Purification of a Novel Protein (ps20) from Urogenital Sinus Mesenchymal Cells with Growth Inhibitory Properties in Vitro*

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David R. Rowley‡‡, Truong D. Dang, Melinda Larsen‡, Michael J. Gerdes, Lauren McBride, and Bing Lu

From the Department of Cell Biology and ‡Cell and Molecular Biology Program, Baylor College of Medicine, Houston, Texas 77030

Our previous studies have characterized mesenchyme-derived proteins to identify biologically active proteins and novel markers for stromal cell paracrine action relative to stromal-epithelial interactions. Previous reports have characterized properties of a growth inhibitory activity (to bladder and prostatic epithelial cells), secreted by U4F fetal rat urogenital sinus mesenchymal cells, not cross-reactive with antibodies to known cytokines, and provisionally termed UGIF. The present study reports the characterization, purification, and biological properties of a 20–21-kDa protein responsible for UGIF activity. The 20–21-kDa protein (termed ps20) was purified to near homogeneity, the amino-terminal sequence was determined, and biological properties were characterized in vitro. Amino-terminal sequence analysis indicated no direct matches or regions of homology with known proteins. Purified ps20 induced a linear and saturable inhibition of [3H]thymidine incorporation in PC-3 prostatic carcinoma cells (half-maximal activity at 2.6 nM), inhibited cell proliferation (increased population doubling time from 19.8 to 25.8 h), and induced a 210% stimulation in the synthesis of secreted proteins. These data suggest that ps20 may be a candidate paracrine effector protein and may play a role in stromal-epithelial cell interactions in the prostate gland.

The induction of epithelial cell growth and differentiation patterns by adjacent mesenchymal cells is a common feature in the organogenesis of many tissues. Such interactions occur in reproductive tissues including seminal vesicle development (1, 2), in the androgen-induced regression of male mammary gland (3), and in estrogen-induced proliferation of mammary gland epithelial (4), vaginal epithelial, and uterine epithelial cells (5). Stromal-epithelial interactions have been particularly well studied in the morphogenesis of fetal urogenital sinus (UGS) to mature prostate gland (6, 7). UGS epithelial cells progress through a specific morphogenesis pattern (prostatic glandular acini) only when recombined with UGS mesenchymal cells (8–10). Similar studies addressing the development of differentiated epithelium in skin (11), gut (12), and lung (13) have shown a likewise potent inductive nature of stromal cells in directing tissue-specific epithelial growth and differentiation patterns.

In postnatal and fully differentiated adult tissues, stromal-epithelial interactions likely maintain histological architecture and differentiated phenotype relative to ongoing modeling and remodeling processes (6). Fully differentiated adult epithelial cells are capable of responding to a heterotypical (different) stroma. In this regard, adult bladder transitional epithelium can be induced by urogenital sinus mesenchyme to change to a prostate-specific epithelial cell phenotype including the expression of prostate-specific proteins, including androgen receptor (14, 15). In addition, abnormal patterns of epithelial cell proliferation and differentiation in neoplastic disease progression are also affected by the origin and type of adjacent stroma. The growth of prostatic Dunning tumor adenocarcinoma was inhibited by 7-fold, and the morphology was altered to a more normal phenotype when recombined with UGS mesenchyme and grown in vivo (16). Similarly, the recombination of normal UGS mesenchyme with bladder transitional cell carcinoma resulted in a change of tumor histopathology to an adenocarcinoma phenotype (17). Moreover, the implantation of UGS mesenchyme directly into the adult mouse prostate gland resulted in an induced hyperplastic phenotype typical of benign prostatic hyperplasia (18). These studies together support the suggestion that stromal-epithelial interactions are likely to be central in modulating the progression and histopathology of epithelial cells in proliferation- and differentiation-related diseases such as cancer and benign hyperplasia (19).

Although stromal cell induction of epithelium is critical for organogenesis and growth control in adult tissues, little is understood regarding paracrine effector molecules, fundamental molecular mechanisms of stromal-epithelial interactions, and the basic biology of UGS mesenchymal cells. Indeed, there is currently no well defined set of parameters or markers to clearly identify the stages of mesenchymal cell differentiation to adult stromal cells (fibroblasts, myofibroblasts, or smooth muscle), or the specific roles of these cell types in continued stromal-epithelial interactions. To identify putative mediators and markers of UGS mesenchymal cell action, we have reported previously the development of organ cultures and mesenchymal cell lines from fetal rat urogenital sinus (20–22). Urogenital sinus mesenchymal cell lines (U4F and U4F1) were adapted into chemically defined medium and characterized for stromal marker proteins (21, 22). Initial studies identified in U4F cell-conditioned medium, a growth inhibitory activity to PC-3 prostatic carcinoma epithelial cells, NBT-II bladder epithelial cells, and My-Lu-1 mink lung epithelial cells in vitro (20, 21). This activity did not cross-react with a battery of neutralizing antibodies to cytokines (including transforming...
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EXPERIMENTAL PROCEDURES

