The N-terminal Module of Thrombospondin-1 Interacts with the Link Domain of TSG-6 and Enhances Its Covalent Association with the Heavy Chains of Inter-α-trypsin Inhibitor*

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We recently found that leukocytes from thrombospondin-1 (TSP1)-deficient mice exhibit significant reductions in cell surface CD44 relative to those from wild type mice. Because TSG-6 modulates CD44-mediated cellular interactions with hyaluronan, we examined the possibility that TSP1 interacts with TSG-6. We showed that recombinant full-length human TSG-6 (TSG-6Q) and the Link module of TSG-6 (Link_TSG6) bind 125I-TSP1 with comparable affinities. Trimeric recombinant constructs containing the N-modules of TSP1 or TSP2 inhibit binding of TSP1 to TSG-6Q and Link_TSG6, but other recombinant regions of TSP1 do not. Therefore, the N-modules of both TSP1 and TSP2 specifically recognize the Link module of TSG-6. Heparin, which binds to these domains of both proteins, strongly inhibits binding of TSP1 to Link_TSG6 and TSG-6Q, but hyaluronan does not. Inhibition by heparin results from its binding to TSP1, because heparin also inhibits TSP1 binding to Link_TSG6 mutants deficient in heparin binding. Removal of bound Ca2+ from TSP1 reduces its binding to full-length TSG-6. Binding of TSP1 to Link_TSG6, however, is enhanced by chelating divalent cations. In contrast, divalent cations do not influence binding of the N-terminal region of TSP1 to TSG-6Q. This implies that divalent cation dependence is due to conformational effects of calcium-binding to the C-terminal domains of TSP1. TSP1 enhances covalent modification of the inter-α-trypsin inhibitor by TSG-6 and transfer of its heavy chains to hyaluronan, suggesting a physiological function of TSP1 binding to TSG-6 in regulation of hyaluronan metabolism at sites of inflammation.

One such extracellular matrix protein that is induced by specific inflammatory signals is thrombospondin-1 (TSP1)1 (reviewed in Refs. 4 and 5). TSP1 plays an important role in early phases of wound repair, and its absence prolongs wound repair in the skin (6). TSP1 is specifically induced in the stroma surrounding some tumors (7) and in inflammatory responses associated with rheumatoid arthritis, glomerulonephritis, atopic dermatitis, atherosclerosis, and restenosis (8–13).

Inflammatory responses in the lungs of mice lacking TSP1 implicate TSP1 as a negative modulator of inflammatory responses, due in part to its ability to activate latent transforming growth factor-β1 (14, 15). Furthermore, TSP1 has direct inhibitory effects on T cell receptor-mediated T cell activation (16) as well as NK cell expansion (17) and dendritic cell activation (18). In contrast, TSP1 primes neutrophils for oxidative burst responses (19) and promotes migration of T cells and other leukocytes (20, 21). A lack of monocyte/macrophage recruitment was proposed to explain the delayed wound repair in TSP1 null mice (6). TSP1 also promotes expansion of inflammatory T cells in rheumatoid synovium (22). Therefore, TSP1 may be both a positive and negative modulator of inflammatory responses. These opposing responses to TSP1 may be partially explained by dissecting the opposing signals arising from engaging the several known TSP1 receptors that are expressed on inflammatory cells (8, 21, 23, 24), but direct interactions with secreted inflammatory modulators should also be considered.

We recently found a deficiency in CD44 expression in leukocytes from TSP1 null mice(25). CD44 is a cellular receptor for hyaluronan (HA) and also has known functions in the regulation of inflammatory responses (26–28). Since to date we have not detected direct interactions between TSP1 and either CD44 or HA, we considered the possibility that TSP1 may interact with other CD44 ligands or HA-binding proteins to influence CD44 expression. HA-binding proteins, also known as hyaladherins, characteristically contain domains related to cartilage link protein (29). TSG-6 (also known as tumor necrosis factor-induced protein-6, TNFIP6 (30)) is a member of this superfamily that attracted our attention based on its known interaction with pentraxin-3 (31), a previous observation that the N-ter-
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EXPERIMENTAL PROCEDURES

