2 and data not shown). After washing of unbound material and blocking, AKR1/CD44+ cells were introduced into the chamber and briefly allowed to adhere to the immobilized substrate at a low fluid shear force. Cell rolling and firm adhesion were assayed at increasing levels of shear (from 0.5 to 8 dyn/cm²) by counting the cells that exhibited a rolling movement and those that remained immobile, respectively, on the substrate at each level of fluid shear. Results are expressed as enhancement ratios, i.e. the number of cells interacting with the TSG-6/HA substrate divided by the number of cells interacting with HA alone at each level of fluid shear, where the value on the HA substrate at the initial (0.5 dyn/cm²) shear force is normalized to 1.0. Mean ± S.E. of values from four experiments are shown. Asterisks denote significant (p < 0.05; Friedman test) increases in the number of cells (rolling or firmly adherent) that interacted with substrates containing TSG-6Q-HA or Link_TSG6-HA as compared with initial interactions with HA alone.

**Fig. 6.** Rolling and adhesion interactions of inducible cells under flow with immobilized substrates containing HA or TSG-6-HA complexes. AKR1/CD44+ΔCY cells (A) and EL4 cells (B), which both express inducible CD44, were assayed for rolling on and firm adhesion to immobilized substrates containing HA alone or complexes composed of TSG-6Q-HA (open bars) or Link_TSG6-HA (hatched bars). The experimental conditions were the same as described for Fig. 5. The results shown are enhancement ratios (mean ± S.E.) at 2 dyn/cm² shear force from 3 experiments. Asterisks indicate significant (p < 0.05) enhancement of rolling interactions on substrates containing protein-HA complexes as compared with the HA substrate. The enhancement ratio on the HA substrate (1.0) is indicated by the dotted line.
This study we have examined the effects of recombinant full-length human TSG-6 (TSG-6Q) and its Link module (Link_TSG6, a high affinity HA binding domain (28, 29, 47)) on HA binding by lymphoid cells expressing CD44. We found that complexes formed between HA and these proteins have CD44 binding properties very different from those of HA alone. Depending on the molar ratio of HA-Q or Link_TSG6 to FL-HA and the absolute concentration of the labeled HA in the complex, binding to CD44 (on a constitutively active cell background) is either inhibited or enhanced as compared with assays done in the absence of protein (Fig. 1). Inhibition is only seen when TSG-6 is present at high molar excess (at > 30:1 w/w ratios to HA). Given the fact that the Link module of TSG-6 binds to HA with higher affinity than CD44 (32), it is perhaps not surprising that TSG-6 effectively competes with CD44 when HA-binding sites are limiting. At higher concentrations of HA and lower protein:HA ratios, binding of the TSG-6-HA complex to CD44 is enhanced compared with the binding of HA alone. This enhancement of HA binding by CD44 was also found to occur under physiologically relevant shear forces, where the presence of TSG-6 proteins in the immobilized HA substrate led to an increase in the number of rolling cells and, to a lesser extent, in the number of tightly adherent cells (Figs. 5 and 6). Reciprocally, binding of soluble HA or TSG-6-HA complexes to cell surface CD44 inhibited the subsequent interaction of cells with immobilized HA, where the protein-HA complex was a more potent antagonist of CD44-mediated cell adhesion than HA alone (Fig. 7).

CD44+ cells that do not constitutively bind HA, such as EL4 and AKR1/CD44ΔCY, were found to exhibit significant ligand binding function after HA preincubation with TSG-6 (Figs. 2–4). In other words, these TSG-6-HA complexes are capable of switching CD44 into an active state, reminiscent of the induction seen with certain antibodies (e.g. IRAWB14). TSG-6, like IRAWB14 (34), retards the dissociation of HA from the cell surface and facilitates binding of low Mr HA fragments (Table 1). The inducing antibody enhances HA binding by direct interaction with CD44 (34, 36). Recent structural studies reveal that the epitope for IRAWB14 is on the opposite face of CD44 from the inducing antibody (34), retards the dissociation of HA from the cell surface and facilitates binding of low Mr HA fragments (Table 1). The inducing antibody enhances HA binding by direct interaction with CD44 (34, 36). Recent structural studies reveal that the epitope for IRAWB14 is on the opposite face of CD44 from the inducing antibody (34), retards the dissociation of HA from the cell surface and facilitates binding of low Mr HA fragments (Table 1).
bilized HA as an adhesion substrate in the absence of inducing stimuli. For example, AKR1/CD44CY cells that fail to bind FL-HA, roll on an HA substrate under flow (37). Similarly, myeloid leukocytes from mouse bone marrow that are virtually unable to bind soluble HA were reported to exhibit attachment to immobilized HA (50). The absence of a requirement for inducing stimuli for CD44-mediated rolling suggests that HA in immobilized form is more favorable for interaction with cell surface CD44 than HA in soluble form. However, in this study we found that significantly more cells rolled on immobilized TSG-6-HA complexes than on HA alone regardless of the constitutive or inducible state of CD44 they expressed. Thus, TSG-6 bound to HA in the immobilized substrate can enhance the CD44-mediated rolling of both constitutive and inducible cells.