Materials — The following materials were purchased: DMEM, Ham's F-12 medium, Hank's solution, penicillin, and streptomycin from Life Technologies, Inc.; Nu-Serum and epidermal growth factor from Collaborative Research (Lexington, MA); fetal bovine serum from HyClone (Logan UT); MCD-110 basal medium, insulin, trypsin type II, crystal violet dye, trypsin blue dye, testosterone, and sodium lauryl sulfate from Sigma; methanol, trichloroacetic acid, EDTA, ammonium carbonate, and ammonium sulfate from Fisher; glacial acetic acid from Chempure (Curtin Matheson, Houston, TX); [3H]Thymidine and [35S]methionine from ICN Radiochemicals (Irvine, CA); aqueous counting scintillant (BSC, no. NBC5104) from Amersham Corp.; Bio-Gel P-100 (100–200 mesh) and P-30 (100–200 mesh) chromatography gels, glycine, and acrylamide from Bio-Rad; diethylaminoethyl cellulose (DE-52) ion exchange resin and glass fibers (934-AH, 2.4 cm) from Whatman, no. 76-003-05 and 24 (no. 76-003-05) well culture plates from Flow Laboratories (McLean, VA); 25 cm² (no. 25101) culture flasks from Falcon (no. 25100) culture flasks from Flow (no. 25100). Crude UGIF activity was isolated from conditioned medium, having biological properties identical to the previously characterized UGIF activity. Based on the cell type of origin and the biological activity in vitro, this protein may be relevant to growth and differentiation mechanisms of stromal-epithelial interactions and may serve as a useful marker for the study of mesenchymal cell ontogeny.

Cell Culture — The U4F urogenital sinus mesenchymal cell line, derived originally from UOS organ explants (20), was cultured in medium BFs (90% DMEM, 5% fetal bovine serum, 5% Nu-Serum, 5 µg/ml insulin, 0.5 µg/ml testosterone, 100 units/ml penicillin, and 100 µg/ml streptomycin), which was replaced every 48 h as previously reported (21). Upon confluence, U4F cultures typically formed multilayered spheroids (multicellular, spherical domes) after 20–30 days in culture, with UGIF activity first detectable in conditioned medium from spheroid-containing cultures (21). To generate sufficient quantities of conditioned medium for analysis of UGIF activity, 75-cm² culture flasks were seeded with U4F cells yielding 100–200 spheroids per flask by 30 days in culture. U4F spheroid cultures were maintained for approximately 3–6 months in this manner with conditioned media collected every 48 h, pooled, quick frozen, and used as starting material for analysis of UGIF activity. For preparative purification of UGIF protein(s), spheroid-containing conditioned medium (MCD-110 basal medium plus 5 µg/ml insulin, 5 µg/ml transferrin, 5 ng/ml sodium selenite, 0.1 µg/ml epidermal growth factor, and 0.5 µg/ml testosterone), formulated empirically to support U4F proliferation and secretion of UGIF activity as reported elsewhere (21). Conditioned medium was collected every 48 h, clarified by centrifugation, and either used immediately or stored at −20°C until subsequent use.

The U4F cell line (ATCC CRL 1435, prostatic carcinoma epithelial) and Mv-Lu-1 cell line (ATCC CCL 64, mink lung epithelial) were received from American Type Culture Collection (Rockville, MD). PC-3 cells were cultured in 93% DMEM/Ham's F-12 medium (1:1), supplemented with 7% fetal bovine serum, and Mv-Lu-1 cells in 90% DMEM, supplemented with 10% fetal bovine serum, with each containing penicillin (55 units/ml) and streptomycin (25 µg/ml). Medium was replaced every 3–4 days. Cultures were grown in a confluent buffer to trypsin-EDTA (0.25% trypsin, 0.025% EDTA in calcium–magnesium-free Hanks' salt solution). Cell viability was established by trypsin blue dye exclusion and cells counted with an improved Neubauer type hemocytometer. All cell lines were routinely tested for mycoplasma contamination (MycoplTest Kit, Life Technologies, Inc.).

Cell Proliferation Assay — Cell growth activity using the [3H]Thymidine incorporation assay following minor modifications of procedures we reported previously (20–23). For the [3H]Thymidine incorporation assay, PC-3 or Mv-Lu-1 target epithelial cells were seeded at 8.1 × 10⁵ cells/148 µl/well in 96-well plates and allowed to attach for 24 h. Wells received a 52-µl aliquot of sample to be tested (previously vials and resolubilized in growth medium in sterile conditions) (200 µl/well final total volume) and were allowed to incubate for an additional 24 h. Cultures were pulsed with [3H]Thymidine (2 µCi/ml) during the final 3 h of incubation. The assay was terminated by fixing cell monolayers in situ with methanol:acetic acid (3:1) (200 µl/well) for 10 min (22°C), washing with 100% methanol (5 min, 22°C) followed by a wash with 5% trichloroacetic acid (5 ml, 4°C), and then washing with methanol (22°C, 200 µl/well). Plates were then allowed to dry for 5–10 min under a heat lamp. Plates could either be stored indefinitely at 22°C or processed immediately. For processing, monolayers were hydrolyzed by 1 N NaOH (200 µl/well, 5 min, 22°C), and aliquots (180 µl) were added to scintillation vials containing 180 µl of a 1 N HCl to neutralize pH, precipitated with trichloroacetic acid, and determined by scintillation counting. Results are presented as the reciprocal of incorporated counts/min (1/cpm) where indicated in the figure legends, to reflect inhibition of [3H]Thymidine incorporation as a peak of activity. Results from all comparative assays are presented as the mean of n ≥3 tests ± S.E. (error bars), and results were analyzed for significance using Student's t-test.

