Stromelysin-3 Is Induced in Tumor/Stroma Cocultures and Inactivated via a Tumor-specific and Basic Fibroblast Growth Factor-dependent Mechanism*

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Stromelysin-3 (STR-3) is a recently characterized matrix metalloproteinase (MMP) with a unique pattern of expression and substrate specificity. Unlike other MMPs, STR-3 is consistently and dramatically overexpressed by multiple epithelial malignancies, including carcinomas of the breast, lung, colon, head and neck, and skin. Recent studies suggest that STR-3 promotes the local establishment of epithelial malignancies, contributing to tumor cell survival and implantation in host tissues; however, STR-3’s mechanism of action remains undefined. STR-3 is a stromal cell product, prompting speculation that infiltrating stromal cells secrete STR-3 in response to tumor-derived factors. To explore this possibility, we developed a tumor/stroma coculture assay in which small cell lung cancer (NSCLC) cell lines were grown on confluent monolayers of normal pulmonary fibroblasts. In these tumor/stroma cocultures, NSCLC cells stimulate normal pulmonary fibroblasts to secrete STR-3 and release extracellular basic fibroblast growth factor. Thereafter, STR-3 is processed at a unique internal sequence via a basic fibroblast growth factor- and MMP-dependent mechanism to a previously unidentified 35-kDa protein that lacks enzymatic activity. 35-kDa STR-3 is the most abundant STR-3 protein in tumor/stroma cocultures and is only detected when normal pulmonary fibroblasts are cultured with malignant bronchial epithelial cells. Therefore, the tumor-specific processing of STR-3 to the 35-kDa protein is likely to be an important regulatory mechanism.

Matrix metalloproteinases (MMP) are zinc-dependent endopeptidases that promote the local invasion and distant metastasis of epithelial malignancies and the neovascularization of tumor cell deposits (1–5) and participate in normal tissue remodeling (6). Currently recognized families of MMPs include collagenases, gelatinases, stromelysins, membrane-type MMPs, and additional single enzymes such as matrilysin and metalloelastase; these enzymes differ in substrate specificity, regulation, tissue-specific expression, and potential interactions with additional MMP family members (2, 3).

Stromelysin-3 (STR-3) (MMP-11) is a recently characterized MMP with a unique pattern of expression and substrate specificity (7, 8). The enzyme was originally isolated on the basis of its overexpression in primary breast cancers and identified as a MMP family member because of its predicted amino acid (aa) sequence (7). Like other MMPs, STR-3 has a highly conserved “pro” domain which is cleaved when the enzyme is converted to its active form. STR-3 also contains a characteristic catalytic domain with a zinc-binding consensus sequence and a “hemopexin” domain with sequence similarity to the heme-binding proteins. However, STR-3 differs from other previously characterized MMPs that are secreted as inactivezymogens. The STR-3 pro domain contains an additional recognition site for the Golgi-associate MMP-1 proteinase convertase, furin (9). Consequently, the ~60-kDa STR-3 proenzyme is processed within the constitutive secretory pathway and released as a ~45-kDa active enzyme (9, 10).

The 45-kDa STR-3 protein is currently thought to be the major active form of the enzyme (9). However, significant questions remain regarding the biological activity of 45-kDa STR-3. Although STR-3 has the characteristic structure of a MMP, its substrate specifically differs markedly from that of other MMP family members (11). A fragment of recombinant murine STR-3 which lacks the C-terminal hemopexin domain displays the properties of a weak metalloproteinase (11). However, the human 45-kDa STR-3 does not degrade classic MMP substrates such as gelatin, casein, and elastin (8). Moreover, the human STR-3 protein contains an amino acid substitution in the highly conserved MMP “met turn” which may alter the activity of the enzyme (12).

To date, the only known substrates for STR-3 are the serine protease inhibitors (serpins), α1-proteinase inhibitor (α1-PI), α1-antitrypsin, and α2-antiplasmin (8). Because α1-PI is the major circulating inhibitor of elastase, the degradation of α1-PI by STR-3 may increase elastase-mediated tissue damage. However, additional MMPs including MMP-1 (tissue collagenase) and MMP-3 (stromelysin-1) also hydrolyze α1-PI (13). Because STR-3 also degrades α2-antiplasmin, the enzyme could also indirectly increase local plasmin levels and promote plasmin-mediated conversion of additional pro-MMPs to their active forms (8). However, the unique aspects of STR-3 regulation,
structure, and function (8, 9, 11, 12, 14) suggest that the protein has additional as yet undefined biological activities in normal tissues and epithelial malignancies.

STR-3 is expressed during normal embryogenesis and the remodeling of certain adult tissues. In human embryos, STR-3 is expressed in developing digits (15); in murine embryos, the enzyme is found during limb, tail, and snout morphogenesis (16). The enzyme is expressed by stromal elements in contact with epithelial cells in normal embryonic and adult tissues. In certain settings, STR-3-positive stromal elements are in contact with epithelial cells undergoing regional apoptosis and selected cell survival. For example, during frog morphogenesis, STR-3 is specifically expressed in small intestine mesenchyme during a time in which primary intestinal epithelial cells undergo apoptosis and replacement by secondary epithelial cells (17). In humans and rodents, STR-3 is expressed in tissues that undergo extensive remodeling such as placenta, uterus, and post-lactation mammary glands (18, 19). For example, female mice who have completed weaning express STR-3 in involuting mammary glands (18). Taken together, these data suggest that specific changes in the viability of normal epithelial cells affect the expression of STR-3 in adjacent stroma.

The settings in which STR-3 is normally expressed provide insights regarding the role of the enzyme in primary tumors. Unlike other MMP family members, STR-3 is consistently and dramatically overexpressed by a variety of primary epithelial malignancies, including carcinomas of the breast, lung, colon, head and neck, and skin (20–25). In our own recent studies, virtually all newly diagnosed primary non-small cell lung carcinomas and precursor lesions and linked with the grade of normal pulmonary fibroblasts. In these tumor/stroma cocultures, STR-3 is specifically expressed in small intestine mesenchyme during the earliest stages of local tumor development in which malignant epithelial cells traverse the basement membrane, invade the surrounding stroma, and directly contact normal stromal elements.

