Monitoring Mouse Prostate Development by Profiling and Imaging Mass Spectrometry*

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Mass spectrometry-based tissue profiling and imaging are technologies that allow identification and visualization of protein signals directly on thin sections cut from fresh frozen tissue specimens. These technologies were utilized to evaluate protein expression profiles in the normal mouse prostate during development (1–5 weeks of age), at sexual maturation (6 weeks of age), and in adult prostate (at 10, 15, or 40 weeks of age). The evolution of protein expression during normal prostate development and maturation were subsequently compared with 15-week prostate tumors derived from genetically engineered mice carrying the Large T antigen gene under regulation of the prostate-specific probasin promoter (LPB-Tag mouse model for prostate cancer). This approach identified proteins differentially expressed at specific time points during prostate development. Furthermore expression of some of these proteins, for example probasin and spermine-binding protein, were associated with prostate maturation, and prostate tumor formation resulted in their loss of expression. Cyclophilin A, a protein found in other cancers, was differentially α-acetylated on the N terminus, and both isoforms appeared during normal prostate and prostate tumor development. Imaging mass spectrometry localized the protein signals to specific prostatic lobes or regions. Thus, tissue profiling and imaging can be utilized to analyze the ontogeny of protein expression during prostate morphogenesis and tumorigenesis and identify proteins that could potentially serve as biomarkers for prostate cancer. Molecular & Cellular Proteomics 7:411–423, 2008.

The proliferative diseases of the human prostate have a major impact on society in terms of morbidity, mortality, and health care. Benign prostatic hyperplasia (BPH)1 appears in nearly all men in advanced age (1), and the lifetime probability of men developing prostate cancer (PCa) is estimated to be one in six (American Cancer Society). Clinically prostate cancer is routinely imaged for prostate cancer detection, tumor staging, planning treatment, and follow-up. Ultrasonography is used for biopsy guidance and brachytherapy seed implantation (2, 3). Endorectal magnetic resonance (MR) imaging is utilized to assess organ-confined tumors. Tumor aggressiveness is further evaluated by MR spectroscopic imaging, and MR imaging with superparamagnetic nanoparticles facilitates in identifying lymph node metastases. Computed tomography evaluates the extent of advanced disease including metastasis to bone (2, 3). These technologies are critical for clinical evaluation. However, complementary approaches such as mass spectrometry-based tissue profiling and imaging are required to begin identifying and evaluating biomarkers that would facilitate in the detection and staging as well as treatment planning and follow-up for PCa.

Mass spectrometry-based tissue profiling and imaging are newly developed technologies that allow the visualization of protein expression directly on thin sections cut from fresh frozen tissue specimens (4–6). Imaging mass spectrometry (IMS) is based on MALDI (7). MALDI matrix is manually (for profiling studies) or automatically (for imaging studies) deposited directly on the tissue sections and analyzed by MALDI time-of-flight MS (supplemental Fig. S1) (4). Typically 300–500 protein signals ranging from 2 to over 70 kDa are detected, providing information on the local proteome composition. When matrix is homogeneously deposited over the entire surface of the section, an image of the proteome can be generated by systematically rastering across the entire section at a fixed resolution (4–6). The mass signal intensities observed can then be plotted as a function of acquisition coordinates and presented as ion intensity images across the tissue section. Thus, a proteome image can be constructed by assembling several hundred protein profiles into an “immunohistochemical” image. Individual m/z species can then be selectively displayed across all the spectra on the tissue section to localize them to a specific region or tissue structure. This technology permits rapid screening and identification of proteins of interest from a tissue section proteome and does not necessitate initial generation of an antibody to a purified protein or synthetic peptide.

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Molecular & Cellular Proteomics 7.2 411
Several key studies analyzing normal mouse organs and tumors including colon (8, 9), prostate (10, 11), and epididymis (12, 13) have been performed using profiling and imaging mass spectrometry. The present study was designed to analyze the evolution of protein expression patterns during normal prostate development by MALDI MS and IMS. Because increased cell proliferation is most prevalent during normal prostate development, it is plausible that proteins involved in the developmental process are reactivated during tumorigenesis. Furthermore increased knowledge of normal mouse prostate biology is fundamental to evaluating the increasing number of mouse models for PCa (14). Normal prostate morphogenesis involves continuous interactions between prostatic stroma and epithelium (15), resulting in the commitment of the prostatic bud boundaries and signaling centers that subsequently invade the mesenchymal pad to induce epithelial elements (16). At birth, the mouse prostate is rudimentary, and branching morphogenesis (15, 17–23) continues until maturation of the prostate gland at 5 weeks of age (15).