For direct cell counting, PC-3 cells were seeded at 4.0 × 10⁵ cells/148 µl/well in 96-well plates and allowed to attach for 24 h. Wells received 52-µl aliquots of test sample and were allowed to incubate for 5 days. Cultures received fresh medium plus/minus test sample every 48 h. At each 24-h interval, cells were released by exposure to 0.25% trypsin, 0.025% EDTA in calcium, magnesium-free Hanks' solution (300 µl/well) for 4 min. To each well were added 70 µl of PC-3 cell growth medium plus 7% fetal bovine serum, 40 µl of the cell suspension was incubated with trypsin blue (4 min, 22°C), and total and viable cells were counted as described previously (20).

Protein Synthesis Assay — Protein synthesis was assayed by incorporation of [35S]methionine according to procedures published previously (20–23). Cultures were seeded identically to the cell culture procedure. On day 2–4 of exposure to test samples, cultures were pulsed with [35S]methionine (10 µCi/ml) for 24 h. The medium (200 µl) was harvested and added to an equal volume of 20% trichloroacetic acid for 1 h at 2°C to precipitate proteins. Aliquots (350 µl) from each sample were added to glass fiber filters (934-AH, 2.4 cm, Whatman) fitted to a filter vacuum manifold (Millipore). Filters were washed sequentially three times with 2 ml of EOB (−20°C) to remove unincorporated label, filters were added to scintillation vials, and incorporated radioactivity was determined by scintillation counting. Cell number was counted from each corresponding set of wells using the direct cell counting method, and results were expressed as disintegrations/min [35S]methionine incorporation/10⁶ cells to standardize results to cell number.

Gel Filtration Chromatography — For analytical studies to assign a protein to the peak of biological activity, 50–200 µl of U4F conditioned medium BFs (serum-containing) were dialyzed against 1 M acetic acid (pH 2.25) and lyophilized. Lyophilized proteins were resolubilized in 1 M acetic acid (1–3 ml) and chromatographed through a...
Bio-Gel P-200 or P-100 gel filtration column (2.5 × 70 cm), equilibrated in 1 M acetic acid, by gravity flow (45 cm), and 3-m fractions were collected. Aliquots (100 µl) were vacuum-dried and resolubilized in 65 µl of medium Bfs and 52-µl aliquots were added to PC-3 cells for [³H]thymidine incorporation assay.

For additional analytical studies and for scaled-up preparative purification of ps20, samples derived from serum-free, chemically defined conditioned medium M₂ were processed through the ion exchange chromatography step as described. Pooled fractions from the biologically active peak were prepared by dialysis (Spectrapore no. 3 tubing, 3,500 M₀ cutoff) against 4 liters of 1 M acetic acid (pH 2.25 overnight at 4 °C). Dialed samples were frozen and lyophilized and either used immediately or stored at −20 °C until use. Lyophilized samples were resolubilized in 1 M acetic acid (1 ml) and applied to either a Bio-Gel P-100 column or P-30 column (1.4 × 70 cm) equilibrated in 1 M acetic acid (pH 2.25). Proteins were eluted with a hydrostatic pressure of 55 cm, and 1.4-ml fractions were collected. Aliquots (100 µl) from each fraction were vacuum-dried in sterile microcentrifuge tubes and resolubilized in 65 µl of medium Bfs, and 52-µl aliquots were added to PC-3 cells for [³H]thymidine incorporation assay.

Reverse Phase High Performance Liquid Chromatography — All samples for HPLC were first chromatographed through either ion exchange chromatography and/or gel filtration chromatography prior to HPLC analysis. Samples (pooled fractions from bioactivity peak) were either vacuum dried or lyophilized and resolubilized in 50% formic acid (0.5 ml). Samples were applied (three consecutive 150-µl applications) to a Waters C-18 reverse phase column fitted to an HPLC system composed of a Waters 22060 Bar underlining and a Waters 215 photodiode array detector. Samples were vacuum-dried, resolubilized in 1 M acetic acid, by gravity flow (45 cm), and 3-ml fractions were collected. Aliquots (100 µl) from each fraction were vacuum-dried, resolubilized in 65 µl of medium Bfs, and 52-µl aliquots were added to either PC-3 cells or Mv-Lu-1 cultures for the [³H]thymidine incorporation assay.

