A Novel Secretory Tumor Necrosis Factor-inducible Protein (TSG-6) Is a Member of the Family of Hyaluronate Binding Proteins, Closely Related to the Adhesion Receptor CD44

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Abstract. TSG-6 cDNA was isolated by differential screening of a λ cDNA library prepared from tumor necrosis factor (TNF)-treated human diploid FS-4 fibroblasts. We show that TSG-6 mRNA was not detectable in untreated cells, but became readily induced by TNF in normal human fibroblast lines and in peripheral blood mononuclear cells. In contrast, TSG-6 mRNA was undetectable in either control or TNF-treated human vascular endothelial cells and a variety of tumor-derived or virus-transformed cell lines. The sequence of full-length TSG-6 cDNA revealed one major open reading frame predicting a polypeptide of 277 amino acids, including a typical cleavable signal peptide. The NH_2-terminal half of the predicted TSG-6 protein sequence shows a significant homology with a region implicated in hyaluronate binding, present in cartilage link protein, proteoglycan core proteins, and the adhesion receptor CD44. The most extensive sequence homology exists between the predicted TSG-6 protein and CD44. Western blot analysis with an antiserum raised against a TSG-6 fusion protein detected a 39-kD glycoprotein in the supernatants of TNF-treated FS-4 cells and of cells transfected with TSG-6 cDNA. Binding of the TSG-6 protein to hyaluronate was demonstrated by coprecipitation. Our data indicate that the inflammatory cytokine (TNF or IL-1)-inducible, secretory TSG-6 protein is a novel member of the family of hyaluronate binding proteins, possibly involved in cell-cell and cell-matrix interactions during inflammation and tumorigenesis.

A recently identified family of hyaluronate (HA)[1] binding proteins includes the cell surface adhesion receptor CD44, cartilage link protein, and proteoglycan core proteins (Neame et al., 1987; Goldstein et al., 1989; Stamenkovic et al., 1989). The conserved, homologous regions include the HA binding domains in all of these proteins (Goetinck et al., 1987; Perin et al., 1987). CD44, also termed Hermes antigen (Jalkanen et al., 1986), Pgp-1 (Hughes et al., 1981), or H-CAM (Culty et al., 1990), is best known as an integral membrane protein responsible for the interaction between lymphocytes and high endothelial cells in the gut-associated lymphoid tissues during lymph node homing (Jalkanen et al., 1986). CD44 is now known to be identical to the cellular hyaluronate receptor (Aruffo et al., 1990; Culty et al., 1990; Miyake et al., 1990), mediating the adhesion of a variety of different cells to HA in the extracellular matrix and on cell surfaces (Underhill, 1989). While CD44 is the principal cell surface receptor for HA, all functions of this adhesion receptor may not depend on its ability to bind HA. Most notably, the function of CD44 as a lymphocyte homing receptor appears to involve the binding of CD44 to mucosal vascular addressins, and not to HA (Jalkanen et al., 1987; Culty et al., 1990). CD44 also acts as a receptor for type I and IV collagen (Carter and Wayner, 1988). The fact that CD44 (or closely related proteins) is (are) expressed on a large variety of mammalian tissues (Underhill, 1989), together with other accumulated evidence, suggest that CD44 mediates many different cell adhesion events involving cell-substratum or cell-cell interactions (Aruffo et al., 1990; Culty et al., 1990; Miyake et al., 1990). The proteoglycan core protein (aggrecan) and link protein are important structural components of the extracellular matrix of cartilage. Aggrecan (Doege et al., 1990*b*) and the closely related proteoglycan core protein of fibroblasts (versican) (Zimmermann and Ruoslahti, 1989) contain at their amino terminus HA binding motifs, which were originally defined in link protein (Doege et al., 1986). In cartilage, aggrecan forms a ternary complex with both HA and link proteins, which determines the structural integrity of the extracellular matrix of cartilage (Hascall and Hascall, 1981; Kimata et al., 1982).

In this paper we report the identification of a new member of the family of HA binding proteins, provisionally termed

1. *Abbreviations used in this paper*: CPC, cetylpyridinium chloride; EDC, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide; HA, hyaluronate; huPBL, human peripheral blood leukocytes; HUVEC, human umbilical vein endothelial cells; IL-1, interleukin 1; TNF, tumor necrosis factor; TSG, TNF-stimulated gene.

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TSG (for TNF-stimulated gene)-6. TSG-6 DNA has been isolated from a cDNA library prepared from tumor necrosis factor-α (TNF-α)-treated human FS-4 diploid foreskin fibroblasts (Lee et al., 1990). TSG-6 mRNA was undetectable in untreated FS-4 cells, but became markedly elevated after treatment with TNF. TSG-6 mRNA was also inducible by interleukin 1 (IL-1), the phorbol ester 12-*O*-tetradecanoylphorbol 13-acetate, calcium ionophore A23187, and double-stranded RNA, but not by various growth factors, interferons, and some other agents (Lee et al., 1990). The relatively selective inducibility of TSG-6 mRNA by the inflammatory cytokines TNF and IL-1 prompted us to sequence the full-length cDNA and analyze TSG-6 mRNA expression in different types of cells. We report here that the protein encoded by TSG-6 cDNA showed a significant sequence homology with members of the family of HA binding proteins, and especially with CD44. Our data indicate that TSG-6 is a TNF (and IL-1)-inducible, secretory HA binding protein. TSG-6 mRNA is readily inducible in normal fibroblasts and peripheral blood leukocytes, but not in a variety of tumor-derived or virus-transformed cell lines. Its high degree of sequence homology with the NH_2-terminal half of CD44, together with its inducibility by TNF and IL-1, suggest that TSG-6 functions as a regulator of cell–cell and cell–matrix interactions during inflammation and tumorigenesis.

Materials and Methods

Cell Cultures

The normal human diploid foreskin fibroblasts (FS-4, FS-48, and FS-49 cell lines), isolated in this laboratory, were maintained in MEM supplemented with 5% FBS. The normal human diploid fetal lung fibroblast line WI-38, and the SV-40-transformed WI-38 cell line (WI-38 VA13) were received from the American Type Culture Collection (Rockville, MD) and grown in Eagles' MEM supplemented with 10% FBS. The SV-40-transformed human skin fibroblast line GM-637 (obtained from the Human Genetic Mutant Cell Repository, Camden, NJ) and the human cutaneous malignant melanoma cell line SK-MEL-19, kindly provided by Dr. Alan Houghton (Memorial Sloan-Kettering Cancer Center, New York), were grown in MEM supplemented with 10% FBS. The human macrophage-like cell line, U937, was grown in RPMI-1640 medium containing 20% FBS. The human rhabdomyosarcoma (A673), human lung carcinoma (A549), and human colon adenocarcinoma cell lines (Colo205 and HT29) were maintained in DME containing 10% FBS. The human umbilical vein endothelial cells (HUVEC) were kindly provided by Dr. Richard Levin (New York University Medical Center, New York) and maintained in α-MEM supplemented with 20% FBS and basic fibroblast growth factor (10 ng/ml; a gift from Dr. D. Moscatelli, Department of Cell Biology, New York University Medical Center). HUVEC cultures were used at passage 5. Mononuclear cells from human peripheral blood leukocytes (huPBL) from the blood of healthy donors were isolated by centrifugation on Ficoll-Hypaque gradients (Isolymph; Gallard-Schlesinger Chemical Manufacturing Corp., Carle Place, NY) and cultured in RPMI-1640 medium containing 10% FBS.

Northern Blot Analysis

Nonadherent U937 cells were treated with TNF (20 ng/ml) for 4 h at a cell density of 10^6 cells/ml. HuPBL (1×10^6 mononuclear cells/ml) were treated with TNF (20 ng/ml), Con A (5 μg/ml), or phytohemagglutinin (2 μg/ml) for 16 h. All other adherent cell lines were used when confluent, and were treated with TNF for 4 h. Cell pellets were prepared, and in most cases total RNA was isolated by the acid guanidium-thiocyanate-phenol-chloroform extraction method (Chomczynski and Sacchi, 1987), except for the normal fibroblast cell lines (FS-4, FS-48, FS-48, and WI-38), for which a method described elsewhere was used (Lin and Vilček, 1987). Northern blots were prepared and probed according to Maniatis et al. (1982). ^{32}P-Labeled probes that detect TSG-6 mRNA and the invariant pHe7 transcripts were prepared with the aid of a nick translation kit (Boehringer Mannheim Corp., Indianapolis, IN).

Sequence Analysis

Among the six λ clones that cross-hybridized with TSG-6cDNA (Lee et al., 1990), clone λ5 had the longest insert (1.4 kb) and was therefore used for full-length cDNA sequence analysis. The 1.4-kb cDNA insert of clone λ5 was subcloned into the EcoRI site of M13mp18 bacteriophage in both orientations. To insure fidelity of sequence determination, directional deletion clones were generated by the ExoIII/S1 method (Henikoff, 1984) in both directions within the M13 clones. The deletion clones were then used to determine the nucleotide sequence, utilizing the Sequenase kit available from United States Biochemical Corp. (Cleveland, OH).