Results from mutagenesis studies indicate that the HA binding activity of Link_TSG6 is required for the enhancement of ligand binding to CD44+ cells, which is consistent with the observation that formation of a TSG-6-HA complex is necessary for enhancement. In addition, amino acids in Link_TSG6 that are not directly involved in HA binding are also implicated in the TSG-6-mediated modulation of CD44 function (i.e. His-4 and His-29; Fig. 8 and Table I). It is possible that these residues (and other amino acids in their vicinity) form a part of a self-association site in the Link module domain, leading to the formation of cross-linked HA fibers.

We have shown here that CD44-expressing cells that do not constitutively bind HA (as is the case for many peripheral leukocytes, (6, 8)) are able to bind TSG-6-HA complexes (Figs. 2–4) and exhibit enhanced rolling on substrates comprising these complexes. This observation suggests a mechanism whereby circulating leukocytes might become adhesive in an inflammatory milieu where TSG-6-HA complexes could be present. Because the expression/synthesis of HA and TSG-6 are up-regulated in blood vessels during inflammation (1–3, 18, 19, 51, 52), the inflammatory environment may provide the proper conditions for activation of “dormant” CD44 by creating a ligand consisting of HA modified by TSG-6. Indeed, CD44-mediated, HA-dependent rolling of T lymphocytes on an endothelial substrate was enhanced when the endothelial cells were stimulated with pro-inflammatory cytokines, which increased the retention of HA on the endothelial monolayer (53). Cytokine treatment could also induce TSG-6 expression in endothelial cells (52).

Although we demonstrate that the interaction of TSG-6 with HA can alter the HA binding function of CD44+ cells in both positive and negative ways, we cannot predict how these effects might influence inflammatory cell function in, for example, an arthritic joint. Are these functions of TSG-6 likely to be pro-inflammatory or anti-inflammatory? It is possible that enhanced cell binding to TSG-6-HA complexes (on the surface of endothelium) facilitates CD44-mediated recruitment of inflammatory cells whose CD44 is in an inducible state, thus exerting a pro-inflammatory effect. On the other hand, a large excess of TSG-6 in the presence of low HA concentrations (Fig. 1B) or binding of soluble TSG-6-HA complexes to the surface of leukocytes (Fig. 7) could inhibit the adhesive interactions of circulating cells with endothelium, thus having an anti-inflammatory outcome.

A further question is how HA signaling through CD44 might be influenced by the presence of TSG-6. It has been reported that CD44 signaling can be initiated by low M, HA (48, 51, 54, 55), and we found that TSG-6 can promote binding of relatively small HA fragments to CD44 (Table I). Although CD44 has been shown to be involved in the migration of leukocytes into inflammatory sites in several in vivo model systems (9–12), further studies will be needed to determine the role of TSG-6 in the local recruitment of CD44+ cells in inflammation and in other CD44-mediated cell functions.

Acknowledgments.—We thank Nicki English for help in preparing the manuscript and Dr. Eva Bajnok, Bara Sarraj, and Wendy Ko for technical assistance.

