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Quantitative Proteomic Analysis of Proteins Released by Neoplastic Prostate Epithelium

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ABSTRACT

Prostate cancer is unusual among neoplasms in that it may be diagnosed at a curable stage through detection of a protein in serum, the serine protease prostate-specific antigen (PSA). PSA is secreted by both normal and neoplastic prostate epithelial cells in response to androgenic hormones and has found widespread use in cancer screening. Because PSA screening is controversial due to sensitivity and specificity issues, efforts continue to focus on the identification and characterization of additional markers that may be used for diagnostic and therapeutic purposes. In this study, we report the application of quantitative proteomic techniques that incorporate isotope coded affinity tag reagents and tandem mass spectrometry to comprehensively identify secreted and cell surface proteins from neoplastic prostate epithelium. LNCaP cells, a prostate tumor-derived cell line that secretes PSA in response to androgen exposure, were grown in a low protein-defined media under androgen-stimulated (A+) and -starved (A-) conditions. Proteomic analysis of the media identified in excess of 600 proteins, 524 of which could be quantified. Nine percent of the proteins had A+/A- ratios > 2.0, including PSA, and 2.5% had ratios < 0.5. A subset of these androgen-regulated proteins appeared to be expressed in abundance. Of these, selected mass spectrometry observations were confirmed by Western analysis. The findings suggest that androgen-mediated release of proteins may occur through the activation of proteolytic enzymes rather than exclusively through transcriptional or translational control mechanisms. On the basis of their known functional roles, several of the abundant androgen-regulated proteins may participate in the progression of neoplastic epithelial cell growth and should be considered as potential serum markers of neoplastic prostate diseases.

INTRODUCTION

Prostate cancer is the most common noncutaneous malignancy affecting males in the United States. It is estimated that 220,900 cases will be diagnosed in 2003 and that 28,900 deaths will occur, making prostate cancer the second leading cause of cancer-related death in men (1). As with many cancers, prostate carcinoma is thought to develop as a consequence of specific genetic mutation(s). Although many of the genetic alterations involve disruption of regulatory mechanisms that facilitate increased cellular life span, other critical changes are required for progression across physical barriers such as basement membranes, escape from immunological surveillance, and stimulation of angiogenesis (2). The replacement of local paracrine signals from supporting stromal tissue with autocrine stimulation can also provide distinct growth advantages (reviewed in Ref. 3). Some of these extracellular processes represent indispensable requirements for disease progression. Their characterization may be useful for understanding the process of carcinogenesis and exploited as diagnostic, prognostic, or therapeutic targets. It is therefore critical that molecules released locally or into the circulation be identified.

In the prostate gland, several abundant secretory proteins are known to be under the control of androgenic hormones. This cohort of androgen-regulated proteins includes prostate-specific antigen (PSA), human glandular kallikrein 2, and γ-glutamyltranspeptidase 1 (4). PSA is a serine protease used widely as a serum marker for the early detection of prostate carcinoma and for determining disease recurrence after therapy. Unfortunately, PSA has suboptimal operating characteristics as a screening test, and there is no clear consensus among expert panels on the use of PSA measurements for the early detection of prostate cancer (5–8). Clinical care would benefit substantially from the identification of markers with more sensitive and specific test characteristics.

Several approaches have been developed to provide a more global characterization of proteins destined for expression on the cell surface or secreted into the extracellular environment. One study used cDNA microarrays to assess transcript levels of mRNAs bound to membrane-associated polysomes and determined that known secreted or membrane proteins encoded by these transcripts were enriched in these fractions (9). Direct approaches for profiling proteins in prostate cancer cell lines (10, 11) and clinical samples (12–16) have centered on two-dimensional PAGE for separation and Edman sequencing or mass spectrometry (MS) for protein identification. Although two dimensional-PAGE experiments have provided interesting findings, the method has many technical and practical limitations that preclude large-scale comprehensive and quantitative analyses (17–20). To address issues of sample throughput and limited sample quantity, surface-enhanced laser desorption/ionization MS has been developed as an alternative for profiling proteins in cancer tissue specimens and patient serum. Profiles of low molecular weight polypeptides generated by surface-enhanced laser desorption/ionization MS analysis have been capable of partitioning benign and malignant prostate disease states with high accuracy (21–23). However, the method suffers from limitations in providing identities for the specific proteins that serve as pathology discriminators.

One approach designed to address issues of sample throughput, protein quantification, and protein identification involves the integration of multidimensional chromatography and automated tandem MS (MS/MS) with a quantitative isotopic dilution reagent termed an isotope coded affinity tag (ICAT; Refs. 24–27). The ICAT reagent affords a dramatic reduction in sample redundancy—and a corresponding increase in throughput—while retaining sample complexity and provides a reporter element allowing for accurate relative protein quantification. This procedure represents a significant improvement over gel-based methods because it is rapid, avoids problems of solubility and extremes of pH, and can identify low abundance proteins. In this study, we report the results of applying quantitative high-throughput MS techniques to characterize the cohort of proteins secreted by prostate cancer cells in an attempt both to characterize the cellular response to androgen and to identify those proteins with characteristics similar to those of PSA, *i.e.*, regulation by androgen and abundant expression in prostate. Several of the identified androgen-regulated secreted proteins represent known constituents of seminal fluid, whereas others have not been explored in the context of normal or neoplastic prostate physiology or pathology.

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MATERIALS AND METHODS

Reagents and Chemicals. All reagents and chemical were purchased from either Sigma (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA) unless otherwise stated. All cell culture reagents were from Life Technologies, Inc. (Gaithersburg, MD) unless otherwise stated. ICAT reagent and monomeric avidin cartridges were purchased from Applied Biosystems (Foster City, CA). All electrophoresis and electroblotting was done with 4–12% precast polyacrylamide NuPAGE NOVEX Bis-Tris gels (Invitrogen, Carlsbad, CA) using polyvinylidene difluoride membrane (Millipore, Bedford, MA). Seventy-five-μm inner diameter $\times$ 360-μm outer diameter-fused silica capillary tubing was from poly-micro Technologies (Phoenix, AZ). Rabbit polyclonal anti-PSA and goat polyclonal anti-insulin-like growth factor binding protein (IGFBP2) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal anti-vascular endothelial growth factor (VEGF) antibodies were purchased from BD PharMingen (San Diego, CA) and NeoMarkers, Inc. (Fremont, CA). Mouse monoclonal anti-hepatocyte activator inhibitor 1 (HAI-1) and anti-matriptase (MAT) were the kind gift of Dr. Chen-Yong Lin (Georgetown University Medical Center). Mouse monoclonal anti-amyloid precursor-like protein 2 (APLP2) was the gift of Dr. Gopal Thinakaran (University of Chicago).