To explore these possibilities in a controlled and easily accessible system, we developed a tumor*stroma* coculture assay in which NSCLC cells are grown on confluent monolayers of normal pulmonary fibroblasts. In these tumor/stroma cocultures, NSCLC cells stimulate pulmonary fibroblasts to secrete STR-3 and release bFGF. Following the release of STR-3 and bFGF, the active 45-kDa STR-3 enzyme is processed at a unique internal sequence via a bFGF- and MMP-dependent mechanism to a major 35-kDa protein that lacks enzymatic activity. Because 35-kDa STR-3 is the most abundant STR-3 protein in tumor/stroma cocultures and is only detected when normal pulmonary fibroblasts are cultured with malignant bronchial epithelial cells, these findings provide additional insights into the regulation and role of STR-3 in epithelial carcinomas.

MATERIALS AND METHODS

Cell Lines—The A549 and SL-6 human NSCLC cell lines were maintained in Dulbecco’s modified Eagle’s medium, 10% FCS, and RPMI, 10% FCS as described previously (26). The SV40-transformed non-tumorigenic human fetal tracheal epithelial cell line, 56 FHTE, was cultured on fibronectin (1 mg/100 ml, Life Technologies, Inc.-coated plates in Dulbecco’s modified Eagle’s medium, 10% FCS (28). Normal human pulmonary fibroblasts derived from fetal (ATCC CCL-153) and adult donors (ATCC CCL-210) were maintained in MEM, 10% FCS (22). All cell lines were incubated at 37 °C with 5% CO2.

Coculture Assays—Direct Cocultures—2 × 105 tumor cells (A549) or SV40-transformed non-tumorigenic tracheal epithelial cells (56 FHTE) were added to confluent monolayers of fetal (CCL-153) or adult (CCL-210) pulmonary fibroblasts in 6-well Falcon plates (Becton Dickinson, Franklin Lakes, NJ). The cocultures were initially incubated for 18 h in MEM, 10% FCS; thereafter, cocultures were washed twice and incubated in serum-free MEM. In selected experiments, the serum-free MEM contained FMA (10 μg/ml) (Sigma), a neutralizing anti-human PDGF-AB polyclonal antibody (25 μg/ml) (UBI, Lake Placid, NY), anti-human bFGF mAb (5 μg/ml) (clone FB-8, Sigma), anti-human EGF-R mAb (5 μg/ml) (C225, gift from J. Mendelsohn, Memorial Sloan Kettering Cancer Center, New York, NY (29)), the reversible MMP inhibitor, BB94 (30–32) (British Biotech, Oxford, UK) in 1% Me2SO, 1% Me2SO, human bFGF mAb (5 μg/ml) (clone FB-8, Sigma), anti-human EGF-R mAb (5 μg/ml) (C225, gift from J. Mendelsohn, Memorial Sloan Kettering Cancer Center, New York, NY (29)), the reversible MMP inhibitor, BB94 (30–32) (British Biotech, Oxford, UK) in 1% Me2SO, 1% Me2SO, alone, or the serine protease inhibitor aprotinin (33) (10 μg/ml) (Sigma).

After 24–72 h, conditioned media from the cocultures were harvested, centrifuged for 10 min at 1200 × g, concentrated ~ 15 × by ultrafiltration (Centricon 10, Amicon, Beverly, MA), and assayed for protein content (DC protein assay, Bio-Rad).

In selected experiments, cells from the tumor/stroma cocultures were washed twice in PBS and lysed at 4 °C in PBS/0.5% Triton X-100. Cell lysates were subsequently incubated for 30 min on a 4 °C rocking platform, centrifuged for 15 min at 4 °C and a 1000 × g to remove insoluble material, and assayed for protein content.

Transwell Cocultures—Confluent monolayers of pulmonary fibroblasts were plated in the lower chambers and NSCLC cells in the upper chamber. After equilibrium was achieved, the pump speed was increased slowly to 15 ml/min to achieve an equal distribution of cells in the chambers. After 24–72 h, conditioned media from the cocultures were harvested, centrifuged for 10 min at 1200 × g, concentrated ~ 15 × by ultrafiltration (Centricon 10, Amicon, Beverly, MA), and assayed for protein content.

Western Blots—Samples of conditioned media and cell lysates were size-fractionated on 10–12.5% SDS-PAGE gels under reducing conditions and transferred to Immobilon P membranes (Millipore, Bedford, MA). The membranes were then incubated for 2 h in blocking buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 0.05% Tween 20, and 5% dry milk) and subsequently incubated for 18 h with selected STR-3 (5ST4A9 or 5ST4C10) (34) or bFGF (clone FB8, Sigma) monoclonal antibodies at final concentrations of 1 μg/ml. After three washes, membranes were incubated with peroxidase-conjugated anti-mouse IgG and developed using enhanced chemiluminescence (Amersham Corp.). Selected autoradiograms were subjected to densitometric analysis (G700 Imaging Densitometer, Bio-Rad).

Centrifugal Elutriation—After A549 NSCLC cells and CCL-153 fibroblasts were directly cocultured as described above, the fibroblasts and tumor cells were separated by centrifugal elutriation (JE-5.0 elutriation system, Beckman Instruments, Inc., Fullerton, CA). Cells from the cocultures were trypanized and washed three times in RPMI 1640 medium containing 1% FCS, 10 mM HEPES, 0.5 mM EDTA, 50 units/ml penicillin, and 50 μg/ml streptomycin at 4 °C. Thereafter, cells were loaded into the elutriation chamber at a pump speed of 8 ml/min (rotor speed 2000 rpm/min) in the same medium. The pump speed was increased slowly to 15 ml/min to achieve an equal distribution of cells in the chamber. After equilibrium was achieved, the pump speed was increased from 20 to 120 ml/min in 5 ml/min increments and 20 sequential
tial 100-ml cell fractions were collected. Collection was completed at a pump speed of 120 ml/min.

To assess the percentage of tumor cells and fibroblasts in each cell fraction, an aliquot of each fraction was added to an individual well of a multichamber glass slide (Lab-Tek chamber slides, Nunc, Naperville, IL). The slides were air-dried and immunostained as described previously (22). Thereafter, the 20 individual 100-ml cell fractions were separately centrifuged and lysed for RNA extraction.

