Identification and Characterization of A Novel Rat Ov-Serpin Family Member, Trespin*

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Serpins are responsible for regulating a variety of proteolytic processes through a unique irreversible suicide substrate mechanism. To discover novel genes regulated by transforming growth factor-β1 (TGF-β1), we performed differential display reverse transcriptase-PCR analysis of NRP-152 rat prostatic epithelial cells and cloned a novel rat serpin that is transcriptionally down-regulated by TGF-β and hence named trespin (TGF-β-repressible serine proteinase inhibitor (trespin)). Trespin is a 397-amino acid member of the ov-serpin clade with a calculated molecular mass of 45.2 kDa and 72% amino acid sequence homology to human bomapin; however, trespin exhibits different tissue expression, cellular localization, and proteinase specificity compared with bomapin. Trespin mRNA is expressed in many tissues, including brain, heart, kidney, liver, lung, prostate, skin, spleen, and stomach. FLAG-trespin expressed in HEK293 cells is localized predominantly in the cytoplasm and is not constitutively secreted. The presence of an arginine at the P1 position of trespin’s reactive site loop suggests that trespin inhibits trypsin-like proteinases. Accordingly, in vitro transcribed and translated trespin forms detergent-stable and thermo-stable complexes with plasmin and elastase but not subtilisin A, trypsin, chymotrypsin, thrombin, or papain. Trespin interacts with plasmin at a near 1:1 stoichiometry, and immunopurified mammal-expressed trespin inhibits plasmin in a dose-dependent manner. These data suggest that trespin is a novel and functional member of the rat ov-serpin family.

The serpins are an expanding superfamily of proteins present in the genomes of viruses, plants, and metazoa (reviewed in Refs. 1 and 2). Serpins are identified by their unique conformation (3, 4), namely a conserved secondary structure containing three β-sheets (designated A, B, and C), α-helices (usually nine), and a reactive site loop (RSL),1 which confers specificity to proteinase recognition. The RSL is composed of ~17 amino acids, which form a flexible region capable of distorting a target proteinase upon entry into the enzyme’s active site. Inhibition occurs after the target proteinase cleaves the serpin RSL (5–8), generating a covalent acyl-enzyme intermediate, which parallels a suicide substrate mechanism (9–11).

The serpin superfamily is divided into 16 different clades based on phylogenetic relationships (1). Clade B members, the ov-serpins, were originally identified by their significant sequence homology to chicken ovalbumin (12). They are competitive inhibitors of serine or cysteine proteinases and can target more than one proteinase through the use of several P1 residues (13, 14). Ov-serpins also share several properties: 1) the absence of an N-terminal signal sequence, 2) the beginning of their amino acid sequences relative to α,-antitrypsin at amino acid position ~23, and 3) the lack of a carbonyl-terminal extension. Human ov-serpins are further characterized by gene structure, namely an exon encoding a polypeptide loop between α helices C and D (i.e. CD loop). The CD loop confers unique characteristics to serpins, such as nuclear localization, and provides a binding motif for ancillary proteins (15–18).

The physiological functions of ov-serpins are not well understood; however, evidence suggests that their roles are diverse. MENT is a nuclear ov-serpin containing a lamin-like chromatin binding domain that induces higher order chromatin assembly when overexpressed (19). Other ov-serpins such as plasminogen inhibitor-9 (PI-9) can inhibit cells from undergoing granzyme B-mediated apoptosis and may regulate host defense against microbial or viral proteinases (20, 21). Bomapin (proteinase inhibitor-10) as well as plasminogen activator inhibitor-2 (PAI-2) confers apoptotic resistance to tumor necrosis factor α in several cell lines (22–24). Maspin (protease inhibitor-5) has been identified as an inhibitor of cell motility and metastasis along with demonstrating anti-angiogenesis properties (25–28). The variety of physiological roles serpins regu-

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1 The abbreviations used are: RSL, reactive site loop; TGF-β, transforming growth factor-β; RT, reverse transcription; IVTT, in vitro translation/transcription; MENT, myeloid and erythroid nuclear termination stage-specific protein; PBS, phosphate-buffered saline; PAI-1 and -2, plasminogen activator inhibitor-1 and -2, respectively; PI-9, proteinase inhibitor 9; TRG, TGF-β-regulated gene; DTT, dithiothreitol; VLK-pNA, d-Val-Leu-Lys-para-nitroanilide; RACE, rapid amplification of cDNA ends; MOPS, 4-morpholinepropanesulfonic acid; SI, stoichiometry of inhibition.

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late foreshadows their potential as novel therapeutic targets for many disease states.

To identify genes whose products may serve as downstream mediators or regulators of transforming growth factor-\(\beta\) signal transduction (reviewed in Refs. 29 and 30), we performed differential display reverse transcription-polymerase chain reaction using NRP-152 rat prostastic cells treated with or without TGF-\(\beta_1\), either alone or in the presence of insulin, which blocks several of TGF-\(\beta_1\)'s effects.\(^2\) NRP-152 is a unique an- drogen receptor-positive basal prostastic epithelial cell line highly sensitive to TGF-\(\beta_1\) (31–34). From these differential display analyses, we have identified and characterized a widely expressed novel rat ov-serpin, trespin, which directly binds and inhibits plasmin with a stoichiometry of 1:1.