In contrast, active cell proliferation continues after 5 weeks in LPB-Tag transgenic mouse tumors expressing Large T antigen under the regulation of the prostate-specific probasin promoter (24, 25). This study utilized transgenic line 12T-7f mice that developed tumors that proliferated rapidly and developed prostatic intraepithelial neoplasia with marked nuclear atypia and locally invasive adenocarcinoma (24). All males carrying the LPB-Tag transgene in a number of transgenic lines developed prostate tumors, indicating that penetrance was 100%. Although this mouse model demonstrates tumor development and progression, it at best mimics human PCa. Anatomically the mouse prostate surrounds the urethra similar to that observed in humans; however, unlike human prostates, the mouse prostate consists of four pairs of distinct prostatic lobes. All lobes were examined in this study. Previous reports have indicated that loss of tumor suppressors and overexpression of androgen receptor, growth factors, and other proteins can induce prostatic hyperplasia and prostatic intraepithelial neoplasia (PIN) (14). The Large T antigen is one of the most effective oncogenes that consistently promotes the development of adenocarcinoma and more invasive PCa in transgenic models (14). Whether SV40 is a causative agent for human cancers remains to be determined; however, SV40 DNA sequences or gene products have been identified in human tumors including mesotheliomas, lymphomas, and brain and bone tumors (26).

In our study, normal mouse prostates were compared with that of LPB-Tag tumors by MALDI profiling and imaging MS to identify proteins common to normal prostatic morphogenesis and tumorigenesis. In the process, proteins, such as probasin and spermine-binding protein, regulated by androgens and expressed in the sexually mature prostate were also identified. Furthermore these secreted proteins were absent in 12T-7f tumors, indicating that expression of differentiated proteins is decreased or lost during tumor cell proliferation. In contrast, cyclophilin A (CypA) was detected with differential α-N-terminal acetylation, and the two CypA isoforms were present during normal prostate and prostate tumor development. CypA is commonly expressed in other cancers (27–29). Thus, profiling and imaging MS can be utilized to identify proteins during the various stages of normal organ and tumor development. These proteins could potentially serve as biomarkers to characterize and identify a given stage during tumorigenesis.

**MATERIALS AND METHODS**

**Animals and Tissues**—CD-1 and LPB-Tag line 12T-7f mice on a CD-1 background were housed in the animal care facility at Vanderbilt University Medical Center in accordance with the National Institutes of Health and institutional guidelines for laboratory animals. CD-1 males and females were purchased from Harlan (Indianapolis, IN). CD-1 mice were crossbred, offspring were euthanized at 1, 2, 3, 4, 5, 6, 10, 15, and 40 weeks of age, and the prostates were dissected as follows. The mouse prostate consists of four pairs of lobes, annotated as anterior prostate (AP), dorsal prostate (DP), lateral prostate (LP), and ventral prostate (VP), which surround the urethra (25). For MALDI MS, the AP, DP, LP, and VP paired lobes were dissected and further separated into single lobes. Single prostatic lobes were snap frozen on dry ice and stored at −80 °C until analyzed.

The LPB-Tag mouse model for prostate cancer has been described in detail elsewhere (24, 25). Briefly the 5'-flanking region of the rat probasin gene designated as the long probasin promoter (-10806 to +28 bp, LPB) was linked to the Large T antigen (Tag) gene and used to generate transgenic mice on a CD-1 background. Tumor incidence in mice carrying the LPB-Tag gene was 100%, and transgene expression occurred specifically in prostate epithelial cells. Several LPB-Tag lines were generated of which the 12T-7f line was one that demonstrated rapid neoplastic prostate growth and progression to lesions similar to human low grade PIN and high grade PIN. Metastases were seen only rarely. For MALDI MS, the prostate lobes from the 12T-7f tumors (15 weeks of age) were individualized as described above with the restriction that dorsal and lateral lobes often fused during tumor development and could not be separated.