REFERENCES
1. Jain, M., He, Q., Lee, W. S., Kashiaki, S., Foster, L. C., Tsai, J. C., Lee, M. E., and Haber, E. (1996) J. Clin. Invest. 97, 596–603
2. Toole, B. P., Wight, T. N., and Tammi, M. I. (2002) J. Biol. Chem. 277, 4593–4596
3. Tammi, M. I., Day, A. J., and Turley, E. A. (2002) J. Biol. Chem. 277, 4581–4584
4. Lesley, J., Hyman, R., English, N., Catterall, J. B., and Turner, G. A. (1997) Glycoconj. J. 14, 611–622
5. Lesley, J., Hyman, R., and Kincade, P. W. (1993) Adv. Immunol. 54, 271–335
6. DeGrendele, H. C., Kosinski, M., Estess, P., and Siegelman, M. H. (1997) J. Immunol. 159, 2549–2553
7. Hatouche, K. S., Hirano, H., Murakami, S., and Hodes, R. J. (1993) J. Immunol. 151, 6712–6722
8. Lesley, J., Howes, N., Perschl, A., and Hyman, R. (1994) J. Exp. Med. 180, 383–387
9. Camp, R. L., Scheinmann, A., Johansson, C., and Puré, E. (1993) J. Exp. Med. 178, 497–507
10. DeGrendele, H. C., Estess, P., and Siegelman, M. H. (1997) Science 278, 672–675
24. Getting, S. J., Mahoney, D. J., Cao, T., Rugg, M. S., Fries, E., Milner, C. M., Wisniewski, H. G., Naime, D., Hua, J. C., Vilcek, J., and Cronstein, B. N. (1996) Br. J. Rheumatol. 35, 10–16

25. Wisniewski, H.-G., Hua, J.-C., Poppers, D. M., Naime, D., Vilcek, J., and Mindrescu, C., Thorbecke, G. J., Klein, M. J., Vilcek, J., and Wisniewski, H.-G. (2000) Arthritis Rheum. 43, 2668–2677

26. Blundell, C. D., Mahoney, D. J., Almond, A., DeAngelis, P. L., Kahmann, J. D., Milner, C. M., and Day, A. J. (2003) J. Biol. Chem. 278, 1155–1158

27. Perschl, A., Lesley, J., English, N., Trowbridge, I., and Hyman, R. (1995) Eur. J. Immunol. 25, 495–501

28. Lesley, J., Schulte, R., and Hyman, R. (1996) Exp. Cell Res. 187, 224–233

29. Zheng, Z., Kato, S., He, Q., Oritani, K., Miyake, K., Lesley, J., Hyman, R., Hamik, A., Parkhouse, R. M. E., Farr, A. G., and Kincade, P. W. (1995) J. Cell Biol. 130, 485–495

30. Hyman, R., Lesley, J., and Schulte, R. (1991) Immunogenetics 33, 392–395

31. Hua, J. C., Naime, D., Vilcek, J., and Cronstein, B. N. (1996) J. Immunol. 156, 1699–1615

32. Skelton, T. P., Zeng, C., Nocks, A., and Stamenkovic, I. (1998) J. Immunol. 160, 1711–1719

33. Underhill, C. B., Chi-Rosso, G., and Toole, B. P. (1983) J. Cell Biol. 96, 130, 557

34. Lesley, J., Hascell, V. C., Tammi, M., and Hyman, R. (2000) J. Biol. Chem. 275, 26967–26975

35. Lesley, J., English, N., Hascell, V. C., Tammi, M. I., and Hyman, R. (2002) in Arthritis and Allied Conditions (Kfilename:bnunia and S. C. G., ed.) Vol. 1, pp. 341–349, Woodhead Publishing Ltd., Cambridge, UK

36. Lesley, J., He, Q., Miyake, K., Hamann, A., Hyman, R., and Kincade, P. W. (1992) J. Exp. Med. 175, 257–266

37. Cîl, I., Lesley, J., Ko, W., Gonda, A., Stoop, R., Hyman, R., and Mikecz, K. (2003) J. Biol. Chem. 278, 11150–11158

38. Tauber, C., Lesley, J., English, N., Trowbridge, I., and Hyman, R. (1995) Exp. Cell Res. 225, 1–16

39. Lesley, J., Aplin, R. T., and Willis, A. C. (1996) Protein Expression Purif. 8, 1–16

40. Kahmann, J. D., Koruth, R., and Day, A. J. (1997) Protein Expression Purif. 9, 315–318

41. Parkar, A. A., Kahmann, J. D., Howat, S. L. T., Bayliss, M. T., and Day, A. J. (1998) FEBS Lett. 428, 171–176

42. Lesley, J., He, Q., Miyake, K., Hamann, A., Hyman, R., Hamik, A., Parkhouse, R. M. E., Farr, A. G., and Kincade, P. W. (1995) J. Cell Biol. 130, 485–495