**RNA Preparation and Analysis**—Total RNAs from specific cell fractions were prepared and analyzed as described previously (35). In brief, total RNAs were isolated by acid guanidinium thiocyanate/phenol/chloroform extraction (RNA STAT-60 kit, Tel-Test, Inc., Friendswood, TX). They were size-fractionated on a 1% agarose gel under denaturing conditions, and transferred to a nylon membrane (Hybond N+, Amersham Corp.). The blot was then hybridized with a 32P-labeled STR-3 cDNA probe as described previously (22).

**a2-Macroglobulin Entrapment Assay—**a2-Macroglobulin entrapment was performed as described previously (8). In brief, aliquots of STR-3-containing conditioned media were incubated with 10 μg of purified human a2-macroglobulin (Sigma) for 18 h at room temperature in the absence or presence of 5 μM of the broad spectrum MMP inhibitor, BB-94. Thereafter, samples were size-fractionated on 10% SDSPAGE gels under non-reducing conditions, blotted, and probed with the anti-STR-3 mAb 5ST4A9 as described above.

**Immunoprecipitation—**Antisera directed against the STR-3 C terminus (RAST Ig) was generated by immunizing two New Zealand rabbits with an ovalbumin-coupled peptide containing the 25 C-terminal STR-3 aa (aa 464–488) (7). Affinity-purified RAST Ig was used to immunoprecipitate STR-3 from the conditioned media of NSCLC (A549)pulmonary fibroblast (CCL-153) cocultures. In brief, 100 μl of 15% conditioned media was incubated for 2 h at 4°C with or without 2 μg of affinity-purified RAST Ig; protein A-Sepharose (25 μg/ml) was added for an additional 30 min at 4°C. Thereafter, samples were centrifuged at 10,000 × g for 2 min and corresponding immunoprecipitates and immunodepleted conditioned media samples were collected. Immunoprecipitates and aliquots of immunodepleted conditioned media were size-fractionated on 10% SDS-PAGE gels under reducing conditions, blotted, and analyzed with the 5ST4A9 STR-3 mAb as described above.

**Purification of 35-kDa STR-3 and Identification of the 35-kDa STR-3 N-terminal Sequence—**Four liters of SL-6-conditioned serum-free media were collected and loaded on a 250-ml dextran sulfate (Sigma) column equilibrated in 20 mM Tris-HCl, pH 7.4, containing 140 mM NaCl, 1 mM CaCl2, and 0.02% Triton X-100. After extensive washing in the equilibration buffer, the column was eluted with ~400 ml of 20 mM Tris-HCl, pH 7.4, 2 mM NaCl, 1 mM CaCl2, and 0.01% Triton X-100.

Thereafter, the STR-3-enriched eluate was dialyzed against 20 mM Tris-HCl, 1 mM CaCl2, 0.01% Triton X-100 and loaded on an anion exchange column made by covalently coupling 1 μg of affinity-purified RAST Ig; protein A-Sepharose (25 μg/ml) was added for an additional 30 min at 4°C. Thereafter, samples were centrifuged at 10,000 × g for 2 min and corresponding immunoprecipitates and immunodepleted conditioned media samples were collected. Immunoprecipitates and aliquots of immunodepleted conditioned media were size-fractionated on 10% SDS-PAGE gels under reducing conditions, blotted, and analyzed with the 5ST4A9 STR-3 mAb as described above.

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Thereafter, the STR-3-enriched eluate was dialyzed against 20 mM Tris-HCl, 1 mM CaCl2, 0.01% Triton X-100 and loaded on an anion exchange column (DEAE-BioGel A, Bio-Rad). After extensive washing in the NaCl buffer (fraction 1), whereas 45-kDa STR-3 was mainly eluted with the 300 mM NaCl buffer fraction 2).

The 35-kDa STR-3-enriched fraction (fraction 1) was subsequently loaded on an immunosorbent column made by coupling 1 mg of immunoaffinity purified RAST Ig to 1 ml of wet protein A beads (Pharmacia Biotech Inc.). After extensive washing in 20 mM Tris-HCl, pH 7.4, 1 mM NaCl, 1 mM CaCl2, the RAST-Ig column was eluted with without 0.1 μg of glycine HCl, pH 2.5. Eluted fractions were immediately adjusted to pH 7.4 and assayed for 35-kDa STR-3 by immunoblotting. 35-kDa STR-3-containing fractions were pooled and concentrated 20 × by ultrafiltration (Centriflo-10, Amicon, Beverly, MA). The concentrated 35-kDa STR-3 sample was size-fractionated by SDS-PAGE, transferred to an Immobilon P membrane, and stained with Amido Black (Sigma). Thereafter, the ~35-kDa STR-3 band was excised and subjected to N-terminal sequence analysis (ABI model 492A, Worcester Foundation for Biomedical Research, Shrewsbury, MA).

**Expression and Purification of Recombinant 45- and 35-kDa STR-3**—To obtain recombinant (r) 35-kDa STR-3 (aa 98–489) was obtained from PMA-treated CCL-153 fibroblasts by reverse transcription-polymerase chain reaction using primers 1 (sense) and 2 (antisense) (primer 1 (sense), 5′ AAGATTCTTCTGCTT-GCTT-TCTGGCCGG 3′ and primer 2 (antisense), 5′ CAGATTCCTGAC-GAAAGTGGTGCG 3′), digested with EcoRI, cloned into the PGEX-4T-1 vector (Pharmacia), and sequenced. An STR-3 cDNA (Gly189-STR-3) encoding the 35-kDa STR-3 protein (aa 189–489) was amplified by polymerase chain reaction from the first construct (Phe98-STR-3) using primers 3 (sense) and 2 (antisense) (primer 1 (sense) 5′ CGGGGATCCGGGGATGTCACATCGAC 3′ and primer 2 (antisense) as noted above), digested with BamHI and EcoRI, ligated into the PGEX-4T-1 vector, and sequenced. Escherichia coli DH5α were transformed with Phe98-STR-3 or Gly189-STR-3 plasmids and subsequently grown in 2 × YT-G medium containing 100 μg/ml ampicillin and 2% glucose at 37°C with shaking. When a cell density corresponding to an A600 of 1.0 reached, recombinant (r) 45-kDa (F98-STR-3) or r35-kDa STR-3 (Gly189-STR-3) expression was induced by adding 0.4 mM isopropyl-1-thio-β-D-galactopyranoside, and the incubation was continued for 18 h at 37°C. Cells harvested from 1-ml bacterial culture were suspended in 50 ml of cold PBS and sonicated for 2 min at 4°C. Proteins were then solubilized in PBS containing 1% Triton X-100 for 30 min at 4°C and fraction 3—

