TSG-6, an Arthritis-Associated Hyaluronan Binding Protein, Forms a Stable Complex with the Serum Protein Inter-α-inhibitor†

Hans-Georg Wisniewski,*,‡ Wilson H. Burgess,§ Joel D. Oppenheim,‡ and Jan Vilček‡

Department of Microbiology and Kaplan Cancer Center, New York University Medical Center, New York, New York 10016, and American Red Cross—Holland Laboratory, Rockville, Maryland 20855

Received February 16, 1994; Revised Manuscript Received April 11, 1994®

ABSTRACT: TSG-6 is a secreted 35-kDa glycoprotein, inducible by TNF and IL-1. The N-terminal portion of TSG-6 shows sequence homology to members of the cartilage link protein family of hyaluronan binding proteins. The C-terminal half of TSG-6 contains a so-called CUB domain, characteristic for developmentally regulated proteins. High levels of TSG-6 protein are found in the synovial fluid of patients with rheumatoid arthritis and some other arthritic diseases. Here we show that TSG-6 readily formed a complex with a protein present in human, bovine, rabbit, and mouse serum. This complex was stable during SDS–PAGE under reducing conditions, and in the presence of 8 M urea. The protein that binds TSG-6 was purified from human serum and identified as inter-α-inhibitor (IαI) by N-terminal microsequencing. Microsequencing of the complex itself revealed the presence of TSG-6 and two of the three polypeptide chains of IαI (bikunin and HC2). Experiments with recombinant TSG-6 and IαI purified from human serum showed that the TSG-6/IαI complex is rapidly formed even in the apparent absence of other proteins at 37 °C, but not at 4 °C. The TSG-6/IαI complex was cleaved by chondroitin sulfate ABC lyase, suggesting that cross-linking by chondroitin sulfate is required for the stability of the complex. Although the functional consequences of TSG-6/IαI complex formation are presently unknown, the fact that TSG-6 forms similar complexes with IαI from several animal species indicates that the components involved in this interaction are conserved in evolution.

TSG-6 (TNF-stimulated gene 6) codes for a secretory 35-kDa glycoprotein that is a member of the hyaladherin family of hyaluronan binding proteins (Lee et al., 1992; Toole, 1991). TSG-6 was originally discovered by differential screening of a cDNA library from TNF-stimulated human diploid fibroblasts (Lee et al., 1990). Sequencing of the cDNA revealed an open reading frame coding for a polypeptide of 277 amino acids including a cleavable signal peptide (Lee et al., 1992). In its N-terminal half, the predicted amino acid sequence shows 36–40% homology to members of the hyaladherin family of proteins that includes the lymphocyte homing/hyaluronan receptor CD44, cartilage link protein, and the proteoglycan core proteins aggrecan and versican (Lee et al., 1992). The C-terminal half of TSG-6 shares 30% sequence homology with the A chain of complement component C1r. This homology region forms a so-called CUB domain which is a motif found in proteins involved in developmental processes (Bork & Beckmann, 1993). Two N-glycosylation consensus sequences are present in TSG-6, and the presence of N-linked carbohydrate was experimentally confirmed (Lee et al., 1992). Like other hyaladherins, TSG-6 protein binds specifically to hyaluronan. TSG-6 expression is tightly regulated, with its transcription in fibroblasts rapidly activated by stimulation with the proinflammatory cytokines IL-1 or TNF-α (Lee et al., 1990, 1993). High levels of TSG-6 protein were found in synovial fluids of patients with rheumatoid arthritis and some other forms of arthritis, whereas no TSG-6 protein was detected in synovial fluids from normal human joints (Wisniewski et al., 1993). In addition, synoviocytes from the joints of rheumatoid arthritis patients showed constitutive TSG-6 expression that was further upregulated by IL-1 and TNF, cytokines that are regularly found in the rheumatoid synovial fluid and tissue (Wisniewski et al., 1993).

Western blot analysis with antibodies to TSG-6 protein revealed, in addition to the 35-kDa band, an unexpected second band with an apparent molecular mass of approximately 120 kDa (Lee et al., 1992; Wisniewski et al., 1992, 1993). (This second band characteristically appeared as a doublet, with a major component of ~120 kDa and a less regularly detectable ~130-kDa component.) Although the nature of what appeared to be a high molecular weight form of TSG-6 protein was not understood, we noted that this form was regularly detected by Western blots in supernatants from TSG-producing cultures maintained in serum-containing medium, but not in serum-free medium. This observation led to the notion that the 120-kDa band may represent a stable complex of TSG-6 with a serum protein (Wisniewski et al., 1992, 1993). The presence of the 120-kDa band was also demonstrated in synovial fluids from arthritis patients (Wisniewski et al., 1993).

Inter-α-inhibitor (IαI)[1] is a member of a family of closely related serum proteins with serine protease inhibitory activity, consisting of IαI, pre-α-inhibitor (PαI), and inter-α-like inhibitor (IαLI) (Enghild et al., 1989; Gebhard et al., 1990; Rouet et al., 1992). The protease inhibitory activity of these proteins resides exclusively in a polypeptide chain termed

†This work was supported by Grant R35CA49731 from the National Cancer Institute and by a grant from the Human Frontier Science Program.

* To whom correspondence should be addressed at the Department of Microbiology, New York University Medical Center, 550 First Ave., New York, NY 10016. Telephone: (212) 263-5315. Fax: (212) 263-7933.

‡ New York University Medical Center.

§ American Red Cross—Holland Laboratory.

® Abstract published in *Advance ACS Abstracts*, May 15, 1994.

[1] Abbreviations: IαI, inter-α-inhibitor; PαI, pre-α-inhibitor; IαLI, inter-α-like inhibitor; HC, heavy chain; MES, 4-morpholineethanesulfonic acid; PTH, phenylthiohydantoin; SAC, heat-killed, formalin-fixed *Staphylococcus aureus* (Immunoprecipitin); PAA, polyacrylamide; TN-5B, BTI-TN-5B1-4 (High Five) insect cells.