43. Hyman, R., Lesley, J., and Schulte, R. (1991) Immunogenetics 33, 392–395

44. Oertli, B., Beck-Schimmer, B., Fun, X., and Wuthrich, R. P. (1998) J. Immunol. 160, 1343–1347

45. DeGrendele, H. C., Estess, P., Picker, L. J., and Siegelman, M. H. (1996) J. Exp. Med. 183, 1119–1130

46. Behr, B., Hefter, C., Bez, S., and Kincade, P. W. (1995) J. Cell Biol. 130, 1233–1252

47. Stoop, R., Gal, I., Gonda, A., Stoop, R., Hyman, R., and Mikecz, K. (2002) J. Cell Biol. 156, 1995–2002

48. Rooney, P., Kumar, S., Ponting, J., and Wang, M. (1995) Int. J. Cancer 60, 632–636

49. Bandman, O., Coleman, R. T., Loring, J. F., Seilhamer, J. J., and Coxs, B. G. (2002) Ann. N. Y. Acad. Sci. 975, 77–90

50. Mohamadzadeh, M., DeGrendele, H., Arzipe, H., Estess, P., and Siegelman, M. (1998) J. Clin. Invest. 101, 97–108

51. McKe, C. M., Penno, M. B., Cowman, M., Bursick, M. D., Strieter, R. M., Bao, C., and Noble, P. W. (1998) J. Clin. Invest. 99, 2403–2413

52. Camenisch, T. D., and McDonald, J. A. (2000) Am. J. Respir. Cell Mol. Biol. 23, 431–433

53. Mikecz, K., Brennan, F. R., Kim, J. H., and Glant, T. T. (1995) Nat. Med. 1, 558–563

54. Brocke, S., Piercy, C., Steinman, L., Weissman, I. L., and Veromaa, T. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 6986–6991

55. Haynes, B. F., Hale, L. P., Patton, K. L., Martin, M. E., and McCallum, R. M. (1991) Arthritis Rheum. 34, 1434–1443

56. Blander, C. D., Mahoney, D. J., Almond, A., DeAngelis, P. L., Kahmann, J. D., Milner, C. M., and Day, A. J. (2003) J. Biol. Chem. 278, 1038–1041

57. Lesley, J., English, N., Hascell, V. C., Tammi, M. I., and Hyman, R. (2002) in Arthritis and Allied Conditions (Kfilename:bnunia and S. C. G., ed.) Vol. 1, pp. 341–349, Woodhead Publishing Ltd., Cambridge, UK

58. Lesley, J., He, Q., Miyake, K., Hamann, A., Hyman, R., and Kincade, P. W. (1992) J. Exp. Med. 175, 257–266

59. Cîl, I., Lesley, J., Ko, W., Gonda, A., Stoop, R., Hyman, R., and Mikecz, K. (2003) J. Biol. Chem. 278, 11150–11158

60. Blander, C. D., Mahoney, D. J., Almond, A., DeAngelis, P. L., Kahmann, J. D., Milner, C. M., and Day, A. J. (2003) J. Biol. Chem. 278, 1038–1041

61. Lesley, J., English, N., Hascell, V. C., Tammi, M. I., and Hyman, R. (2002) in Arthritis and Allied Conditions (Kfilename:bnunia and S. C. G., ed.) Vol. 1, pp. 341–349, Woodhead Publishing Ltd., Cambridge, UK

62. Lesley, J., He, Q., Miyake, K., Hamann, A., Hyman, R., and Kincade, P. W. (1992) J. Exp. Med. 175, 257–266

63. Cîl, I., Lesley, J., Ko, W., Gonda, A., Stoop, R., Hyman, R., and Mikecz, K. (2003) J. Biol. Chem. 278, 11150–11158

64. Blander, C. D., Mahoney, D. J., Almond, A., DeAngelis, P. L., Kahmann, J. D., Milner, C. M., and Day, A. J. (2003) J. Biol. Chem. 278, 1038–1041

65. Lesley, J., English, N., Hascell, V. C., Tammi, M. I., and Hyman, R. (2002) in Arthritis and Allied Conditions (Kfilename:bnunia and S. C. G., ed.) Vol. 1, pp. 341–349, Woodhead Publishing Ltd., Cambridge, UK