SDS-PAGE Electroelution — Samples were vacuum-dried, resublimed in Laemmli sample buffer (24) and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) following the procedures of Laemmli (24). Samples were heated (5–10 min, 95 °C), 60–100-µl sample aliquots loaded per lane, and electrophoresed through the stacking gel at 40 mA×m and through the running gel (15% acrylamide) at 70-mA×m continuous current. Proteins were visualized using silver staining procedures as described previously (22). Protein determinations of highly purified samples below detectable range with Bradford analysis (<1 µg) were determined by comparative analysis of sample lane with sets of protein standards 50–500 ng/lane in SDS-PAGE gels and an assignment was made based on relative staining density after silver staining. For renaturation of activity after SDS-PAGE, gels were washed in double-distilled water, protein standards position was determined by incubation in 1 M KCl at 4 °C for visualization of opaque bands, and protein bands of interest were precisely excised in 1–2-mm slices. Excised proteins were renatured following minor modifications of the guanidine-HCl method (25) or were directly eluted from gels with 1 M acetic acid overnight (agitation, 22 °C), vacuum-dried, and processed for biological assay as described.

Protein Sequencing — Substantially purified ps20 protein samples were electrophoresed through SDS-PAGE gels as described, except 100 mM thiglycolic acid was added to the cathode buffer, and gels were preelectrophoresed for 20 min at 50 V to reduce amino-terminal blockage. The gel was incubated in transfer buffer (25 mM Tris-borate, 192 mM glycine, pH 8.5) for 5 min and assembled with Immobilon-P or Pro-Blott PVDF membranes in a Bio-Rad Trans-Blot cell apparatus according to the manufacturer’s recommendations. Proteins were electroblotted using a Bio-Rad system for 18 h at 4 °C. Proteins were visualized by Coomassie staining, the ps20 protein bands were excised, and the sequence was determined using Applied Biosystems 477A and 773A protein sequencers.

RESULTS

Assignment of UGIF Activity to a Protein Species — Initial studies were conducted to determine whether a putative protein species or set of proteins could be assigned to the peak or peaks of UGIF biological activity in order to assess feasibility of purification and focus on a particular protein for preparative purification. Accordingly, gel filtration chromatography and reverse phase HPLC were used to analyze UGIF activity, and eluted proteins were analyzed with SDS-PAGE. UGIF activity was harvested from U4F mesenchymal cell-conditioned medium following conditions reported previously (20, 21). UGIF activity in conditioned medium was first detectable from confluent U4F cultures having small multicellular spheroids, which formed at approximately 25 days of culture, and activity continued to increase with the development of larger U4F cell spheroids as reported previously (21). Identical to previous reports, UGIF activity in CM collected from these long term cultures did not cross-react with neutralizing antibodies to a variety of cytokines including transforming growth factor-βs, interleukin-6, and interferons (21).

Ammonium sulfate precipitation of proteins from conditioned medium was used as a first step to concentrate samples. Approximately 90% of growth inhibitory activity precipitated within the 20–40% saturation range of ammonium sulfate (data not shown), which was then used for subsequent procedures. For analysis of UGIF bioactive proteins, all chromatography buffers were formulated to be both volatile (no salt residues upon vacuum drying) and bacteriostatic (sterile, non-supportive of bacterial growth) so that aliquots from gel filtration or HPLC columns could be vacuum-dried in sterile vials and used directly for biological assay (addition to target epithelial cells in culture) without additional steps of dialysis and sterilization of sample. This strategy allowed for the biological assay of hundreds of fractions per day. For initial analysis of UGIF biological activity, precipitated proteins from conditioned Bfs medium (conditioned by U4F cells for 48 h) and unconditioned control Bfs medium were resolubilized in ammonium carbonate buffer. The initial chromatography step to analyze biological activity utilized ion exchange chromatography. Samples were dialyzed against 20 mM ammonium carbonate (pH 8.85), applied to and eluted from a DE-52 anion-exchange column according to the methods under “Experimental Procedures.” Fig. 1 shows the biological activity and A290 protein elution profiles. The elution of a major growth inhibitory peak was observed from the 48-h conditioned medium preparations. In comparison, the control (fresh medium) sample did not produce a growth inhibitory peak and exhibited an otherwise similar baseline activity and A290 elution pattern. These studies indicated the UGIF activity peak was produced by U4F cells and was not a constitutive component of fresh Bfs medium.