Preparation of Bacterial Fusion Proteins

To express a bacterial fusion protein of TSG-6, we used an EcoRI cDNA insert from clone λ6 (Lee et al., 1990). Clone λ6 contains a cDNA insert that lacks 402 bp at the 5'end and 4 bp at the 3' end of the TSG cDNA sequence shown in Fig. 1 *A*. An EcoRI-BamHI (406 bp) restriction fragment (which encodes the portion of TSG-6 open reading frame spanning from Ile^{115} to Asp^{248}) was isolated from the EcoRI cDNA insert of clone λ6 and cloned into the same restriction sites in the polylinker downstream of, and in frame with, a portion (37 kD) of the *Escherichia coli* TrpE open reading frame in the pATH21 vector (Sprindler et al., 1984), resulting in the TrpE/TSG-6 expression plasmid pATH-TSG-6. The same restriction fragment (EcoRI-BamHI) was also inserted into the pEX34A bacterial expression vector, resulting in the MS2/TSG-6 expression plasmid pEX-TSG-6. pEX34A is a derivative of pEX29 (Klinkert et al., 1985; Strebel et al., 1986), which permits the production of foreign proteins fused to the NH_2-terminal part of the MS2 polymerase and controlled by the temperature-inducible λP_L promoter. The two expression plasmids (pATH-TSG-6 and pEX-TSG-6) were transfected into competent *E. coli* HB101 and *E. coli* ΔHΔtrp cells (Remaut et al., 1981), respectively. Cells transformed with pATH-TSG-6 were grown and induced by the addition of 3-β-indolacrylic acid (Sigma I1625) (Sprindler et al., 1984), and *E. coli* transformed with pEX-TSG-6 were induced by raising the incubation temperature (Remaut et al., 1981). Purification of both fusion proteins was done essentially as described by Strebel et al. (1986). Briefly, cells from a IL culture were pelleted and washed with TEN (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 0.5 M NaCl), lysed with lysozyme (5 mg/ml), and finally broken by sonication. Insoluble material was recovered by centrifugation (30 min, 20,000 *g*) and extracted sequentially with 20 ml of 3 M urea and 5 ml of 7 M urea each for 30 min at 37°C. The 7 M urea extract containing the fusion protein was further purified by preparative 10% SDS-PAGE. After electrophoresis the fusion protein was excised from the gel, electroeluted, and concentrated as needed. The purity of the electroeluted fusion protein was checked on analytical gels. After the second round of electroelution, we obtained highly purified fusion proteins with no detectable *E. coli* protein bands on SDS-PAGE (data not shown).

Preparation and Purification of Polyclonal Antisera Specific for TSG-6 Protein

Rabbits were first immunized with ~200 μg of the TrpE/TSG-6 fusion protein suspended in Freund's complete adjuvant, and boosted at intervals of 2–3 wk with the same amount of protein in Freund's incomplete adjuvant. All injections were performed subcutaneously, except for the final boost which was done intravenously. Rabbits were bled 6 d after immunizations. Sera were analyzed by immunoblotting as described (Towbin et al., 1979). To obtain antibodies specific for the TSG-6-coded domains in the TrpE/TSG-6 fusion protein, the antiserum was purified on an affinity column to which the MS2/TSG-6 fusion protein was coupled. The affinity column was prepared as follows. 5 mg of purified MS2/TSG-6 fusion protein was dialyzed extensively against 0.5 M NaCl and mixed with 3 ml of EAH-Sepharose 4B beads (Pharmacia Fine Chemicals, Piscataway, NJ). After adjusting pH to 4.5, 40 mg 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) (Aldrich Chemical Co., Milwaukee, WI)/ml H_2O was added dropwise under constant stirring. The coupling reaction was then allowed to proceed overnight under constant stirring. 200 μl acetic acid was then added and the beads were incubated for another 4 h to block the remaining amino groups on the matrix. The matrix material was washed several times alternately with a buffer containing 0.1 M acetate, pH 4.0,

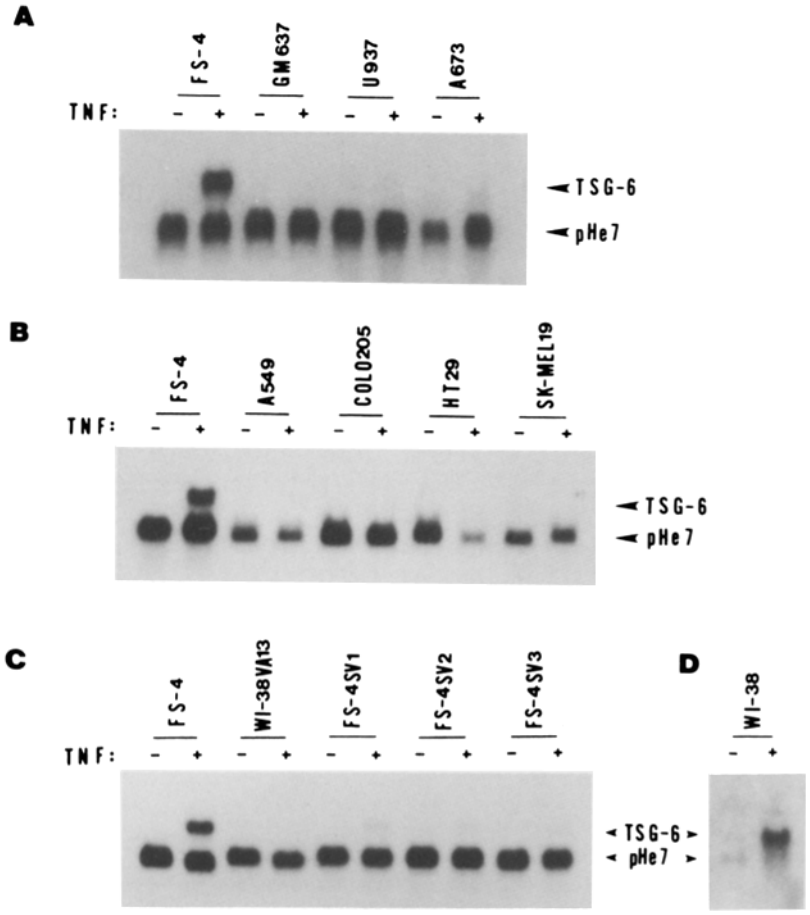

Figure 1. Northern blot analysis of TSG-6 mRNA expression in various types of cells. Cultures were incubated for 4 h with control medium or TNF (20 ng/ml). Total RNA was isolated and fractionated on 1% formaldehyde-agarose gels, transferred to membranes, and hybridized with ^{32}P-labeled TSG-6 cDNA and with the ^{32}P-labeled pHe7 cDNA insert specific for an invariant ~1.0-kb mRNA species. (*A*) FS-4, human diploid foreskin fibroblasts; GM-637, SV-40 virus-transformed human skin fibroblasts; U937, human macrophage-like histiocytic lymphoma cell line; A673, human rhabdomyosarcoma. (*B*) A549, human lung carcinoma; Colo-205, human colon adenocarcinoma; HT29, human colon adenocarcinoma; SK-MEL19, human cutaneous malignant melanoma. (*C*) WI-38 VA13, SV-40 virus-transformed human fetal lung fibroblasts; FS-4 SV1, FS-4 SV2, and FS-4 SV3 are FS-4 lines immortalized by transfection of SV-40 large T antigen (Wolchok J., G. Pecoraro, and J. Vilček, unpublished results). (*D*) WI-38, human diploid fetal lung fibroblasts.

0.5 M NaCl and a buffer containing 0.1 M $NaHCO_3$, pH 8.3, 0.5 M NaCl, and finally suspended and stored in TBS (20 mM Tris-HCl, pH 7.5, 0.5 M NaCl). For affinity purification of antiserum, 0.5 ml of MS2/TSG-6 Sepharose beads, equilibrated with TBS containing 0.05% Tween-20 (TTBS), was mixed and incubated with 1 ml of antiserum at 4°C overnight under constant rotation. The reaction mixture was then washed three times with 3 ml TTBS. To elute bound antibody specific for the TSG-6 protein, 1 ml of 0.1 M glycine-HCl buffer, pH 2.5, was added to the beads, and the supernatant was collected and immediately neutralized with solid Tris.

Stable Transfection of TSG-6 cDNA into GM-637 Cells

An expression plasmid pSV-TSG-6 was constructed by replacing the β-tubulin isotype, Mβ1 coding region in the plasmid pSVβ1 (Lewis et al., 1987) with the full-length TSG-6 cDNA. To exploit suitable restriction enzyme sites for easier cloning we used the M13mp18 vector carrying the full-length TSG-6 cDNA at the EcoRI site, in either the sense or antisense orientation with respect to *lac* promoter (P_{lac}). A HindIII-NcoI fragment containing the 5′ region of TSG-6 cDNA was isolated from the antisense construct and a NcoI-KpnI fragment containing the 3′ region of TSG-6 cDNA was isolated from the sense construct. Both fragments were ligated into the HindIII/KpnI cleaved plasmid pSVβ1. The resulting expression construct (pSV-TSG-6) was used to cotransfect the GM-637 cell line with pRSVneo (Gorman et al., 1983), a plasmid that contains the bacterial neomycin resistance gene linked to RSV LTR, by $CaPO_4$-DNA precipitation (Graham and Van der Eb, 1973). The transfected cells were given a 30-s glycerol shock by treatment with 15% glycerol, and further incubated for 16 h. Thereafter, the cell monolayers were replated in medium containing G418 (800 μg/ml) to select for cells expressing the neomycin resistance marker. Colonies were isolated from transfection plates with the aid of cloning rings, subcloned in 24-well plates, and expanded to monolayer cultures. Multiple independent transfectants were selected and tested for the expression of TSG-6 mRNA by Northern blot analysis. The GSV-L5 line, found to express the highest level of TSG-6 mRNA, was used in the experiments.