MATERIALS AND METHODS

Cell Culture—The NRP-152 rat prostacic epithelial cell line (31) was maintained in GM2 culture medium (Dulbecco’s modified Eagle’s medium/Ham’s F-12 supplemented with 5% fetal bovine serum, 5 \(\mu\)g/ml insulin, 10 ng/ml cholera toxin, 20 ng/ml epidermal growth factor, and 0.1 \(\mu\)M dexamethasone) in Nunc 80-cm\(^2\) tissue culture flasks. The cells were kept at 37 \(^\circ\)C in a 95% air, 5% \(\text{CO}_2\) environment and at subconfluence (every 3–4 days) passaged 1:40. All experiments were performed under low serum conditions, where NRP-152 cells were cultured in Dulbecco’s modified Eagle’s medium/Ham’s F-12 supplemented with 15 \(\mu\)g/ml HEPES (pH 7,5), 1% calf serum (HyClone), 10% dexamethasone, 50 \(\mu\)g/ml penicillin, 0.1 \(\mu\)M dexamethasone, HEK293, a human embryonic kidney cell line, was grown in Dulbecco’s modified Eagle’s medium/Ham’s F-12 with 10% fetal bovine serum. THP-1, a human monocyte leukemia cell line, was grown in Dulbecco’s modified Eagle’s medium/Ham’s F-12 with 10% fetal bovine serum. THP-1, a human monocyte leukemia cell line, was grown in RPMI supplemented with 10% heat-inactivated fetal bovine serum, 0.05 \(\mu\)g/ml gentamycin. RBL-1, a rat basophilic leukemia cell line, was grown in Dulbecco’s modified Eagle’s medium/F-12 supplement- ed with 10% calf serum and 400 \(\mu\)g/ml gentamycin.

Differential Display—Following the method of Zhao et al. (35), differential display RT-PCR was performed using total RNA prepared from either untreated NRP-152 cells (control) or cells treated with TGF-\(\beta_1\) (10 ng/ml) for 24 h in the absence or presence of insulin (5 \(\mu\)g/ml). The RNA for differential display was isolated by a modified RNeasy method (36). The \(^{32}\)P-labeled PCR products were then resolved through a denaturing polyacrylamide gel, and the selected bands were reamplified and cloned into pCR-TRAP (GenHunter).

Northern Blot Analysis—Total RNA was extracted from cells using an RNeasy Total RNA kit (Qiagen) and resolved through a 1% agarose gel. Equal loading of the gel was confirmed by ethidium bromide staining of 28 S ribosomal RNA. The RNA was then transferred to a Nytran membrane (Schleicher & Schuell). Nytran membranes were cross-linked using ultraviolet radiation and slot-blotted with 1 \(\mu\)g each of cDNA and prehybridized for 4 h at 60 \(^\circ\)C. The membranes were then washed (2 \times SSC, 0.1 \(\mu\)g/ml) at room temperature; 2 \times SSC, 0.1 \(\mu\)g/ml, 30 min at 60 \(^\circ\)C; 0.5 \times SSC, 0.1 \(\mu\)g/ml, 60 min at 60 \(^\circ\)C; 0.1 \times SSC, 0.1 \(\mu\)g/ml, 30 min at 60 \(^\circ\)C and exposed to a phosphor screen. ImageQuant was used to quantitate differences in mRNA expression.

Nytran membranes used contained cDNA probes for trespin and \(\beta\)-actin. The 1.17-kb trespin cDNA probe was designed to hybridize to bases 79–1251 of trespin mRNA, whereas the 0.35-kb \(\beta\)-actin probe was complementary to exons 2 and 3 of the rat \(\beta\)-actin gene. A 1.4-kb fragment of the pcDNA3 expression vector, prepared by digestion with EcoRI, was also used for nonspecific DNA binding.

RT-PCR—Reverse transcription was performed using murine leukemia virus reverse transcriptase and random hexamer primers (Gene-Amp; PerkinElmer Life Sciences) with 0.2 \(\mu\)g of total RNA from each tissue. The cDNA was then PCR-amplified (40 cycles of 94 \(^\circ\)C for 30 s, 55 \(^\circ\)C for 30 s, 72 \(^\circ\)C for 60 s) using AmpliTaq DNA polymerase (Gene-Amp) in a 20- \(\mu\)l reaction buffer (10 \(\text{mM}\) Tris, pH 8.0, 5 \(\text{mM}\) MgCl2, 40 \(\text{ng}\) of PCR polymerase, and 2 \(\mu\)M dNTPs) and centrifuged 15 min at 15,000 \(\times \text{g}\). Complexes were resolved in 4 \(\times\) Tris-Acetate-EDTA buffer and transferred to a nylon membrane. The membranes were then washed (2 \times SSC, 0.1 \(\times\) SSC, 30 min at room temperature; 2 \times SSC, 0.1 \(\times\) SSC, 10 min at 60 \(^\circ\)C; 0.5 \(\times\) SSC, 0.1 \(\times\) SSC, 60 min at 60 \(^\circ\)C) and exposed to a phosphor screen. ImageQuant was used to quantitate differences in mRNA expression.