Materials—TSP1 was purified from human platelets obtained from the National Institutes of Health Department of Transfusion Medicine, under protocols approved by the National Institutes of Health Institutional Review Board, and was stored at −70 °C in 0.2 mM Tris, pH 7.6, 0.15 M NaCl, 0.1 mM CaCl₂, 20% (w/v) sucrose (38). Monomeric and trimeric recombinant regions of TSP1 and TSP2 expressed in insect cells were prepared as described (39, 40). TSP proteins/domains were labeled with 125I using IODO-GEN (Pierce) as described previously (41). Recombinant full-length TSG-6Q and Link_TSG-6Q were prepared as described (42–44). Link_TSG-6Q mutants with either impaired HA binding (Y12F and Y59P) or heparin binding (K34A/K54A and K20A/K32A/K41A) activities were constructed and characterized as described previously (45, 46). Biotinylated-Link_TSG-6Q was prepared as described previously (47).

High molecular weight HA was obtained from Biotechnology General, Rehovot, Israel. The heparin sodium salt from porcine intestinal mucosa (MW ~ 12,000) was purchased from Pierce. Heparin coupled to bovine serum albumin was purchased from Sigma.

Solid Phase Binding Assays—Immulon® 2 HB (TerumoLabsystems, Franklin, MA) microtiter strips with breakaway wells were coated directly with 50 μl of the indicated concentrations of TSG-6Q or Link_TSG-6Q. For competitive binding studies, 10 μl HA (50 μg/ml) was preincubated with a 6-fold molar excess of TSP1 (6 μg) for 1 h at 37 °C prior to addition of purified Iα (8 μg) and a 14-mer HA oligosaccharide (HA14, 2673 Da; 10 μg), i.e. to give final concentrations of 0.25 μM TSG-6Q, 1.6 μg TSP1, 1.8 μg Iα, and 150 μg HA₁4 in 20 μl HEPES-HCl, pH 7.5, 10 mM Tris-HCl, pH 7.6, 150 mM NaCl, 5 mM MgCl₂, 5 mM CaCl₂, 10% (w/v) sucrose. After incubating for the indicated times and temperatures, the reaction mixture was analyzed by Tris-Tricine SDS-gel electrophoresis.

Data Analysis—Self-displacement binding experiments were analyzed using Scalfit version 2.4 of the LIGAND program (49).

RESULTS

TSP1 Binds to the Link Module of TSG-6—Human platelet TSP1 was labeled using 125I and tested for binding to human TSG-6 immobilized on plastic (Fig. 1). As shown in Fig. 1A, at pH 7.3 in the presence of physiological salt concentrations and divalent cations, TSP1 bound minimally to immobilized full-length TSG-6Q at 4 °C. Dose-dependent binding, however, markedly increased with temperature and was highest at 37 °C.

TSG-6 is an ~35-kDa secreted protein composed of contiguous Link and CUB modules (30). The TSG-6 Link module binds to HA (50, 51), chondroitin 4-sulfate (47), the G₁ domain of aggrecan (52), heparin (46, 53), and pentraxin-3 (31). The function of the CUB module of TSG-6 remains unknown, although the fact that it is highly conserved between species suggests that it is essential for at least someactivities of TSG-6. To determine which domain of TSG-6 mediates binding of TSP1, we examined binding of TSP1 to the HA binding domain of TSG-6 (Link_TSG-6Q). 125I-TSP1 bound to immobilized Link_TSG-6Q in a similar temperature- and dose-dependent manner as to TSG-6Q (Fig. 1B). Therefore, the Link domain of TSG-6 is sufficient for TSP1 binding, but we cannot exclude additional interactions of TSP1 with the CUB domain.

To test the influence of calcium ions, which modulate the conformation and some ligand binding properties of TSP1 (54, 55), on its binding to TSG-6, 5 mM EDTA was added to chelate free Ca²⁺ (Fig. 1, C and D). EDTA decreased binding of TSP1 to TSG-6Q—4-fold at each temperature examined (Fig. 1C). Binding of TSP1 to Link_TSG-6Q, in contrast, was somewhat increased in the absence of divalent cations (Fig. 1D).