For IMS, the urogenital organs including prostate, seminal vesicles, and bladder attached to the urethra were removed. The seminal vesicles were carefully dissected, and the prostate (with urethra and bladder intact) was positioned such that the dorsal prostatic surface was placed onto the Optimum Cutting Temperature embedding medium (OCT) block. Cryosections were cut from the ventral prostatic surface and initially contained cross-sections of DP, LP, and VP, whereas deeper sections contained primarily AP and DP.

**Mass Spectrometry Profiling of Proteins**—Sections from the individualized fresh frozen prostate lobes were cut at −15 °C in a cryostat (30). To minimize contamination from the embedding medium (OCT polymer compound), the lobes were simply deposited on top of an OCT drop prior to its complete solidification. To minimize contamination from the blade, the disposable cryostat blade was rinsed with both methanol and acetone to remove any residual oil. The sections were cut at a thickness of 12 μm and thaw-mounted on gold-coated flat MALDI target plates. Prior to matrix application, the sections were allowed to dry in a desiccator for 1 h under house vacuum. Discrete spots of matrix (sinapinic acid prepared at 20 mg/ml in 50:50 acetonitrile, 0.2% trifluoroacetic acid solution) were then manually deposited on the sections using an automatic pipette by overlaying two 300-nl drops to increase matrix crystal density (supplemental Fig. S1).

MALDI MS analyses of the individualized prostate lobes were per-
formed using an Applied Biosystems Voyager DE-STR time-of-flight mass spectrometer equipped with a N₂ laser. MS data acquisition was manually performed at a repetition rate of 3 Hz by averaging signals from 1000 consecutive laser shots (four series of 250 shots). The instrument was operated in the linear mode configuration under delayed extraction condition with a source bias of 25 kV with optimum ion focusing at a m/z of about 15,000. The MS data were acquired in the m/z range between 2000 and 70,000. The MS data were further processed using some of the advanced functions of the Applied Biosystems Data Explorer software. Background was first subtracted using the Advanced Baseline Correction function (peak width, 40; flexibility, 0.5; degree, 0.1), and the data were smoothed using a nine-point Gaussian function. Finally the data were internally calibrated using the doubly and singly charged ion species from β-hemoglobin at m/z 7809.5 and 15,617.9, respectively. Comparison of signal intensity variations for the different lobes and across time points was performed using ClinProTools 2.1 (Bruker Daltonics, Billerica, MA). The mass spectra were realigned and normalized using the total ion current, and the standard deviations for common spectral features were calculated (31).

Whole Prostate Imaging Mass Spectrometry—IMS analyses were performed on sets of two 3- and 6-week control and 10-week 12T-7f cancerous mouse prostates. Because of the mouse prostate size (in particular from the 12T-7f prostate) and physiognomy, the samples were fully embedded in OCT prior to sectioning for each sample. Three sections cut at different heights were imaged. Sections were cut at a thickness of 10 μm and thaw-mounted on MALDI-TOF MS-compatible indium-tin oxide-coated glass slides (32). The OCT compound was removed by rinsing the sections in graded ethanol and water with the following protocol (4): 30 s in 70% ethanol, 30 s in 95% ethanol, 30 s in 70% ethanol, 30 s in deionized water, 30 s in 70% ethanol, and finally 30 s in 95% ethanol. Removal of the OCT compound has been found to be crucial for the overall quality of the MALDI MS data (4, 30). The first two rinses in 70 and 95% ethanols are aimed at "fixing" the proteins in the sections prior to the removal of the OCT compound, which is highly soluble in water. This limits in large part the solubilization or delocalization of proteins from the surface of the section. Furthermore the ethanol rinse also solubilize lipids and physiological salts from the section resulting in the detection of MALDI protein signals with higher yields. Sections were then fully dried in a desiccator for about 5 min prior to matrix deposition. When dried, matrix was automatically printed over the whole surface of the sections using an acoustic reagent multiphoton laser induced decay in collaboration with Labcyte Inc. (33). Prior to the printing of the matrix, the sections were seeded with a very thin layer of sinapinic acid matrix as described previously (33). After the seeding step, matrix (sinapinic acid prepared as mentioned above) droplets (~120 pl) were ejected from the spotter and collected in a array format on the section (supplemental Fig. S1). For the 3- and 6-week control prostate sections, matrix was printed with a center-to-center spacing of 150 μm. For the 12T-7f prostate section, matrix was printed with a center-to-center spacing of 200 μm. To generate a homogeneous field of matrix crystals at each printing coordinate, five passes of one drop were printed.