Development of pcDNA3-FLAG-trespin, pCEP4-FLAG-trespin, and pcDNA3-FLAG-bomapin Vectors—The hemagglutinin tag and translational enhancer element was excised from pcDNA3 by digestion with HindIII and BamHI and replaced with 5’-HindIII-Kozak-FLAG-BamHI-3’ cDNA made by annealing complementary oligonucleotides (Integrated DNA Technologies, Inc.). Trespin (codons 2 to stop) was amplified with primers containing BamHI and EcoRI ends and ligated to BamHI- and EcoRI-digested pcDNA3-FLAG to generate pcDNA3-FLAG-trespin. pCEP4-FLAG-trespin was generated by excision of FLAG-trespin from pcDNA3-FLAG-trespin using complete NcoI digestion, followed by limited digestion with HindIII and ligation with HindIII and NcoI-digested pCEP4. The bomapin coding sequence (codons 2 to stop) was PCR-amplified from THP-1 total RNA with primers containing BamHI and ClaI ends and ligated into BamHI and ClaI-digested pcDNA3-FLAG-bomapin. Constructs were sequenced for confirmation.

Proteinase Binding Assays—\(^3^5\)S-Labeled trespin and bomapin were generated using a T7-coupled in vitro transcription/translation system (Promega) with pcDNA3-FLAG-trespin (or pcDNA3-FLAG-bomapin) as template. 1 \(\mu\)l of in vitro transcribed/translated (IVTT) reaction was combined with the indicated nanogram amounts of proteinases (plasmin, elastase, subtilisin A, papain, and thrombin fragment, 1-chloro-3-tosylamido-7-amino-2-heptanone-treated trypsin, 0.1 \(\mu\)l trypsin, 0.1 \(\mu\)l of chymotrypsin, and 1-t-oxychloro-2-phenylthethyl chloromethyl ketone-treated trypsin from Worthington) in Tris-buffered saline (pH 7.4) in a total volume of 15 \(\mu\)l. Samples were incubated at 37 \(^\circ\)C for 15 min and then heated to 100 \(^\circ\)C for 10 min in the presence of 2% SDS and 100 mM DTT. Complexes were resolved in 4–12% NuPAGE gradient gels (Invitrogen) using 1× MOPS buffer (Invitrogen) and transferred

\(^2\) D. Danielpour, unpublished results.
to nitrocellulose before exposing to a phosphor screen for 48 h. Images were generated by an Amersham Biosciences PhosphorImager and ImageQuant Software.

**Purification of FLAG-trespin**—HEK293 cells (2.0 × 10^7/100-mm plate) were transfected with 10 μg of amino-terminal FLAG-tagged trespin in pCEP4 (Invitrogen) (pCEP4-FLAG-trespin) or pCEP4 control using a standard calcium phosphate co-c precipitate method, and stable clones were selected with 300 μg/ml hygromycin (Invitrogen). A pool of stable clones expressing FLAG-trespin (or pCEP4 control) were grown to subconfluence in 10% fetal bovine serum/Dulbecco’s modified Eagle’s medium/Ham’s F-12 in 15 × 150-mm² plates. Cells were lysed in NETT (150 mM NaCl, 1 mM EDTA, 10 mM Tris pH 7.4, 1% Triton X-100) containing protease inhibitors (Complete tablets without EDTA; Roche Molecular Biochemicals), and the lysates were centrifuged at 14,000 rpm for 10 min before immunoprecipitation with anti-FLAG M2-agarose (Sigma) overnight at 4 °C. Immune complexes were washed twice with each NETT and Tri-buffered saline, pH 7.4. FLAG-trespin was eluted at 4 °C from the resin with five 2-ml washes (30 min each of 100 μg/ml FLAG peptide (Sigma) in Tri-buffered saline, concentrated to 1 mg of amino-terminal FLAG-tagged trespin with each NETT and Tris-buffered saline, pH 7.4. FLAG-trespin was resolved in a 4–15% NuPAGE gel, 14-kDa cut-off membrane) against 5 mM HEPES for 48 h (buffer changed every 16 h). Purified FLAG-trespin was quantified by a microtiter BCA protein assay (Pierce) and Coomassie Blue staining of a 4–12% NuPAGE gel, using bovine serum albumin standards.

**Subcellular Fractionation and Conditioned Medium**—One 150-mm² dish of stable transfected pCEP4-FLAG-trespin HEK293 cells were trypsinized, resuspended, and quantified. 25% of the cell suspension was used for whole lystate, and 75% was used for nuclear extract. For preparation of nuclear extract, cells were centrifuged at 4000 rpm for 10 min and resuspended in 2 ml of lysis buffer (10 mM HEPES, pH 7.5, 2 mM EDTA, 5 mM MgCl₂, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml each leupeptin, aprotinin, antipain, and 4–aminomethylbenzenesulfonyl fluoride). Cells were incubated on ice for 20 min, homogenized with 10 passes through a 25-gauge needle, and centrifuged at 4000 rpm for 10 min at 4 °C. The pellet was washed twice with 1 ml of lysis buffer and resuspended with 300 μl of lysis buffer. Samples were incubated for 1 h at ~8 °C, thawed, and centrifuged at 14,500 rpm for 30 min at 4 °C; supernatant is the nuclear extract. For whole cell lystate, cells were harvested, resuspended in lysis buffer, and immediately incubated at ~80 °C for 1 h. The thawed lysate was then centrifuged at 14,500 rpm for 30 min at 4 °C; supernatant is the whole cell lystate. The same volume of nuclear extract and whole cell lystate (equal number of nuclei and whole cells per ml) was subjected to Western blot analysis, and FLAG-trespin was detected as mentioned above. Proliferating cell nuclease restriction fragment expression (clone NA03, 1:250; Oncogene Research) was used as loading control.