Partial Enrichment of TSG-6 Protein by Affinity Chromatography on Sepharose-bound HA

Sepharose-bound HA was prepared essentially as described by Tengblad (1979), except that the water-soluble coupling agent EDC was used in judicious quantities to prevent excess modification of the carboxylic groups in HA. We used 10 μmol EDC/100 mg HA per ml of EAH-Sepharose, so that ~1 out of 25 carboxylic groups in HA would be coupled to the amino groups on the beads. For the enrichment of the TSG-6 protein, 300 ml of the conditioned medium from GSV-L5 cultures containing 10% serum was adjusted to pH 5.0 by adding 3 M sodium acetate (pH 4.6) and then applied onto an HA-Sepharose column (2 ml bed volume) which had been equilibrated with PBS containing 100 mM sodium acetate, pH 5.0 (APBS). Nonspecifically bound material was eluted with 15 ml APBS. Specifically bound proteins were eluted with APBS containing 2 M NaCl plus 4 M guanidine-HCl, dialyzed against PBS, and then stored frozen until used.

Coprecipitation of TSG-6 Protein with Glycosaminoglycans by Cetylpyridinium Chloride

To analyze the binding of TSG-6 protein to HA and other glycosaminoglycans, TSG-6 protein–glycosaminoglycan complexes were precipitated by cetylpyridinium chloride (CPC). 25-μl aliquots of the TSG-6 protein partially enriched on HA-Sepharose were mixed with 40 μg (in a final volume of 120 μl in PBS) of: HA (H4015; Sigma Chemical Co.), chondroitin 4,6-sulfate (C8529; Sigma Chemical Co.), dermatan sulfate (C4259; Sigma Chemical Co.), heparan sulfate (H7640; Sigma Chemical Co.), heparin

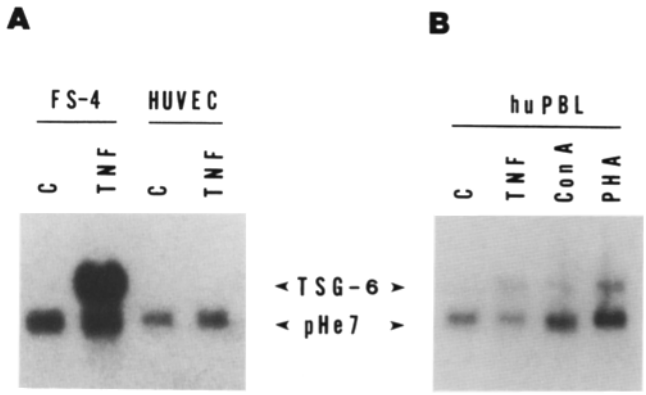

Figure 2. Northern blot analysis of TSG-6 mRNA expression in HUVEC and mononuclear cells isolated from huPBL. Total RNA was isolated and subjected to Northern blot analysis as in Fig. 1. (*A*) FS-4 cells and HUVEC were incubated for 4 h with control medium (*C*) or TNF (20 μg/ml). (*B*) HuPBL were incubated for 16 h with control medium (*C*) or TNF (20 ng/ml), Con A (5 μg/ml), or PHA (2 μg/ml).

(H3125; Sigma Chemical Co.), or dextran sulfate (17-0340-01; Pharmacia Fine Chemicals). After 1 h incubation at 37°C, 50 μl of 2.5% CPC was added to the samples and the mixtures were stirred and then incubated for another 1 h at 37°C. After incubation, the mixtures were centrifuged for 15 min in a centrifuge (Eppendorf Inc., Fremont, CA) at maximum speed. The pellets were carefully rinsed twice with 1% CPC containing 20 mM NaCl. The pellets were then dissolved in 100 μl SDS-PAGE sample buffer (60 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 7% β-mercaptoethanol, 0.001% bromphenol blue). The supernatants were also collected and processed for electrophoresis. After fractionation on SDS-PAGE (12% polyacrylamide), TSG-6 protein present in the pellets and supernatants was visualized by Western analysis with the aid of affinity-purified anti-TSG-6 antibody.

Results

Expression of TSG-6 mRNA in Different Types of Cells

TSG-6 cDNA was originally isolated from a cDNA library prepared from TNF-treated cultures of normal human diploid FS-4 foreskin fibroblasts (Lee et al., 1990). To determine what other cell types express TSG-6 message, we examined a large number of cell lines for the presence of TSG-6 mRNA by Northern blot analysis (Fig. 1). In each of the cell lines RNA from both untreated and TNF-treated cultures was examined. TSG-6 mRNA was not detectable in any of the cell lines without TNF treatment. TSG-6 mRNA became readily detectable after TNF treatment in FS-4 cells (Fig. 1, *A–C*) and in the WI-38 line of normal human fetal lung fibroblasts (Fig. 1 *D*). TNF treatment produced a similar increase in steady-state TSG-6 mRNA levels in two other normal human foreskin fibroblast lines, designated FS-48 and FS-49 (data not shown). However, in none of the tumor-derived cell lines (U937, A673, A549, Colo205, HT29, and SK-MEL19) or SV-40-transformed fibroblast lines (GM-637 and WI-38VA13) was TSG-6 mRNA detectable after TNF treatment (Fig. 1, *A–C*). We also examined the inducibility of TSG-6 mRNA in cloned lines derived from the FS-4 cell line after transfection with SV-40 large T antigen. Interestingly, these cell lines, which display altered growth characteristics but do not appear to be fully transformed, showed much lower levels of TSG-6 mRNA after TNF treatment than the parental FS-4 cells (Fig. 1 *C*). The lack of TSG-6 mRNA induction seen in the fully transformed cells and the decreased inducibility in the SV-40 T antigen–transfected FS-4 lines were not the result of a general lack of responsiveness of these cells to TNF. For example, TSG-14 mRNA (Lee et al., 1990) was more strongly inducible by TNF in the SV-40 T antigen–transfected FS-4 lines than in the original FS-4 cells (data not shown). Hence, it appears that induction of TSG-6 mRNA is suppressed in transformed cells.

We also examined the inducibility of TSG-6 mRNA by TNF in normal HUVEC. The latter cells are known to be highly responsive to TNF and several genes inducible by TNF in normal fibroblasts were also inducible in HUVEC cultures (Collins et al., 1986; Holzman et al., 1990). We were therefore surprised to find that TNF treatment failed to induce the appearance of TSG-6 mRNA in these cells (Fig. 2 *A*). However, we were able to demonstrate an increase in TSG-6 mRNA levels in cultures of freshly isolated peripheral blood human mononuclear cells after treatment with TNF or after treatment with the T cell mitogens Con A or phytohemagglutinin (Fig. 2 *B*). Although the cell type producing TSG-6 mRNA in the heterogeneous cultures of mononuclear cells has not been identified, the latter results show that induction of TSG-6 mRNAs is not restricted to fibroblasts.

TSG-6 cDNA Codes for a Putative Protein with Homology to Lymphocyte Homing Receptor CD44 and the Cartilage Link Protein Family

Full-length TSG-6 cDNA was found to be 1,414 bases long, apparently consisting of a 68-base 5′-untranslated region, a continuous open reading frame of 831 bases, and a 3′ AT-rich untranslated region (~75% A+T content) (Fig. 3 *A*). Within the 3′-untranslated region are multiple mRNA destabilization consensus sequence motifs AUUUA (Shaw and Kamen, 1986), which may account for the decline in the TSG-6 mRNA level seen after 4 h or more of continuous treatment with TNF (Lee et al., 1990). A consensus polyadenylation signal (AATAAA) is also located at the 3′ end. The largest open reading frame predicts a polypeptide of 277 amino acids with an estimated molecular weight of 31,200. No other open reading frame with a significant length was found. The putative initiation methionine is followed by 11 consecutive hydrophobic amino acids, followed by a charged region, suggesting that this portion might form a cleavable signal peptide (Fig. 3 *B*). Following the (-3,-1) rule (von Heijne, 1984), the putative signal sequence cleavage site will be located after Gly^{19}. This would result in a mature form of the protein with a predicted molecular weight of 28,895 and a calculated pI of 7.0. In addition, the predicted TSG-6 protein sequence contains two potential sites of N-linked glycosylation (Asn^{118}-Arg-Ser and Asn^{258}-Thr-Ser). The sequence Ser^{44}-Gly preceded by an acidic amino acid (Glu^{41}) is indicative of a potential chrondroitin sulfate linkage site (Zimmermann and Ruoslahti, 1989). It is also worth noting that there is an aspartic acid–rich region (residue Asp^{179} to Asp^{217}) in addition to a serine- and threonine-rich region at the COOH-terminal end; the latter region includes six consecutive Ser/Thr residues (Thr^{259} to Thr^{264}), which may

A

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-68                                                                ga att cgc   -61
-60  act gct ctg aga att tgt gag cag ccc cta aca ggc tgt tac ttc act aca act gac gat    -1