Fractions from ion exchange representing the major peak of eluted biological activity (delineated by the bar underlining fractions in Fig. 1) were collected and pooled. The pooled sample was dialyzed against 1 M acetic acid (pH 2.5, 4 liters) overnight at 4 °C. Dialyzed samples were quick frozen, lyophilized, and used either directly or stored at −20 °C. UGIF activity prepared in this manner was further analyzed by gel filtration chromatography for assignment of size using a variety of buffer conditions including 1 M acetic acid, ammonium carbonate, and ammonium acetate. Of these conditions, gel filtration chromatography in 1 M acetic acid reduced the interaction with column matrix optimally and allowed for a reproducible recovery of an activity peak as shown in Fig. 2. Biological activity was detected as a single major peak, eluting consistently in the calculated 18–20-kDa size range (position of molecular mass markers is shown across the top of graph, Fig. 2). SDS-PAGE analysis of eluted fractions (Fig. 2, lower panel) showed the elution pattern of a 20–21-kDa protein to be directly correlated with the elution peak of biological activity (fraction 56, arrow). To further establish the correlation of this protein species with peak activity, additional samples were pooled, chromatographed through C18 reverse phase HPLC columns, and eluted with a linear gradient of acetonitrile as shown in Fig. 3. In direct agreement with gel filtration, the
major peak of UGIF activity from HPLC was associated with a protein of approximately 20–21 kDa as analyzed by SDS-PAGE (Fig. 3, lower panel, arrow, peak fraction 93).

To facilitate the scale up of preparations to allow for the purification of the 20–21-kDa species, a chemically defined (serum-free) growth medium (medium M₃) was developed empirically to lower protein complexity in the starting conditioned medium. The M₃ medium supported U4F spheroid growth and production of UGIF activity as described under "Experimental Procedures." To determine whether the 20–21-kDa protein was responsible for UGIF activity in conditioned M₂ medium, biological activity eluted as a single peak and fractions were pooled and processed for analytical gel filtration chromatography and HPLC as described in Figs. 2–3. Fresh medium preparations were negative for biological activity. Bottom panel shows the corresponding A₂₈₀ pattern of total protein.

**Fig. 1. Ion exchange chromatography.** Proteins from U4F cell 48-h conditioned medium (serum-containing media Bfs) or volume-matched fresh Bfs (as control) were precipitated with (NH₄)₂SO₄ and chromatographed through a DE-52 anion exchange column. Aliquots (100 µl) from each fraction were vacuum-dried and assayed for inhibition of [³H]thymidine incorporation in PC-3 cells as described under "Experimental Procedures." Activity was plotted as reciprocal of incorporated counts/min (1/cpm) to illustrate inhibition of [³H]thymidine incorporation as a peak of activity. With conditioned medium samples, activity eluted as a single peak and fractions were pooled and processed for analytical gel filtration chromatography and HPLC as described in Figs. 2–3. Fresh medium preparations were negative for biological activity. Bottom panel shows the corresponding A₂₈₀ pattern of total protein.

**Fig. 2. Analytical gel filtration chromatography.** Proteins from U4F mesenchymal cell conditioned medium (Bfs medium) were precipitated with (NH₄)₂SO₄, chromatographed through DE-52 as described in Fig. 1, and analyzed with gel filtration chromatography, and fractions were assayed for biological activity with PC-3 cells as described in Fig. 1 and under "Experimental Procedures." Upper panel, elution profile of biological activity from P-100 gel filtration column. Biological activity eluted as a single peak (maximum activity at fraction 56) associated with the 18–20-kDa size region. The elution position of molecular size markers are shown across the top of the graph: bovine serum albumin (66 kDa), chymotrypsinogen A (27.5 kDa), soybean trypsin inhibitor (20.1 kDa), and lysozyme (14.3 kDa). Lower panel, SDS-PAGE analysis of eluted fractions. Fractions (underlined by bar, top panel) from gel filtration were vacuum dried, electrophoresed through a 15% acrylamide gel, and stained with the silver method as described. The elution pattern of a 20–21-kDa species (arrow, fraction 56, bottom panel) correlated directly the position of eluted bioactivity peak. Molecular size markers are shown in lanes 1 and 10: myoglobin fragment I (8.16 kDa), myoglobin (16.9), soybean trypsin inhibitor (20.1 kDa), carbonic anhydrase (29 kDa), ovalbumin (43 kDa), and bovine serum albumin (66 kDa).
likely represent variable degrees of 20-21-kDa protein breakdown products, as they were derived from previous step 20-21-kDa protein was monomeric in structure as analyzed in either staining. The purification procedure as described yielded approximately 700 ng to 1 µg) pooled from multiple purification preparations was electrophoresed through SDS-PAGE gels, blotted to PVDF membranes, and analyzed for amino-terminal sequence as described under "Experimental Procedures." A single sequence was detected with unambiguous assignments made for positions 1–14 and 19–28 as follows: NH2-Thr-Trp-Glu-Ala-Met-Leu-Pro-Val-Arg-Leu-Ala-Glu-Lys-Ser-Xaa-Xaa-Xaa-Xaa-Val-Ala-Ala-Thr-Gly-Xaa-Arg-Gln-Pro-His. Analysis with PDB, SwissProt, PIR, SPUpdate, GenPept, and GUPdate data bases indicated no regions of direct match or homology with previously characterized proteins. The 20–21-kDa protein was hereafter referred to as ps20 (20-kDa prostate stromal protein).