The secretion of trespin from HEK293-FLAG-trespin cells was compared with the content of total cellular FLAG-trespin as follows. Conditioned medium (treated with 1% Triton X-100 and protease inhibitor mixture) and whole cell lystate (treated similarly) from 10⁷ cells were each mixed overnight with 50 μl of anti-FLAG-agarose. Resin was then washed three times with PBS plus 1% Triton X-100 and eluted with a total of 0.2 ml of 0.1 mg/ml FLAG peptide, as described by the manufacturer. Equal volumes of each eluted fraction were analyzed by Western blot.

**Thermostability of Trespin**—0.5 μg of purified FLAG-trespin was suspended in 20 μl of PBS and heated to the indicated temperatures for 5 min (39). Samples were then centrifuged at 14,000 rpm for 30 min at 4 °C. The supernatant was combined with SDS loading buffer and resolved in a 4–12% NuPAGE gel with 1× MOPS buffer, transferred to nitrocellulose, and subjected to Western blot analysis with anti-FLAG (clone M1; Sigma) as described (40).

**Binding Stoichiometry**—Active plasmid concentration was determined by titrating the plasmid substrate n-Val-Leu-Lys-pRNA (VLK-pRNA; Sigma) to measure the turnover rate, using a spectrophotometric method similar to Chase and Shaw (41). Plasmid (100 nM) was incubated with the indicated concentrations of FLAG-trespin (0–88.4 nM) in PBS for 30 min at 37 °C. Residual plasmid activity was measured (A₄₀⁵ with a Tecan Spectra Mini microplate reader and WinSelect 3.0 software) after a 20-min incubation with VLK-pRNA (1 mM final concentration) at 37 °C. The stoichiometry of inhibition was determined by dividing the velocity of inhibited reaction (V/V₀) by the ratio of FLAG-trespin to plasmid (IL/Iₑ). Linear regression analysis was performed by GraphPad Prism 3.0 to extrapolate the inhibitor and enzyme ratio resulting in 100% inhibition.

**Proteinase Inhibition Studies**—Under pseudo-first order conditions, plasmid (100 nM), VLK-pRNA (1 mM final concentration), and the indicated nanomolar concentrations of FLAG-trespin (0–88.4 nM) were combined simultaneously in PBS (total reaction volume was 100 μl). Plasmid activity proceeded at 37 °C, and the rate of product formation was recorded (A₄₀⁵) as described above.

**RESULTS**

**Cloning of Trespin**—Differential display RT-PCR was used to identify and isolate genes regulated by TGF-β1 in NRP-152 cells. This was done with RNA isolated from NRP-152 cells treated with or without 10 ng/ml TGF-β1 for 24 h either alone or in the presence of 5 μg/ml insulin, which blocks several effects of TGF-β1. Following differential display screening with 20 primer sets, we identified the 3’-end of a novel gene, which we initially named TGF-β-regulated gene 13 (TRG-13). Expression of TRG-13 is decreased by TGF-β1 in a manner that is blocked in the presence of insulin (Fig. 1A, doublet is observed due to denaturing electrophoresis of double-stranded DNA). We confirmed these results by Northern blot analysis of total RNA from NRP-152 cells, using a [32P]dCTP-labeled cDNA probe prepared by reamplification of the original 300-bp fragment eluted from the differential display gel. TRG-13 migrates as a single 1.4-kb transcript and is down-regulated at early as 5 h after TGF-β1 exposure (Fig. 1B).

To obtain full-length TRG-13 cDNA, we performed 5’ RACE RT-PCR using oligonucleotide primers complementary to the 300-bp differential display product. From these reactions, we amplified a PCR product that contains the entire coding region of TRG-13. This product, totaling 1345 nucleotides, has 1.2 kb of coding region with 78 and 73 bp of 5’- and 3’-flanking untranslated regions, respectively. Analysis of this sequence with a nucleotide BLAST search revealed that TRG-13 shares high sequence similarity with serpins, with greatest similarity to the human bone marrow serpin bomapin (~80%) (42). cDNA alignment of TRG-13 to bomapin allowed us to identify the open reading frame and the ATG codon representing the translation start site (43). Although TRG-13 shares significant nucleotide and amino acid sequence similarity with other serpins, it is down-regulated by TGF-β1, which contrasts with the dogmatic view of TGF-β influence on proteolysis (44, 45).

TGF-β1 has been shown to regulate mRNA expression through both transcriptional and post-transcriptional mechanisms. To determine whether the decrease in trespin mRNA produced by TGF-β1 occurred through loss of gene transcription, we measured trespin mRNA production in NRP-152 cells by nuclear run-on assay. The amount of trespin mRNA produced in NRP-152 cells treated with 10 ng/ml TGF-β1 for 24 h was ~20% that of untreated control cells (Fig. 1C), demonstrating that TGF-β1 lowers trespin mRNA levels by decreasing transcription of the trenspin gene. For this reason, we have renamed this serpin trespin (for TGF-β-repressible gerine proteinase inhibitor).

**Trotter Sequence Analysis**—Based on its deduced sequence, trespin consists of 397 amino acids (Fig. 2A) with a predicted molecular mass of 45.2 kDa. Whereas trespin protein has 72% sequence identity with human bomapin, it also shows about 40% identity with other serpins, including human PAI-2 (46), chicken MENT (19), and human PI-9 (47, 48) (Fig. 2B). Amino acids are shaded according to similarities (red and yellow are most homologous to trespin) by ESPript 2.0. Trespin’s predicted fold (Fig. 3) is based on the alignment of structures with Protein Data Bank identification numbers 1BY7 (human PAI-2), 1JJR (human PAI-2 complexed with RSL peptide), 1HLE (equine leukocyte elastase inhibitor), and 1OVA (chicken ovalbumin). The two most divergent regions are 1) reactive loop 350–364, which is built based on secondary structure prediction (Swiss model) and steric considerations, and 2) loop 64–75, which cannot be built without experimental data. However,

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loop 64–75 is modeled after a similar 10VA loop where possible. Even so, the in silico structure determination suggests that trespin exhibits a classical serpin fold with nine a helices, three β sheets, and an exposed RSL.