  1  ATG ATC ATC TTA ATT TAC TTA TTT CTC TTG CTA TGG GAA GAC ACT CAA GGA TGG GGA TTC    60
  1  Met Ile Ile Leu Ile Tyr Leu Phe Leu Leu Leu Trp Glu Asp Thr Gln Gly Trp Gly Phe   20

 61  AAG GAT GGA ATT TTT CAT AAC TCC ATA TGG CTT GAA CGA GCA GCC GGT GTG TAC CAC AGA   120
 21  Lys Asp Gly Ile Phe His Asn Ser Ile Trp Leu Glu Arg Ala Ala Gly Val Tyr His Arg    40

121  GAA GCA CGG TCT GGC AAA TAC AAG CTC ACC TAC GCA GAA GCT AAG GCG GTG TGT GAA TTT   180
 41  Glu Ala Arg Ser Gly Lys Tyr Lys Leu Thr Tyr Ala Glu Ala Lys Ala Val Cys Glu Phe    60
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181  GAA GGC GGC CAT CTC GCA ACT TAC AAG CAG CTA GAG GCA GCC AGA AAA ATT GGA TTT CAT   240
 61  Glu Gly Gly His Leu Ala Thr Tyr Lys Gln Leu Glu Ala Ala Arg Lys Ile Gly Phe His    80

241  GTC TGT GCT GCT GGA TGG ATG GCT AAG GGC AGA GTT GGA TAC CCC ATT GTG AAG CCA GGG   300
 81  Val Cys Ala Ala Gly Trp Met Ala Lys Gly Arg Val Gly Tyr Pro Ile Val Lys Pro Gly   100

301  CCC AAC TGT GGA TTT GGA AAA ACT GGC ATT ATT GAT TAT GGA ATC CGT CTC AAT AGG AGT   360
101  Pro Asn Cys Gly Phe Gly Lys Thr Gly Ile Ile Asp Tyr Gly Ile Arg Leu Asn Arg Ser   120
                                                                 ==========

361  GAA AGA TGG GAT GCC TAT TGC TAC AAC CCA CAC GCA AAG GAG TGT GGT GGC GTC TTT ACA   420
121  Glu Arg Trp Asp Ala Tyr Cys Tyr Asn Pro His Ala Lys Glu Cys Gly Gly Val Phe Thr   140

421  GAT CCA AAG CGA ATT TTT AAA TCT CCA GGC TTC CCA AAT GAG TAC GAA GAT AAC CAA ATC   480
141  Asp Pro Lys Arg Ile Phe Lys Ser Pro Gly Phe Pro Asn Glu Tyr Glu Asp Asn Gln Ile   160

481  TGC TAC TGG CAC ATT AGA CTC AAG TAT GGT CAG CGT ATT CAC CTG AGT TTT TTA GAT TTT   540
161  Cys Tyr Trp His Ile Arg Leu Lys Tyr Gly Gln Arg Ile His Leu Ser Phe Leu Asp Phe   180

541  GAC CTT GAA GAT GAC CCA GGT TGC TTG GCT GAT TAT GTT GAA ATA TAT GAC AGT TAC GAT   600
181  Asp Leu Glu Asp Asp Pro Gly Cys Leu Ala Asp Tyr Val Glu Ile Tyr Asp Ser Tyr Asp   200

601  GAT GTC CAT GGC TTT GTG GGA AGA TAC TGT GGA GAT GAG CTT CCA GAT GAC ATC ATC AGT   660
201  Asp Val His Gly Phe Val Gly Arg Tyr Cys Gly Asp Glu Leu Pro Asp Asp Ile Ile Ser   220

661  ACA GGA AAT GTC ATG ACC TTG AAG TTT CTA AGT GAT GCT TCA GTG ACA GCT GGA GGT TTC   720
221  Thr Gly Asn Val Met Thr Leu Lys Phe Leu Ser Asp Ala Ser Val Thr Ala Gly Gly Phe   240

721  CAA ATC AAA TAT GTT GCA ATG GAT CCT GTA TCC AAA TCC AGT CAA GGA AAA AAT ACA AGT   780
241  Gln Ile Lys Tyr Val Ala Met Asp Pro Val Ser Lys Ser Ser Gln Gly Lys Asn Thr Ser
                                                                 ==========

781  ACT ACT TCT ACT GGA AAT AAA AAC TTT TTA GCT GGA AGA TTT AGC CAC TTA taa aaa aaa   840
261  Thr Thr Ser Thr Gly Asn Lys Asn Phe Leu Ala Gly Arg Phe Ser His Leu              277

841  aaa agg atg atc aaa aca cac agt gtt tat gtt gga atc ttt tgg aac tcc ttt gat ctc   900

901  act gtt att att aac att tat tta tta ttt ttc taa atg tga aag caa tac ata att tag   960

1021 gca tag aaa taa caa gcg tta aca ttt tca tat ttt ttt ctt tca gtc att ttt gta ttt  1080

1081 gtg gta tat gta tat atg tac cta tat gta ttt gca ttt gaa att ttg gaa tcc tgc tct  1140

1141 atg tac agt ttt gta tta tac ttt tta aat ctt gaa ctt tat gaa cat ttt ctg aaa tca  1120

1201 ttg att att cta caa aaa cat gat ttt aaa cag ctg taa aat att cta tga tat gaa tgt  1260

1261 ttt atg cat tat tta agc ctg tct cta ttg ttg gaa ttt cag gtc att ttc ata aat att  1120

1321 gtt gca ata aat atc ctt cgg aat tc                                              1346
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B

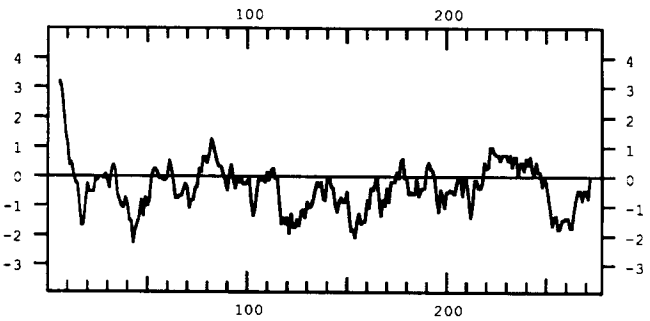


Figure 3. Nucleotide sequence of TSG-6 cDNA and deduced amino acid sequence. (*A*) Nucleotide and amino acid residues are numbered from the first methionine of the major open reading frame. The putative signal sequence is underlined (*thick line*). Potential glycosylation sites for N-linked glycans are shown by double broken lines. Potential chondroitin sulfate linkage site and consensus sequence are shown by star with single broken line. Also underscored are the mRNA decay consensus sequence motifs ATTTA (*thin line*) and the polyadenylation signal (AAAA). These sequence data are available from EMBL/GenBank/DDBJ under accession number M31165. (*B*) Hydropathic profile of the deduced amino acid sequence of TSG-6 using the algorithm of Kyte and Doolittle (1982).

