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 NH₂-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., 1990b) 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...
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 NH₂-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 Eagle's 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 (Isopaque, Nyegaard, Norway) 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 10⁶ cells/ml. HuPBL (1 x 10⁶ mononuclear cells/ml) were treated with TNF (20 ng/ml), Con A (5 μg/ml), or phorbol myristate acetate (PMA) (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-49, and WI-38), for which a method described elsewhere was used (Lin and Vileik, 1987). Northern blots were prepared and probed according to Maniatis et al. (1982). 32P-Labeled probes that detect TSG-6 mRNA and the invariant

pH7 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-6 cDNA (Lee et al., 1990), clone X5 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 X5 was subcloned into the EcoRI site of M13mp8 bacteriophage in both orientations. To insure fidelity of sequence determination, directional deletion clones were generated by the Exonuclease III method (Henikoff et al., 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 EcorRI cDNA insert from clone X6 (Lee et al., 1990). Clone X6 contains a cDNA insert that lacks 402 bp at the 5'end and 4 bp at the 3' end of the TSG-6 cDNA sequence shown in Fig. 1A. An EcorRI-BamHI (406 bp) restriction fragment (which encodes the portion of TSG-6 open reading frame spanning from Ile11 to Asp25) was isolated from the EcoRI cDNA insert of clone X6 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 NH₂-terminal part of the MS2 polymerase and controlled by the temperature-inducible Apf 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 3β-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 1L 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 Antiserum 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 antisera 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, 4.0 mg (3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) (Aldrich Chemical Co., Milwaukee, WI) 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.
Stable Transfection of TSG-6 cDNA into GM-637 Cells

An expression plasmid pSV-TSG-6 was constructed by replacing the β-tubulin isotype, Mß1 coding region in the plasmid pSVß/l (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.). A HindIII-Ncol fragment containing the 5' region of TSG-6 cDNA was isolated from the antisense construct and a Ncol-Kpnl fragment containing the 3' region of TSG-6 cDNA was isolated from the sense construct. Both fragments were ligated into the HindIII/ Kpnl cleaved plasmid pSVß/l. The resulting expression construct (pSV-TSG-6) was used to cotransfect the GM-637 cell line, in either the sense or antisense orientation with respect to lac promoter (P.). 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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 charac-

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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' A+T-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^284-Thr-Ser). The sequence Ser^44-Gly preceded by an acidic amino acid (Glu^44) is indicative of a potential chondroitin sulfate linkage site (Zimmermann and Ruoslahti, 1989). It is also worth noting that there is an aspartic acid-rich region (residue Asp^79 to Asp^116) in addition to a serine- and threonine-rich region at the COOH-terminal end; the latter region includes six consecutive Ser/Thr residues (Thr^299 to Thr^304), which may
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 linkagesite and consensus sequence are shown by star with single broken line. Also underlined are the RNA decay consensus sequence motifs ATTTA (thin line) and the polyadenylation signal (AAA). 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 NH₂-terminal half of TSG-6 protein (Gly34 to Cys227) was aligned with the 2-B domain (Gly158 to Cys252) of human link protein (Doege et al., 1990a) 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 (Gly32 to Cys115) (Goldstein et al., 1989; Stamenkovic et al., 1989), versican (Gly109 to Cys181) (Zimmermann and Ruoslahti, 1989), and aggrecan (Gly148 to Cys257) (Doege et al., 1990b). Residues common to at least three sequences were boxed. (B) A portion of the NH₂-terminal sequence of TSG-6 protein (Arg33 to Pro145) was aligned with a region (Arg32 to Pro166) 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 (Gly139 to Phe269) was aligned with a portion (Gly44 to Phe139) of the sequence of complement C1r (Leytus et al., 1986) using the FASTA program.
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 NH₂-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., 1990a), and the human proteoglycan core proteins versican (Zimmermann and Ruoslahti, 1989) and aggrecan (Doege et al., 1990b), with the positions of four cysteine residues conserved in all of these proteins (Fig. 4A). Based on the alignment shown in Fig. 4A, 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 (CysB' to Pro98) 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). This expression construct was used to transfected 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. 1A). 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. 6A (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 "GN4r"). 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.

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 transfected 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. 1A). 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.

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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 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 undisassociated 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-
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).

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

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**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.
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.

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. 4A 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 Vlček, 1987; Dinarello, 1988; Vlček 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 chemoattractant and activating factor (a cytokine structurally related to IL-8), and the metalloprotease 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 (Dudina and Hardingham, 1989; Doege et al., 1990a), and the proteoglycan core proteins versican (Zimmermann and Ruoslahti, 1989) and aggrecan (Doege et al., 1990b). The homologous region, thought to comprise the HA binding domain (Goetinck et al., 1987; Perin et al., 1987; Cultry 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 \( \sim 30\% \)), Stamenkovic et al. (1989) calculated that the probability of an equal or better match occurring between unrelated proteins was \(<10^{-10}\). 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 polyionic 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 \( \beta1-3 \) and \( \beta1-4 \) 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 \( \alpha1-4 \) and \( \beta1-4 \) bonds but shares with HA the content of \( N \)-acyethylglucosamine, 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; Cultry 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

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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; Shimnei 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, Günther 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–matrix interactions during inflammation and tumorigenesis.

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