0006-2960/94/0433-7423$04.50/0

bikunin (Gebhard et al., 1989, 1990) that is shared by all members of this family. The different proteins are formed by the linkage of bikunin to one or two of three heavy chains (HC1, HC2, HC3) which show 38–54% amino acid sequence homology to each other (Bourguignon et al., 1993). IαI consists of bikunin linked to both HC1 and HC2, PαI consists of bikunin linked to HC3, and IαLI is a complex of bikunin with HC2 (Enghild et al., 1989; Gebhard et al., 1990; Rouet et al., 1992). A chondroitin 4-sulfate chain whose reducing end is linked to Ser^{10} of bikunin (Enghild et al., 1991; Chirat et al., 1991) cross-links the polypeptide chains of these complex protein molecules, which are all stable in SDS–PAGE under reducing conditions. Here we show that the earlier recognized 120-kDa complex reactive with antibodies to TSG-6 protein (Lee et al., 1992; Wisniewski et al., 1992, 1993) consists of TSG-6 and components of IαI, cross-linked by a glycosaminoglycan chain.

EXPERIMENTAL PROCEDURES

Materials. Chrondroitin sulfate ABC lyase from *Proteus vulgaris* (EC 4.2.2.4) and hyaluronate lyase from *Streptomyces hyalurolyticus* (EC 4.2.2.1) were purchased from Sigma, rabbit anti-human IαI immunoglobulin was from Dako (Glostrup, Denmark), and [^{35}S]methionine/[^{35}S]cysteine (Tran^{35}S-label) was purchased from ICN. Immunoprecipitin (SAC) was from GIBCO BRL, and EX-CELL 300 and EX-CELL 400 medium was from JRH Biosciences (Lenexa, KS). Albumin Removal Affini-Filters were from Affinity Technology, New Brunswick, NJ, Centricon-10 concentrators were from Amicon, and PVDF membranes (Immobilon-P) were from Millipore. FPLC equipment and separation matrices were from Pharmacia. All other chemicals were purchased from commercial suppliers and were of analytical or molecular biology grade.

Production and Purification of Recombinant Human TSG-6 Protein. Recombinant *Autographa californica* nuclear polyhedrosis virus (genus *Baculovirus*) containing human TSG-6 cDNA (Wisniewski et al., 1992) was used for the infection of High Five insect cells from *Trichoplusia ni* (BTI-TN-5B1–4; purchased from Invitrogen, San Diego, CA). For high-level expression and purification of TSG-6 protein, TN-5B insect cells were grown in serum-free EX-CELL 400 medium. The cell culture medium was replaced 24 h after inoculation of ~70–80% confluent cultures with recombinant virus and collected again 48 h later. For purification of TSG-6 protein, cleared culture supernatant was directly loaded on a MonoS (HR5/5) column equilibrated with 20 mM MES, pH 6.5. Bound protein was eluted with a linear gradient (20 mL) from 0 to 1 M NaCl in 20 mM MES, pH 6.5. Fractions containing the bulk of TSG-6 protein (0.45–0.65 M NaCl) were pooled, concentrated in Centricon-10 units, and applied onto a Superdex 75 (HR10/30) column equilibrated with 20 mM MES, pH 6.5, 0.5 M NaCl. This resulted in the recovery of ≥95% pure TSG-6 protein as judged by silver staining of SDS–PAGE gels. About 1 μg of pure protein was recovered per 1 mL of culture supernatant. Microsequencing of the purified TSG-6 protein established Trp^{18} as the N-terminus of the mature secreted glycoprotein (data not shown and Table 2), which is in good agreement with the predicted cleavage site of the signal peptide sequence (Von Heijne, 1984, 1985).

Analysis of TSG-6 Binding to Carrier Protein. To determine the presence of TSG-6 binding protein, samples to be analyzed were mixed with an equal volume of TSG-6-containing serum-free supernatants of TN-5B insect cells infected with recombinant *Baculovirus* and incubated at 37 °C for 30–60 min. (Purified TSG-6 protein was used in some experiments, as indicated.) Thereafter, the mixtures were analyzed for the presence of the 120-kDa complex by Western blotting with purified rabbit antibody to TSG-6 as described earlier (Wisniewski et al., 1993). For detection of IαI epitopes by Western blotting, membranes were incubated with a rabbit anti-human IαI antibody (Dako) at a 1:2000 dilution for 1 h.

Immunoprecipitation. Human HepG2 hepatoma cells producing IαI constitutively (Bourguignon et al., 1989) were grown in serum-free EX-CELL 300 medium. [^{35}S]Methionine (Tran^{35}S-label) was added to about 75% confluent HepG2 cell cultures in a 25 cm^2 flask (500 μCi/culture), and the culture supernatant was collected after 24 h. The ^{35}S-labeled HepG2 culture supernatant (300 μL) was incubated with 16 μg of purified recombinant TSG-6 protein or with buffer for 1 h at 37 °C. Samples were precleared with 150 μL of 10% SAC (heat-killed, formalin-fixed *Staphylococcus aureus*). Rabbit anti-TSG-6 antiserum or preimmune serum from the same rabbit (5 μL) was added to the supernatants and incubated for 3 h at 37 °C. Thereafter, 150 μL of 10% SAC was added and incubated for 30 min at room temperature. The supernatants were removed, and the pellets were washed 3 times with 1 mL of 20 mM Tris, pH 7.5, 0.5 M NaCl, 0.02% Tween-20. Pellets were resuspended in 40 μL of SDS–PAGE sample buffer (reducing) and incubated for 3 min in a boiling water bath. The supernatants were removed and analyzed by SDS–PAGE on 10% PAA gels and fluorography.