IMS data were acquired using a Bruker Daltonics Autoflex MALDI time-of-flight mass spectrometer equipped with smart beam laser technology (34). The instrument was operated in the linear mode configuration under delayed extraction condition with a source potential of 20 kV with optimum ion focusing at ~m/z 15,000. The MS data were acquired in the m/z range between 2000 and 90,000. The acquisition method was calibrated using the doubly and singly charged ion species from β-hemoglobin at m/z 7809.5 and 15,617.9, respectively, before the start of each acquisition. MS data from each of the matrix spots were automatically acquired at a repetition rate of 200 Hz using the FlexImaging software (Bruker Daltonics) by averaging ion signals resulting from 250 consecutive laser shots. The MS profiles were assembled using home-built software into a data format importable into the Biomap software (Novartis, Basel, Switzerland) used to visualize the ion images.

**Protein Identification**—Protein identifications were carried out with a two-step top-down directed approach. This involved the extraction of proteins from ~100 μg of tissue with added Halt protease inhibitor mixture including EDTA in 1.0 ml of T-PEER Tissue Protein Extraction Reagent (Pierce) according to the manufacturer’s instructions. Briefly the tissue sample was Dounce-homogenized at 200 rpm for 2 min on ice followed by sonication with a cell disruptor for 4×15 s (Fisher Scientific) and 10-min centrifugation at 10,000 ×g at 4°C to clarify the solution. The protein supernatant mixture was transferred to a fresh tube and quantified using a BCA colorimetric test (Pierce). TFA was then added at a concentration of 0.1% to 800 μg of protein in ~500 μl of sample buffer and syringe-loaded onto a trap column (C₂, Vydac, 2.5 mm) and then washed for 10 min with 0.1% TFA at 0.2 ml/min. A standard dual solvent linear organic gradient was used to separate the proteins on a 150 × 2.5-mm analytical column (C₂, Vydac) attached to the previously loaded guard column on an HPLC instrument (Shimadzu) at a flow rate of 0.5 ml/min. The linear portion of the gradient consisted of increasing buffer B (acetonitrile, 0.1% TFA) from buffer A (double distilled H₂O, 0.1% TFA) 20–45% over 35 min and then to 70% over another 15 min while collecting fractions every 30 min in a 96-deep well polystyrene plate (Whatman). The sample plate was dried down in a spin vacuum apparatus (Thermo) to dryness, brought up in 50 μl of 70% acetonitrile, and co-spotted onto a MALDI target plate with sinapinic acid matrix solution (20 mg/ml in 50:50 acetonitrile, 0.1% TFA). An LC-MALDI run was carried out using WARP-LC software to run the Bruker Autoflex II mass spectrometer in linear mode. Those fractions containing peaks of interest were then dried down, brought up in a non-reducing/denaturing solution, and subjected to heat followed by separation on a one-dimensional Tricine 10–20% gel (Invitrogen). Staining was carried out with colloidal Coomassie stain (Invitrogen) and visualized at 700 nm with an IR scanner (Odyssey, Licor). Those spots that correlated with the mass of interest were excised and digested with trypsin gold (Promega) according to the manufacturer’s instructions.

Peptide mapping and MS/MS were carried out on an Ultraflex III MALDI-TOF/TOF mass spectrometer (Bruker Daltonics) in automated mode following spotting samples mixed with α-cyano-4-hydroxycinnamic acid (5 mg/ml in 50% acetonitrile, 0.1% TFA, 5 mM ammonium acetate). Settings included the use of fuzzy control for laser adjustment and acquisition with 800 shots for the peptides mass fingerprints followed by MS/MS on the top 10 most intense ions with 400 shots for precursor and 1200 shots for accumulation of fragments induced by laser-induced decay. Resultant spectra were analyzed in BioTools and sent through MASCOT using a unimouse database with search windows corresponding to 0.2 dalton for the precursor and 0.35 dalton for the resultant fragments. The peak lists for MS and MS/MS were generated automatically in batch mode with Flex Control Version 3.0 and Flex Analysis Version 3.0 (Bruker Daltonics) using a proprietary “Top-Hat” base-line tool along with the “SNAP” peak detection algorithm, which was set to include peaks with signal to noise above 4.0 and quality factor above 100, followed by an automated calibration with neighboring peptide calibration standards (Bruker Daltonics). Peptide fingerprints and fragment information were searched through MASCOT 2.1, using trypsin digestion with up to one misdigestion permitted, global modifications of carbamidomethyl on cysteine with variable oxidation on methionine, a mass window of 0.20 Da for MS and 0.35 Da for MS/MS, against a mouse subset of the Uniref database with 131,631 entries created January 12th, 2007 that was formed to speed
Monitoring Mouse Prostate Development by Imaging MS