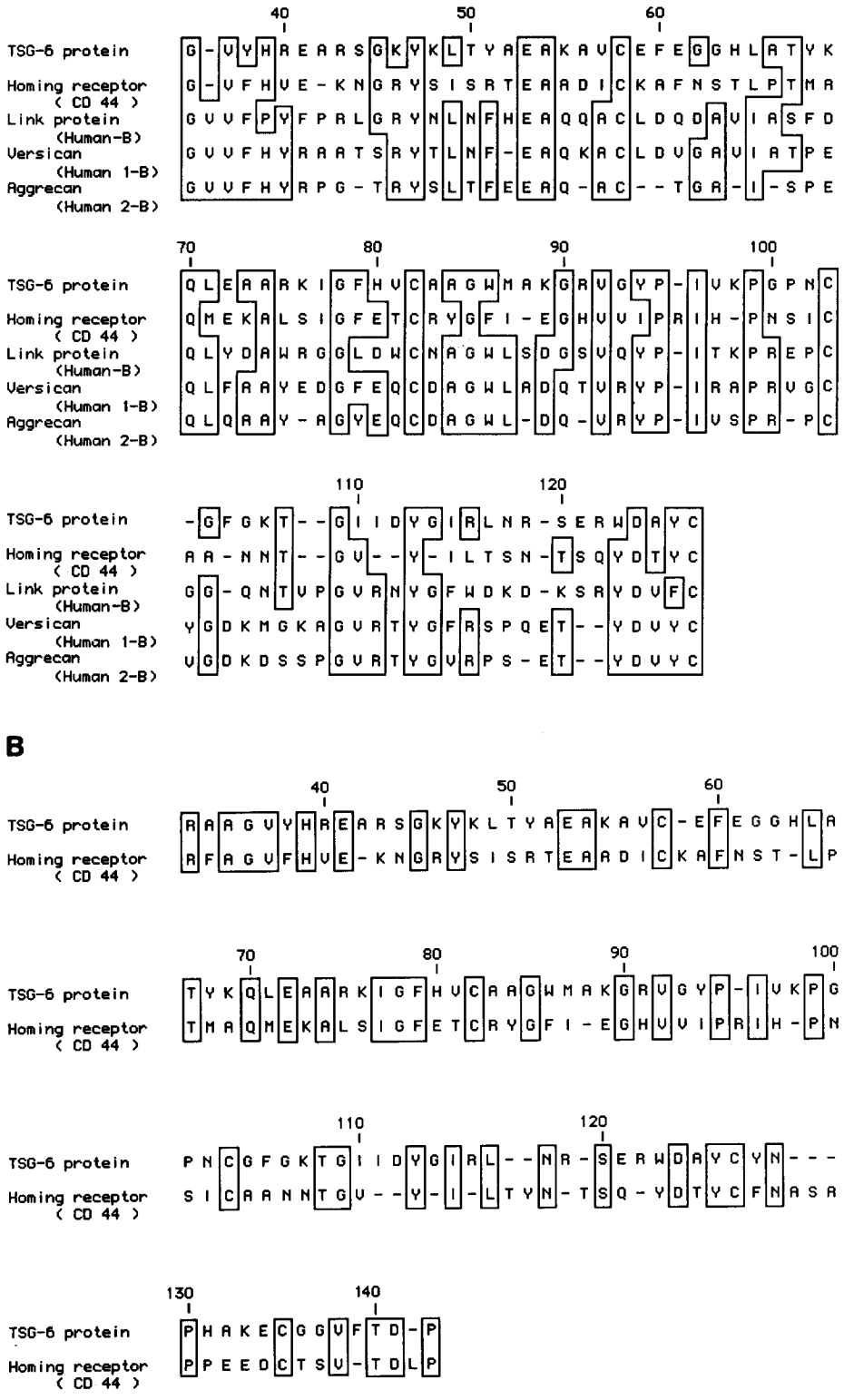


Figure 4. Amino acid sequence comparison of the TSG-6 protein with other known proteins. (*A*) A portion of the NH_2-terminal half of TSG-6 protein (Gly^{36} to Cys^{127}) was aligned with the 2-B domain (Gly^{158} to Cys^{252}) of human link protein (Doege et al., 1990*a*) using a combination of the BestFit and FASTA programs available from the Genetics Computer Group of the University of Wisconsin, Madison, WI (Devereux et al., 1984). The other sequences, which were previously shown to share a common domain, were aligned with the TSG-6 protein sequence manually, based on earlier alignments of CD44 (Gly^{32} to Cys^{118}) (Goldstein et al., 1989; Stamenkovic et al., 1989), versican (Gly^{149} to Cys^{118}) (Zimmermann and Ruoslahti, 1989), and aggrecan (Gly^{478} to Cys^{571}) (Doege et al., 1990*b*). Residues common to at least three sequences were boxed. (*B*) A portion of the NH_2-terminal sequence of TSG-6 protein (Arg^{33} to Pro^{142}) was aligned with a region (Arg^{29} to Pro^{136}) of CD44 using the BestFit program (gap weight, 0.5; length weight, 0.1). Note that the alignment is slightly different from the alignment shown in *A* in which homologies among all members of the family were highlighted. (*C*) A portion of the COOH-terminal sequence of TSG-6 protein (Gly^{136} to Phe^{240}) was aligned with a portion (Gly^{16} to Phe^{116}) of the sequence of complement C1r (Leytus et al., 1986) using the FASTA program.

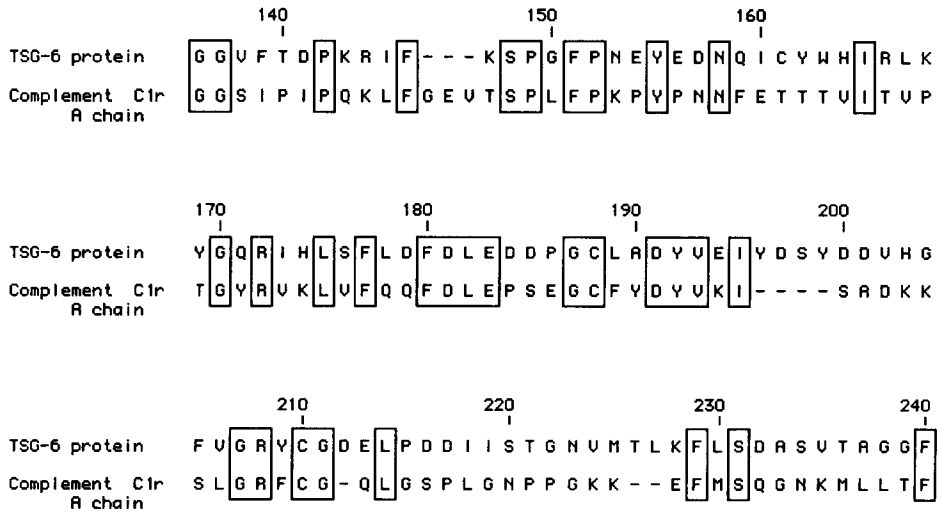


Figure 4.

serve as acceptors for O-linked carbohydrates (Tomita et al., 1978).

Comparison of the amino acid sequence deduced from the TSG-6 cDNA with the protein sequences available in databases revealed interesting homologies (Fig. 4). The NH_2-terminal half of the putative TSG-6 gene product shows a high degree of homology with the human lymphocyte homing receptor/HA receptor CD44 (Goldstein et al., 1989; Stamenkovic et al., 1989), human cartilage link protein (Dudhia and Hardingham, 1989; Doege et al., 1990*a*), and the human proteoglycan core proteins versican (Zimmermann and Ruoslahti, 1989) and aggrecan (Doege et al., 1990*b*), with the positions of four cysteine residues conserved in all of these proteins (Fig. 4 *A*). Based on the alignment shown in Fig. 4 *A*, this portion of the predicted TSG-6 protein (residues 36–127) shows ~37% identity with CD44, 36% identity with cartilage link protein, and 40% identity with the proteoglycan core proteins versican and aggrecan. The highest degree of homology (~60%) exists between a limited region (Cys^{81} to Pro^{99}) of the TSG-6 cDNA-encoded protein and a corresponding region of link protein and proteoglycan core proteins. The conserved basic amino acids in this region have been implicated in an ionic-type interaction with the negatively charged uronic acid residues in HA (Hardingham et al., 1976; Lyon, 1986; Goetinck et al., 1987). No significant sequence homology was found between the TSG-6 protein and most other members of this family upstream of Gly^{36} or downstream of Cys^{127} in TSG-6. However, sequence homology between the TSG-6 and CD44 proteins extends over wider portions of the two molecules. An alignment of residues 33–142 of TSG-6 with a homologous portion of CD44 reveals 37% identity (Fig. 4 *B*). In addition, the COOH-terminal half of TSG-6 (Fig. 4 *C*) shows ~30% sequence homology with the α-fragment of complement C1r A chain (Leytus et al., 1986).

The predicted secondary sequence of the TSG-6 protein (Fig. 5) reveals that the region homologous to CD44, link protein, and the proteoglycan core proteins forms an extended loop involving two disulfide bonds, conserved in all of these proteins. This loop structure was shown to form the HA binding domain of link protein (Goetinck et al., 1987), and is also thought to be responsible for HA recognition in the other proteins in this family (Perin et al., 1987; Culty et al., 1990).

TSG-6 Is a Secretory Protein

As a first step toward the characterization of the protein coded for by the TSG-6 cDNA, we immunized rabbits with an *E. coli*-derived fusion protein composed of portions of the *E. coli* TrpE and TSG-6 proteins. The resulting antibody was purified by adsorption and elution on a Sepharose column to which a MS2/TSG-6 fusion protein was coupled (see Materials and Methods). We also constructed a constitutive expression plasmid pSV-TSG-6 containing the complete TSG-6 coding region (see Materials and Methods). This expression construct was used to transfect the GM-637 cell line, together with the pRSVneo plasmid containing the bacterial neomycin resistance gene. The GM-637 line was earlier shown not to express detectable endogenous TSG-6 mRNA (see Fig. 1 *A*). After clonal isolation, the GSV-L5 line that constitutively expressed a high level of TSG-6 mRNA was chosen for the analysis of the presence of TSG-6 protein.

Fig. 6 A (*top*) shows that no immunoreactive band was detected by Western blot analysis of a concentrated culture supernatant of GM-637 cells transfected with plasmid pRSVneo alone (marked "GN4"). In contrast, two immunoreactive bands were present in the supernatant of the GSV-L5 line, transfected with the TSG-6 cDNA-containing expression vector. The faster migrating band, with an apparent molecular mass of 39 kD on SDS-PAGE, may represent the glycosylated form of the TSG-6 protein. (The molecular mass of the mature TSG-6 protein based on the primary sequence is ~29 kD.) In addition, a slower migrating immunoreactive band (>110 kD) was detected.