Partial Purification of the TSG-6 Binding Protein from Human Serum for N-Terminal Microsequencing. Protein precipitated between 40% and 55% saturation with ammonium sulfate from 40 mL of fresh human serum was dissolved in 10 mL of PBS and dialyzed against 50 mM KH_2PO_4, pH 7.0, 50 mM NaCl. Four aliquots were passed through Affini-filter cartridges for albumin removal (Affinity Technology). Each cartridge was washed with 5 mL of the same buffer and eluted with 5 mL of 50 mM KH_2PO_4, pH 7.0, 0.5 M NaCl. The eluates of four cartridges were pooled, dialyzed against 20 mM Tris, pH 7.5, 50 mM NaCl, and loaded on a MonoQ column (HR5/5, Pharmacia) using a Pharmacia FPLC system. Protein was eluted with a linear gradient (16 mL) from 50 mM to 1 M NaCl in 50 mM Tris, pH 7.5. Fractions containing TSG-6 binding activity were pooled and concentrated in Centricon-10 units to a final volume of 200 μL. This material was further separated by FPLC on a Superdex 200 column (HR10/30). The column was equilibrated with 50 mM Na_2HPO_4, pH 7.0, 150 mM NaCl and run at a flow rate of 0.5 mL/min. Fractions containing TSG-6 binding activity were pooled and concentrated in Centricon-10 units. The material was further separated by SDS–PAGE under reducing conditions on a 4–15% PAA gel and transferred in methanol-free transfer buffer at 200 mA for 1 h to a PVDF membrane. Staining with Coomassie Blue R250 revealed only one protein band greater than 200 kDa which was used for microsequencing.

Purification of IαI from Human Serum. IαI was purified from human serum according to Salier et al. (1980), with some modifications. FPLC on Q Sepharose Fast Flow was used instead of DEAE-Sephacel chromatography. Chelating Sepharose Fast Flow was used for zinc chelate chromatography. Phenyl Superose was used for hydrophobic chromatography, and Superdex 200 was used for size-exclusion chromatography instead of Sephacryl-300. The IαI recovered was essentially pure as judged by SDS–PAGE and silver staining.

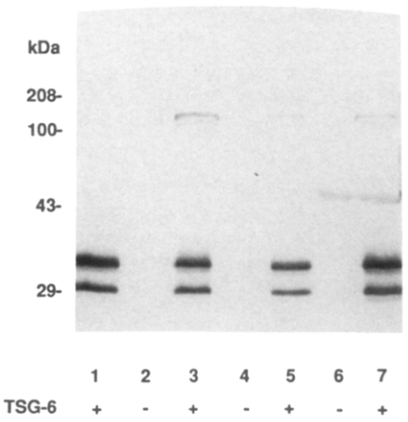

FIGURE 1: TSG-6 forms a stable complex with a serum protein. Fetal bovine serum (*lanes 2, 3*), serum-free supernatant of human HepG2 cells (*lanes 4, 5*), or mouse serum (*lanes 6, 7*) was incubated in the absence (*lanes 2, 4, 6*) or presence (*lanes 3, 5, 7*) of recombinant human TSG-6 for 1 h at 37 °C. All samples were then subjected to Western blot analysis with rabbit antiserum to TSG-6. Lane 1 is a TSG-6 control. The lower (29 kDa) TSG-6 band represents nonglycosylated protein present in variable amounts in preparations of TSG-6 protein from insect cells infected with recombinant *Baculovirus*.

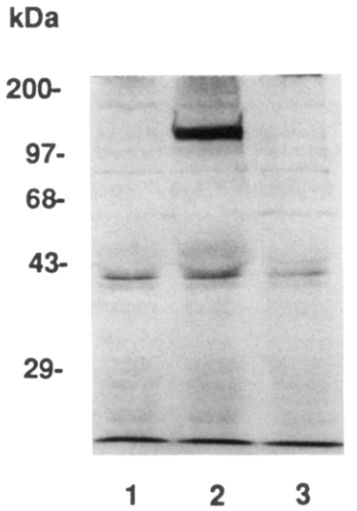

FIGURE 2: Immunoprecipitation of a ^{35}S-labeled 120-kDa protein complex by anti-TSG-6 antiserum. ^{35}S-Labeled HepG2 culture supernatant was preincubated for 1 h at 37 °C with (*lanes 2, 3*) or without (*lane 1*) unlabeled purified recombinant TSG-6 protein and precipitated with rabbit anti-TSG-6 immune serum (*lanes 1, 2*) or preimmune serum (*lane 3*) from the same rabbit. The samples were then analyzed by SDS–PAGE in a 10% PAA gel and fluorography.

Protein Sequencing. Coomassie Blue-stained protein bands on the PVDF membranes were cut from the blots and placed directly into a micro-cartridge of an Applied Biosystems Model 473A protein sequencer. Automated Edman degradations were performed using standard cycles with gas phase delivery of TFA. Data collection and reduction were performed using Applied Biosystems Model 610 software.

Microsequencing of the TSG-6/IαI Complex. Purified recombinant TSG-6 (2.5 μg) was incubated with 2.6 μg of IαI purified from human serum for 1 h at 37 °C. After SDS–PAGE in an 8% PAA gel under reducing conditions, protein was transferred to a PVDF membrane in methanol-free transfer buffer at 200 mA for 1 h. The membrane was stained with Coomassie Blue R250, and the newly formed 120-kDa band (not present in the IαI or TSG-6 preparation) was excised for microsequencing.

RESULTS

Binding of TSG-6 Protein to a Protein Present in Mammalian Sera and in Supernatants of Human HepG2 Hepatoma Cells. Western blot analysis of serum-free culture supernatants of TN-5B insect cells infected with recombinant *Baculovirus* encoding human TSG-6 revealed the presence of a 32-kDa band reactive with antibody to TSG-6 (Figure 1, lane 1). Recombinant human TSG-6 protein produced in insect cells migrates somewhat faster than the 35-kDa TSG-6 protein from human cells (Wisniewski et al., 1993; data not shown) possibly due to a different extent of glycosylation. A second band recognized by antibody against TSG-6, with the apparent molecular mass of 29 kDa, probably represents unglycosylated TSG-6 protein. When TSG-6-containing culture supernatants were incubated at 37 °C in the presence of fetal bovine serum (lane 3), mouse serum (lane 7), or serum-free culture supernatant of human HepG2 hepatoma cells (lane 5), an additional 120-kDa band became readily apparent. A band of identical electrophoretic mobility appeared after incubation of recombinant TSG-6 protein with human or rabbit serum (data not shown).