Cell Culture. To determine the capacity of a low protein defined media to support cell survival, LNCaP human prostate cancer cells (American Type Culture Collection, Manassas, VA) were grown for 3 days in phenol red-free RPMI 1640 supplemented with 10% fetal bovine serum at 37°C until 75% confluent. The cells were washed three times with PBS and changed to a low protein defined media based on a previously published serum-free media protocol for LNCaP cells (28), consisting of phenol red-free RPMI 1640 supplemented with Insulin-Transferrin-Selenium-A, 1 ng/ml epidermal growth factor, 0.1 nM T3, 5 μg/ml fetuin (Sigma), and 10 mM HEPES (pH 7.2). Cells were observed for morphological changes and cell death for 7 days. Media samples were taken after 24 and 48 h for PSA analysis by Western blot. After 3 days, the media was changed to phenol-free RPMI 1640 supplemented with 10% fetal bovine serum, and the cell growth was observed for another 7 days.

For the proteomic analysis, LNCaP cells were grown to 75% confluence in 175-cm^2 dishes. The cells were washed three times with PBS and changed to the low protein defined media supplemented with either 1 nM of the synthetic androgen R1881 (NEN/Life Sciences Products) or ethanol vehicle control. Cells were cultured for 72 h before the conditioned media was collected.

Processing of Conditioned Media. Conditioned media (145 ml $\times$2) was removed to 250-ml centrifuge tubes (Corning, Cambridge, MA) and centrifuged at 3500 rpm for 10 min at 4°C to remove cells and cell debris. All sample handling was done on ice. The samples were concentrated to 5 ml over a membrane pressure concentrator with a 3-kDa cutoff (Millipore) and subsequently desalted over a 10-ml Econo-Pac column (Bio-Rad, Bedford, MA). The samples were then further concentrated to 1.75 ml over membrane pressure concentrator with a 3-kDa cutoff (Millipore). Protein concentrations were measured using a Bradford assay (Bio-Rad).

ICAT Labeling, Digestion, Peptide Chromatography, and Microcapillary Liquid Chromatography-Electrospray Ionization- MS/MS. Protein samples were processed essentially as described previously (25, 29). To prepare proteins for labeling, solutions containing 2 mg of protein from the conditioned media of the A+ and A- treated cells were independently adjusted to a final SDS concentration of 0.05%, 50 mM Tris-HCl (pH 8.3), and 5 mM EDTA. The protein samples were denatured by boiling for 5 min. Upon cooling, urea was added to a final concentration of 6 M. Proteins were reduced using 5 mM Tri(2-carboxyethyl)phosphine hydrochloride for 30 min at 37°C. Proteins were labeled with ICAT reagent at a concentration of 1 mM for 90 min at room temperature using the isotopically heavy reagent for the A+ sample and the isotopically light reagent for the A- sample. DTT was added to a final concentration of 5 mM to quench unreacted ICAT reagent. Both samples were diluted 1:5 in 20 mM Tris-HCl (pH 8.3). Completeness labeling was determined by SDS-PAGE. Labeled samples were combined and digested overnight at 37°C using 160 μg of trypsin (Promega, Madison, WI). The resulting peptide mixture was acidified with 10% phosphoric acid and separated on an Integral 100Q separation system (Applied Biosystems) using cation-exchange chromatography with a 100 $\times$ 4.6-mm column containing 5 μm of particle Polysulfoethyl-A resin with 300 Å pore (PolyLC, Columbia, MD). The buffers used were: buffer A, 20 mM KH_2PO_4, 25% acetonitrile (pH 3.0); and buffer B, 20 mM KH_2PO_4, 350 mM KCl, 25% acetonitrile (pH 3.0). A flow rate of 700 μl/min was run with a gradient of 0–30% buffer B over 30 min, then 20–100% B over 20 min. Thirty 90-s fractions were collected. To enrich for ICAT-labeled peptides, individual factions were passed over columns containing monomeric avidin (Applied Biosystems). The fractions were prepared by evaporating the acetonitrile in a vacuum centrifuge. The volume of each sample was corrected to 1 ml with 2$\times$ PBS (1$\times$ PBS = 150 mM NaCl, 5 mM Na_2HPO_4, 1.7 mM KH_2PO_4 at pH 7.2). The pH of each fraction was raised to >8.0 using 500 mM ammonium bicarbonate. The peptides were loaded on the column by hand injection using a 1-ml syringe. The columns were washed with 1 ml of PBS and 1 ml of 50 mM ammonium bicarbonate (pH 8.3) in 20% methanol. Biotinylated peptides were eluted using 1.3 ml of 30% acetonitrile and 0.4% trifluoroacetic acid. The last 1 ml of eluate was collected. The eluate was reduced in volume to 5 μl using low pressure centrifugation. Total volume was raised to 20 μl with buffer A of the reverse-phase chromatography (0.5% acetic acid, 0.005% heptaflourobutyric acid, and 5% acetonitrile) solvent system. Two μl of each sample were loaded onto a fused silica capillary column packed to a bed length of 10 cm with C18 spherical silica 5-μm mean particle size Monitor resin (Column Engineering, Ontario, CA). The loaded column was washed isocratically for 10 min with buffer A. Elution of peptides was performed using a linear gradient 10–35% buffer B (0.5% acetic acid, 0.005% heptaflourobutyric acid, and 100% acetonitrile) over 75 min at a flow rate of 300 nl/min using a HP 1100 solvent delivery system. MS, including collision induced dissociation, was performed in an automated fashion using the dynamic exclusion option on an LCQ classic mass spectrometer (Thermo-Finnigan, San Jose, CA) equipped with a in-house built microspray device (24).