Expression of Trespin in Rat Tissues and Cellular Localization—Consistent with its expression in the NRP-152 prostatic epithelial cell line, we found trespin is constitutively secreted into the extracellular matrix. The nuclear versus cytoplasmic distribution of trespin was determined by fractionation of HEK293 cells stably expressing FLAG-trespin, followed by Western blot analysis using an anti-FLAG M1 antibody. Expression of FLAG-trespin in duplicate cultures was assayed (Fig. 5A, upper panel) in a nuclear fraction compared with whole cell extracts. The same number of nuclei and whole cells were analyzed to allow for direct comparison; the Western blot detection of proliferating cell nuclear antigen is shown as loading control (Fig. 5A, lower panel). These analyses indicated that FLAG-trespin is primarily localized to the cytoplasm, with only a minor species in the nucleus, which greatly contrasts with bomapin, a predominantly nuclear protein.

To measure if trespin was secreted, the total conditioned medium (Fig. 5B) and whole cell lysate from a confluent 150-mm dish of HEK293-FLAG-trespin cells were each purified with immobilized anti-FLAG M2, and an equal volume of each eluted fraction was analyzed by Western blot. No FLAG-trespin was detectable in the conditioned medium from the HEK293-FLAG-trespin clones, suggesting that trespin is not constitutively secreted.

Survey for Target Proteinases—Serpins normally form complexes with target proteinases that are resistant to reducing agents, SDS and heat (50, 51). The presence of an arginine at the reactive center (Fig. 2) suggests that trespin inhibits trypsin-like proteinases. To discover targets that trespin binds and potentially regulates, we screened a panel of serine proteinases (subtilisin A, trypsin, chymotrypsin, thrombin, plasmin) and potentially regulates, we screened a panel of serine proteinases (subtilisin A, trypsin, chymotrypsin, thrombin, plasmin) for the ability to form SDS-stable, DTT-stable, and thermostable complexes with trespin. Trespin complexes with thrombin and weakly with trypsin. To confirm the reported bomapin results and directly compare the proteinase specificity of these serpins to further substantiate that these proteins are not homologues. As mentioned above, trespin forms complexes with elastase and plasmin; data from Riewald and Schleef (42) show that bomapin complexes with thrombin and weakly with trypsin. To confirm the reported bomapin results and directly compare the proteinase specificities of these serpins to further substantiate that these proteins are not homologues.

The above RT-PCR results indicated lung, skin, and stomach tissues have the greatest trespin expression. Since lung has the highest trespin mRNA expression, we probed rat (Fig. 4C, left panel) and human (Fig. 4C, right panel) lung poly(A)+ RNA (2 μg/lane) Northern blots to compare trespin and bomapin expression in this tissue. Similar to Fig. 4B, trespin is expressed in rat lung, whereas the human lung blot was negative for bomapin expression, consistent with previously published results (42). As control for the bomapin probe and hybridization conditions, we performed Northern blot analysis of two leukemic cell lines of rat basophilic and human monocytic origin, RBL-1 and THP-1, respectively (Fig. 4D). THP-1 demonstrated high bomapin expression, as suggested by Riewald et al. (49), and trespin expression was present in RBL-1 (Fig. 4C). Evidence suggests that bomapin is not expressed in nonmonocytic leukocytes (such as basophils), and trespin’s expression in RBL-1 provides further support that it is not the rat homologue of bomapin.

Ov-serpins have unique cellular localization, and many are secreted into the extracellular matrix. The nuclear versus cytoplasmic distribution of trespin was determined by fractionation of HEK293 cells stably expressing FLAG-trespin, followed by Western blot analysis using an anti-FLAG M1 antibody. Expression of FLAG-trespin in duplicate cultures was assayed (Fig. 5A, upper panel) in a nuclear fraction compared with whole cell extracts. The same number of nuclei and whole cells were analyzed to allow for direct comparison; the Western blot detection of proliferating cell nuclear antigen is shown as loading control (Fig. 5A, lower panel). These analyses indicated that FLAG-trespin is primarily localized to the cytoplasm, with only a minor species in the nucleus, which greatly contrasts with bomapin, a predominantly nuclear protein.

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Although the tissue expression patterns and cellular localization of trespin and bomapin are different, we wanted to compare the proteinase specificities of these serpins to further substantiate that these proteins are not homologues. As mentioned above, trespin forms complexes with elastase and plasmin; data from Riewald and Schleef (42) show that bomapin complexes with thrombin and weakly with trypsin. To confirm the reported bomapin results and directly compare the proteinase specificities of these serpins to further substantiate that these proteins are not homologues. As mentioned above, trespin forms complexes with elastase and plasmin; data from Riewald and Schleef (42) show that bomapin complexes with thrombin and weakly with trypsin. To confirm the reported bomapin results and directly compare the proteinase specificities of these serpins to further substantiate that these proteins are not homologues.
FIG. 2. Trespin amino acid sequence and homology to other ov-serpin members. A, deduced amino acid sequence of trespin. The arrow indicates the putative P1–P1′ scissile bond. B, comparison of trespin amino acid sequence with serpins: human bomapin, human PAI-2, chicken MENT, and human PI-9. The canonical reactive site loops and scissile bond are designated.
100% of trespinIVTT was complexed at near 1:1 stoichiometry.