The 120-kDa Band Represents a Complex of TSG-6 with a Distinct Protein. In order to show that the newly formed 120-kDa band revealed by Western blot analysis is indeed a complex of TSG-6 with a distinct protein, and not a TSG-6 oligomer whose formation is promoted by serum, we employed immunoprecipitation. When a supernatant from HepG2 cells cultured in serum-free medium in the presence of [^{35}S]-methionine was incubated with unlabeled purified recombinant TSG-6 protein and immunoprecipitated with a rabbit antiserum to TSG-6, a labeled 120-kDa molecule was precipitated (Figure 2). This immunoprecipitation was specific because incubation with preimmune serum from the same rabbit or incubation of ^{35}S-labeled HepG2 supernatants with immune serum in the absence of TSG-6 protein failed to precipitate a labeled 120-kDa molecule. [^{35}S]Methionine incorporation into a molecule specifically recognized by an antibody to TSG-6 indicates that the 120-kDa molecule is indeed a complex of TSG-6 protein with another distinct protein that is constitutively produced and secreted by human HepG2 cells.

Partial Purification and Identification of the TSG-6 Binding Protein from Human Serum. Fractionation of normal human serum by ammonium sulfate precipitation showed that proteins precipitated between 40% and 55% saturation contained most of the TSG-6 binding activity, although significant binding was also detected in the fraction precipitated at an ammonium sulfate saturation of 40% (data not shown). The purification procedure used for the isolation of the binding protein and the Western blot-based assay used for the detection of TSG-6 binding protein are described under Experimental Procedures. During Affini-filter chromatography, most of the TSG-6 binding activity eluted at 0.5 M NaCl together with residual albumin (data not shown). FPLC on a MonoQ column proved to be very efficient for further purification of the TSG-6 binding protein. SDS–PAGE followed by silver staining revealed that incubation of fractions from MonoQ-FPLC with recombinant TSG-6 protein resulted in the partial disappearance of a >200-kDa band and the appearance of a new band at 120 kDa (Figure 3). This finding suggests that the human TSG-6 binding protein is greater than 200 kDa in size and hence considerably greater than its complex with TSG-6. During FPLC on Superdex 200, the TSG-6 binding protein eluted with a retention volume corresponding to a molecular mass of about 270 kDa. Fractions containing TSG-6 binding activity were concentrated

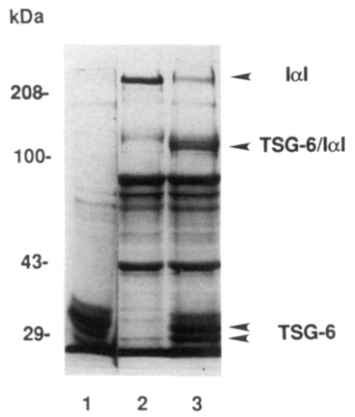

FIGURE 3: Changes in the band pattern of a fraction of human serum proteins after incubation with purified TSG-6 protein. A partially purified preparation of TSG-6 binding protein from human serum (after fractionated ammonium sulfate precipitation, Affini-Filter chromatography, and FPLC on MonoQ) was incubated in the absence (*lane 2*) or presence (*lane 3*) of purified TSG-6 protein at 37 °C for 1 h. Lane 1 is a TSG-6 control. The samples were separated by SDS–PAGE in a 10% PAA gel under reducing conditions and silver-stained.

Table 1: N-Terminal Amino Acid Sequence of the Human TSG-6 Binding Protein Determined by Microsequencing

position	amino acid residues	
1	A	S
2	V	L
3	L	P
4	P	E
5	Q	G
6	E	–[a]
7	E	–
8	E	–
9	G	–
10	–	–
11	G	–
12	G	–

[a] Not determined.

about 80-fold before SDS–PAGE which was performed on 4–15% PAA gradient gels or 8% PAA gels under reducing conditions. Proteins were transferred electrophoretically to PVDF membranes. CBB staining revealed the presence of only one band greater than 200 kDa which was cut for microsequencing.

Microsequencing of the TSG-6 binding protein resulted in double signals for the first five cycles and one signal for each of the following seven cycles. Comparison of the resulting sequences with sequences stored in protein databases revealed that they identify 2 of the 3 chains of the human inter-α-(trypsin) inhibitor (IαI): the bikunin chain of IαI was represented by its 12 N-terminal amino acids whereas the heavy chain 2 (HC2) was represented by its 5 N-terminal amino acids (Table 1). It is noteworthy that Ser^{10} of the bikunin chain could not be identified. No signals corresponding to the heavy chain 1 (HC1) of IαI were retrieved. The microsequencing data along with the molecular mass of ~250 kDa (determined by SDS–PAGE) indicate that the TSG-6 binding protein is IαI, rather than IαLI whose molecular mass is 130–140 kDa (Enghild et al., 1989; Rouet et al., 1992). Subsequent microsequencing of another preparation of IαI purified from human serum allowed the identification of HC1 besides HC2 and bikunin. However, the detected amount of HC1 was substantially lower than that of the two other chains on a molar basis (data not shown).

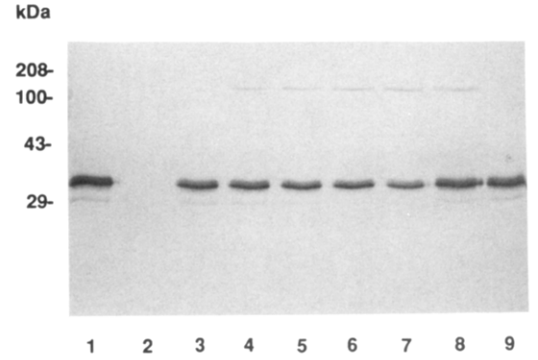

FIGURE 4: Time course and temperature dependence of the formation of the TSG-6/IαI complex. Purified recombinant TSG-6 protein (*lane 1*) and IαI purified from human serum (*lane 2*) were mixed and incubated for 2 min (*lane 3*), 5 min (*lane 4*), 10 min (*lane 5*), 15 min (*lane 6*), 30 min (*lane 7*), or for 60 min (*lane 8*) at 37 °C or for 60 min at 0 °C (*lane 9*). The reaction mixtures were then separated by SDS–PAGE and subjected to Western blot analysis with rabbit anti-TSG-6 serum.