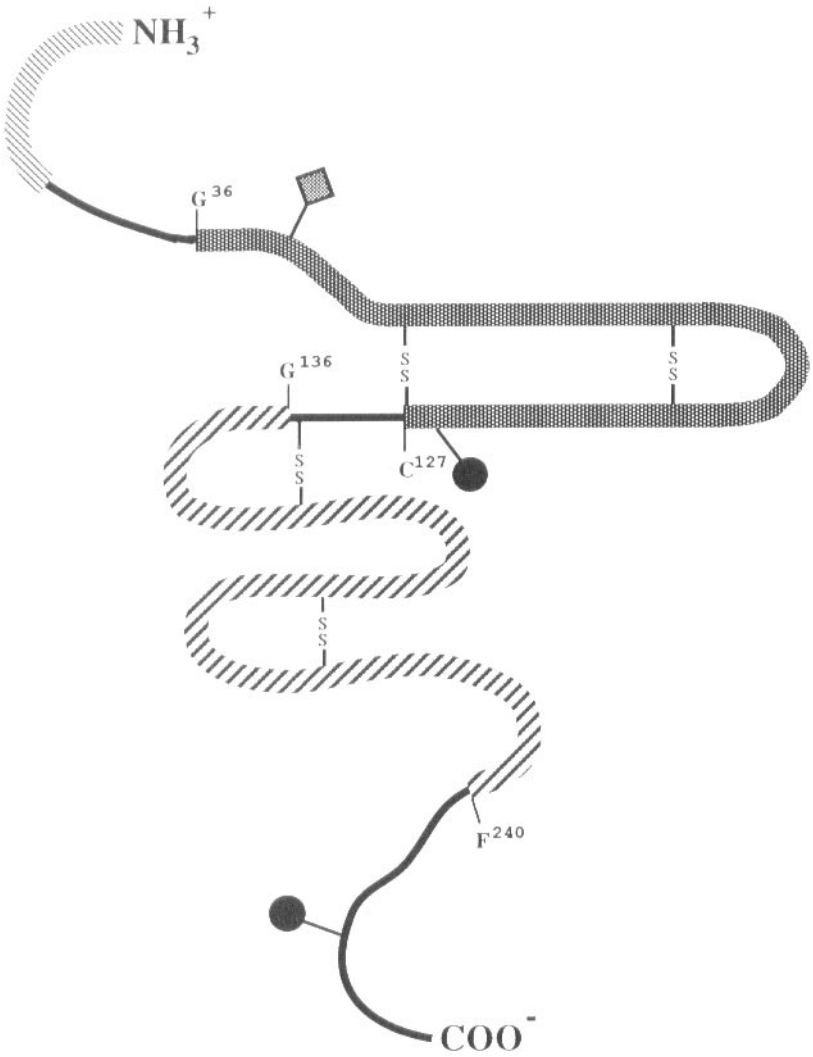


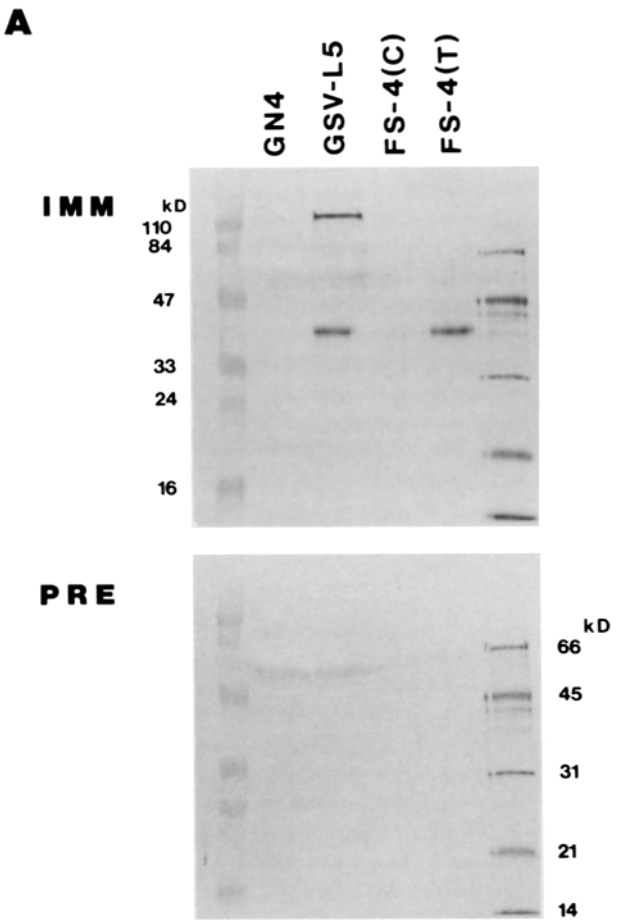
Figure 5. Schematic diagram of the putative secondary structure of TSG-6 protein. The predicted signal peptide, the domain Gly^{36} to Cys^{127} showing homology with the HA binding regions of CD44, link proteins, and proteoglycan core proteins, and the domain Gly^{136} to Phe^{240} showing homology to complement C1r α fragment are highlighted by cross-hatching. Also depicted are two potential N-glycosylation sites (*ball and stick*) and a potential chondroitin sulfate linkage site (*square box and stick*). Disulfide linkages (Cys^{58}–Cys^{127} and Cys^{82}–Cys^{103}) are based on the known disulfide bonds in the HA binding domains of link protein and other proteoglycan core proteins. Disulfide linkage (Cys^{188}–Cys^{210}) is based on the known disulfide bond in complement C1r. Predicted disulfide bond formation between Cys^{135} and Cys^{161} is hypothetical because no cysteine residue corresponding to Cys^{161} in the TSG-6 protein is present in complement C1r.

To determine whether the TSG-6 protein was also detectable in the FS-4 fibroblast line, cultures were either stimulated with 20 ng/ml TNF or left untreated. After 24 h the medium was collected and concentrated ~100-fold in an Amicon apparatus (Amicon, Beverly, MA). Samples were subjected to Western blot analysis, side-by-side with the samples of cultures transfected with the TSG-6 expression vector (Fig. 6 *A*, *top*). A major 39-kD band and a minor >110-kD band were detected by the affinity-purified antiserum in the culture supernatant of TNF-treated FS-4 cells (lane marked FS-4 (T)), but not in control FS-4 cultures (marked FS-4 (C)). The two bands seen in the supernatant of TNF-stimulated FS-4 cells were identical to the bands detected in the medium of GSV-L5 cultures. As also seen with GSV-L5 cells (cf. Fig. 6, *A* and *B*), the proportion of the 39-kD and >110-kD bands detected in the media from TNF-treated FS-4 cells varied from one experiment to another (not shown). No bands were detected in either GSV-L5 cultures or TNF-stimulated FS-4 cells when identically processed preimmune serum from the same rabbit was used in Western blot analysis (Fig. 6 *A*, *bottom*). Taken together, these results indicate that both the 39-kD and the >110-kD bands detected by the immune serum represent products of the TSG-6 gene. Subsequent analysis indicated that the apparent molecular mass of the >110-kD band is ~120 kD (see Fig. 7), suggesting that it may represent either an undissociated trimeric form of TSG-6 or a complex of TSG-6 with another protein. Since SDS-PAGE separations were run under reducing conditions (7% β-mercaptoethanol), it is unlikely that intermolecular disulfide linkage accounted for the formation of the 120-kD form.

While a 39-kD immunoreactive band was readily detectable in the concentrated culture medium of GSV-L5 cells, a cell extract from the same cultures failed to reveal the presence of TSG-6 protein (Fig. 6 *B*). Similarly, we were unable to detect the TSG-6 protein in extracts of TNF-treated FS-4 cultures (not shown). These data suggest that, once synthesized, TSG-6 protein is rapidly secreted into the culture medium. These findings are in agreement with the presence of a cleavable signal peptide, predicted from the deduced amino acid sequence (Fig. 3, *A* and *B*).

TSG-6 Is a Glycoprotein

Inasmuch as the deduced amino acid sequence of the TSG-6 protein predicts two potential N-glycosylation sites, the presence of N-linked carbohydrate was analyzed by *N*-glycosi-



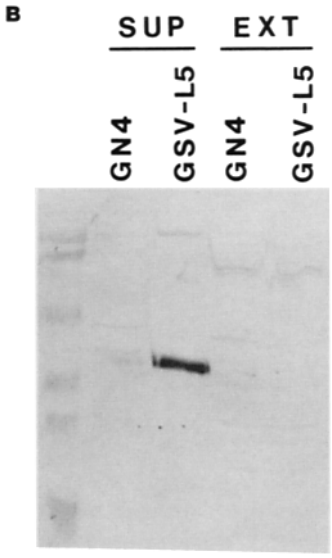


Figure 6. Detection of TSG-6 protein in cultures of TNF-treated FS-4 cells and in GM-637 cells transfected with TSG-6 cDNA (GSV-L5) by Western blot analysis. (*A*) Serum-free medium collected from TNF-treated FS-4 cells or TSG-6 cDNA-transfected GSV-L5 cells was concentrated ~100-fold. The samples were analyzed by SDS-PAGE (12% polyacrylamide). In the upper panel (*IMM*), bands were developed with the aid of anti-TSG-6 antibody purified by affinity chromatography as described in Materials and Methods. In the lower panel (*PRE*), bands were developed with similarly processed pre-immune serum from the same rabbit. GN4, supernatant of GM-637 cells transfected with pRSVneo; GSV-L5, supernatant of GM-637 cells transfected with pSV-TSG-6; FS-4 (C), supernatant of untreated FS-4 cells; FS-4 (T), supernatant of FS-4 cells treated for 24 h with TNF (20 ng/ml). Molecular weight standards are indicated to the left of the top panel and to the right of the bottom panel. (*B*) Western blot analysis showing that TSG-6 protein is detectable in the culture medium of GSV-L5 cells, but not in cell lysates. Serum-free medium collected from cultures of GN4 cells or GSV-L5 cells was concentrated ~100-fold in an Amicon apparatus (PM10 membrane). To prepare lysates, cells from a 175-cm^2 flask were treated with ~0.5 ml SDS-PAGE sample buffer (60 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 7% β-mercaptoethanol, and 0.001% bromphenol blue). The samples were then subjected to Western blot analysis with the aid of affinity-purified anti-TSG-6 antibody. Sup, supernatants of GN4 or GSV-L5 cultures, concentrated ~100-fold; Ext, lysates of GN4 or GSV-L5 cells.

dase F treatment. TSG-6 protein from GSV-L5 cell culture supernatants was first partially purified by affinity chromatography (see Materials and Methods) and then treated with 16.7 U/ml *N*-glycosidase F at 37°C for different times. A 3-h incubation with the enzyme resulted in the disappearance of the 39-kD band and the appearance of two bands with apparent molecular masses of 36 and 33 kD, respectively (Fig. 7). After overnight treatment with 16.7 U/ml *N*-glycosidase F the 36-kD band was no longer detectable and all of the 39-kD form was apparently converted into the 33-kD band (not shown). These data suggest that the 36-kD band corresponds to a partially deglycosylated form, and the 33-kD band to a monomeric form of TSG-6 protein completely devoid of N-linked carbohydrate. These results are consistent with the predicted presence of two N-glycosylation consensus sequences in the TSG-6 protein (Figs. 3 *A* and 5). Glycosidase treatment also resulted in a decrease of the higher molecular weight band recognized by antibodies to the TSG-6 protein, from an apparent molecular mass of ~120 kD to ~110 kD (Fig. 7).