Biological Activity of ps20—To confirm growth inhibitory activity was endogenous to the ps20 protein, renaturation experiments were conducted with purified ps20 protein extracted from SDS-PAGE gels of samples generated from HPLC steps. Samples purified following the above steps were electrophoresed through an SDS-PAGE gel. The ps20 protein band was precisely excised (1-mm width gel band) as well as control gel bands containing either no loaded protein or protein controls (11-kDa range). The samples were either electroeluted from the gel slices, dialyzed, and renatured following modifications of the guanidine-HCl method (25), or were passively eluted from crushed gel slices in 1 M acetic acid and dialyzed following procedures described under "Experimental Procedures." The ps20 protein renatured with either of these procedures produced significant (p < 0.01) growth inhibitory activity relative to the control (Fig. 4). The purification and activity analyses focused exclusively on the 20–21-kDa species.

The fourth purification step utilized reverse phase HPLC owing to the utility of HPLC in separating proteins of similar size based on hydrophobic properties. Pooled fractions from gel filtration chromatography (front consistent peak, Fig. 5, fractions 52–55) were vacuum-dried, resolubilized in 50% formic acid, and analyzed with reverse phase HPLC as described under "Experimental Procedures." The column was eluted with a shallow gradient of acetonitrile to produce optimal separation of major peak versus inconsistent minor peak proteins. Fig. 6 shows the biological activity elution profiles and the corresponding SDS-PAGE analysis associated with peak activity. Biological activity eluted as a well defined and consistently observed front peak (fraction 20–23, peak = fraction 22), which was directly correlated with the elution pattern of the 20–21-kDa protein species (Fig. 6, lower panel), purified to near homogeneity as determined by SDS-PAGE analysis and silver staining. The purification procedure as described yielded approximately 600–650 ng of the 20–21-kDa protein from 600 ml of conditioned medium. Subsequent analyses showed a consistent purification to near homogeneity and indicated the 20–21-kDa protein was monomeric in structure as analyzed in either reducing or nonreducing SDS-PAGE conditions. Similar to observations from the previous gel filtration step, a second broad peak or set of peaks (fractions 25–34) of less well defined and highly variable elution patterns and activity levels, eluted at higher acetonitrile concentrations. These inconsistent peaks likely represent variable degrees of 20–21-kDa protein breakdown products, as they were derived from previous step 20–21-kDa protein peak fractions and were observed only in scaled-up preparations in later stages of purification.

For microsequence analysis, the 20–21-kDa protein (approximately 700 ng to 1 µg) pooled from multiple purification preparations was electrophoresed through SDS-PAGE gels, blotted to PVDF membranes, and analyzed for amino-terminal sequence as described under "Experimental Procedures." A single sequence was detected with unambiguous assignments made for positions 1–14 and 19–28 as follows: NH2-Thr-Trp-Glu-Ala-Met-Leu-Pro-Val-Arg-Leu-Ala-Glu-Lys-Ser-Xaa-Xaa-Xaa-Xaa-Val-Ala-Ala-Thr-Gly-Xaa-Arg-Gln-Pro-His. Analysis with PDB, SwissProt, PIR, SPUpdate, GenPept, and GUPdate data bases indicated no regions of direct match or homology with previously characterized proteins. The 20–21-kDa protein was hereafter referred to as ps20 (20-kDa prostate stromal protein).

Biological Activity of ps20—To confirm growth inhibitory activity was endogenous to the ps20 protein, renaturation experiments were conducted with purified ps20 protein extracted from SDS-PAGE gels of samples generated from HPLC steps. Samples purified following the above steps were electrophoresed through an SDS-PAGE gel. The ps20 protein band was precisely excised (1-mm width gel band) as well as control gel bands containing either no loaded protein or protein controls (11-kDa range). The samples were either electroeluted from the gel slices, dialyzed, and renatured following modifications of the guanidine-HCl method (25), or were passively eluted from crushed gel slices in 1 M acetic acid and dialyzed following procedures described under "Experimental Procedures." The ps20 protein renatured with either of these procedures produced significant (p < 0.01) growth inhibitory activity relative to the control (Fig. 4).
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Fig. 5. Preparative gel filtration chromatography. Upper panel, the eluted peak of biological activity from column chromatography using serum-free conditioned medium (48 h, medium Mn, preparative volumes, ≥600 ml) were chromatographed through a P-30 gel filtration column and aliquots assayed for biological activity with PC-3 cells as described in the text and under “Experimental Procedures.” Activity eluted in two peaks. An early front peak consistently eluted at fraction 53, in the 18–21-kDa size region (molecular size markers shown across top, as in Fig. 2). A set of secondary, variable and inconsistent peaks eluted in fractions 58–63 as shown. Lower panel, fractions (bar region, upper panel) were analyzed by SDS-PAGE and silver staining. Note the elution pattern of the 20–21-kDa protein (fraction 53, arrow) directly correlated with the front activity peak as shown in the upper panel. The front peak material was pooled (fractions 52–55) and used for reverse phase HPLC (Fig. 6). Molecular size markers are shown in lane 1 and as indicated in Fig. 2.