Data Analysis. MS/MS spectra were searched using the SEQUEST (30) search tool against a database composed of the International Protein Index human sequence database downloaded from the European Bioinformatics Institute.[3] Bovine (TRYPBOVIN) and porcine trypsin (TRYPPIG) sequences from Swiss-Prot were appended to the human database. The database search results were validated using the PeptideProphet program (31, 32). This program assigns to each peptide identification a probability that it has been correctly identified based upon its SEQUEST scores and features of the assigned peptide, including the number of triptych termini. A total of 70,642 individual spectra assigned a probability of correct assignment > 0.05 were retained. Quantification of the ratio of each protein (isotopically heavy *versus* light) was made using an in house program called ASAPRatio (33). Using a second program, ProteinProphet (32, 34), the assigned peptides were grouped according to corresponding protein. The probability of a correct protein assignment was computed by this program. Proteins that were represented by numerous peptides, high percentage sequence coverage, or extremely strong single ion elution profiles were classified as abundant. Each protein identification was assigned a cellular location based on information available from Swiss-Prot, Locus link, or other publicly available databases. Information on the in-house software used is available on line.[4]

Gel Electrophoresis and Immunoblotting. LNCaP cell lysate was made by adding 0.5% SDS, 50 mM Tris-HCl (pH 8.0), 5 mM EDTA directly onto two equally dense 6-cm dishes of LNCaP cells cultured in the defined media (with and without androgen) and scraping the liquid into a 1.7-ml tube. The sample was sonicated for 10 s on ice and boiled for 5 min. Concentrated media samples were loaded as follows: Coomassie staining (5 μg/lane) and immunoblotting (20 μg/lane) except in the case of the APLP2 blot where only 2 μg/lane were used. Ten μl/lane LNCaP lysate used to test the APLP2 antibody. All gels were reducing with the exception of HAI-1 and matriptase (MAT). Proteins were electroblotted using the Novex Mini-Cell and polyvinylidene difluoride. All blocking and probing with primary and secondary antibody was done 5% nonfat powdered milk in T- Tris-buffered saline [25 mM Tris (pH 9), 150 mM NaCl, and Tween 0.5%] for 1 h using the antibody conditions below. Membranes were visualized using enhanced chemiluminescence using Hyperfilm ECL (Amersham Pharmacia Biotech, Piscataway, NJ). Primary antibody dilution conditions: anti-HAI-1 (1:2000); MAT (1:2000); anti-VEGF (BD Pharmingen; 1:500); anti-VEGF (NeoMarkers, Inc.; 1:200); anti-APLP2 (1:2500); and anti-IGFBP2 (1:250). Secondary antibodies purchased from Santa Cruz Biotechnology were all used at 1:10,000.

[3] Internet address: ftp://ftp.ebi.ac.uk/pub/databases/IPI/current/ipi.HUMAN.fasta.gz.

[4] Internet address: http://www.systemsbiology.org/Default.aspx?pagename=proteomicssoftware.

RESULTS

LNCaP Cells Regulate PSA Expression in a Defined Low Protein Media. The LNCaP cell line is an androgen-sensitive prostate cancer line derived from a lymph node metastasis in a human subject (35, 36). This cell line is used extensively in prostate cancer research because it is one of only a few lines that recapitulates the biochemical characteristics of prostate cancer. LNCaP cells are usually cultured in the presence of 10% FCS. Because the goal of this project was to identify proteins released by LNCaP cells, medium without added serum was required to avoid overwhelming cell-derived proteins with exogenously added calf serum proteins. Hedlund and Miller (28) reported that the LNCaP cell line was successfully cultured for both short- and long-term periods in a serum-free media supplemented with growth factors and albumin. Their original formulation called for albumin and fetuin at concentrations 2 and 0.5 mg/ml, respectively. In our modified low protein defined media (LPDM), the albumin and hydrocortisone were eliminated and the fetuin was used at a 100-fold lower concentration (5 μg/ml). Insulin, transferrin, pyruvate, and selenium were added using a commercially available premixed mixture to the approximate values in the original reference. LNCaP cells grown in RPMI with 10% FCS then changed to LPDM were found to survive with minimal cell death upwards of 1 week. Cells assumed a spindle or neuronal shape after 3 days in LPDM, but the addition of FCS-supplemented media restored normal proliferation and morphology and permitted passage. LNCaP cells were tested by changing the media to either LPDM or RPMI base media plus charcoal-stripped FCS (lacking androgen) with and without androgen supplementation using 1 nM R1881. PSA production was androgen regulated (Fig. 1) in LPDM over 72 h.

Identification of Proteins in LNCaP Cell-Conditioned Media. We used chromatographic fractionation, MS/MS, and ICAT to identify and quantify proteins secreted by the LNCaP prostate adenocarcinoma cell line. Media from cells grown for 72 h in the presence (A+) or absence (A-) of the synthetic androgen R1881 were directly compared. The cultured cell supernatants were concentrated, and ~4 mg of total protein were isolated from each of the A+ and A- samples. Two mg of each sample were alkylated with the ICAT reagent on cysteine side chains using ICAT-d8 (heavy) to label the A+ sample and the ICAT-d0 (light) to label the A- sample. After labeling, the samples were combined, digested with trypsin, and the resulting peptide mixtures were separated into 30 fractions using cation exchange high-performance liquid chromatography. The ICAT-labeled peptides were isolated by avidin affinity chromatography.

Each cation exchange fraction was subjected to microcapillary liquid chromatography followed by electrospray ionization-MS/MS. This analysis produced 70,642 individual peptide spectra, which were compared against the International Protein Index human sequence database using the SEQUEST software program (30). Each peptide assignment was given a probability score using the PeptideProphet software (31), and all peptide assignments with $P > 0.05$ were assigned a D0/D8 quantification using the ASAPRatio software (33). The results were then assigned a protein identification using the ProteinProphet software (34). A total of 517 unique proteins was both quantifiable and had identification Ps > 0.9 and is available as a supplement on line.[5] A table of the 123 proteins that were identified but not accurately quantified is also available online at the same address.

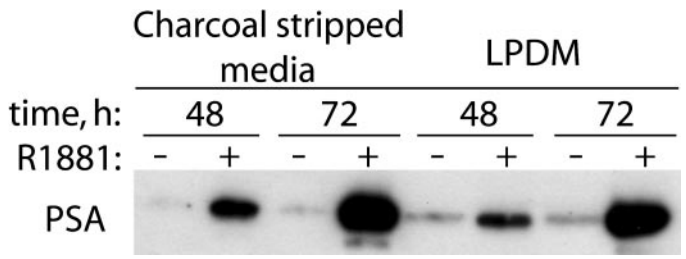

Fig. 1. Androgen regulation of PSA expression in standard media and low protein-defined media (LPDM). LNCaP cells grown to near confluence were changed to either RPMI 1640 containing either 10% charcoal-stripped FCS or LPDM. Cells were grown in the presence of absence of 1 nM R1881. Samples of media were withdrawn at 24 and 48 h and probed with anti-prostate-specific antigen (PSA) antibody after SDS-PAGE and electroblotting.