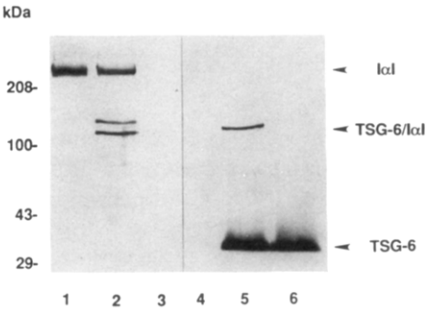

FIGURE 5: Antibodies to IαI or TSG-6 protein recognize a 120-kDa complex formed after incubation of purified IαI and TSG-6 protein. Purified IαI was incubated in the absence (*lanes 1, 4*) or presence (*lanes 2, 5*) of purified TSG-6 protein at 37 °C for 10 min. Lanes 3 and 6 contain purified TSG-6 protein without IαI. All samples were separated by SDS–PAGE on 8% PAA under reducing conditions. For Western blot analysis, lanes 1–3 were developed with anti-IαI, and lanes 4–6 were developed with anti-TSG-6 antibody.

Rapid Complex Formation between Recombinant TSG-6 Protein and IαI Purified from Human Serum at 37 °C. The 120-kDa TSG-6/IαI complex formed readily when purified TSG-6 protein and purified IαI were incubated together at 37 °C but not at 0 °C (Figure 4). At 37 °C, complex formation was detectable within 2 min, and the reaction appeared to be complete by 10 min. At 0 °C, however, little or no TSG-6/IαI complex was formed within 1 h (Figure 4). Monospecific rabbit antisera against either TSG-6 protein or IαI (Figure 5) detect the 120-kDa complex in Western blots, indicating the presence of both TSG-6 and IαI epitopes in a stable complex. Besides the formation of the TSG-6/IαI complex of 120 kDa, incubation of TSG-6 protein with IαI resulted in the appearance of yet another band with a molecular mass of ~130 kDa (Figure 5, lane 2) which was detected by anti-IαI but not by anti-TSG-6. This IαI derivative appears to be a byproduct of the reaction of TSG-6 with IαI. It should be noted that the 120-kDa TSG-6/IαI complex is formed by purified TSG-6 and IαI proteins in the apparent absence of other proteins.

Composition of the TSG-6/IαI Complex. The 120-kDa complex, formed by incubating together purified TSG-6 protein and IαI, was isolated and identified by SDS–PAGE in an 8% PAA gel, electrotransfer to a PVDF membrane, and CBB staining. Microsequencing of the isolated band corresponding to the complex revealed the presence of TSG-6 protein, bikunin, and heavy chain 2 (HC2) of IαI in nearly

Table 2: N-Terminal Amino Acid Sequences of the TSG-6/IαI Complex Determined by Microsequencing

position	amino acid residues			yield (pmol of PTH)		
	bikunin	HC2	TSG-6			
1	A	S	W	10	12	15
2	V	L	G	10	12	12
3	L	P	F	9	11	9
4	P	G	K	9	15	8
5	Q	E	D	7	12	13
6	E	S	G	11	8	13
7	E	E	I	13		9
8	E	E	F	15		9
9	G	M	H	11	9	6
10	S	M	N	8	12	13

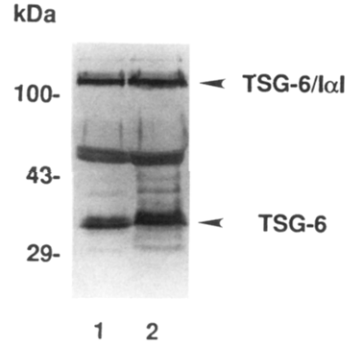

FIGURE 6: Treatment of the TSG-6/IαI complex with 8 M urea. 100 μL of a TN-5B insect cell culture supernatant containing TSG-6 protein was incubated with 100 μL of a 1:50 dilution of human serum for 1 h at 37 °C. Thereafter, one 100-μL aliquot was mixed with 2 mL of 8 M urea and concentrated in a Centricon-10 unit to 100 μL (*lane 2*); the other aliquot was left untreated (*lane 1*). The reaction mixtures were then separated by SDS–PAGE and subjected to Western blot analysis with anti-TSG-6 antibody.

equimolar ratios (Table 2). Interestingly, Ser^{10} of the bikunin chain, which was not detectable during microsequencing of IαI (see Table 1), was found in an equimolar amount in the TSG-6/IαI complex. The unexpected appearance of a serine residue in position 10 could reflect the presence of an additional chain or partial modification of one or more N-termini. A less likely possibility is that Ser^{10} of the bikunin chain, which is glycosylated in IαI, might become deglycosylated in the process of TSG-6/IαI complex formation.

The Stable Cross-Link in the TSG-6/IαI Complex Is Provided by a Glycosaminoglycan Chain. The stability of the 120-kDa TSG-6/IαI complex during SDS–PAGE under reducing conditions raises the question of the nature of the interaction between TSG-6 and the other components of the complex. Addition of 8 M urea before SDS–PAGE did not affect the 120-kDa complex (Figure 6). The effect of treatment with 8 M guanidine hydrochloride could not be fully evaluated because the detectability of both the TSG-6 band and the TSG-6/IαI complex in Western blots was significantly diminished. However, the TSG-6/IαI complex was still detectable after treatment with 8 M guanidine hydrochloride (data not shown). Taken together, these data reflect the high stability of the TSG-6/IαI complex, and the formation of a covalent bond cannot be ruled out.