At the highest mass of plasmin (100 pmol), increasing linearly (data not shown) up to 100 pmol of plasmin. Also, no other proteins in the eluted material shown.

A specific bands detectable by SDS-PAGE and Coomassie Blue staining.

Also, it appears that trespinIVTT may serve as a plasmin cleavage substrate in the presence of high plasmin activity, which may occur before or after complex formation. In contrast, elastase did not exhibit a classical dose-response inhibition profile (Fig. 7B), with the majority of complex formation detectable at 20 pmol of elastase and little to zero detectable complex formation at <20 pmol elastase. Even so, the majority of trespinIVTT was cleaved at 100 pmol of elastase (with minimal complex observed), suggesting that it preferentially serves as an elastase substrate.

**Trespin Inhibits Plasmin Activity in a Dose-dependent Manner.**—Due to the classical dose-response profile between plasmin and trespin, we wanted to observe if purified trespin could inhibit plasmin activity in vitro. We developed a stable mammalian expression system (pCEP4-FLAG-trespin) in HEK293 cells and purified FLAG-trespin from these cells by large scale immunoprecipitation with anti-FLAG-agarose and gentle elution with 100 μg/ml competitor FLAG peptide. The eluted FLAG-trespin was highly pure (~95%) with minimal nonspecific bands detectable by SDS-PAGE and Coomassie Blue staining (Fig. 8A). Also, no other proteins in the eluted material were recognized by the FLAG antibody (Fig. 8B), as demonstrated by the control immunoprecipitation lacking FLAG immunoreactive bands. To ensure that the purified FLAG-trespin was in its active conformation, we obtained a thermal denaturation profile. Native serpins, due to their metastable conformation, undergo a temperature-induced transition (i.e. precipitate out of solution) with a melting temperature of \( T_m = 60-70^\circ C \) and no other detectable transition up to 125 °C (1, 52). Cleaved or latent forms exhibit a sharp, highly cooperative unfolding transition at a much higher temperature of \( T_m = 124^\circ C \). Fig. 8C shows that FLAG-trespin rapidly precipitates at \( -60-70^\circ C \), suggesting that it is properly folded and in the appropriate conformation for further analyses.

- Classical serpins exhibit a stoichiometry of inhibition (SI) of 1:1 with their target proteinase. The SI \( (k_i + k_h)/k_h) \) determines whether the serpin-proteinase complex partitions down the pathway leading to the formation of a covalent inhibitory complex (k_i) or if parallel substrate pathways (k_h) predominate (53). A SI of 1 suggests that the formation of inhibitory complexes predominates, whereas a SI of >1 indicates the substrate pathway exceeds the classical dose-response profile between plasmin and trespin, the indicated concentrations of FLAG-trespin (0–168.8 nM) were combined with 100 nM plasmin in PBS and incubated for 20 min at 37 °C. Following the addition of plasmin substrate, D-Val-Leu-Lys-pNA (final concentration 1 mM),
Fig. 5. *Trespin* is primarily localized to the cytosol and is not secreted. A, intracellular and extracellular distribution of *trespin* was determined by fractionation of HEK293 cells stably expressing FLAG-trespin, followed by Western blot analysis. Expression of FLAG-trespin (upper panel) in duplicate cultures was assayed in both nuclear and whole cell lysate. An equal number of nuclei and whole cells were loaded per lane, and proliferating cell nuclear antigen (PCNA) is shown as loading control (lower panel). B, to measure if *trespin* was secreted, total conditioned medium and whole cell lysate from a confluent 150-mm dish of stable HEK293-FLAG-trespin cells were each purified with immobilized anti-FLAG M2, and an equal volume of each eluted fraction was analyzed by Western blot using anti-FLAG M1 antibody. Total cell lysate is shown as control (+ control). Analyses are representative of three independent experiments.

Residual plasmin activity was recorded using a microplate reader (A405) after a 20-min incubation at 37 °C. The fractional activity (A405 of inhibited reaction (V0/A405 control reaction (V0))) was plotted against the ratio of FLAG-trespin ([I]0) to plasmin ([E]0). Linear regression analysis was performed to extrapolate the inhibitor and enzyme ratio resulting in 100% inhibition (x intercept), and we obtained an SI of 0.85 (Fig. 9A), similar to the expected SI of 1:1. The inset (same x and y axes) contains data points from above and includes [I]0/[E]0 ratios of >1. Data points with [I]0/[E]0 ratios of >1 are on the x axis (due to 100% inhibition) and bias the linear regression analysis; therefore, they were omitted in determining the stoichiometry of inhibition.

To further substantiate that *trespin* inhibits plasmin through irreversible inactivation, we performed a progress curve method analysis (54, 55). 100 nM plasmin was simultaneously combined with VLK-pNA (1 mM final substrate) and the indicated concentrations of FLAG-trespin (0–84.4 nM) in PBS. Plasmin activity (A405) was recorded using a microplate reader and plotted against time (s). Fig. 9B demonstrates that FLAG-trespin inhibits plasmin activity in a dose-dependent manner. Furthermore, the initial inactivation (<300 s) proceeds at a linear rate; however, the inactivation pathway (i.e. plasmin inhibition by *trespin*) predominates as the rate of product formation is followed. These results demonstrate that *trespin* inhibits plasmin by directly binding to plasmin’s active site, resulting in functional inactivation, and not through an allosteric mechanism.