**RESULTS**

**STR-3 Is Induced in Tumor/Stroma Cell Cocultures**—In previous studies, STR-3 was overexpressed by stromal cells in primary NSCLC of all stages and pathologic subtypes (22). To determine whether STR-3 was secreted by stromal cells in response to tumor-derived factors, an in vitro assay was developed in which NSCLC cell lines were cocultured with normal pulmonary fibroblasts for 1–3 days in serum-free media. Thereafter, conditioned media from the tumor/stroma cocultures were size-fractionated, immunoblotted, and analyzed for STR-3 (Fig. 1).

When cultured alone, NSCLC cell lines such as A549 (tumor, T) secrete virtually no STR-3 (Fig. 1, lanes 4 and 8). Normal fetal (CCL153) and adult (CCL-210) pulmonary fibroblasts that are cultured alone for 1–3 days also secrete little 45- or 60-kDa STR-3 (Fig. 1, lanes 1 and 5). In marked contrast, tumor/stroma cocultures secrete increased quantities of the 60-kDa proform and the 45-kDa active enzyme (CCL-153, 5.5 × base line and CCL-210, 24.5 × base line, Fig. 1, lanes 3 and 7). Similar results were obtained when pulmonary fibroblasts were cocultured with additional NSCLC cell lines (data not shown).

**Fibroblasts Are the Primary Source of STR-3 in Tumor/ Stromal Co-cultures**—To determine which cells secreted STR-3 in the tumor/stroma cocultures, the CCL-153 fibroblasts were separated from A549 NSCLC cells after 24 h of coculture using centrifugal elutriation. The relative percentages of fibroblasts and tumor cells in the elutriated cell fractions were estimated by keratin immunostaining. Fraction 1 contained the lowest percentage of keratin-positive cells (18% positive) and fraction 2 the highest percentage of keratin-positive cells (88% positive), indicating that these two fractions represented excellent sources of fibroblast- and tumor cell-enriched RNAs.

For these reasons, RNAs from fibroblast- and tumor cell-enriched fractions (fractions 1 and 20, respectively) were prepared and analyzed by Northern blot for STR-3 (Fig. 2, lanes 4 and 5); RNAs from CCL-153 fibroblasts and A549 NSCLC cells cultured separately (Fig. 2, lanes 1 and 3) or directly cocultured and harvested together (Fig. 2, lane 2) were also analyzed. As expected, STR-3 transcripts were significantly more abundant.

620
in tumor/stroma cocultures than in fibroblasts or tumor cells cultured separately (Fig. 2, compare lane 2 with lanes 1 and 3). STR-3 transcripts were also significantly more abundant in the fibroblast-enriched fraction than in the tumor cell-enriched fraction. Taken together, these data identify the fibroblasts as the primary source of STR-3 in the tumor/stroma cocultures.

A Previously Unidentified 35-kDa STR-3 Protein Is the Major Form of STR-3 in Tumor/Stroma Cocultures—Although the fibroblast-derived mature active 45-kDa STR-3 enzyme was present in conditioned media from tumor/stroma cocultures, the major form of STR-3 was a previously unidentified 35-kDa protein (Fig. 1, lanes 3 and 7). This 35-kDa STR-3 protein was of particular interest because it constituted ~70% of all STR-3 in conditioned media from prolonged (day 3) cocultures (Fig. 1, lane 7).

The 35-kDa STR-3 Protein Is Processed at the N Terminal and Lacks a Portion of the Catalytic Domain—To determine whether the newly identified 35-kDa STR-3 protein was a processed form of the larger active enzyme, conditioned media from tumor/stroma cocultures were initially immunoprecipitated with protein A alone or with an antiserum directed against the C-terminal STR-3 peptide (RAST). Immunoprecipitates and aliquots of immunodepleted conditioned media were subsequently immunoblotted and analyzed with an antibody directed against the STR-3 hemopexin domain (5ST4A9). Control protein A immunoprecipitates (lane 1), RAST-STR-3 immunoprecipitates (lane 2), and aliquots of conditioned media following immunoprecipitation with protein A alone (lane 3) or RAST (lane 4) are included. B, α2-macroglobulin entrapment. Conditioned media from day 3 A549 tumor/CCL-153 stroma cocultures were incubated for 18 h as follows: alone at 4 °C (lane 1); alone at 37 °C (lane 2); in combination with 10 μg of purified α2-macroglobulin at 37 °C (lane 3); or in combination with α2-macroglobulin and the competing broad spectrum metalloproteinase inhibitor (BB-94, 10 μM) at 37 °C (lane 4). Thereafter, samples were size-fractionated, immunoblotted, and analyzed for STR-3.

The 35-kDa STR-3 protein is processed at the N terminus and does not entrap α2-macroglobulin. A, 35-kDa STR-3 N-terminal processing. Conditioned media from day 3 A549 tumor/CCL-153 stroma cocultures were initially immunoprecipitated with protein A alone or with an antiserum directed against the C-terminal STR-3 peptide (RAST). Immunoprecipitates and aliquots of immunodepleted conditioned media were subsequently immunoblotted and analyzed with an antibody directed against the STR-3 hemopexin domain (5ST4A9). Control protein A immunoprecipitates (lane 1), RAST-STR-3 immunoprecipitates (lane 2), and aliquots of conditioned media following immunoprecipitation with protein A alone (lane 3) or RAST (lane 4) are included. B, α2-macroglobulin entrapment. Conditioned media from day 3 A549 tumor/CCL-153 stroma cocultures were incubated for 18 h as follows: alone at 4 °C (lane 1); alone at 37 °C (lane 2); in combination with 10 μg of purified α2-macroglobulin at 37 °C (lane 3); or in combination with α2-macroglobulin and the competing broad spectrum metalloproteinase inhibitor (BB-94, 10 μM) at 37 °C (lane 4). Thereafter, samples were size-fractionated, immunoblotted, and analyzed for STR-3.