up searches on a local server. Cutoff scores of less than 50 for peptides and 100 for proteins were not considered; this is higher than expected to be statistically relevant by MASCOT but in our experience found to be more reliable. All peptide hits were evaluated visually as well. As these samples were highly purified from multidimensional isolations, a reverse database was not included to determine false positives as is commonly used in large data sets. Post-translational identification was determined by including an N-terminal acetylation as a variable modification in RapiDeNovo software carried out in the BioTools Version 3.0 Suite. The proteins analyzed in this study matched with a single identification following the database search in MASCOT with high scores, and the individual peptides in which MS/MS were carried out were found to associate with a single form of the protein as opposed to varied similar proteins in the same family as well.

Antibodies and Other Reagents—Anti-CypA rabbit antiserum was purchased from Upstate Cell Signaling Solutions (Lake Placid, NY). Anti-β-actin antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). All secondary antibodies were purchased from Zymed Laboratories Inc. (San Francisco, CA). Protein Extraction and Western Blot Analysis—Tissue extracts from CD-1 (3 and 15 weeks) and 12T7-f (15 weeks) prostates were prepared in lysis buffer (20 mM HEPES (pH 7.4), 300 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.1% Triton X-100, 0.5 mM DTT, 20 mM β-glycerophosphate, 0.1 mM NaVO₃, 0.5 mM PMSF, 1 mM leupeptin, and 5 μg/ml aprotinin). This suspension was homogenized with a Polytron homogenizer and centrifuged at 15,000 × g for 30 min, and the supernatant was collected and stored at −80 °C. All protein concentrations were determined using the BCA protein assay kit (Pierce).

Protein Sampling and Western Blot Analysis—Proteins were transferred to polyvinylidene difluoride membranes (Bio-Rad). Membranes were blocked with 5% milk for 1 h followed by incubation with primary anti-CypA (1:10,000 dilution) antibody at 4 °C overnight. The membrane was washed in Tween 20/TBS, and peroxidase-conjugated secondary antibody was diluted 1:10,000 for common spectral features across the different prostate developmental time points. This is illustrated in supplemental Fig. S2, A–C, for the 6-week DP lobe where intensity variations for three signals across the studied mass range are given. For each lobe, similar results were also obtained when comparing signal intensity variations for common spectral features across the different prostate developmental time points. This is illustrated in supplemental Fig. S2D for the DP signal detected at m/z 5444. Supplemental Fig. S2, E and F, also shows the intensities observed for two protein signals that displayed clear expression variations across the developmental time point studied (see Figs. 1–5).

Figs. 1–5 present the evolution of the resulting protein profiles for selected mass ranges where intensity differences were observed as a function of the different time points investigated as well as for the 15-week-old 12T-7f tumors. Some signals such as m/z 5444 (consistent with the molecular weight of COX7C) (12) and 6046 were expressed irrespective of age and were also clearly seen in the 15-week-old 12T-7f tumors, indicating that these proteins were constitutively expressed in normal prostate and prostate tumors (Figs. 1 and 2). Other signals such as m/z 4937 (consistent with the molecular weight of thymosin β10), 4965 (consistent with the molecular weight of thymosin β4) (6, 12), and 6225 were only expressed across all lobes during the first 2 weeks of life, suggesting that these proteins could play a role in early prostate development (Figs. 1 and 2). Although formal identification of these proteins is potentially important to further understand prostate development, the amount of tissue required to extract sufficient protein for purification and subsequent identification necessitates breeding large numbers of mice and is beyond the scope of this study.