Fig. 6. Preparative HPLC chromatography. Upper panel, fractions from gel filtration chromatography as shown in Fig. 5 were pooled (fractions 52–55) and used for reverse phase HPLC as described in the text and under “Experimental Procedures.” Shown is the elution profile of biological activity with PC-3 cells and acetotriulur elution gradient. Biological activity eluted with a consistently observed front peak (fraction 22) and highly variable secondary peak region (fractions 25–34). Lower panel shows the SDS-PAGE analysis and silver staining of proteins in entire eluted fractions (bar region). The elution pattern of the 20–21-kDa protein correlated directly with the initial peak (fractions 20–23) and was purified to near homogeneity. Molecular size markers (shown in lane 1) are as indicated in Fig. 2.

to controls as shown in Fig. 7. Renaturation of ps20 extracted with 1 M acetic acid produced a 15.4% inhibition of \( ^{3}H \)thymidine incorporation over control (Fig. 7A). Guanidine-HCl renaturation of ps20 (Fig. 7B) produced up to a 30.3% inhibition relative to controls. With either procedure in any particular experiment, renaturation efficiency after SDS-denaturation was maximally 15–20% relative to activity levels of samples prior to SDS-PAGE, similar to expected efficiencies of renaturation for proteins extracted and processed from denaturing SDS-PAGE gels (25).

To determine biologically active concentrations, purified ps20 was used for dose-response assays with target PC-3 cells, and exhibited linear and saturable dose-response curve as shown in Fig. 8. Half-maximal activity was observed at 55 ng/ml (2.62 nM) under these conditions. Maximal (saturable) inhibition was observed at 6.3–8 nm. To correlate inhibition of \( ^{3}H \)thymidine incorporation with inhibition of cell proliferation, PC-3 cells were incubated with 7.2 nm ps20 or vehicle control for 5 days, and cells were counted every 24 h as shown in Fig. 9A. Under these conditions, PC-3 cell proliferation was inhibited by 52–60% relative to control at any particular time point, in general agreement with the maximal percent inhibition of \( ^{3}H \)thymidine incorporation (approximately 70%) at 7.2 nm shown in Fig. 8. Under these conditions, the population doubling time of subconfluent PC-3 cells (days 1–3) was increased from an average of 19.8 h in control cultures to 25.8 h in ps20-treated cultures. In addition, ps20-treated cultures attained confluence at lower cell densities (days 3–5), due to a change in cell shape to a larger, more spread-out phenotype as discussed below. The ratios of viable to nonviable cells were identical between control and ps20 treated cultures indicating the results were not due to increased cell death or toxicity, in agreement with our previous reports using crude UGIF activity (21). The inhibition of cell proliferation was associated with a stimulation of protein synthesis. As shown in Fig. 9B, purified ps20 (7.2 nm) stimulated the synthesis of secreted proteins from PC-3 cells by 210.4% relative to control on a per cell basis. In addition, the ps20-treated (7.2 nm) PC-3 cells assumed a larger spread-out cell shape with increased pseudopodia and filopodia cell extensions compared to control cultures (data not shown). The effects of purified ps20 on growth inhibition, stimulation of protein synthesis, and alteration in morphology are each consistent with previous reports on UGIF activity in crude conditioned medium (20, 21). Based on these data, the activity ascribed to ps20 protein likely accounts for the previously observed UGIF activity in crude conditioned medium.

DISCUSSION

Data presented in this study shows the characterization and purification of a novel 20–21-kDa protein (termed ps20) derived from fetal urogenital sinus mesenchymal cells and exhibiting growth inhibitory and protein synthesis stimulatory activities in vitro in a dose-dependent and saturable manner, suggesting responses are mediated through saturable pathways. Amino-terminal sequence information revealed a unique sequence with no matches to proteins in data bases, indicating ps20 represents a novel protein species. Additional studies beyond the scope of the present report are required to generate probes and determine the specific pattern of ps20 expression in mesenchymal cell ontogeny, the molecular mechanisms of action in stromal-epithelial interactions, and the extent of target interactions on mesenchyme-epithelium interactions.
paracrine factors, matrix molecules, and direct cell-cell communication each are likely to contribute to the overall mechanisms of stromal induction of epithelial phenotype. The actions of ps20 may be a component of any such mechanism. Although cell-type specificity. Results here report the activity of ps20 with PC-3 (human prostatic carcinoma) and Mv-Lu-1 (mink lung epithelial) assay target cells. Our previous studies with crude preparations showed identical activities with NBT-II (rat bladder epithelial cells) and Y-79 cells (human retinoblastoma cells), in addition to PC-3 and Mv-Lu-1 cells (20, 21). The full range of specific cell types responsive to ps20 activities is not yet known and will require greater yields of native ps20 or recombinant protein to address fully.