Fig. 1. STR-3 is induced and processed to a major 35-kDa protein in tumor/stroma cell cocultures. Confluent fibroblasts (F) (A, CCL-153; B, CCL-210) were cultured alone (F), with 10 ng/ml PMA (F + PMA), or with A549 NSCLC cells (F + T) for 1–3 days in serum-free medium. NSCLC cells (T) were also cultured alone under the same conditions. Thereafter, conditioned media from the tumor/stroma cocultures, tumor cells, and fibroblasts were size-fractionated, immunoblotted, and analyzed for STR-3 using the 5ST4A9 monoclonal antibody. The ~60-kDa STR-3 proenzyme, the 45-kDa mature active enzyme, and the newly identified 35-kDa protein are indicated.

Fig. 2. Fibroblasts are the primary source of STR-3 in tumor/stroma cocultures. Confluent CCL-153 fibroblasts (10<sup>6</sup> cells) and A549 NSCLC cells (10<sup>6</sup> cells) were cocultured 24 h in serum-free medium, trypsinized, and separated by centrifugal elutriation. 20 cell fractions were collected and analyzed by immunohistochemistry for keratin expression. The fibroblast-enriched fractions (fraction 1, 18% keratin staining and the tumor cell-enriched fraction; fraction 20, 88% keratin staining) were selected for analysis of STR-3 transcripts. Fibroblasts and tumor cells that were cultured separately and directly cocultured unseparated tumor/stromal cells were also analyzed. Total RNAs (40 μg) from CCL-153 fibroblasts (lane 1) or A549 tumor cells (lane 3) cultured alone, directly cocultured and unseparated tumor/stromal cells (lane 2), and coculture cell fractions enriched for fibroblasts (lane 4) or tumor cells (lane 5) were size-fractionated, blotted, and hybridized to a <sup>32</sup>P-labeled STR-3 probe. The ~2.2-kilobase pair STR-3 transcripts are indicated (arrow).

Fig. 3. The 35-kDa STR-3 protein is processed at the N terminus and does not entrap α2-macroglobulin. A, 35-kDa STR-3 N-terminal processing. Conditioned media from day 3 A549 tumor/CCL-153 stroma cocultures were initially immunoprecipitated with protein A alone or with an antiserum directed against the C-terminal STR-3 peptide (RAST). Immunoprecipitates and aliquots of immunodepleted conditioned media were subsequently immunoblotted and analyzed with an antibody directed against the STR-3 hemopexin domain (5ST4A9). Control protein A immunoprecipitates (lane 1), RAST-STR-3 immunoprecipitates (lane 2), and aliquots of conditioned media following immunoprecipitation with protein A alone (lane 3) or RAST (lane 4) are included. B, α2-macroglobulin entrapment. Conditioned media from day 3 A549 tumor/CCL-153 stroma cocultures were incubated for 18 h as follows: alone at 4 °C (lane 1); alone at 37 °C (lane 2); in combination with 10 μg of purified α2-macroglobulin at 37 °C (lane 3); or in combination with α2-macroglobulin and the competing broad spectrum metalloproteinase inhibitor (BB-94, 10 μM) at 37 °C (lane 4). Thereafter, samples were size-fractionated, immunoblotted, and analyzed for STR-3.

The 35-kDa STR-3 protein is processed at the N terminus and lacks a portion of the catalytic domain—To determine whether the newly identified 35-kDa STR-3 protein was a processed form of the larger active enzyme, conditioned media from tumor/stroma cocultures were initially immunoprecipitated with an antiserum directed against the C-terminal STR-3 peptide (RAST). RAST-STR-3 immunoprecipitates were subsequently immunoblotted and analyzed with an antibody directed against the STR-3 hemopexin domain (5ST4A9). Fig. 3A includes RAST-STR-3 (lane 2) and control immunoprecipitates (lane 1) and aliquots of conditioned media following immunodepletion with RAST (lane 4) or protein A alone (lane 3). The RAST antiserum removes the majority of 35-kDa STR-3 from tumor/stroma cell-conditioned media, indicating that 35-kDa STR-3 contains the full C terminus in addition to the 55ST4A9 epitope from the hemopexin domain (Fig. 3A, lanes 2 and 4).
To determine whether 35-kDa STR-3 undergoes additional processing at the N terminus, a duplicate blot of the indicated samples was analyzed with an antibody directed against the STR-3 catalytic domain (SST4C10). Although the 45-kDa active enzyme reacted with SST4C10, 35-kDa STR-3 was not identified by this antibody (data not shown). Taken together, these data indicate that 35-kDa STR-3 is present at the N terminus and that this smaller STR-3 protein lacks the SST4C10 epitope from the catalytic domain.

35-kDa STR-3 Does Not Entrap α2 Macroglobulin—Because 35-kDa STR-3 contains the C-terminal hemopexin domain but lacks a portion of the N-terminal catalytic domain, we compared the biological activity of 35-kDa STR-3 and the mature 45-kDa active enzyme using an α2-macroglobulin entrapment assay (8). α2-Macroglobulin is a broad range protease inhibitor which complexes with all of the previously characterized metalloproteinases, including 45-kDa STR-3 (8).

In Fig. 3B, conditioned media from tumor/stroma cocultures were incubated as follows: alone at 4 °C (lane 1); alone at 37 °C (lane 2); in combination with α2-macroglobulin at 37 °C (lane 3); or in combination with α2-macroglobulin and a competing broad-spectrum metalloproteinase inhibitor (BB-94) at 37 °C (lane 4). There is a modest decrease in 45-kDa STR-3 and no change in 35-kDa STR-3 when samples are incubated at 37 °C rather than 4 °C (Fig. 3B, lanes 1 and 2). When samples are incubated with α2-macroglobulin at 37 °C, there is the expected decrease in 45-kDa STR-3 secondary to entrapment of the enzyme (Fig. 3B, lane 3); however, incubation with α2-macroglobulin does not reduce the quantity of 35-kDa STR-3 (Fig. 3B, lane 3). Consistent with this observation, BB-94 reversed the α2-macroglobulin entrapment of 45-kDa STR-3 without affecting 35-kDa STR-3 (Fig. 3B, lane 4). Taken together, these data provide preliminary functional evidence that 35-kDa STR-3 activity lacks the metalloproteinase activity of the larger 45-kDa enzyme.