## Discussion

In this report, we have identified a novel member of the rat ov-serpin family that is down-regulated by TGF-β1 using differential display RT-PCR. *Trespin* is expressed in a variety of tissue types and forms SDS-stable, DTT-stable, and thermostable complexes with elastase and plasmin. FLAG-trespin exhibits a classical dose-response inhibition profile with plasmin (Fig. 9B), and complex formation between FLAG-trespin and plasmin is detectable at 1 pmol (Fig. 7A).

Three-dimensional structural prediction by a Swiss Model Protein Data Bank homology screen (Fig. 3) and steric considerations demonstrated that *trespin* has a classical serpin fold with nine α-helices, three β-sheets, and canonical carboxyl-terminal RSL. *Trespin* lacks an amino-terminal signal sequence, suggesting that it is not part of the secretory pathway, and Fig. 5B further suggests that *trespin* is not normally secreted. However, analysis of the amino acid sequence revealed the presence of potential N-linked glycosylation sites (NX(T/S)) at Asn177, Asn301, Asn329, Asn331, Asn334, and Asn383 that may allow trespin, like PAI-2 (29, 30), to be secreted under certain conditions. The stimuli that promote potential cellular relocalization and/or secretion of *trespin* are under investigation.

Protein motif analysis indicated that *trespin* shares an identical nuclear targeting sequence (KKRK) with bomapin (16) at amino acids 74–77. However, analysis of the trespin sequence by PSORT (56) predicted trespin to be primarily cytoplasmic with minor percentages targeted to nuclear and mitochondrial compartments; in contrast, PSORT prediction and data suggest that bomapin is localized to the nucleus (16). Cellular fractionation experiments (Fig. 5A) in our laboratory demonstrate that FLAG-trespin is predominantly cytoplasmic in HEK293 cells, consistent with PSORT predictions and preliminary data with endogenous trespin in NRP-152 cells (data not shown).
It is important to note that although functional domains between serpins are highly conserved, this similarity does not indicate similar function or homologous genes and is not a useful method to predict homologous serpins. Several human serpins within the ovalbumin and heat shock 47 clades exhibit high degrees of RSL homology, such as the squamous cell carcinoma antigen serpins 1 and 2 (65% homologous RSLs) and SERPINH1 and SERPINH2 (100% homologous RSLs); furthermore, trespin and bomapin have 67% RSL homology, which does not suggest that these proteins are homologues. Accordingly, interspecies homologues of the same serpin, such as PAI-2, usually demonstrate 100% RSL conservation, whereas the structural backbone may vary by 25%. Serpins evolved through gene duplication, which results in highly homologous proteins with specificity not necessarily conferred through sequence but rather by tissue-specific expression and regulation. 

A recent review by Silverman et al. (1) notes that a linear relationship between human and murine (and assumed other species) is not likely and that once all human serpins have been identified, proper nomenclature of nonhuman serpins (i.e. following the established rules of serpin nomenclature by the Second International Symposium on the Structure and Biology of Serpins and the HUGO Gene Nomenclature Committee), including rat trespin, will ensue.

Each member of the ov-serpin family has a unique and sometimes limited tissue expression. Trespin is likely to function in multiple tissues as indicated by its expression pattern, in contrast to human bomapin, which has been detected only in the bone marrow by multiple tissue Northern blot analyses and RT-PCR screens (49). Results suggest that primary cells and established cell lines from monocytic lineage have significant bomapin expression (49), with elevations in patients with acute monocytic leukemia and no detectable expression in the peripheral blood. On the contrary, we detected trespin expression in RBL-1, a basophilic cell line, which is representative of one cell type in peripheral blood, and in other tissues (prostate, lung, etc.). This result, in conjunction with protease specificity (Fig. 6B) and other mentioned differences, strongly suggests that trespin is not the rat homolog of bomapin but instead is a novel rat serpin.

Alignment of the trespin amino acid sequence with bomapin and other serpins (PAI-2, PI-9, and MENT) indicated a unique reactive center of trespin between amino acids 346 (P17) and 367 (P5’). Within the reactive site loop, the amino acid sequence of the hinge region (P17–P8) is highly conserved among serpins shown to inhibit serine proteinase activity (57–59). Trespin is identical to the consensus sequence for the hinge region at every residue except for the P9 position, suggesting that it functions as an inhibitory serpin. Of the amino acids present in the reactive site, the P1 residue is most critical in determining the proteinase specificity of an inhibitory serpin (2, 60). Trespin, like bomapin and PAI-2, contains arginine at its P1 position, suggesting that it interacts with trypsin-like serine proteinases. We analyzed a variety of trypsin-like serine proteinases and determined that elastase and plasmin formed stable complexes with trespin (Fig. 6A). Although elastase formed a stable complex with trespin, the profile obtained (Fig. 7B) did not indicate a dose-dependent inhibitory response. This may be the result of trespin binding to a region of elastase other
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Fig. 9. Trespin inhibits plasmin in a dose-dependent manner. A. The stoichiometry of inhibition for FLAG-trespin with plasmin was determined by preincubating 100 nM plasmin with the indicated concentrations of FLAG-trespin (0–88.4 nM) for 37 min at 37 °C. VLK-pDNA (1 mM final concentration) was added, and the reaction was incubated for 20 min at 37 °C. Residual plasmin activity was recorded using a microplate reader (A405). The fractional activity was the product formation [V]0 divided by the control reaction [V]0. Data points with [V]0/[E]0 ratios of >1 are plotted on the x axis (due to 100% inhibition) and bias the linear regression analysis; therefore, they are omitted in determining the stoichiometry of inhibition. B, a progress curve method analysis was used to demonstrate that FLAG-trespin inhibits plasmin in a dose-dependent manner. 100 nM plasmin was simultaneously combined with VLK-pDNA (1 mM final substrate) and FLAG-trespin (0 nM ( ), 4.42 nM ( ), 8.84 nM ( ), 17.68 nM ( ), 26.52 nM ( ), 35.36 nM ( ), 44.2 nM ( ), and 88.4 nM ( )) in the presence of PBS. Plasmin activity (A405) was recorded using a microplate reader for the indicated times and plotted against time (s). These analyses are representative of three independent experiments.