The in vitro responses elicited by the ps20 protein are consistent with a potential function in stromal-epithelial interactions involving tissue growth and differentiation control in the prostate gland. Extracellular matrices produced by stromal cells, direct cell-cell contact, and secretion of paracrine-acting effectors have each been shown to induce and/or facilitate tissue-specific gene expression and epithelial cell phenotype in a wide variety of tissues. Accordingly, the combined influences of paracrine factors, matrix molecules, and direct cell-cell communication each are likely to contribute to the overall mechanisms of stromal induction of epithelial phenotype. The actions of ps20 may be a component of any such mechanism. Although extracellular protein which also stimulates protein synthesis by purified ps20. Panel A, PC-3 cells were incubated with either 7.2 nM purified ps20 (maximal active concentration) or vehicle control for 5 days and counted each 24 h as described under “Experimental Procedures.” Purified ps20 produced a 52–60% decrease in PC-3 cell proliferation, increased population doubling time from 19.8 to 25.8 h (days 1–3), and achieved confluence at a lower cell density. Panel B, PC-5 cells were incubated with 7.2 nM purified ps20 or vehicle control for 3 days and pulsed with [35S]methionine (10 μCi/ml) the final 24 h, cells were counted, and proteins from the medium were assayed for [35S]methionine incorporation as described under “Experimental Procedures.” Presented are disintegrations/min of incorporated [35S]methionine/10^4 cells. Values are mean of n = 3 separate determinations, ±S.E.

FIG. 7. Renaturation of ps20 activity from SDS-PAGE. The ps20 protein isolated by HPLC was electrophoresed through SDS-PAGE, and the ps20 protein band was excised and renatured by the guanidine HCl method or the gel crushed and proteins extracted with 1 M acetic acid as described in the text and under “Experimental Procedures.” Shown in each panel is the inhibition of [3H]thymidine incorporation (1/cpm) in PC-3 cells induced by renatured ps20 (column C) relative to controls (columns A, B, and D) as described below. Panel A, gel extracted in 1 M acetic acid. A, media control; B, extracted gel (no protein) control; C, extracted ps20 protein; D, acetic acid control. Panel B, gel-extracted proteins were renatured with the guanidine HCl method. A, media control; B, extracted gel control (no protein); C, extracted ps20 protein; D, extracted gel control (arbitrary 11-kDa protein band). Values are mean of n = 3 separate determinations ± S.E. Column C is statistically significant (p < 0.01) in each experiment.

FIG. 8. Dose-response of purified ps20 activity. The ps20 protein was purified as described in Figs. 1–6, and final protein yield was determined as described under “Experimental Procedures.” Increasing concentrations of purified ps20 were added to PC-3 cells, and cells were assayed for [3H]thymidine incorporation as described under “Experimental Procedures.” Incorporated [3H]thymidine (cpm) was plotted as a function of ps20 concentration. One-half maximal activity was determined at 2.62 nM with maximal activity at 6.3–8 nM. Values are mean of n = 3 separate determinations, ±S.E.
required to determine primary structure and assess likely biological actions. In addition, the characterization of ps20 expression patterns and regulatory pathways are required to define molecular regulatory mechanisms and assess possible functions in stromal cell biology. The ps20 protein may or may not represent a paracrine effector growth regulatory protein involved in receptor-mediated pathways. Alternatively, the ps20 protein may function as a matrix component laid down by mesenchyme or as an external cell membrane protein involved in cell-cell or cell-matrix adhesion, thereby affecting proliferation/differentiation. However, it should be cautioned that the present report addresses biological properties in vitro only, and no data yet exist to indicate a similar activity or properties of ps20 in the fetal urogenital sinus or normal prostate gland in vivo.

Progress to understand both prostate organogenesis and prostate disease progression requires the elucidation of genes and proteins involved in stromal cell biology and the mechanisms of stromal-epithelial interactions. The ps20 protein may be of significance in assessing any potential role of this protein in prostatic diseases, typified by alterations in epithelial cell proliferation, differentiation, and patterns of stromal cell histology. The observation that ps20 growth inhibits the PC-3 carcinoma cell line derived from a human prostatic adenocarcinoma points to the possibility that ps20 actions may affect prostatic carcinoma progression. Understanding the expression patterns of ps20 in prostatic disease (benign prostatic hyperplasia and prostatic carcinoma) and defining molecular mechanisms of action will be of importance in assessing any potential role of this protein in prostatic disorders.

Of additional interest is the possibility that ps20 may represent a new marker for stromal cell ontogeny and functional differentiation. Our preliminary studies to date\(^2\) indicate that significant ps20 expression is limited to mesenchymal cells and adult stromal cells expressing smooth muscle differentiation marker proteins and is not observed in either fibroblast or epithelial cells. Recent studies indicate that prostate smooth muscle represents the major androgen-regulated stromal cell type in the postnatal prostatic gland (26, 27). Prostate smooth muscle cells evolve from androgen receptor-positive mesenchymal cells, such as U4F cells, in the immediate proximity to developing pockets of epithelial islands (28). Accordingly, ps20 expression may correlate with ontogeny of mesenchymal differentiation to smooth muscle and may provide insight to mechanisms of mesenchymal/smooth muscle actions.

This report serves as the initial characterization of the ps20 protein and associated biological properties. The full extent of ps20 activity and relevance to prostate gland biology awaits more extensive characterization of cDNA encoding this protein and determination of specific biological activity in vivo.

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