Interactions between TSP1 and TSG-6 could also be detected when TSP1 was immobilized, and TSG-6 binding was detected by ELISA using an antibody that binds to epitopes distant from the Link domain (i.e. RAH1 (48)). By using this approach, dose-dependent binding of TSG-6 and Link_TSG-6Q to immobilized TSP1 was observed (Fig. 2A). TSG-6 antibodies were used to define further the TSP1-binding site. Antibody A38 inhibited...
TSG-6 binding to TSP1, but antibody Q75 did not (Fig. 2B).
Notably, antibody A38 also inhibits HA binding (56), TSG-6/H18528 complex formation, and cumulus-oocyte complex expansion (57); antibody Q75 does not affect any of these functions.

Both of the above assays required surface adsorption of one of the two proteins on polystyrene, which is known in some cases to alter protein conformation and may partially denature the adsorbed protein (58, 59). To confirm that TSP1 and TSG-6 can interact in the absence of such potential perturbations, we used pull-down assays to detect formation of complexes between TSG-6 and TSP1 in solution. The TSG-6 antibody Q75 was used for this purpose because it did not perturb binding in the solid phase assay (Fig. 2B). Based on detection by Western blotting, TSP1 was pulled down by the Q75 antibody only in the presence of TSG-6Q (Fig. 2C). Conversely, biotinylated Link_TSG6 was pulled down by a TSP1 antibody recognizing an epitope distant from its TSG-6 binding domain (see below) only in the presence of soluble TSP1 (Fig. 2D).

Quantitative analysis of competitive binding experiments using unlabeled TSP1 showed that in the presence of divalent cations at 37 °C, TSP1 bound with a $K_d$ of 310 nM to immobilized full-length TSG-6 (Table I). In the absence of divalent cations, the affinity of binding decreased ~10-fold. In contrast, binding of TSP1 to Link_TSG6 at 37 °C was at an ~3-fold higher affinity (80 nM) than to the full-length protein. The $K_d$ value decreased further to 30 nM in the absence of divalent cations. These data indicate an important role of divalent cations in TSP1-TSG-6 interactions and suggest that the CUB domain of TSG-6 may sterically restrict TSP1 binding.

The N-module of TSP1 Mediates TSG-6 Binding—TSP1 is a homotrimeric glycoprotein composed of 150-kDa subunits. Each monomer contains an N-terminal globular module (N), a disulfide-mediated oligomerization site (o), a vWC-like module (C), three properdin/type 1 repeats (P), three epidermal growth factor like-like repeats (E); seven tandem aspartate-rich, Ca$^{2+}$-binding repeats (Ca); and a C-terminal globular domain (G) (see Fig. 3E). Several recombinant regions of TSP1 were examined to define the TSG-6 binding domain (Fig. 3). 125I-TSP1 binding to TSG-6 (Fig. 3A) or Link_TSG6 (Fig. 3B) was inhibited by a trimeric N-terminal construct (NoC1) but not by any recombinant TSP1 proteins tested that lacked the N-terminal domain (CP123, P3E123, and E3CaG-1). Lack of inhibition by CP123 suggested that the TSG-6-binding site is in the N-module rather than the C-module, but lack of inhibition by CP123 could also be due to a requirement for trimeric C module for binding to TSG-6.

Within the TSP family, TSP1 and TSP2 are most closely related in their domain organization and primary sequences. TSP1 and TSP2 share the highest degree of sequence identity in their C-terminal regions (60), but the N-modules of human TSP1 and TSP2 are only 36% identical. Despite this divergence, a trimeric recombinant region of TSP2 equivalent to NoC1 (NoC2) also inhibited TSP1 binding to Link_TSG6, albeit with ~4-fold lower potency (Fig. 3B). The N-module of TSP1 contains binding sites for heparin (61), calreticulin (62), and several $\beta_1$ integrins (21, 63, 64). Mutants of NoC1 (D162A) and NoC2 (D158A) were available that disrupt a sequence recognized by $\beta_1$ integrins (21). Both of these mutants inhibited binding of 125I-TSP1 to Link_TSG6 with comparable activities.
as the native NoC1 (Fig. 3D). Therefore, the TSG-6-binding site appears to be distinct from this α4β1 integrin-binding site in TSP1 and TSP2.