It is known that the three polypeptide chains of IαI are cross-linked by a chondroitin sulfate chain (Enghild et al., 1989; Jessen et al., 1988; Balduyck et al., 1989). It has been shown that bikunin, HC2 of IαLI, and HC3 of PαI are covalently bound to chondroitin 4-sulfate (Enghild et al., 1989, 1991, 1993). To determine if cross-linking via chondroitin sulfate is also required for the stability of the TSG-6/IαI complex, purified TSG-6 protein and IαI were first incubated

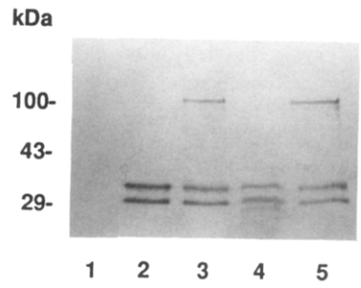

FIGURE 7: Treatment of the TSG-6/IαI complex with chondroitin sulfate ABC lyase or hyaluronidase. Partially purified IαI was incubated in the absence (*lane 1*) or presence of purified TSG-6 protein (*lanes 3–5*) at 37 °C for 1 h. The sample containing both IαI and TSG-6 protein was divided into aliquots and further incubated without enzyme (*lane 3*), with 800 milliunits of chondroitin sulfate ABC lyase (*lane 4*), or with 1.6 units of hyaluronidase (*lane 5*). Lane 2 is a TSG-6 control without IαI. The reaction mixtures were separated by SDS–PAGE and analyzed by Western blotting with anti-TSG-6 antibody.

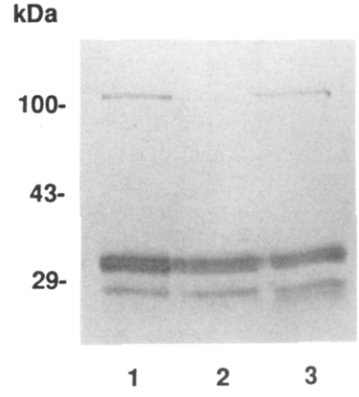

FIGURE 8: TSG-6/IαI complex formation by IαI and TSG-6 proteins pretreated with chondroitin sulfate ABC lyase. Four micrograms of purified IαI and 3 μg of purified TSG-6 protein were incubated separately for 16 h at 37 °C in the absence or presence of 30 milliunits of chondroitin sulfate ABC lyase. Thereafter, control IαI was mixed with control TSG-6 protein (*lane 1*), chondroitinase-pretreated IαI was mixed with control TSG-6 protein (*lane 2*), and control IαI was mixed with chondroitinase-pretreated TSG-6 protein (*lane 3*). The reaction mixtures were incubated for 1 h at 37 °C before SDS–PAGE and Western blot analysis with anti-TSG-6 antibody.

together to allow the formation of the complex and then treated with chondroitin sulfate ABC lyase from *Proteus vulgaris* or hyaluronidase from *Streptomyces hyalurolyticus*. Treatment with chondroitin sulfate ABC lyase resulted in complete disappearance of the complex whereas hyaluronidase had no effect (Figure 7).

Chondroitin Sulfate Associated with IαI Is Required for the Formation of the TSG-6/IαI Complex. In order to determine the effect of chondroitin sulfate ABC lyase on either TSG-6 protein or IαI separately, purified TSG-6 protein and purified IαI from human serum were incubated with a limited amount of chondroitinase for 16 h and then mixed with untreated IαI or TSG-6 protein, respectively. Western blotting revealed that IαI preincubated with chondroitin sulfate ABC lyase was no longer able to form a complex with untreated TSG-6 protein (Figure 8). On the other hand, pretreatment with chondroitinase had little effect on the ability of TSG-6 protein to react with untreated IαI (Figure 8). A slight decrease of the amount of TSG-6/IαI complex formed by chondroitinase-pretreated TSG-6 (Figure 8) can be explained by carry-over of the enzyme into the final TSG-6–IαI incubation mixture because the chondroitinase could not be selectively inactivated. Limiting chondroitinase activity to the necessary minimum was essential in this experiment in

order to prevent IαI inactivation during the final incubation. This finding suggests that IαI, but not TSG-6 protein, has a chondroitinase-sensitive structure required for the formation of the TSG-6/IαI complex.

DISCUSSION

We showed earlier that TSG-6 synthesis is rapidly induced in human diploid fibroblasts and peripheral blood mononuclear cells after stimulation with the inflammatory cytokines TNF and IL-1 (Lee et al., 1990, 1992; Wisniewski et al., 1993). The affinity of TSG-6 protein for hyaluronan suggests a possible association with the extracellular matrix and cartilage (Lee et al., 1992). High levels of TSG-6 protein in the synovial fluid of patients with rheumatoid arthritis and constitutive TSG-6 expression by cultured synovial cells from rheumatoid joints *in vitro* that was further enhanced by TNF and IL-1, suggested a role for TSG-6 in inflammatory diseases of connective tissue and cartilage (Wisniewski et al., 1993). Little is known, however, about the actions of TSG-6 at the molecular or cellular level. Here we show that recombinant human TSG-6 protein is readily incorporated into a stable 120-kDa complex if incubated with human, fetal bovine, rabbit, or mouse serum. Isolation and microsequencing of the human TSG-6 binding protein allowed its identification as IαI, an extensively studied serum protein.

IαI is a complex protein in which the bikunin chain is linked to HC1 and HC2 (Enghild et al., 1989; Gebhard et al., 1990; Rouet et al., 1992). Yet, sequence analysis of the TSG-6 binding protein purified from human serum revealed only the bikunin chain and HC2 (Table 1), and no indication for the presence of HC1 was obtained. Subsequent sequencing of another preparation of IαI purified from human serum resulted in the detection of very weak signals for the sequence of HC1, corresponding to about one-tenth of the other two chains on a molar basis. Other investigators who reported N-terminal sequencing data for IαI also received incomplete and divergent sequences for HC1 when sequencing the unmodified IαI molecule (Enghild et al., 1989; Jessen et al., 1988; Malki et al., 1992). Nevertheless, little doubt exists that the TSG-6 binding protein is IαI because IαI is the only member of this protein family with a molecular mass greater than 200 kDa. IαLI, which consists of bikunin and HC2, the two chains we detected by microsequencing, has a molecular mass of only 130–140 kDa (Enghild et al., 1989; Rouet et al., 1992). In addition, we found that purified IαI forms what appears to be the same 120-kDa complex with TSG-6 protein as whole serum (Figures 4 and 5). While there is no doubt that IαI is the TSG-6 binding protein we have isolated, it is possible that IαLI or PαI also can bind TSG-6 protein.