The Generation of 35-kDa STR-3 Requires Tumor-specific Interactions with Surrounding Stromal Cells—To determine whether malignant epithelial cells are required for the generation of 35-kDa STR-3, we cocultured pulmonary fibroblasts with either NSCLC (Fig. 4, A and C, lanes 4–6) or SV40 immortalized non-tumorigenic tracheal epithelial cells (Fig. 4, A and C, lanes 1–3) and assayed STR-3 in the resulting conditioned media. As previously demonstrated (Fig. 1), CCL-153 fibroblasts constitutively secrete higher levels of STR-3 than the CCL-210 fibroblasts (compare Fig. 4, A, lanes 1 and 4 with C, lanes 1 and 4).

The induction of STR-3 was over 5-fold greater in cocultures of pulmonary fibroblasts and NSCLC cells than in cocultures of fibroblasts and SV40-immortalized non-tumorigenic tracheal epithelial cells (compare Fig. 4, A and C, lane 5 versus 2, and C, lane 5 versus 2). In addition, although 35-kDa STR-3 was the most abundant STR-3 protein in tumor/stroma cocultures (Fig. 4, A and C, lane 5), 35-kDa STR-3 was undetectable in cocultures of SV40-immortalized tracheal epithelial cells and pulmonary fibroblasts (Fig. 4, A and C, lane 2). These data indicate that the induction of 45-kDa STR-3 is more efficient in tumor/stroma cocultures and that the generation of 35-kDa STR-3 is tumorspecific and dependent upon the interaction between malignant bronchial epithelial cells and pulmonary fibroblasts. For these reasons, we attempted to identify the factors responsible for the generation of 35-kDa STR-3.

The Generation of 35-kDa STR-3 in Tumor/Stroma Cocultures Requires bFGF—Because factors including PDGF, EGF, and bFGF increase STR-3 transcript abundance in normal pulmonary fibroblasts in vitro (7, 22), we evaluated the potential role of these factors in STR-3 induction and processing in the tumor/stroma cocultures. As indicated in Fig. 4 (A and C), tumor/stroma cocultures were performed in the presence or absence of neutralizing antibodies directed against PDGF, the EGF receptor, or bFGF. None of these neutralizing antibodies reduced the quantities of 45-kDa STR-3 in conditioned media from tumor/stroma cocultures (Fig. 4, A and C, compare lanes 5, 8, 11, and 14); PDGF and EGF receptor neutralizing antibodies also had no effect on the generation of 35-kDa STR-3.
The generation of 35-kDa STR-3 and the release of bFGF do not require direct tumor/stroma cell contact. NSCLC cells (A549) and pulmonary fibroblasts (CCL-153) were directly cocultured (D) or cocultured in a transwell apparatus which permits only the diffusion of soluble factors (TW). In both types of cocultures, fibroblasts were cultured alone (F) or with tumor cells (F + T); tumor cells were also cultured alone (T). A, conditioned media from the direct (D) and transwell (TW) day 3 cocultures was size-fractionated, immunoblotted, and analyzed for STR-3 (lanes 1–3) and transwell (TW) cocultures (fibroblasts alone, lane 4; fibroblasts from the lower chamber of a coculture, lane 5; tumor cells from the upper chamber of a coculture, lane 6; and tumor cells alone, lane 7). Cell lysates were size-fractionated, immunoblotted, and analyzed for bFGF.

Because extracellular bFGF was implicated in the generation of 35-kDa STR-3, additional aliquots of conditioned media from the day 3 tumor/stroma cocultures (Fig. 4A and C, NA) were also assayed for bFGF content (Fig. 4, B and D). Although neither fibroblasts nor NSCLC cells that were cultured alone released detectable quantities of bFGF (Fig. 4, B and D, lanes 1 and 3), tumor/stromal cocultures released readily detectable bFGF (Fig. 4, B and D, lane 2).

The Generation of 35-kDa STR-3 and the Release of bFGF Do Not Require Direct Tumor/ Stromal Cell Contact: Transwell Assays—To determine whether bFGF induction/release and bFGF-mediated processing of STR-3 require physical contact between NSCLC cells and pulmonary fibroblasts, the two cell types were cocultured in a transwell apparatus which permits only the diffusion of soluble factors. As indicated in Fig. 5A (lanes 2 and 5), 35-kDa STR-3 and bFGF were equally abundant in conditioned media from transwell and direct tumor/stroma cocultures.

Because bFGF release was not dependent upon tumor/stromal cell contact, the transwell cocultures were also used to identify the cellular source of bFGF. Fibroblasts and tumor cells from transwell cocultures were separately lysed and analyzed for bFGF (Fig. 5B, lanes 5 and 6); direct fibroblast/tumor cell cocultures were similarly evaluated (Fig. 5B, lane 2). It is readily apparent that the fibroblasts are the primary source of bFGF in tumor/stroma cocultures (Fig. 5B, compare lanes 5 and 6) and that the fibroblasts produce increased bFGF following their exposure to NSCLC cells (Fig. 5B, compare lanes 4 and 5, lanes 1 and 2).

The generation of 35-kDa STR-3 is inhibited by BB-94. Normal fetal (CCL-153, A) or adult (CCL-210, B) pulmonary fibroblasts (F, lanes 1, 4 and 7), fibroblasts and tumor cells (A549) (F + T, lanes 2, 5, 8), or tumor cells (T, lanes 3, 6, and 9) were cultured in the absence (Control, lanes 1–3) or presence of 10 µg/ml BB94 (BB94, lanes 4–6) or 10 µg/ml aprotinin (Aprotinin, lanes 7–9) for 3 days. Thereafter, conditioned media were size-fractionated, immunoblotted, and analyzed for STR-3.