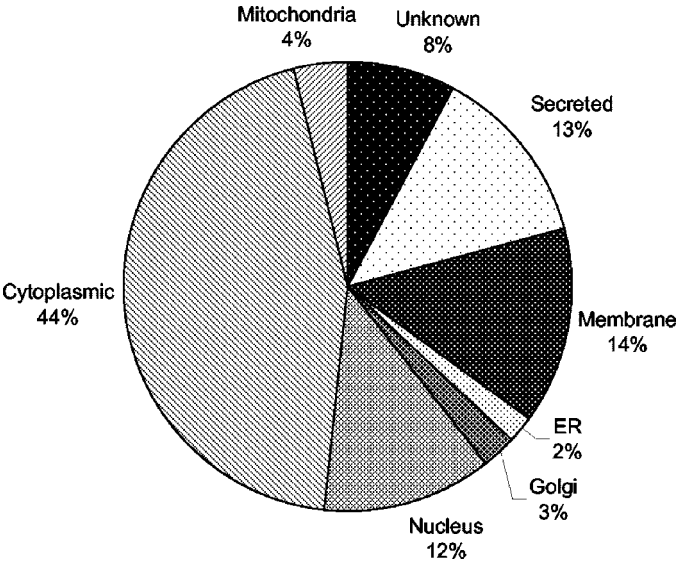

Fig. 2. Cellular distribution of proteins identified in LNCaP-conditioned media.

Cellular Localization of Identified Proteins. Annotation for the identified and quantified proteins was obtained from the Swiss-Prot and LocusLink databases, and these were used to assign a cellular location. Twenty-seven percent of the proteins identified in this study have previously been assigned to compartments exposed to the extracellular environment (membrane and extracellular; Fig. 2), whereas putative intracellular proteins (cytoplasmic and nuclear) made up 56% of all protein identifications. Five percent were assigned to the endoplasmic reticulum or Golgi. Eight percent of the proteins had no assigned cellular location.

Identification of Androgen-Regulated Proteins. PSA is one of the most striking examples of a hormonally regulated protein. This characteristic may relate both to its prostate tissue restriction and to tissue-specific mechanisms of regulating proliferation and differentiation. To identify proteins that mimic the androgen-regulated and potentially prostate-restricted characteristics of PSA expression, we focused our attention on proteins with abundance levels affected by the androgen content of the media. For each identified protein, the relative abundance between A+ and A- samples was calculated. The distribution of all A+/A- protein ratios conformed to a bell-shaped curve centered about unity (Fig. 3) with 88% of the ratios located between 0.5 and 2.0. The majority of proteins with ratios outside of this range was more abundant in the A+ cell media relative to the A- cell media (9% of all proteins), whereas only 2.5% of all proteins were reduced in abundance >2-fold in response to androgen exposure.

The LNCaP cell line, as with normal and neoplastic prostate epithelium *in vivo*, is known to secrete PSA in response to androgens. The results of our high-throughput study using mass spectrometry and ICAT analysis confirmed these findings. The absolute abundance of

[5] Internet address: http://personal.systemsbiology.net/dmartin/.

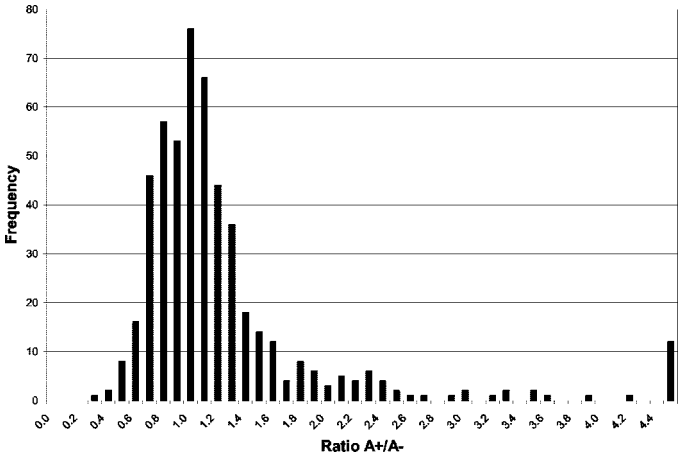

Fig. 3. Distribution of protein abundance ratios in LNCaP-conditioned media after androgen exposure. Ratios calculated for all proteins identified were normalized to the median for the group and plotted. A number > 1 means the protein was more abundant after androgen exposure.

PSA was demonstrated by the large number of spectra found for its peptides (37), including 3 of 4 cysteine-containing tryptic peptides and an additional 14 cysteine-containing peptides with a single missed triptych cleavage. Four noncysteine containing PSA peptides were also identified. The average A+/A- ratio for PSA was 3.5, a result consistent with observations obtained by Coomassie and Western analysis (Fig. 4). In addition to PSA, 51 proteins were found to have ratios significantly outside of the distribution of the general population (Tables 1 and 2). These proteins varied with respect to the number of peptides found/protein, and the quality of single ion chromatograms that were used to calculate their ratios.

The distribution of proteins with abundance levels affected by androgen exposure differed substantially when comparing their subcellular locations. The abundance ratios of proteins found in the cytoplasm and nucleus overwhelmingly fell in a narrow range around one, indicating little influence of androgenic hormones on their expression. Only 6 of 272 (2.2%) cytoplasmic proteins, 0 of 71 nuclear proteins, and none of 20 mitochondrial proteins exhibited ≥2-fold changes in abundance. In contrast, 15 of 68 (22%) of the extracellular proteins and 29 of 74 (39%) membrane proteins were up- or down-regulated by ≥2-fold after androgen exposure. In proteins of undetermined cell location, 2 of 40 (5%) were outside this range. Of 16 proteins with matches to bovine entries in the database and thus presumably present in the growth serum, one, a bovine RNase pancreatic precursor protein, was found to have a ratio of >2.0.

Transferrin was added to the base media and therefore should have been equally divided between the samples. The ratio for transferrin was 0.75 compared with the median protein ratio (decreased in media from androgen stimulated cells). This may have been due to the increased utilization of transferrin by A+ cells or by increased metabolic activity in cells stimulated with androgen, resulting in an increase in global protein production causing dilution of the transferrin. Both of these possibilities are consistent with the stimulatory affects of androgen on prostate cancer cells.

Abundance Estimates of Proteins in LNCaP-Conditioned Media. PSA represents a useful protein for prostate cancer screening in part because of its copious expression by prostate epithelium. The LNCaP cells mimic this behavior as demonstrated by the visualization of a protein band corresponding to PSA on a Coomassie-stained polyacrylamide gel separation of LNCaP cell proteins in defined media (Fig. 4*A*). We sought to identify potential protein markers of prostate epithelium with levels of expression similar to PSA and therefore created a subclass of identified proteins categorized as abundant in the LNCaP cell-conditioned media. Although it is not possible to definitively determine the absolute quantity of proteins from the data obtained, those proteins corresponding to peptides sampled at frequencies significantly above the norm were labeled abundant. Proteins with multiple peptide spectra that were seen in consecutive cation exchange fractions were also put into this group.