To confirm specific binding of the N-terminal domains of TSP1 to TSG-6, we examined direct binding of 125I-NoC1 to TSG-6 proteins (Fig. 4). 125I-NoC1 bound with similar dose dependence to full-length TSG-6Q (Fig. 4A) and Link_TSG6 (Fig. 4B). We also used this assay to examine further the basis for the divalent cation dependence of the TSP1-TSG-6 interaction. NoC1 lacks the known Ca2⁺-binding sites of TSP1, which are located in the type 3 repeats and C-terminal domain (54, 65). Unlike full-length TSP1 (Fig. 1), NoC1 binding to TSG-6Q was identical in the presence and absence of divalent cations (Fig. 4A). Binding of NoC1 to Link_TSG6 was slightly higher in the absence of cations (Fig. 4B). These results indicate that the divalent cation dependence observed for full-length TSP1 binding to TSG-6Q is not because of direct effects of divalent cations on either TSG-6 or its interaction with the N-modules of TSP1; NMR titration studies have shown that there is no specific binding of Ca2⁺ ions to the TSG-6 Link module (3).

Effects of Heparin on TSP1-TSG-6 Interactions—TSP1 is a known heparin-binding protein, which is primarily mediated by the N-module (61, 66). Heparin, which is very similar in structure to highly sulfated regions of heparan sulfate, is an excellent model for studying heparan sulfate-protein interactions. The Link module of TSG-6 also interacts specifically with the C-terminal regions of TSP1 (99). Bound Link_TSG6 was detected by electrophoresis on 12% acrylamide Tris-HCl SDS gels and blotting using streptavidin-peroxidase and ECL.

### Table 1

| TSG-6/Bio-Link | PBS Ca²⁺/Mg²⁺ at 37 °C | PBS/EDTA at 37 °C | PBS Ca²⁺/Mg²⁺ at 22 °C |
|---------------|------------------------|-------------------|------------------------|
| Link_TSG6     | 8.0 ± 3.1 × 10⁻⁶        | 3.0 ± 1.2 × 10⁻⁶  | 1.13 ± 0.01 × 10⁻⁷     |
| Full-length TSG-6Q | 3.1 ± 0.6 × 10⁻⁷      | -2.4 × 10⁻⁶       | 1.2 ± 0.1 × 10⁻⁷       |

V. A. Higman and A. J. Day, unpublished data.
heparin, via a site distinct from its HA-binding surface (30, 46, 53). Therefore, heparin could potentially inhibit interactions of TSP1 with TSG-6 by binding to either of these proteins.

To define further the molecular basis for the binding of TSP1 to TSG-6, we compared interactions between TSP1 and wild type Link_TSG6 with that of Link_TSG6 mutants that have significantly reduced heparin-binding properties (Fig. 5A). Folded mutants K34A/K54A and K20A/K32A/K41A, which have ~60 and ~10% of wild type activity (46), respectively, bound better to TSP1 than did wild type Link_TSG6. This suggested that the TSP1-binding site on TSG-6 is distinct from the heparin-binding site.

In contrast, binding of 125I-TSP1 to immobilized wild type Link_TSG6 was potently inhibited by heparin with an IC50 less than 1 μg/ml (Fig. 5B). However, binding of TSP1 to the Link_TSG6 mutants K34A/K54A or K20A/K32A/K41A was inhibited by heparin with identical IC50 values to that of wild type (Fig. 5B). Because the K20A/K32A/K41A mutant has about 90% reduced heparin binding, this result suggested that heparin inhibits primarily via its interaction with TSP1. However, this inhibition could also be explained by co-aggregation of trivalent labeled TSP1 with its multivalent ligand heparin in solution. Thus, inhibition by soluble heparin in the former assay may not be due to direct competition by heparin for a TSG-6-binding site on TSP1. This potential artifact could be circumvented by immobilizing heparin and by examining the ability of TSG-6 to inhibit binding of 125I-TSP1. By using this approach, we found that binding of 125I-TSP1 to plates coated with heparin covalently coupled to BSA could be fully inhibited by Link_TSG6 (Fig. 5C). Because the Link_TSG6 mutants with impaired heparin binding (46) exhibited similar dose dependence as the wild type, we conclude that heparin inhibits TSP1 binding to TSG-6 by interaction with the heparin-binding site in the N-module of TSP1. The heparin and TSG-6-binding sites
on TSP1 either overlap or are situated so that heparin binding sterically inhibits binding of TSG-6. Our data also suggests that TSP1 and heparin recognize separate sites on the Link module of TSG-6.