Numerous other signals were progressively detected across all lobes as the prostate develops and matures. This is the case for the signals at m/z 5242, m/z 6122 (consistent with the molecular weight of serine protease inhibitor Kazal-type 3)
(35), m/z 18,394 (consistent with the molecular of probasin) (10), and m/z 24,702 (Figs. 1–4). These signals typically were very weak at week 4 and became clearly present at week 5 when prostate branching morphogenesis was complete. Furthermore, expression continued in the growth-quiescent adult prostate. The signal at m/z 6122 was barely detected in AP and found to be expressed in higher abundance in DP, LP, and VP. The signal at m/z 18,394 was detected throughout the lobes, but it was expressed in higher abundance in DP and LP. In DP, m/z 18,394 was detected as early as week 3. The signal observed at m/z 24,702 was detected as early as 2–3 weeks of age and was found to be more abundant in AP and DP (Fig. 4). Thus, the evolution of differential protein expression with age and between prostatic lobes could be traced by MALDI MS. Interestingly none of these signals were detected in the lobes of 15-week-old 12T-7f tumors. One group of signals between m/z 21,800 and 24,200 was clearly specific to the ventral lobe of the prostate (Fig. 5). Similar signals have
been identified previously and characterized from the mouse prostate and correspond to the glycosylated forms of the spermine-binding protein (SBP) (10). Again these signals were not seen in the ventral lobe of 15-week-old 12T-7f tumors, indicating that the expression of differentiated proteins was down-regulated during cell proliferation.

The protein signals seen in Fig. 3 consisting of a doublet at m/z 17,841 and 17,883 were present at all ages in both the normal prostate and in 12T-7f prostate tumors. These signals were identified as two isoforms of CypA (see below). The two forms of CypA are separated by 42 Da consistent with the presence of an α-N-acetyl group on the N terminus of the protein (12). The evolution in intensity of CypA was more difficult to follow over time as it appeared to be progressively masked by the presence of an additional signal at m/z 17,874 that emerged at about the week 4 time point and progressively increased with time. This was particularly obvious in AP and DP. In contrast, the CypA signal was recovered...
Interestingly the signal intensity ratio maturation as illustrated by the evolution of the CypA signals relatively constant with time and do not disappear with prostate prostate was analyzed. Overall the CypA signals appear relatively a clean doublet when the 15-week-old 12T-7f cancerous prostate tumors, indicating that the non-acetylated CypA form was less prominent in PCa. Although variations in the abundance variation of the non-acetylated for the 1–4-week-old prostate but 0.77 ± 0.03 for 12T-7f tumors, indicating that the non-acetylated CypA form was less prominent in PCa. Although variations in the abundance of CypA could not be determined from the profiling data, the variation of the non-acetylated versus acetylated ratio and the fact that CypA has been previously seen to be associated with cancer (discussed below) has motivated us to more specifically investigate its expression in both normal and cancer mice (see Figs. 9–11).

Identification of CypA—The results related to the identification and N-terminal characterization of CypA are presented in Fig. 6. Two distinct HPLC fractions were identified containing the signals at m/z 17,841–17,882 (Fig. 6, A and B) consistent with those observed in the tissue profiles. From both fractions, after trypsin enzymatic digestion, mapping of the resulting peptides by MALDI MS (Fig. 6, C and D), and database searching, CypA was unambiguously identified with Mowse scores over 80 and sequence coverages of over 70% (see supplemental Figs. S3 and S4). Further the observed molecular weight for CypA corresponded exactly with the theoretical molecular weight 17,840 computed based on the amino acid sequence after removal of the N-terminal methionine (Swiss-Prot accession number P17742). To fully characterize the position of the acetylation event on the N terminus of CypA, MALDI MS/MS measurements were performed on the signals observed at m/z 2006.0 and 2048.0, respectively, matched to the non-acetylated and acetylated N-terminal peptides (Fig. 6, E and F). Based on observed fragmentation patterns, N-terminal CypA acetylation was found to be unambiguous (supplemental Figs. S5 and S6). These findings are in excellent agreement with a previous characterization of CypA from the mouse epididymis (12).