than the active site or proteolytic degradation of trespin by elastase, which generates trespin fragments with altered conformation(s) or affinities for elastase. The dose-dependent degradation of FLAG-trespin by elastase is obvious in Fig. 7B, which suggests that trespin may be a potential elastase substrate.

Most serpins characterized inhibit proteinases through a suicide substrate-like mechanism with the exception of maspin (61). From a screen to find target proteinases that trespin binds, only plasmin exhibited dose-dependent complex formation (Fig. 7A). Serpins usually exhibit a 1:1 stoichiometry with their target proteinase, and deviations from this ratio are often observed by altering the length of the RSL (62). Enzyme catalysis studies demonstrate trespin neutralizes plasmin with a stoichiometry of inhibition of 0.85 (Fig. 9A). The 15% deviation from the ideal 1:1 ratio may be due to the inherent error of the assays used to determine the concentration of immunopurified FLAG-trespin or active plasmin. Further support for the possibility that FLAG-trespin inhibits plasmin was obtained by a progress curve method analysis. This analysis demonstrates that FLAG-trespin inhibits plasmin in a dose-dependent manner (Fig. 9B) and that the FLAG-trespin inactivation pathway predominates as catalysis proceeds.

We have not yet determined whether trespin is expressed in peripheral blood (except for the basophilic cell line, RBL-1; Fig. 4D) or in other tissues where plasmin activity is evident. However, our data indicating that trespin inhibits plasmin provide fresh insight to the active site(s) of proteinases which trespin may regulate. Due to the significant degree of amino acid homology to bomapin, we were interested in determining whether trespin exhibited similar functional characteristics to bomapin, in light of the differences in tissue expression and cellular localization. Bomapin has been shown to form stable complexes with thrombin and trypsin (42); however, we cannot detect the formation of any trespin complex with these proteinases (Fig. 6, A and B). Also, the only physiological role of bomapin described is resistance to tumor necrosis factor α-induced apoptosis (22) in HeLa cells, a cell line that does not express endogenous bomapin. Even so, we investigated a similar role for trespin and found no protection from tumor necrosis factor α-induced apoptosis in HeLa cells by both transient transfection and stable clone assays (data not shown), further substantiating that bomapin and trespin have distinct physiological roles.

In multiple tumor types through genetic and epigenetic mechanisms, the TGF-β pathway is susceptible to loss or mutation of either TGF-β receptors or Smad proteins (63). The consequence of these alterations is the escape from the tumor-suppressive effects of TGF-β, namely cell cycle arrest, apoptosis, and perhaps increased tumor burden induced by local immune suppression, enhanced metastatic potential, or angiogenesis (64, 65). In the nontumorigenic basal prostatic epithelial cell line, NRP-152, our laboratory in collaboration with Dr. Lalage Wakefield’s group has demonstrated a potential tumor suppressor role for the TGF-β type II receptor (66). NRP-152 cells respond to TGF-β with classical effects: extracellular matrix secretion, cell cycle arrest, and apoptosis (32). Other unique and well characterized properties of these cells make them an ideal model for prostatic carcinogenesis and to explore novel pathways regulated by TGF-β.

The literature describes few cases of serpin regulation by TGF-β, such as PAI-1, a direct transcripational target of TGF-β1 (44, 45, 67), and PAI-2 (68, 69). PAI-1 deposition into the extracellular matrix is increased by TGF-β1, along with a down-regulation of the PAI-1 targets: urokinase-type and tissue-type plasminogen activators (45). The protein level of PAI-2 can be regulated by TGF-β1, but data suggest that this effect may be cell type-dependent and occurs through nontranscriptional mechanisms (68, 69). The overall effect of the above TGF-β1 responses is a dramatic decrease in extracellular proteolytic activity. On the contrary, trespin is transcriptionally down-regulated by TGF-β1 (Fig. 1C) and is found to be only intracellular (Fig. 4C). Trespin may protect against cellular injury induced by a variety of stimuli such as leakage of potent proapoptotic enzymes like granzyme B or cathepsin G in the cytoplasm or mispackaged lysosomal or secretory granule enzymes. Trespin’s ability to complex with elastase and plasmin, along with its expression in RBL-1, suggests a potential role in fibrinolysis or similar cascades. Of interest to our laboratory is the impact trespin will have on the biology of TGF-β signal transduction and responses.

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Identification and Characterization of A Novel Rat Ov-Serpin Family Member, Trespin
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