The generation of 35-kDa STR-3 is inhibited by BB-94. Normal fetal (CCL-153, A) or adult (CCL-210, B) pulmonary fibroblasts (F, lanes 1, 4 and 7), fibroblasts and tumor cells (A549) (F + T, lanes 2, 5, 8), or tumor cells (T, lanes 3, 6, and 9) were cultured in the absence (Control, lanes 1–3) or presence of 10 µg/ml BB94 (BB94, lanes 4–6) or 10 µg/ml aprotinin (Aprotinin, lanes 7–9) for 3 days. Thereafter, conditioned media were size-fractionated, immunoblotted, and analyzed for STR-3.

The generation of 35-kDa STR-3 is inhibited by BB-94. Normal fetal (CCL-153, A) or adult (CCL-210, B) pulmonary fibroblasts (F, lanes 1, 4 and 7), fibroblasts and tumor cells (A549) (F + T, lanes 2, 5, 8), or tumor cells (T, lanes 3, 6, and 9) were cultured in the absence (Control, lanes 1–3) or presence of 10 µg/ml BB94 (BB94, lanes 4–6) or 10 µg/ml aprotinin (Aprotinin, lanes 7–9) for 3 days. Thereafter, conditioned media were size-fractionated, immunoblotted, and analyzed for STR-3.
MMP-dependent mechanism to a major previously undescribed 35-kDa protein which differs in biological activity from 45-kDa STR-3 (Fig. 7).

35-kDa STR-3 Purification and Analysis of N-terminal Sequence—Because the 35-kDa STR-3 lacks the 5ST4C10 epitope from STR-3 catalytic domain and fails to entrap α2-macroglobulin (Fig. 3B), this major STR-3 protein is unlikely to have the enzymatic activity of its 45-kDa precursor. To specifically compare mature active 45-kDa STR-3 with the 35-kDa processed protein, we purified 35-kDa STR-3 and identified its N terminus.

35-kDa STR-3 Purification—An unusual NSCLC cell line, SL-6, was used in the large scale purification of 35-kDa STR-3. Unlike the majority of NSCLC cell lines that do not secrete STR-3, SL-6 cells secrete STR-3 and process the mature active enzyme to 35-kDa protein in absence of normal pulmonary fibroblasts (Fig. 8A, lane 1). In SL-6 cells, the processing of STR-3 to a 35-kDa protein is also bFGF- and MMP-dependent; 35-kDa STR-3 is less abundant when SL-6 is cultured in presence of a neutralizing bFGF mAb (Fig. 8A, lane 2) or concentrations of ≥1 μg x BB-94 (Fig. 8, A and B). In SL-6 cells cultured with increasing amounts of the MMP inhibitor (BB-94), the concentrations of both 60-kDa pro-STR-3 and mature 45-kDa STR-3 increase as that of the 35-kDa STR-3 decreases, confirming the precursor/product relationship between the larger and smaller STR-3 proteins (Fig. 8B).

N-terminal Sequence of 35-kDa STR-3—To determine the N-terminal sequence of 35-kDa STR-3, the protein was purified from SL-6 conditioned media using a three-step procedure (see “Materials and Methods”). The highly purified STR-3 sample contained a single major ~35-kDa band after SDS-PAGE, transfer to an Immobilon membrane, and Amido Black staining (data not shown). This 35-kDa STR-3 band was excised and subjected to N-terminal sequencing. Three N-terminal sequences were identified in the broad ~35-kDa STR-3 band (Fig. 9). These N-terminal sequences, an 190–195, 192–194, and 194–199 from full-length STR-3, yield a protein with the calculated mass of ~35-kDa.

The identification of three related N-terminal 35-kDa STR-3 sequences suggests that the protein may be cleaved at a single site and subsequently degraded by an autocatalytic mechanism (37, 38) or by additional enzymes in SL-6 conditioned media. In this regard, it is of interest that the most N-terminal 35-kDa STR-3 sequence (Glu190-Tyr194) is preceded by a non-polar glycine residue (aa 189) and a unique highly polar 5-aa sequence (KTHRE, aa 184–188) (Fig. 9). Although the KTHRE sequence is completely conserved in human, murine, and amphibian STR-3, the sequence differs in its entirety from the analogous non-polar aa sequence (GPGIG) in other MMP family members (Fig. 9, inset) (39). These data suggest that the tumor-specific and bFGF-mediated processing of STR-3 occurs via a mechanism unique to STR-3.

35-kDa STR-3 Lacks Specific N-terminal Residues Required for MMP Enzymatic Activity—Of note, 35-kDa STR-3 lacks specific N-terminal residues that have been implicated in MMP-mediated cleavage. These missing aa include a highly conserved Ala (Ala178 in STR-3) that plays an important role in the reaction mechanism and the secondary zinc ligands Asp164, Asp166, His179, and a calcium ligand Asp 171 that contribute to the structural integrity of MMPs (40, 41). Taken together, the epitope mapping (Fig. 3A) and preliminary structural and functional analyses (Figs. 3B and 9) suggest that 35-kDa STR-3 lacks components of the catalytic domain necessary for enzymatic activity.

![Fig. 8](image_url) SL-6 cells secrete STR-3 and process the mature active enzyme to a 35-kDa protein via a bFGF- and MMP-dependent mechanism in the absence of fibroblasts. The NSCLC cell line, SL-6, was cultured for 24 h in serum-free medium in the absence (A and B, control lane 1) or the presence of 5 μg/ml anti-bFGF mAb (A, abFGF, lane 2) or the indicated concentrations of BB-94 (B, lane 3 and B, lanes 2–8).