Evidence that the reaction between TSG-6 and IαI indeed yields a complex of TSG-6 with one or more polypeptide chains of IαI is provided by the immunoprecipitation data (Figure 2). Further information about the composition of the TSG-6/IαI complex is derived from Western blot analysis. Antisera specific for either TSG-6 protein or IαI detected a 120-kDa band newly formed upon incubation of TSG-6 protein and IαI with each other (Figure 5), suggesting the presence of TSG-6 and IαI epitopes in the complex. This was confirmed by microsequencing of the TSG-6/IαI complex (Table 2) which revealed the presence of three chains: TSG-6 protein, bikunin, and HC2. The signals of all three chains are represented in nearly equimolar amounts, suggesting that the complex contains one of each polypeptide chain. No signals corresponding to HC1 could be detected. The molecular mass of the complex is surprisingly low if one considers the molecular masses of the incorporated polypeptides. HC2 has a molecular mass of 70 kDa (Enghild et al., 1989), the reported molecular mass of bikunin is 26–70 kDa depending on the extent of glycosylation (Gebhard et al., 1990; Rouet et al., 1992), and that of recombinant TSG-6 protein is 32 kDa. The fact that the apparent molecular mass of the 120-kDa complex is less than the sum of its components suggests that some additional modifications, such as deglycosylation or limited proteolytic cleavage, might take place. Alternatively, changes of the gross structure of the complex due to incorporation of TSG-6 could have a substantial effect on its apparent molecular mass.

The unusual stability of the TSG-6/IαI complex raises the question of the nature of the bonds linking its components. The resistance of the complex to boiling in 2% SDS and 5% β-mercaptoethanol as well as to 8 M urea makes any noncovalent hydrophobic or hydrophilic bond unlikely. In addition, the strict temperature dependence of TSG-6/IαI complex formation (Figure 4) suggests that the reaction involves an activated transition state and supports the notion that a covalent bond is formed. However, an unusually stable noncovalent association of TSG-6 with the glycosaminoglycan chain of the complex cannot be ruled out. The polypeptide chains of IαI are cross-linked by a chondroitin 4-sulfate chain (Enghild et al., 1989; Jessen et al., 1988; Balduyck et al., 1989). The sensitivity of the TSG-6/IαI complex to chondroitin sulfate ABC lyase (Figure 7) and the inability of chondroitinase-pretreated IαI to form the complex (Figure 8) suggest that the chondroitin 4-sulfate chain cross-linking the polypeptide chains of IαI is also required for the formation of the 120-kDa complex. It has been shown that the chondroitin 4-sulfate chain of IαI is bound to Ser^{10} of bikunin via a common Gal-Gal-Xyl oligosaccharide (Enghild et al., 1989, 1991; Chirat et al., 1991). An unusual ester bond has been shown to cross-link the α-carboxylic group of the HC2 C-terminal Asp^{648} of IαLI to C-6 of an internal *N*-acetylgalactosamine of the chondroitin 4-sulfate chain (Enghild et al., 1993). A similar bond cross-links the C-terminal Asp^{618} of HC3 to chondroitin 4-sulfate in PαI (Enghild et al., 1991, 1993). Analysis of the cDNAs of the three heavy chains showed that all have the conserved consensus sequence VXXDPHFII, supposed to determine the cleavage site for the C-terminal propeptide (Bourguignon et al., 1993) after the aspartic acid residue. This cleavage generates the free α-carboxylic group of the now C-terminal aspartic acid residue which forms the ester bond to an internal *N*-acetylgalactosamine of chondroitin 4-sulfate, two reactions that may be closely coupled. Interestingly, TSG-6 also features a core of the consensus sequence $VXXDP^{249}$. Hence, it is conceivable that TSG-6 forms a direct covalent bond to the chondroitin 4-sulfate chain of IαI. Additional studies are required to determine the molecular structures formed and the exact nature of the interactions leading to the formation of the exceedingly stable TSG-6/IαI complex.

Although the trypsin-inhibitory activity of IαI has been known for a long time (Heide et al., 1965), little is known about the functions of the different members of the IαI family. However, disease-associated presence in various tissues and fluctuations seen in the serum levels of IαI and IαI-related proteins suggest an involvement in pathologic processes. Daveau et al. (1993) reported a distinct pattern of changes in serum concentrations of the different members of the IαI family during acute inflammation. Proteins identical with, or closely related to, the bikunin chain of IαI have been detected in the stroma and the surrounding connective tissue of malignant tumors (Yoshida et al., 1989), in brain tissue of

patients with Alzheimer's disease (Yoshida et al., 1991), and in serum and urine of patients with inflammatory disease, cancer, and leukemias (Rudman et al., 1976; Franck & Pedersen, 1983; Chawla et al., 1990). A link between IαI and rheumatoid arthritis was suggested over 20 years ago when Becker and Sandson (1971) found IαI associated with hyaluronan in the synovial fluid of patients with rheumatoid arthritis, whereas no IαI was detectable in control synovial fluids. This finding was confirmed and extended to show that IαI associates *in vitro* with hyaluronan isolated from the synovial fluid of healthy subjects (Hutadilok et al., 1988). Huang et al. (1993) showed recently that in the presence of serum the two heavy chains of IαI become covalently associated with hyaluronan.