Proteins in this abundant category included those added exogenously to the growth media: transferrin and fetuin. These proteins were represented by 1806 and 232 individual MS/MS events, respectively. In part, this was because of chromatographic elution profiles demonstrating a tailing in both cation exchange and reverse-phase chromatography because of overloading that led to repetitive sequencing of the same peptides despite the dynamic exclusion function of the mass spectrometer. Also seen in abundance were six proteins known to be present in the bovine serum used to culture the cells before changing to the serum-free growth media. These six proteins were represented by a combined 400 individual MS/MS events. Fifty LNCaP-derived proteins were categorized as abundant and are listed in Table 3. Normal and neoplastic prostate epithelial cells have previously been reported to secrete some of these such as PSA and IGFBP2 (37, 38), but many have not been associated with prostate physiology or pathology. Most of the abundant cytosolic proteins derived from the LNCaP cells are structural and metabolic proteins that are common, highly expressed intracellular proteins. Of the cytosolic proteins, two, Quiescin (39) and selenium binding protein 1, stand out from the group as nonmetabolic proteins with potentially interesting functions related to cancer cell behavior.

Confirmation of ICAT-MS Expression Results. Proteins with changes in abundance in response to androgens as determined by ICAT-MS were additionally studied by Western analysis. Of particular interest were those proteins expected to be secreted or expressed on the plasma membrane. Whenever possible, attempts were made to acquire antibodies that were directed toward extracellular epitopes. Antibodies tested include those against Kunitz-type protease inhibitor

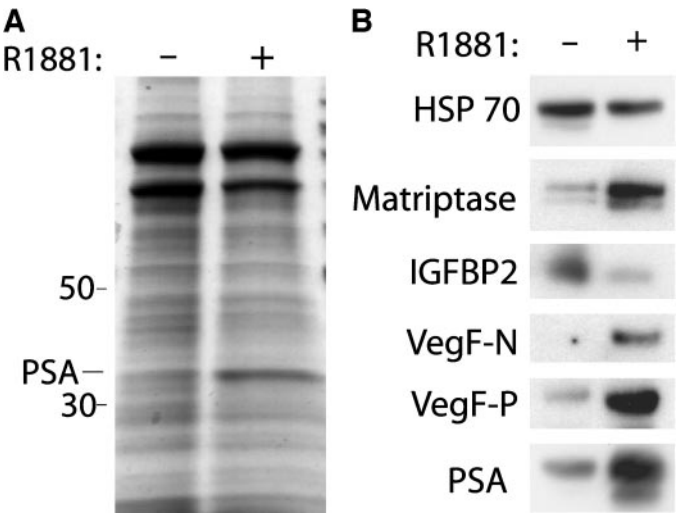

Fig. 4. Analysis of protein expression in LNCaP cells grown in defined culture conditions. Androgen-regulated PSA expression was observed in the low protein-defined media as determined by Coomassie stain (*A*) and Western analysis (*B*). Heat shock protein 70 (HSP 70) and Coomassie staining were used as loading controls. Proteins were chosen that demonstrated change in abundance in the androgen-supplemented media. Immunodetection was performed using two different antibodies recognizing vascular endothelial growth factor (VEGF; NeoMarkers, Inc. and BD PharMingen) and antibodies recognizing matriptase and insulin-like growth factor binding protein 2 (IGFBP2). Both anti-VEGF antibodies have immunoreactivity at 28 kDa with much stronger signal seen in the presence of androgen. The matriptase reactivity is seen at the expected 64 kDa. The addition of androgen reduced the IGFBP2 immunoreactivity.

Table 1 *Proteins with increased abundance in LNCaP conditioned media after androgen exposure*

Proteins with an A+/A− ratio >2.0 are annotated. The table gives the gene symbol, protein name, A+/A− ratio calculated using the software ASAPratio for the identified peptides, the SD of the ratio, the percent protein coverage, the number of unique peptides found, and the cellular location based on the SwissProt and LocusLink databases (M, membrane; S, secreted; C, cytoplasmic; N, nuclear; GOL, Golgi; ?, unknown). The probability of correct protein identification as calculated by the ProteinProphet software was 0.99 or greater for all proteins.

Gene symbol	Protein name	A+/A− ratio	SD	Unique peps	Cell location
TNFRSF10B	TNF receptor superfamily member 10B precursor	17.17	4.58	6	M
BSG	Basigin precursor	12.88	5.02	5	M
TFRC	Transferrin receptor protein 1	7.81	2.27	3	M
F11R	Junctional adhesion molecule 1 precursor	7.36	1.68	4	M
IGFBP3	Insulin-like growth factor binding protein 3 precursor	7.08	2.72	3	S
LDLR	Low-density lipoprotein receptor precursor	6.57	1.64	12	M
ANGPT2	Angiopoietin-2 precursor	6.08	1.40	1	S
IGSF8	EWI2	5.43	1.10	4	M
SCOTIN	Scotin-BAB71152, unnamed protein	5.39	3.63	1	?
KLK2	Glandular kallikrein 2 precursor	5.34	3.42	6	S
JAG1	Jagged 1 precursor	4.92	1.43	23	M
APLP2	Amyloid-like protein 2 precursor	4.17	2.06	9	M
SPINT1	Kunitz-type protease inhibitor 1 precursor	3.89	1.08	22	S
LRIG1	Membrane glycoprotein LIG-1	3.59	1.39	3	M
KLK3	Prostate-specific antigen precursor	3.50	0.66	37	S
VEGF	Vascular endothelial growth factor A precursor	3.49	0.24	3	S
LGMN	Legumain precursor	3.24	0.33	2	C
ALCAM	CD166 antigen precursor	3.21	0.38	7	M
KIAA1265	Hypothetical protein KIAA1265	3.13	1.24	3	M
ACVR1B	Activin A type IB receptor	2.97	0.41	4	M
PLXNB3	Plexin-B3	2.95	1.00	2	M
IL6R	Interleukin-6 receptor α chain precursor	2.84	0.72	3	M
C1orf8	Protein C1orf8 precursor	2.66	0.10	2	M
ST14	Suppressor of tumorigenicity 14	2.60	0.74	10	M
PLAB	Prostate differentiation factor	2.47	0.34	6	S
NOTCH2	Neurogenic locus notch homologue protein 2 precursor	2.45	0.40	2	M
MLP	MARCKS-related protein	2.38	0.93	4	C
B2M	β-2-microglobulin precursor	2.34	0.48	14	S
NRP1	Neuropilin-1 precursor	2.33	0.36	27	M
NEO1	Neogenin precursor	2.27	0.03	5	M
PSAP	Proactivator polypeptide precursor, contains: saposin A	2.26	0.35	1	S
CDH1	E-cadherin	2.23	1.69	15	M
ATOX1	Copper transport protein ATOX1	2.21	0.22	3	GOL
ADAM10	ADAM10	2.20	0.46	10	S
IGF2R	Cation-independent mannose-6-phosphate receptor precursor	2.20	0.60	16	M
TNFRSF10D	TNF receptor superfamily member 10D precursor	2.19	0.10	3	M
PLXNB1	Plexin-B1/SEP receptor precursor	2.18	0.51	2	M
SEMA4A	Semaphorin 4A precursor	2.18	0.48	4	M
VLDLR	Very low-density lipoprotein receptor precursor	2.09	0.05	1	M
GALNT1	Polypeptide *N*-acetylgalactosaminyltransferase	2.09	0.52	3	GOL
EFNA5	Ephrin-A5 precursor	2.05	0.39	9	M
PTPRS	protein tyrosine phosphatase, receptor type, σ precursor	2.03	0.49	11	M
APP	Amyloid β A4 protein precursor	2.02	0.34	24	M