![Fig. 9](image_url) N-terminal sequence of 35-kDa STR-3. Three N-terminal sequences (aa 190–195, 192–194, and 194–199) were identified in the broad ~35-kDa STR-3 band. The most N-terminal 35-kDa STR-3 sequence (Glu190-Tyr194) is preceded by a non-polar glycine residue (aa 189) and a highly polar 5-aa sequence (KTHRE, aa 184–188) (Fig. 9). Although the KTHRE sequence is completely conserved in human, murine, and amphibian STR-3, the sequence differs in its entirety from the analogous non-polar aa sequence (GPGIG) in other MMP family members (inset) (39).
We have developed a tumor/stroma coculture assay in which NSCLC cells stimulate normal pulmonary fibroblasts to release STR-3 and the potent angiogenic peptide, bFGF (Fig. 7). In these cocultures, STR-3 is processed via a tumor-specific and bFGF-dependent mechanism to yield a major 35-kDa protein that lacks enzymatic activity (Fig. 7). Because 35-kDa STR-3 is the most abundant STR-3 protein in tumor/stroma cocultures and is only detected when normal pulmonary fibroblasts are cultured with malignant bronchial epithelial cells, these findings provide additional insights into the regulation and potential role of STR-3 in epithelial carcinomas.

STR-3 differs from other MMP family members in several key ways as follows: 1) the mature enzyme does not degrade common MMP substrates (8, 11); 2) the human STR-3 protein contains an amino acid substitution in the highly conserved MMP met turn which may alter its enzymatic activity (12); and 3) STR-3 undergoes tumor-specific processing at a highly conserved sequence that is specific to STR-3 (Fig. 9). In addition, large quantities of recombinant STR-3 are required to degrade the only described native substrate, α1-PI (Ref. 8 and Fig. 10). Taken together, these data suggest that STR-3 may not function in a manner analogous to other previously characterized MMPs and that the protein may have an as yet unidentified function.

The currently described coculture assays provide important additional clues regarding the unique nature of STR-3. In the tumor/stroma cocultures, tumor cells stimulate normal stromal cells to secrete STR-3 and mediate the processing of 35-kDa STR-3. Although 35-kDa STR-3 is the major form of the enzyme in conditioned media from tumor/stroma cocultures (Figs. 1 and 4), 35-kDa STR-3 was not previously detected in conditioned media from normal pulmonary fibroblasts or STR-3 Cos cells transfectants (8, 27). This is not surprising because the generation of 35-kDa STR-3 requires an interaction between tumor and stromal cells (Fig. 4). These observations underscore the utility of an in vitro assay which mimics the in vivo setting in which tumor cells invade the basement membrane and come into direct contact with normal stromal elements.

We performed the coculture assays with two types of normal pulmonary fibroblasts and either malignant bronchial epithelial cells or non-tumorigenic tracheal epithelial cells (Fig. 4). When normal pulmonary fibroblasts are cocultured with malignant bronchial epithelial cell lines, 45-kDa STR-3 is secreted and processed to the major 35-kDa protein. When fibroblasts are cocultured with non-tumorigenic tracheal epithelial cells, there is reduced but detectable secretion of 45-kDa STR-3; however, there is no detectable processing to 35-kDa STR-3 protein (Fig. 4, A and C, lane 2). The demonstrated enzymatic activity of STR-3 is so different from that of other MMPs that it remains to be determined whether 45-kDa STR-3 is functioning as a classic MMP in vivo. Furthermore, the unique nature of STR-3 processing and tumor-specific and bFGF-dependent generation of 35-kDa STR-3 prompt speculation regarding additional functions for the processed protein.

Our analyses of STR-3 induction and processing in tumor/stroma cocultures indicated that bFGF increased the processing but not the induction of STR-3. Although recombinant bFGF increased STR-3 transcript abundance in normal pulmonary fibroblasts in previous in vitro studies (7, 22), neutralizing bFGF antibodies did not inhibit the induction and secretion of STR-3 in tumor/stroma cocultures (Fig. 4, A and C, lane 14). However, neutralizing bFGF antibodies dramatically decreased the processing of STR-3 to the major 35-kDa protein in these assays (Fig. 4, A and C, lane 14). Because bFGF mediates the processing of STR-3 to the enzymatically inactive 35-kDa protein in tumor/stroma cocultures, we analyzed bFGF release in these conditions. As indicated in Figs. 4 and 5, tumor/stroma coculture increased the production and release of fibroblast-derived bFGF.

bFGF stimulates the growth and metastasis of many types of tumors (42–49) and promotes tumor angiogenesis (42, 50, 51). There are several alternatively spliced 18–23-kDa bFGF isoforms that are thought to have different mechanisms of action; the low molecular mass 18-kDa bFGF isoform is most likely to be released and to interact with high affinity cell-surface receptors (52, 53). Consistent with these observations, 18-kDa bFGF is the primary isoform detected in conditioned media from tumor/stroma cocultures (Fig. 5A). However, bFGF lacks a classical leader sequence and appears to be released by novel secretory mechanisms (50, 54). For these reasons, the tumor/stroma cocultures may represent a useful model system in which to analyze potential mechanisms of bFGF release.

Because bFGF stimulates the production of multiple proteinases including MMPs and serine proteinases of the plasmin activator/plasmin system (2, 3, 36), we explored the possibility that bFGF-mediated STR-3 processing occurred via an additional MMP or plasmin. The broad spectrum MMP inhibitor (BB-94) inhibited the generation of 35-kDa STR-3 in tumor/stroma cocultures indicating that bFGF-mediated processing of 35-kDa requires additional MMP activity. In tumor/stroma cocultures, the major processed form of STR-3 occasionally appears as a ~37/35-kDa STR-3 doublet (Figs. 4 and 5), prompting speculation that STR-3 may undergo initial MMP-mediated cleavage and subsequent additional processing. The identification of three related 35-kDa STR-3 N-terminal amino acid sequences (Fig. 9) is also consistent with this observation. Recently, other MMP family members have also been reported to undergo initial MMP-mediated cleavage and subsequent autocatalytic processing (37, 38).

In summary, the data derived from the tumor/stroma coculture assay provide additional evidence regarding the unique nature of STR-3. STR-3 differs from other MMP family members in its initial activation (9), substrate specificity, and biological activity (9, 10) and near-uniform overexpression in epithelial malignancies (20–25). The current studies demonstrate...
that in tumor/stroma cocultures, STR-3 is also processed at a unique internal sequence via a tumor-specific and bFGF- and MMP-dependent mechanism to a major 35-kDa protein that lacks enzymatic activity. The regulatory nature of the major 35-kDa STR-3 protein and the relationship between bFGF and STR-3 in tumor/stroma cocultures and primary epithelial malignancies will be of further interest.

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