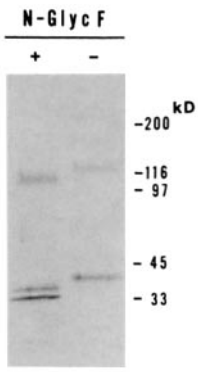


Figure 7. Reduction in the apparent molecular size of TSG-6 protein after *N*-glycosidase F treatment. TSG-6 protein, partially purified by affinity chromatography (see Materials and Methods), was incubated with 16.7 U/ml *N*-glycosidase F (*left lane*) or without enzyme (*right lane*) at 37°C for 3 h. Western blot analysis was performed after SDS-PAGE on a 4–15% polyacrylamide gradient gel.

In view of the presence of a potential chondroitin sulfate linkage site, as noted above (Figs. 3 *A* and 5), we also examined the effect of chondroitin sulfate ABC lyase (chondroitinase ABC) on the apparent molecular size of TSG-6 protein produced in GSV-L5 cultures. The latter treatment resulted in no demonstrable change in the migration pattern of the 120- or the 39-kD band recognized by the antibody to TSG-6 (data not shown), suggesting the absence of a linked chondroitin sulfate moiety.

TSG-6 Is a Hyaluronate Binding Protein

In view of the high degree of homology with the domain responsible for HA binding in CD44 and the cartilage link

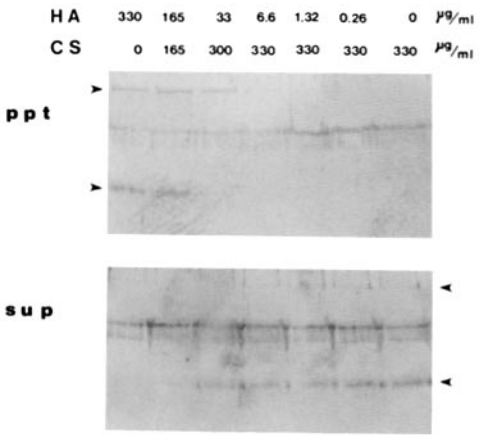


Figure 8. Effect of varying concentrations of hyaluronate on the coprecipitation of TSG-6 protein by CPC. A constant amount of TSG-6 protein partially enriched by affinity chromatography on HA coupled to Sepharose was mixed with varying concentrations of HA for 1 h and processed as described in Materials and Methods. Constant glycosaminoglycan concentration (330 μg/ml) was maintained by adding varying amounts of chondroitin sulfate as a carrier. Western analysis was performed to visualize TSG-6 protein bound to HA in the precipitate (*ppt*) as well as unbound TSG-6 protein in the supernatant (*sup*). The 120- and 39-kD bands of the TSG-6 protein are marked with arrows.

protein family (Figs. 4 *A* and 5), it seemed plausible that the TSG-6 protein too is an HA binding protein. To test this notion we first determined whether TSG-6 protein would bind to HA immobilized on Sepharose beads. It has been known that HA binding proteins, such as proteoglycan core protein and cartilage link protein, can be purified by affinity chromatography on immobilized HA (Tengblad, 1979). Since HA is a linear polysaccharide built from repeating disaccharide units consisting of D-glucuronic acid and *N*-acetyl-D-glucosamine, every carboxylic group of D-glucuronic acid unit in HA can be potentially modified by the coupling agent EDC and immobilized to the amino group of EAH-Sepharose. To prevent complete modification of the carboxylic groups in HA we used a subeffective concentration of EDC (10 μmol/100 mg of HA), so that ~1 out of every 25 carboxylic groups would be modified and coupled to the functional amino groups of EAH-Sepharose. Using this approach, we were able to effectively enrich the TSG-6 protein from the conditioned medium of GSV-L5 cultures by column affinity chromatography on EAH-Sepharose coupled with HA (HA-Sepharose). In addition, we were able to partially block the binding of TSG-6 protein to HA-Sepharose by adding soluble HA, but not by soluble chondroitin sulfate, when the two glycosaminoglycans were used at the same concentration of 1 mg/ml (data not shown).

To address more directly the specific interaction between TSG-6 protein and HA we attempted to coprecipitate TSG-6 protein with HA and other glycosaminoglycans (chondroitin 4,5-sulfate, dermatan sulfate, heparan sulfate, and heparin). It has been shown that glycosaminoglycans can be precipitated by cationic detergents such as CPC as a result of bulky salt formation between the two molecules (Scott, 1961). We reasoned that if the TSG-6 protein interacts with HA through specific recognition of sugar residues as well as through an ionic interaction, then the TSG-6 protein could be coprecipitated more efficiently with HA than with other glycosaminoglycans. This prediction was essentially borne out. TSG-6 protein from a culture supernatant of GSV-L5 cultures was enriched by affinity chromatography on HA-Sepharose, as described in Materials and Methods. A constant amount of the enriched TSG-6 protein was incubated with varying concentrations of HA and/or chondroitin sulfate (Fig. 8). CPC was then added to all samples and the resulting precipitates were sedimented by centrifugation. The pellets as well as the supernatants were then analyzed for the presence of TSG-6 protein by Western blotting. HA produced a concentration-dependent coprecipitation of the 120- and 39-kD bands of TSG-6. Addition of chondroitin sulfate caused no coprecipitation, nor did the presence of chondroitin sulfate affect coprecipitation with HA. Since chondroitin sulfate carries a stronger negative charge than HA, this result suggests that the binding of TSG-6 to HA is not due solely to a simple ionic interaction.

Similar coprecipitation experiments were also carried out with TSG-6 and dermatan sulfate, heparan sulfate, and heparin (not shown). When used at concentrations up to 2.5 mg/ml, dermatan sulfate (like chondroitin sulfate) produced no detectable coprecipitation of the TSG-6 protein (not shown). Heparan sulfate and heparin, though clearly less effective than HA when used at the same concentrations, at higher concentrations produced a partial coprecipitation of the 120-kD band with little or no coprecipitation of the 39-kD band of the TSG-6 protein. Taken together, these results suggest that interaction of TSG-6 protein with HA involves a recognition of sugar residues specific to HA, perhaps together with an ionic-type interaction.

Discussion

TNF-α and the functionally related cytokines IL-1α and IL-1β play major roles in the inflammatory processes (Balkwill, 1989). Although many different cell types can be induced to produce TNF-α, IL-1α, and IL-1β, in the intact organism their main sources appear to be monocytes and macrophages (Le and Vilček, 1987; Dinarello, 1988; Vilček and Lee, 1991). All three cytokines are characteristically produced at sites of inflammation; they play beneficial roles as immunoregulators, mediators of host resistance, and promoters of tissue repair. On the other hand, overproduction of TNF or IL-1 during inflammation can lead to tissue damage and systemic toxicity (Balkwill, 1989). Earlier we reported the isolation of eight distinct cDNAs from a cDNA library prepared from TNF-α-treated normal human FS-4 foreskin fibroblasts (Lee et al., 1990). All eight cDNAs correspond to mRNAs whose levels are either undetectable or very low in untreated FS-4 cells, and are significantly increased upon the exposure of cells to either TNF or IL-1. Partial sequencing of these cDNAs revealed that four of them represent known genes, namely, IL-8 (a cytokine whose major role is thought to be chemotactic activity for neutrophils and T cells), monocyte chemotactic and activating factor (a cytokine structurally related to IL-8), and the metalloproteinases collagenase and stromelysin, respectively (Lee et al., 1990). Subsequently, a fifth cDNA (TSG-37) was found to be identical to metallothionein II (Lee, T. H., and G. W. Lee, unpublished data). The three remaining cDNAs could

not be matched with DNA sequences available in databanks, suggesting that they correspond to novel genes.