HA and TSP1 Bind to Distinct Sites on Link_TSG6—The HA-binding site of Link_TSG6 has been mapped, and five key residues (i.e. Lys-11, Tyr-12, Tyr-59, Phe-70, and Tyr-78) that contribute to HA binding have been identified on a single face of the Link module (45, 51, 67). Two Link_TSG6 mutant constructs that have wild type folds were selected for comparison. Mutations of Tyr-12 or Tyr-59 (mutants Y12F and Y59F) significantly reduce HA binding activity (1 and 4% of wild type binding) (45, 68). As shown in Fig. 6A, these mutant proteins exhibit the same TSP1 binding efficiency as wild type protein. These findings suggested that TSP1 interacts with an area of the Link module of TSG-6 distinct from the HA-binding site.

Addition of HA did not inhibit binding of TSP1 to immobilized TSG-6Q at pH 7.3 (results not shown). However, some interactions of TSG-6 with HA are highly pH-dependent, with maximal binding at pH 6 and a dramatic reduction in activity at neutral pH (52), and TSG-6 also binds to aggrecan with a similar pH dependence. Given the reported pH dependence of TSG-6 interactions with HA, these experiments were repeated at pH 6.0. We first examined the effect of pH on the interaction TSP1 with TSG-6 and Link_TSG6 (Fig. 6B). Remarkably, 125I-TSP1 binding to both TSG-6Q- and Link_TSG6-coated plates increased with decreasing pH values between 7.5 and 5.5. We then explored the possibility that interaction of TSP1 with TSG-6 could be inhibited by HA in a pH-dependent manner. As shown in Fig. 6C, however, binding of 125I-TSP1 to Link_TSG6 slightly increased in the presence of unlabeled HA at both pH 6.0 and pH 7.0. Similar results were obtained for binding of TSP1 to Link_TSG6 mutants defective in HA binding (Fig. 6D). These results support the conclusion that TSP1 and HA recognize separate sites on Link_TSG6. In addition, we have shown that TSP1 does not bind detectably to HA itself (data not shown), suggesting that TSG-6 can mediate interactions between TSP1 and HA by binding simultaneously to both ligands.

TSP1 Enhances the Interaction between TSG-6 and Interα-inhibitor in the Absence of HA—To define functional consequences of TSP1 interactions with TSG-6, we examined the effects of this complex formation on the known molecular in-
Figure 6. Effect of hyaluronan and pH on TSP1 binding to Link_TSG6. A, binding of 125I-TSP1 was determined to substrates coated using the indicated concentrations of Link module mutants deficient in hyaluronan binding (Y, Y12F; T, Y59F) or wild type Link_TSG6 ( ). Background values are subtracted from each data point (256 ± 13.6). B, the pH dependence of 125I-TSP1 binding to immobilized TSG-6Q ( ) or Link_TSG6 ( ). TSP1 binding was determined at the indicated pH values in two buffer systems (sodium acetate, solid lines with closed symbols; Na-HEPES, dashed lines with open symbols). C, 125I-TSP1 binding to immobilized Link_TSG6 was determined in the presence of the indicated concentrations of HA at a pH optimal for HA binding to TSG-6 (pH 6.0, ■) and at pH 7.0 ( ). Data are presented as a% of the control specific binding determined at each pH in the absence of HA. D, TSP1 binding was determined to immobilized wild type Link_TSG6 ( ) or the indicated Link_TSG6 mutants with reduced HA binding activity (dotted lines with open symbols) in the presence of the indicated concentrations of HA. Data are presented as a% of the specific TSP1 binding determined for each Link protein in the absence of HA. For all panels, the results (mean counts/min ± S.E.) shown are representative of two independent experiments performed in triplicate.

Discussions

We have identified TSG-6 as an additional ligand for the N-terminal region of TSP1. This interaction is sensitive to the conformation of TSP1, which is regulated by binding of Ca2+. We have previously shown that TSP1 binds to TSG-6 through its heavy chains, and that this binding is essential for the transfer of TSG-6 to HA. In this study, we have further characterized the interaction between TSP1 and TSG-6 by analyzing the binding of TSP1 to different forms of TSG-6, and by determining the effect of pH and hyaluronan on the binding of TSP1 to TSG-6.