1 (SPINT1) precursor also called hepatocyte growth factor activator (HAI-1), suppressor of tumorigenicity 14 also called MAT, VEGF, IGFBP2 precursor, and APLP2.

Western analysis confirmed the expression ratios determined by the ICAT method (Fig. 4*B*). For each of the proteins tested, there was at least one immunoreactive band that showed definite signal change in the defined media according to androgen concentration that was consistent with mass spectrometry data. Heat shock protein 70, which was unchanged by proteomic analysis, was unchanged by immunoblot. Immunodetection was performed using two anti-VEGF antibodies, both of which demonstrated increased signal at 28 kDa. In addition, one of these antibodies showed increased signal at 14 kDa under androgen stimulated conditions (data not shown). Reactivity toward suppressor of tumorigenicity 14 (MAT) protein was observed at the expected 64 kDa, and levels were increased after the addition of androgen. The androgen exposure caused a decrease in the amount of IGFBP2 immunoreactivity in androgen-stimulated conditions. The ICAT-MS results for APLP2 were initially difficult to confirm by Western analysis, presumably because of the extensive posttranslational modification (40) of this protein that resulted in significant smearing. However, probing of whole cell lysate with anti-APLP-2 antibody demonstrated clear evidence of regulation by androgen (Fig. 5). APLP-2 was also categorized in the abundant group and immunoblotting was consistent with this finding as the initial analyses of

Table 2 *Proteins with decreased abundance in LNCaP-conditioned media after androgen exposure*

Proteins with an A+/A− ratio <0.5 are listed. The table gives the gene symbol, protein name, A+/A− ratio calculated using the software ASAPratio for the identified peptides, the SD of the ratio, the percent protein coverage, the number of unique peptides found, and the cellular location based on the SwissProt and LocusLink databases (M, membrane; S, secreted; C, cytoplasmic; N, nuclear). The probability of correct protein identification as calculated by the ProteinProphet software was 1.0 for all proteins.

Gene symbol	Protein name	A+/A− ratio	SD	Unique peps	Cell location
TIMP2	Metalloproteinase inhibitor 2 precursor	0.50	0.15	10	S
C1R	Complement C1r component precursor	0.48	0.09	9	S
ADAM15	ADAM 15 precursor	0.48	0.08	6	M
IGFBP2	Insulin-like growth factor binding protein 2 precursor	0.45	0.04	38	S
FN1	fibronectin 1 isoform 1 preproprotein	0.44	0.03	30	S
HSPCB	Heat shock protein HSP 90-β	0.42	0.11	6	C
LOC284781	Similar to hypothetical protein MGC3077	0.38	0.06	4	C
MMP10	Stromelysin-2 precursor	0.37	0.02	5	S

Table 3 *Abundant proteins in LNCaP cell-conditioned media*

Proteins found to have large numbers of peptides, peptides spread among multiple cation exchange fractions, very strong single ion trace intensities, and multiple noncysteinal peptides were considered abundant. The table gives the gene symbol, protein name, A+/A− ratio calculated using the software ASAPratio for the identified peptides, the SD of the ratio, the percent protein coverage, the number of unique peptides found, and the cellular location based on the SwissProt and LocusLink databases (M, membrane; S, secreted; C, cytoplasmic; N, nuclear; ER, endoplasmic reticulum; ?, unknown). The probability of correct protein identification as calculated by the ProteinProphet software was 1.0.

Gene symbol	Protein name	A+/A− ratio	SD	% protein coverage	Unique peps	Cell location
TF	Serotransferrin precursor	0.82	0.23	67.6	212	S
IGFBP2	Insulin-like growth factor binding protein 2 precursor	0.39	0.13	42.4	38	S
KLK3	Prostate specific antigen precursor	3.50	0.66	58.2	37	S
CLSTN1	Calsyntenin-1 precursor	0.58	0.09	20.6	35	M
FN1	Fibronectin 1 isoform 1 preproprotein	0.44	0.22	12.7	30	S
NRP1	Neuropilin-1 precursor	2.10	0.78	27.0	27	M
ALDOA	Fructose-bisphosphate aldolase A	1.13	0.15	38.0	27	C
ADAMTS1	ADAMTS-1 precursor	1.50	0.88	24.1	26	S
LAMA5	Laminin α-5 chain precursor	0.65	0.09	8.2	26	S
PPFIA2	Protein tyrosine phosphatase, receptor type, F, isoform 2 precursor	1.14	0.16	11.6	26	M
LAMC1	Laminin γ-1 chain precursor	1.04	0.14	11.5	24	S
APP	Amyloid β A4 protein precursor	2.02	0.34	13.8	24	M
TPI1	Triosephosphate isomerase	0.95	0.20	47.2	24	C
JAG1	Jagged 1 precursor	4.92	1.43	16.8	23	M
FASN	Fatty acid synthase	1.83	0.44	11.5	23	C
SPINT1	Kunitz-type protease inhibitor 1 precursor	4.06	2.55	20.3	22	S
AGRN	AGRIN precursor (Fragment)	0.98	0.15	11.3	20	S
QSCN6	Quiescin	0.63	0.10	16.2	20	C
CKB	Creatine kinase, B chain	1.55	0.72	28.3	20	C
SELENBP1	Selenium binding protein 1	0.53	0.23	26.7	20	?
APOH	Beta-2-glycoprotein I precursor	0.94	0.13	37.7	19	S
LAMB2	Laminin beta-2 chain precursor	0.98	0.31	13.0	18	S
FLNB	β-Filamin	1.06	0.21	8.4	18	M
VCP	Transitional endoplasmic reticulum ATPase	1.15	0.15	22.0	17	C
IGF2R	Cation-independent mannose-6-phosphate receptor precursor	2.20	0.60	8.1	16	M
PGK1	Phosphoglycerate kinase 1	1.02	0.11	16.5	16	C
PARK7	RNA-binding protein regulatory subunit	1.02	0.21	29.1	16	C
CST3	Cystatin C precursor	1.18	0.33	26.0	15	S
APEX1	DNA-(apurinic or apyrimidinic site) lyase	1.00	0.09	39.1	15	N
CDH1	E-cadherin	2.23	1.69	6.6	15	M
SORD	Sorbitol dehydrogenase	1.08	0.05	25.3	15	C
B2M	Beta-2-microglobulin precursor	2.34	0.48	41.2	14	S
SPON2	Spondin 2	0.97	0.05	21.8	14	S
CALR	Calreticulin precursor	0.97	0.16	24.2	13	ER
LOC345561	Peptidyl-prolyl *cis-trans* isomerase A	1.05	0.09	28.4	13	C
MDK	Midkine precursor	1.35	0.32	44.8	12	S
APLP2	Amyloid-like protein 2 precursor	4.17	2.06	10.2	9	M
EFNA5	Ephrin-A5 precursor	2.05	0.39	19.3	9	M
PLAB	Prostate differentiation factor	2.47	0.34	16.6	6	S