In this paper we describe the characterization of one of the novel cDNAs, termed TSG-6. The largest open reading frame predicted a polypeptide of 277 amino acids, including a characteristic hydrophobic signal peptide sequence (Fig. 3). The putative amino acid sequence deduced from the TSG-6 cDNA revealed a significant degree of homology with a conserved region present in a recently identified family of proteins (Fig. 4) that includes the HA receptor/lymphocyte homing receptor CD44 (Goldstein et al., 1989; Stamenkovic et al., 1989), cartilage link protein (Dudhia and Hardingham, 1989; Doege et al., 1990*a*), and the proteoglycan core proteins versican (Zimmermann and Ruoslahti, 1989) and aggrecan (Doege et al., 1990*b*). The homologous region, thought to comprise the HA binding domain (Goetinck et al., 1987; Perin et al., 1987; Culty et al., 1990), is tandemly repeated in the cartilage link protein and proteoglycan core proteins, while being represented only once in CD44 (Goldstein et al., 1989; Stamenkovic et al., 1989) as well as in TSG-6 (Figs. 3 and 5). The degree of sequence homology between TSG-6 and the other human proteins of this family is of a similar magnitude as the homology of the other members of this family to each other. Based on a similar alignment of the sequences of human CD44 with rat and chicken cartilage link proteins (showing a homology of ~30%), Stamenkovic et al. (1989) calculated that the probability of an equal or better match occurring between unrelated proteins was $<10^{-15}$. Interestingly, the homology between TSG-6 and CD44 extended over regions of the two proteins that showed no significant homology with the other members of this protein family (Fig. 4B). This finding suggests a closer evolutionary relationship of TSG-6 to CD44 than to the cartilage link and proteoglycan core proteins. The homologous sequences in the TSG-6 and CD44 proteins may have diverged from the same ancestral gene before duplication of this domain in the proteoglycan core and cartilage link proteins, or they may have been derived from one of these domains (Goldstein et al., 1989).

Antibodies generated against a bacterial fusion protein of TSG-6 were used to demonstrate the presence of TSG-6 protein in the culture medium of cells transfected with a TSG-6 expression vector or in FS-4 fibroblasts treated with TNF (Fig. 6). The 39-kD band defined by the antibodies apparently represents the fully glycosylated mature monomeric form of TSG-6 protein. The generation of two faster migrating bands (36 and 33 kD, respectively) after treatment with *N*-glycosidase F (Fig. 7) is consistent with the presence of two potential N-glycosylation sites in the coding sequence. The apparent molecular weight of TSG-6 protein after prolonged treatment with *N*-glycosidase F (33 kD) is somewhat greater than the predicted molecular weight of 28,895 of the unglycosylated mature polypeptide. This small discrepancy could be due to incomplete deglycosylation (e.g., presence of O-linked carbohydrate) or to slightly anomalous migration characteristics on SDS-PAGE.

A higher molecular weight form with the apparent size of ~120 kD was also detected by the anti-TSG-6 antibody in the culture media of GM-637 cells transfected with TSG-6 cDNA and of TNF-treated FS-4 cells (Figs. 6 *A*, 7, and 8). Although all SDS-PAGE separations were done under reducing conditions, the proportions between the 120- and 39-kD forms varied from experiment to experiment. The 120-kD form may represent an undissociated complex of TSG-6 with a putative carrier molecule or a tightly held TSG-6 homotrimer.

Since the predicted sequence of the protein indicated that TSG-6 is a member of the family of HA binding proteins, we sought to determine whether the TSG-6 protein too can bind HA. TSG-6 protein secreted into the medium of GM-637 cells transfected with a TSG-6 expression vector was partially purified by adsorption and elution on Sepharose-bound HA. That binding to HA involves more than a simple ionic interaction is supported by the experiments in which we demonstrated coprecipitation of the TSG-6 protein with HA by the cationic detergent CPC (Fig. 8). These experiments indicate that CPC, which interacts with the polyanionic glycosaminoglycans through an ionic-type interaction, did not cause dissociation of the complexes formed between TSG-6 and HA. The failure of CPC to coprecipitate TSG-6 mixed with chondroitin-4,5-sulfate or dermatan sulfate suggests a weaker and less specific interaction between TSG-6 and these glycosaminoglycans. It is interesting that TSG-6 protein was not coprecipitated with chondroitin-4,5-sulfate and dermatan sulfate (Fig. 8 and data not shown), even though they share with HA the common structural feature of alternating β1-3 and β1-4 bonds and have an even higher charge density because of their sulfation. On the other hand, heparan sulfate, which differs from HA considerably by having alternating α1-4 and β1-4 bonds but shares with HA the content of *N*-acetylglucosamine, seems to interact with TSG-6 protein stronger than some other glycosaminoglycans, though not as strongly as HA (not shown).

What is the biological function of the TSG-6 protein? The final answer to this question will have to await the results of experiments with purified TSG-6 protein. In view of its homology with CD44 and demonstrated ability to interact with HA, secretory TSG-6 protein could act as a competitive inhibitor of the interaction between CD44 and its ligand(s). Although CD44 is thought to represent the principal HA receptor, expressed on a wide variety of cells (Stoolman, 1989; Underhill, 1989), some functions of CD44 may not depend on its ability to bind HA (Carter and Wayner, 1988; Culty et al., 1990). Hence it is conceivable that the biological functions of TSG-6 also are not limited to situations in which binding to HA is involved. Even if interaction with HA would not be central to its biological function, binding of TSG-6 to HA might act as a means to efficiently concentrate TSG-6 in the extracellular matrix, in the vicinity of its target cells. Future experiments will seek to determine whether TSG-6 can compete with CD44 for binding to HA, and also whether TSG-6 can affect lymphocyte homing mediated by the interaction of CD44 with a still incompletely characterized mucosal vascular addressin (Jalkanen et al., 1987; Stoolman, 1989).

Inducibility by TNF or IL-1 suggests a role for TSG-6 in inflammation. In BCG-induced granulomatous inflammation, TNF released from macrophages plays a crucial role in the aggregation and activation of macrophages, leading to the development of granulomas (Kindler et al., 1989). In addition, an increase in HA was found in the early stage of the BCG-induced granulomatous response in the rabbit lung (Love et al., 1979) and in delayed-type hypersensitivity reactions (Shannon and Love, 1980; Campbell et al., 1982). The interaction between HA and CD44 is thought to be involved

in macrophage aggregation (Green et al., 1988). Hence, it is conceivable that the TSG-6 protein, induced by TNF or IL-1 in the microenvironment of inflammatory granulomas, modulates the aggregation and activation of macrophages mediated by the HA-CD44 interaction. It is also of interest that TNF and IL-1 increase the amount of HA secreted by human synovial fibroblasts (Butler et al., 1988). Local accumulation of HA may facilitate cell migration to sites of injury, modulate cell proliferation, and regulate inflammatory and immune cell responses by effects on antigen and/or mediator binding (Laurent and Fraser, 1986; Whiteside and Buckingham, 1989; Meyer et al., 1990). TSG-6 also could play a role in the modulation of these processes.

The presence of a shared HA binding domain in TSG-6, cartilage link protein, and proteoglycan core proteins suggests a possible involvement of TSG-6 in the structural organization of the extracellular matrix of connective tissue. The structural integrity of cartilage is maintained by the ternary complex involving proteoglycan, link protein, and HA (Hascall and Hascall, 1981). Cartilage breakdown occurs in rheumatoid arthritis, osteoarthritis, and other joint diseases as a result of destabilization of the ternary complex. TNF and IL-1 are known to cause proteoglycan degradation, leading to cartilage breakdown (Saklatvala, 1986; Shinmei et al., 1989). It is conceivable that TSG-6 protein induced by TNF or IL-1 during inflammation could interact with link protein or proteoglycan core protein through the shared homologous domain, resulting in a destabilization of the proteoglycan aggregates.

Since both TNF and IL-1 are mitogenic for normal fibroblasts (Schmidt et al., 1982; Sugarman et al., 1985; Vilček et al., 1986), generation of TSG-6 could be related to the processes of wound healing, tissue repair, and fibrosis. It is intriguing that none of the transformed cells examined by us was found to contain demonstrable TSG-6 mRNA (Fig. 1). The lack of TSG-6 expression in tumor cell lines contrasts with the high level of CD44 mRNA expression in many transformed cells (Stamenkovic et al., 1989). The involvement of HA and the cellular HA receptor in transformation, invasive growth, and metastasis has been well established (Knudson et al., 1989). Invasiveness of tumor growth correlates with increased amounts of HA in the tumor and in the interface to the surrounding connective tissue (Toole et al., 1979). Virus-transformed cells showed an increased HA binding capacity compared with the parental cell lines (Underhill and Toole, 1981). A highly invasive bladder carcinoma also showed a high degree of HA binding (Nemec et al., 1987). Finally, Günthert et al. (1991) recently showed a causal relationship between the expression of a novel splicing variant of CD44 and the metastatic potential of rat tumor cell lines. Inasmuch as TSG-6 binds to HA, it is conceivable that TSG-6 is involved in the modulation of these processes, possibly as a competitive inhibitor of CD44 binding to HA.

In conclusion, we have identified a novel inflammatory cytokine-inducible gene, termed TSG-6, coding for a protein that includes a unique loop domain homologous to a sequence in the HA receptor/lymphocyte homing receptor CD44, cartilage link protein, and proteoglycan core proteins. Mature TSG-6 is a secreted protein whose apparent molecular mass is 39 kD in monomeric form. It is glycosylated at two N-linked glycan acceptor sites. We have shown that the TSG-6 protein binds HA. Together these characteristics suggest that TSG-6 plays a role in cell–cell or cell–cell matrix interactions during inflammation and tumorigenesis.

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