TSP1 Enhances Transfer of TSG-6 Heavy Chains to HA—The TSG-6-HC complex is an intermediate in the covalent transfer of HC to HA (37). By incubating an HA oligosaccharide (HA14) with purified Iol and TSG-6Q that was preincubated in the presence or absence of TSP1, we found that TSP1 also enhances the TSG-6-mediated transfer of Iol heavy chains onto HA (Fig. 7C). Formation of the HC-HA14 product was enhanced at 30 and 120 min in the presence of TSP1, and increased loss of intact Iol was evident in the presence of TSP1 at the latter time point.
were analyzed by SDS-gel electrophoresis on 7.5% TSP1 buffer diluted to a final volume of 50 μl.

Heavy chain transfer onto HA14 (H18528) evidenced by the increased amount of the bikunin (H9262) enhanced TSG-6 (mean S.D.).

Normalized to the mock-treated serum signal at each time point analyzed using ImagePro software. Integrated densities are expressed in arbitrary units.

**TSP1** enhances TSG-6-mediated Iol degradation (TSG-6 HC complex formation) and transfer of HC onto HA. A, full-length recombinant TSG-6Q (at 5 μg/ml final concentration; 0.17 μM based on a molecular mass of 30 kDa) was preincubated for 1 h at 37 °C with TSP1 (at 100 μg/ml final concentration; 0.67 μM based on a subunit molecular mass of 150 kDa) or mock-incubated with the corresponding TSP1 buffer diluted to a final volume of 50 μl in DPBS with divalent cations. TSG-6Q alone, TSG-6Q precomplexed with TSP1, or mock-incubated TSP1 was subsequently incubated with 0.5 μl of Iol-containing fetal bovine serum for 10–60 min at 37 °C. A, reaction mixtures were analyzed by SDS-gel electrophoresis on 7.5% Iol or 12% Tris-HCl acrylamide gels (TSG-6) followed by Western blotting with goat anti-TSG-6 antibody or with rabbit antiserum to Iol and exposed at subsaturating densities were analyzed using ImagePro software. Integrated densities are expressed normalized to the mock-treated serum signal at each time point (mean ± S.D.). C, Coomassie Blue-stained SDS-PAGE gel showing enhanced TSG-6 HC complex formation at 30 and 120 min (as evidenced by the increased amount of the bikunin HC byproduct (37)) and heavy chain transfer onto HA(14) (i.e. to form HC-HA(14)) following preincubation with TSP1 for 1 h at 37 °C.

Depletion of Ca²⁺ reduces the affinity of TSP1 for TSG-6 - 10-fold. One premise for examining this interaction was that the N-module of TSP1 belongs to the pentraxin family, and pentraxin-3 is a known TSG-6 ligand (31). Indeed, we found that the pentraxin module of TSP1 and presumably the same module of TSP2 interact with the Link module of TSG-6. The TSG-6-binding site in pentraxin-3 has not been defined, however, so we cannot conclude that pentraxin-3 and TSPs interact with TSG-6 through their paralogous domains. As reported previously for pentraxin-3 (31, 36), mutation of residues in TSG-6 required for HA binding does not inhibit TSP1 binding, and we further demonstrate that HA does not inhibit TSP1 interaction with TSG-6. TSP1 binding to TSG-6 is also maintained at pH values permissive for TSG-6 binding to HA (52). Thus, TSG-6 may mediate formation of trimolecular complexes containing HA and TSP1 or TSP2. In contrast, heparin binding to the N-module of TSP1 potently inhibits the same interaction, suggesting that heparan sulfate proteoglycans may be negative modulators of this interaction. Finally, we demonstrate that TSP1 is an enhancer of the activity of TSG-6 to covalently associate with heavy chains of Iol and to catalyze the covalent modification of HA by Iol (36, 37, 70–73). Insights into other potential functions of the TSP1-TSG-6 interaction may come from examining their patterns of expression. Both proteins have limited expression in normal tissues but are rapidly induced by inflammatory responses. One inflammatory site where these two proteins are clearly co-expressed is in synovial fluid. TSP1 is induced in rheumatoid synovium (74) and accumulates on the surface of fibroblast-like synoviocytes, where it engages T cells through their CD47 receptor (8). Similarly, TSG-6 is elevated in arthritic synovial fluid (75) and joint tissues (76) and is induced by interleukin-17 on fibroblast-like synoviocytes of rheumatoid arthritis patients (77). TSG-6 shows consistent anti-inflammatory properties in mouse models of inflammation/arthrosis (68, 78–84), whereas implantation of hydron pellets containing TSP1 in rats during adjuvant-induced arthritis increased inflammation (85). Therefore, TSP1 and TSG-6 may have opposing functions in rheumatoid arthritis.