APLP-2 in concentrated media produced extraordinary signal strength. Additional blots had to be performed on diluted samples. For another protein, Kunitz-type protease inhibitor 1, the 40- and 20-kDa immunoreactive signals were increased in androgen-supplemented defined media, in agreement with the proteomic data. In whole cell lysates, this protein appeared at its full length (M_r 55,000) and as a truncated species, neither of which differed according to androgen addition, suggesting that the appearance of these fragments was due to an androgen activated proteolytic activity (Fig. 6). LNCaP cells were recultured under identical conditions for confirmation of the Western blots. All of the changes observed using immunoblotting were seen in this repeat experiment (data not shown).

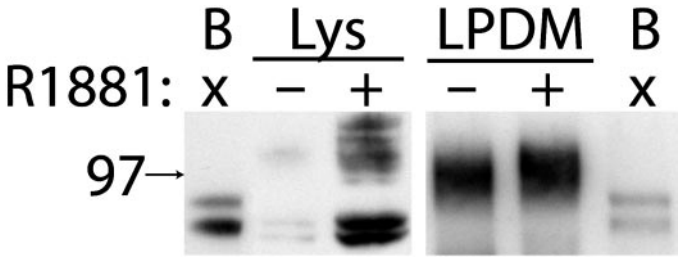

Fig. 5. Western analysis of APLP2 expression. Whole cell lysate (Lys) and low protein-defined media (LPDM) from LNCaP cells grown for 24 h in LPDM +/− R1881 was separated by SDS-PAGE and transferred to polyvinylidene difluoride. The membrane was probed with antibodies recognizing APLP2. APLP2 protein levels in whole cell Lys increased after androgen exposure, APLP2 regulation in defined media was difficult to quantitate because of protein smearing, presumably because of extensive posttranslational modification. Whole brain extract (*B*) was used as a positive control.

DISCUSSION

In this study, we have demonstrated the feasibility and use of using high-throughput quantitative proteomics to identify proteins of potential relevance to the extracellular environment of neoplastic prostate epithelium. More than 500 proteins were identified, and of these, 52 were found to be regulated by androgenic hormones. As with other database search strategies, this analysis is limited to finding only those peptides in the database and only those that are unmodified. As expected, PSA and glandular Kallikrien 2 were up-regulated by androgen. However, most of the other androgen-regulated proteins identified are novel findings. For many of these proteins, the androgen-mediated mechanism(s) resulting in the observed abundance changes is not known. For some proteins, the regulation may be at the level of transcription, as is the case for PSA. Other mechanisms may involve posttranscriptional events resulting in altered message or protein stability. Our findings suggest that in the context of cell surface or secreted proteins, the enzymatic activity of androgen-regulated proteases influence the extracellular concentrations and activities of substrate proteins by cleavage-release or biochemical activation.

Although the primary objective of this study was to identify secreted or cell surface proteins expressed in prostate epithelial cells, the identified proteins included many intracellular proteins from both the

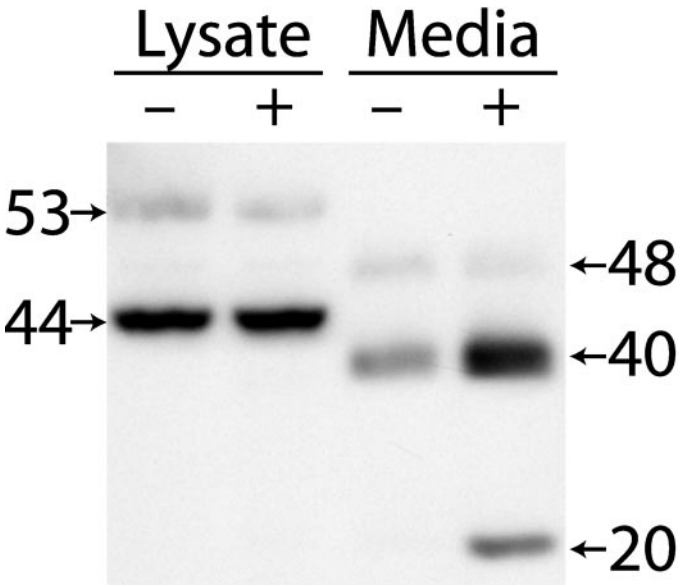

Fig. 6. Western analysis of Kunitz-type protease inhibitor 1 precursor (HAI-1): low protein-defined media and whole cell lysate were prepared as described with and without androgen stimulation. Increased HAI-1 immunoreactivity at 40 and 19 kDa was observed in the media after androgen exposure (low protein-defined media). Androgen regulation of HAI-1 was not observed in whole cell lysates.

cytoplasmic and nuclear compartments. In the context of a diagnostic disease marker, abundant cell type-specific proteins may serve as viable indicators as intracellular constituents are transiently found in the circulation because of cell death and the subsequent elimination of cellular waste products. Similarly, during the course of this experiment, it is likely that a portion of the LNCaP cell population died as a result of abortive replication or apoptosis, events which would cause the constitutive release of intracellular proteins into the media. A previous study has shown that 10% of LNCaP cells grown in a protein-free defined media underwent apoptosis at 72 h (41). In contrast to the extracellular proteins, the group of intracellular proteins demonstrated little response to androgens. This may be the result of preferential identification of intracellular housekeeping proteins that are abundant and less likely to be regulated by androgen and also reflects the physiological prostate cellular response to androgenic hormones that involves the up-regulation of cellular secretory activity rather than changes in other specific metabolic functions. Furthermore, nearly all residual bovine serum proteins were distributed at about the same ratio as the cytoplasmic proteins.