Mouse Prostate Protein Imaging—In parallel to the acquisition of lobe-specific protein profiles, protein images were acquired from whole mouse prostate sections for the 3- and 6-week-old development time points as well as for 10-week-old 12T-7f prostate tumors. Because the mouse prostate has a complex architecture in a three-dimensional volume, two or three parallel sections were cut at different heights within the prostate to investigate the protein signatures from all of the different lobes for each prostate specimen. After sectioning, rinsing, and matrix deposition, the sections were imaged by MALDI MS. After image acquisition, the matrix was removed, and the sections were stained with hematoxylin and eosin (H&E) for histological evaluation and alignment with the ion images. Fig. 7 presents examples of different ion images obtained from the 6-week-old CD-1 prostate. The H&E images represent the top, middle, and bottom sections of the prostate. In all three sections, the urethra as well as the bladder can be seen. In the top section, only the LP and VP lobes are visualized. In the middle section, VP tissue is no longer present, but all of the other prostate lobes are visualized. In the bottom section, only AP and DP can consistently be observed. From the IMS analysis of each section, on average 150 different ions images were obtained, all originating from different protein species. Ion intensity images are presented for m/z 5444 (COXO), 9592, and 18,394 (probasin) across all sections. Fig. 7 also presents ion images for proteins detected throughout the prostate (top, middle, and bottom sections). The m/z 5444 signal is consistently detected throughout the different lobes of the prostate as well as in the inner wall of the bladder but has a significantly stronger intensity in the smooth muscle layer surrounding the urethra. The signals at m/z 9592 and 18,394 are clearly lobe-specific; m/z 9592 is detected in the AP, DL, and LP with the strongest signature in LP, whereas m/z 18,394 is also absent in VP but has a very strong signature in DP and LP. For m/z 5444 and 18,394, the relative ion intensities are very consistent with the relative intensities observed in Figs. 1 and 3, respectively.

Within the same data sets, numerous protein signals were found to be lobe-specific. Fig. 8 presents several such examples observed from the three sections cut from the 6-week-old mouse prostate. Fig. 8A presents ion images from protein signals predominantly observed in VP from the top section such as for m/z 6122 (serine protease inhibitor Kazal-type 3)
and for the group of signals between \( m/z \) 21,800 and 24,200 (SBP). These findings again correlate with the intensities observed for the same signals in Figs. 3 and 5, respectively. Fig. 8, B and C, represents ion images from the middle section where protein signals predominantly originate in LP, although \( m/z \) 18,073 also demonstrates significant intensity in DP as can be seen in Fig. 8E (bottom section). Fig. 8, D and E, represents ion images from various protein signals predominantly observed in AP and DP from the middle and bottom sections. An overlapping protein at \( \sim m/z \) 17,874 appearing around week 5 of prostate development determined that CypA signal expression at \( m/z \) 17,840 and 17,882 could not reliably be monitored. In contrast, clear ion images were obtained for the CypA signals from the week 3 development time point as well as from 12T-7f tumors (Fig. 9). CypA ion density maps were acquired at a resolution of 150 \( \mu \)m from two parallel cross-sections (top and bottom) from a 3-week prostate for both the non-acetylated and \( \alpha \)-N-terminal acetylated forms of CypA at \( m/z \) 17,840 and 17,882, respectively (Fig. 9A). Both images are correlative and indicate the presence of CypA within all of the prostate lobes. Similar results were obtained for 12T-7f prostate tumors (Fig. 9B). Because of the much larger dimension of the tumors, IMS was performed at a resolution of 200 \( \mu \)m. Both forms of CypA were co-expressed, and expression was only seen within the four prostate lobes.

**Immunohistochemistry and Western Blot Analyses of CypA—** Immunohistochemical and Western blot analyses were performed to confirm the MS profiling and imaging results obtained for CypA. Unlike MALDI MS, which can identify posttranslational processing, the CypA antibody only detects total CypA protein levels. Antibodies that distinguish between \( \alpha \)-N-terminal acetylated and non-acetylated CypA are currently not available. In 15-week CD-1 epithelial cells, CypA staining occurred in a punctate pattern, and expression was primarily nuclear with limited cytoplasmic expression (Fig. 10). In stromal cells, however, staining was predominantly cytoplasmic, and nuclei contain little to no CypA expression.