The possible functional implications of complex formation between TSG-6 and IαI are still elusive. Though TSG-6 has been detected in human serum, it is most prominent at sites of local inflammation (Wisniewski et al., 1993; and unpublished results). Therefore, TSG-6/IαI interaction probably occurs most readily at inflammatory sites such as the synovial tissue in rheumatoid arthritis, where both TSG-6 and IαI are found at high concentrations together with hyaluronan. Indeed, synovial fluids from arthritis patients contained both the free 35-kDa form and the 120-kDa complexed form of TSG-6 protein (Wisniewski et al., 1993). Recently, the rabbit homologue of TSG-6 has been cloned and characterized as a developmentally regulated protein (Feng & Liau, 1993), supporting the idea that TSG-6 might play a role in developmental processes. Tissue remodeling could be the common denominator of TSG-6 association with developmental and inflammatory processes. The fact that human TSG-6 forms complexes of equal size with IαI from different mammalian species indicates that the molecular structures involved in the interaction are well conserved among mammals, perhaps reflecting selective pressure due to functional and structural constraints.

ACKNOWLEDGMENT

We thank Brian Hampton for microsequencing, Gene Liau for helpful discussion, Angel Feliciano for technical assistance, and Ilene Totillo for manuscript preparation.

REFERENCES

Balduyck, M., Laroui, S., Mizon, C., & Mizon, J. (1989) *Biol. Chem. Hoppe-Seyler 370*, 329–336.
Becker, A., & Sandson, J. (1971) *Arthritis Rheum. 14*, 764–766.
Bork, P., & Beckmann, G. (1993) *J. Mol. Biol. 231*, 539–545.
Bourguignon, J., Sesboüé, R., Diarra-Mehrpour, M., Daveau, M., & Martin, J.-P. (1989) *Biochem. J. 261*, 305–308.
Bourguignon, J., Diarra-Mehrpour, M., Thiberville, L., Bost, F., Sesboüé, R., & Martin, J.-P. (1993) *Eur. J. Biochem. 212*, 771–776.
Chawla, R. K., Lawson, D. H., & Travis, J. (1990) *J. Cell Biochem. 42*, 207–217.
Chirat, F., Balduyck, M., Mizon, C., Laroui, S., Sautiere, P., & Mizon, J. (1991) *Int. J. Biochem. 23*, 1201–1203.
Daveau, M., Rouet, P., Scotte, M., Faye, L., Hiron, M., Lebreton, J.-P., & Salier, J.-P. (1993) *Biochem. J. 292*, 485–492.
Enghild, J. J., Thøgersen, I. B., Pizzo, S. V., & Salvesen, G. (1989) *J. Biol. Chem. 264*, 15975–15981.
Enghild, J. J., Salvesen, G., Hefta, S. A., Thøgersen, I. B., Rutherfurd, S., & Pizzo, S. V. (1991) *J. Biol. Chem. 266*, 747–751.
Enghild, J. J., Salvesen, G., Thøgersen, I. B., Valnickova, Z., Pizzo, S. V., & Hefta, S. A. (1993) *J. Biol. Chem. 268*, 8711–8716.
Feng, P., & Liau, G. (1993) *J. Biol. Chem. 268*, 9387–9392, 21453.
Franck, C., & Pedersen, J. Z. (1983) *Scand. J. Clin. Lab. Invest. 43*, 151–155.
Gebhard, W., Schreitmüller, T., Hochstrasser, K., & Wachter, E. (1989) *Eur. J. Biochem. 181*, 571–576.
Gebhard, W., Hochstrasser, K., Fritz, H., Enghild, J. J., Pizzo, S. V., & Salvesen, G. (1990) *Biol. Chem. Hoppe-Seyler 371*, Suppl. 13–22.
Heide, K., Heimburger, N., & Haupt, H. (1965) *Clin. Chem. Acta 11*, 82–85.
Huang, L., Yoneda, M., & Kimata, K. (1993) *J. Biol. Chem. 268*, 26725–26730.
Hutadilok, N., Ghosh, P., & Brooks, P. M. (1988) *Ann. Rheum. Dis. 47*, 377–385.
Jessen, T. E., Faarvang, K. L., & Ploug, M. (1988) *FEBS Lett. 230*, 195–200.
Lee, T. H., Lee, G. W., Ziff, E. B., & Vilček, J. (1990) *Mol. Cell. Biol. 10*, 1982–1988.
Lee, T. H., Wisniewski, H.-G., & Vilček, J. (1992) *J. Cell Biol. 116*, 545–557.
Lee, T. H., Klampfer, L., Shows, T. B., & Vilček, J. (1993) *J. Biol. Chem. 268*, 6154–6160.
Malki, N., Balduyck, M., Maes, P., Capon, C., Mizon, C., Hau, K. K., Tartar, A., Fournet, B., & Mizon, J. (1992) *Biol. Chem. Hoppe-Seyler 373*, 1009–1018.
Rouet, P., Daveau, M., & Salier, J.-P. (1992) *Biol. Chem. Hoppe-Seyler 373*, 1019–1024.
Rudman, D., Chawla, R. K., Hendrickson, L. J., Vogler, W. R., & Sophianopoulos, A. J. (1976) *Cancer Res. 36*, 1837–1846.
Salier, J. P., Martin, J. P., Lambin, P., McPhee, H., & Hochstrasser, K. (1980) *Anal. Biochem. 109*, 273–283.
Toole, B. P. (1991) in *Cell Biology of Extracellular Matrix* (Hay, E. D., Ed.) 2nd ed., pp 305–341, Plenum Press, New York.
Von Heijne, G. (1984) *J. Mol. Biol. 173*, 243–251.
Von Heijne, G. (1985) *J. Mol. Biol. 184*, 99–105.
Wisniewski, H.-G., Lee, T. H., Lee, S., & Vilček, J. (1992) in *Physiology and Pathophysiology of Cytokines* (Mantovani, A., & Ghezzi, P., Eds.) pp 149–155, Biomedical Press, Augusta, GA.
Wisniewski, H.-G., Maier, R., Lotz, M., Lee, S., Klampfer, L., Lee, T. H., & Vilček, J. (1993) *J. Immunol. 151*, 6593–6601.
Yoshida, E., Sumi, H., Maruyama, M., Tsushima, H., Matsuoka, Y., Sugiki, M., & Mihara, H. (1989) *Cancer 64*, 860–869.
Yoshida, E., Yoshimura, M., Ito, Y., & Mihara, H. (1991) *Biochem. Biophys. Res. Commun. 174*, 1015–1021.