In addition to PSA, which was 3.5-fold more abundant after androgen exposure, several other proteins found to change in abundance after androgen addition were also found in high abundance in the media (Table 1). These including neuropilin-1, Amyloid β A4, Jagged 1, Kunitz-type protease inhibitor 1, amyloid-like protein 2, ephrin-A5, prostate differentiation factor, cation-independent mannose-6-phosphate receptor precursor, and E-cadherin. The paired attributes of androgen-dependent release and a high concentration in media make these proteins some of the more interesting potential diagnostic targets.

Another interesting category of proteins are those with androgen-regulated release that may influence neoplastic cell growth. These include VEGF, IGFBP2, and fatty acid synthase. Levels of VEGF increased >3-fold after androgen treatment. VEGF is a secreted mediator of vascular endothelial cell growth thought to be critical for the progression of malignancy (reviewed in Refs. 42, 43). VEGF has previously been shown to be regulated by androgens in prostate epithelium (44–49) and is present at very high levels in seminal plasma (50). Our results also indicated that levels of IGFBP2 decreased in the LNCaP media after androgen treatment. These findings are in agreement with other published studies. A recent study of LNCaP cells reported that cellular levels of IGFBP-2 mRNA and protein were up-regulated after androgen starvation (51) and increases in IGBP2 levels in rat (52, 53) and human (51) prostate after the removal of androgens is also described. Clinically, immunohistochemical analysis of tissue microarrays demonstrated high expression of IGFBP2 protein in 100% of the hormone refractory clinical tumors (54). Another protein known to be regulated by androgens, fatty acid synthase, increased by 1.8-fold in our experiments. Fatty acid synthase has been shown to be regulated at the level of transcription (55, 56), and our findings are in agreement. This enzyme is not known to be membrane associated or secreted. Thus, it is likely that fatty acid synthase levels are abundant enough within LNCaP cells that release after cell death resulted in detectable levels.

Several of the differentially expressed proteins identified in this study have been reported to be released from the plasma membrane by a protease-mediated mechanism termed ectodomain shedding (reviewed in Refs. 57, 58). Kunitz-type protease inhibitor 1 precursor (HAI-1) is an example of a shed protein that was increased ~3.9-fold in the extracellular milieu after the addition of androgen. HAI-1 is widely expressed in both normal and malignant cells (59), and encodes a Kunitz type serine protease inhibitor with a COOH-terminal transmembrane domain known to inhibit two serine proteases: hepatocyte growth factor activator and MAT. Hepatocyte growth factor activator serves to activate hepatocyte growth factor, a known mitogen for prostate, hepatic, and other cell types (59, 60). Membrane bound HAI-1 is shed from the membrane in 58-, 40-, and 25-kDa fragments. The smaller fragments retain a Kunitz domain and are inhibitory to MAT (59, 61). Western analysis confirmed the ICAT findings and demonstrated that the addition of androgen results in the increased production of immunoreactive 40- and 20-kDa HAI-1 fragments, whereas the expression of larger immunoreactive forms in whole cell lysates remained constant. This suggests that the addition of androgen results in an enzymatic activity that promotes the shedding of HAI-1 rather than a mechanism involving transcriptional regulation.

Suppressor of tumorigenicity 14 (MAT) encodes a serine protease with extracellular matrix-degrading properties that is postulated to play a role in breast cancer invasion (61–64). MS with the ICAT reagent identified 7 different peptides and made four quantification measurements indicating a 2-fold change in MAT levels. Western analysis confirmed this finding. In addition to higher amounts of the full-length MAT protein, the addition of androgen resulted in higher levels of two MAT fragments of ~64 kDa. These fragments are similar in size to those reported by Satomi *et al.* (65) after MAT was activated by trypsin proteolysis. These data suggest that like HAI-1, the changes in MAT abundance may be the result of an androgen-regulated proteolytic activity. In addition to HAI-1 and MAT, six other proteins identified in this study, APLP-2, the LDL receptor (66), Jagged 1 (66), Notch 2 (67, 68), E-cadherin (69), and the IL6 receptor (70, 71) are also known to undergo ectodomain shedding, although none have been reported as responsive to androgenic hormones. Ongoing studies are aimed toward the identification of the protease(s) responsible for the shedding of these proteins.

We identified several proteins released from prostate cancer cells in response to androgens that are known to mediate cellular responses of proliferation and apoptosis. Eight individual peptides corresponding to death receptor 5 were identified and their quantification indicated a 12-fold increase in death receptor 5 abundance after androgen treatment. Death receptor 5 is a critical mediator of apoptosis after signaling by the ligand tumor necrosis factor-related apoptosis-inducing

ligand (reviewed in Refs. 72, 73). The shedding of the extracellular domain has unknown consequences and has not been reported in the context of androgen regulation. Androgens also increased the levels of Jagged 1, a ligand for the Notch receptor, by 3.5-fold. Previous studies have identified low levels of Jagged1 mRNA in both LNCaP cells grown at steady-state conditions and in mouse models of prostate adenocarcinoma (74). Jagged1 protein identification is virtually certain with database matches of 14 Jagged1 peptides and subsequent confirmation by Western analysis (data not shown). Notch signaling has been shown to be responsible for cell fate determination during normal development and is implicated in human T-cell leukemia (75, 76). Activation of the Notch pathway in prostate cells has been shown to inhibit cell proliferation (74). Thus, hormone-mediated alterations in Jagged1 levels could provide a mechanism explaining the proliferative shut-off phenomenon described in some studies of cellular responses to androgens (77, 78). It is also suggestive that these two androgen-regulated proteins, along with APLP-2, have been linked to processing by intramembranous γ-secretase (79–82), which could potentially explain the release of fragments into the growth media.

In summary, we have used a high-throughput proteomics approach to identify and quantify both known and novel proteins released by prostate cancer cells after androgen stimulation. This analysis benefited from the implementation of automated sample processing and new data analysis tools, which currently allow an experiment of this type to be completed within just a few months. Many of the proteins identified with this method may serve as mediators of intracellular responses to exogenous stimuli. Identifying these proteins serves as a first step in determining their role in normal and pathological conditions. As has been extensively documented for PSA, exploiting the secretory or cell surface attributes of cancer cells may identify potential targets for improved diagnosis or therapy of neoplastic prostate disease.

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