TSP1 has also been extensively studied in inflammation associated with cardiovascular disease. TSP1 is strongly induced during the intimal hyperplasia associated with atherosclerosis and in mechanical injury, diabetes, and hypercholesterolemia (reviewed in Ref. 13). TSP1 is a positive regulator of vascular smooth muscle cell proliferation and motility. Although less well characterized in cardiovascular disease, TSP-6 is also strongly induced in the neointima following balloon injury of rat iliac arteries, and expression of TSG-6 in vascular smooth muscle cells stimulates their proliferation (86); TSG-6 is one of only a small number of genes up-regulated in arterial smooth muscle cells in response to mechanical strain (87). Relevant to the divalent cation dependence we observe for TSP1 binding to TSG-6, a polymorphism in TSP1 associated with familial premature coronary heart disease decreases Ca²⁺ binding to the protein (88).

TSP1 and TSP2 have well documented suppressive activities in cancer (reviewed in Refs. 7 and 89), but TSG-6 has not been studied in this inflammatory context. One common link may be the shared regulation of TSP1 and TSG-6 by p53. TSG-6 was identified as a p53-dependent gene expressed following irradiation (90); and TSP1 expression is positively regulated by wild type but not mutant p53 (91).

Phenotypes of TSP and TSG-6 transgenic mice suggest other potential overlapping functions of these proteins. TSP1 null mice have a lung inflammatory phenotype (14); TSP2 null mice have prolonged inflammation in delayed type hypersensitivity reactions (92), and TSG-6 null mice have several inflammatory abnormalities (83). TSG-6 null mice exhibit female infertility because of a defect on cumulus-oocyte complex expansion (70), as do bikunin null (93, 94) and pentraxin-3 null mice (31, 95). TSP1 null mice have a mild fertility impairment, but the basis has not been defined.⁴ In rats, TSP1 is expressed in early

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⁴ S. A. Kuznetsova and D. D. Roberts, unpublished results.
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anternal phase in the granulosa cells of antral follicles and after ovulation is localized to the developing corpus luteum (96). TSP2 is also expressed in granulosa cells, and both TSP1 and TSP2 are increased in response to luteinizing hormone stimulation. In a growing follicle, TSP is distributed uniformly in the follicular basement membrane and in scattered threadlike masses within the granulosa cell layer (97).

The interactions with TSP1 and TSP2 described here may provide new clues to the mechanism for the potent inhibitory action of TSG-6 in models of acute and chronic inflammation. A large body of evidence supports the conclusion that some anti-inflammatory actions of this protein may involve mechanisms independent of IL-1 or HA (30, 68, 83, 84, 98). Based on the evidence that TSP1 can both inhibit and exacerbate different aspects of specific inflammatory responses, TSG-6 interactions should be considered in efforts to define the molecular bases of these biological activities of TSP1.

Further study is also needed to define the mechanism for TSP1 binding to TSG-6 and to determine whether TSP1 binds to any other HA-binding proteins by recognizing conserved features in their Link modules. Given that pentraxin-3 and related regions of two TSPs recognize the Link module of TSG-6, it will also be of interest to determine whether binding of Link modules is a more widespread property of the pentraxin family.

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*Thrombospondin-1 Interactions with TSG-6*
The N-terminal Module of Thrombospondin-1 Interacts with the Link Domain of TSG-6 and Enhances Its Covalent Association with the Heavy Chains of Inter-α-trypsin Inhibitor

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