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**Fig. 6. Identification and characterization of cyclophilin A.** MALDI MS protein profiles from the HPLC fractions containing the mass signals at \( m/z \) 17,841 (A) and \( m/z \) 17,883 (B). C and D, MALDI MS peptide mass fingerprinting from each fraction, respectively, obtained after tryptic digestion. MALDI MS/MS spectra of the non-acetylated (\( m/z \) 2006) (E) and acetylated (\( m/z \) 2048) (F) CypA N-terminal peptide are shown.
CypA staining was similar in 12T-7f prostate tumors; however, the intensity of nuclear and cytoplasmic CypA immunoreactivity increased, suggesting that CypA expression was elevated in the tumors (Fig. 10L). In addition, similar cellular localization and expression levels were observed in 3-week CD-1 and 12T-7f pros-
tates (data not shown). These observations correlate with the CypA expression profiles generated by MALDI MS and IMS. CypA expression was also examined in human BPH and prostate adenocarcinoma tissue sections. As anticipated, CypA expression was similar in the BPH and tumor sections (Fig. 10, C and F, respectively). In epithelial cells, CypA expression was punctate, strongly nuclear, and cytoplasmic, whereas CypA expression was predominantly cytoplasmic in stromal cells.

Western blot analyses were performed to compare the levels of CypA expression in normal prostate with that in 12T-7f tumors (Fig. 11A). CypA appeared as a doublet in normal CD-1 prostates (3 and 15 weeks) as well as in 12T-7f tumors. Whether these bands correlate with the acetylated and non-acetylated forms is not known because antibodies that recognize N-terminal acetylation are not available. CypA levels (upper band) increased >2-fold in the mature 15-week compared with the 3-week developing CD-1 prostate (Fig. 11B). In addition, CypA expression increased nearly 5-fold in 15-week 12T-7f tumors compared with age-matched normal controls, correlating with the increase in CypA expression observed by immunohistochemical analysis. Expression levels of the lower band increased in parallel with a 2.6-fold increase in 15-week 12T-7f compared with 15-week controls.

**DISCUSSION**

MALDI-TOF and IMS have been utilized to analyze the ontogeny of protein expression in the developing and adult prostate as well as in 12T-7 prostate tumor specimens. These data show that increases and decreases in protein expression occur as a continuum during normal prostate development.
For example, the signals at m/z 4937, m/z 4965, and m/z 6225 were expressed with greater intensities during weeks 1 and 2, suggesting that these proteins functioned specifically during this stage in prostate development. However, these signals were not seen in the 12T-7f prostate tumors. In contrast, signals corresponding to androgen-regulated proteins such as probasin and SBP progressively increased during prostate development, reaching adult levels as the prostate sexually matured. Most if not all of the signals showing this intensity trend were absent in 12T-7f tumors, indicating that normal protein synthesis decreased with tumor cell dedifferentiation into a more proliferative phenotype. IMS significantly complemented MALDI MS, demonstrating that SBP expression was VP lobe-specific, whereas probasin expression was prostate-specific, being absent in bladder and urethra. IMS also revealed that the protein masking the CypA signal essentially occurred in the AP (data not shown). Thus in combination, MALDI-MS and IMS can be utilized to identify the combinatorial patterns in which proteins are expressed during the different stages in prostatic development. Furthermore they could identify the ontogeny of proteins during tumorigenesis.

In addition to protein expression, IMS also allows the detection of posttranslational modifications events. This is one of the strong points of the technology whereas exact mass measurements between neighboring mass signals may lead to the confirmation or identification of secondary protein processes. In this study, a clear change in the proportion of α-N-acetylated and non-acetylated CypA was observed between the normal developing mouse prostate and 12T-7f prostate tumors. Posttranslational modifications influence processes such as organogenesis, development, growth, reproduction, and tumorigenesis by controlling stimulus-specific functions. Eighty to ninety percent of all mammalian proteins are acetylated; yet the role of this acetylation is not clear. In one study, α-N-acetylation shifted activity of the proopiomelanocortin-derived proteins between endorphinergic and melanocortinergic actions, which are often antagonistic (36). Our MS analysis of CypA indicates that the initial methionine residue is removed, and the adjacent valine residue can be acetylated. The differential abundance of this modification is demonstrated in normal prostate development and prostate cancer, suggesting that acetylated and non-acetylated CypA has specific functions in these processes. Although only 3% of yeast proteins are acetylated on the valine residue, ~45% of mammalian proteins have valine residues modified by an acetyl group, and valine is thought to be partially modified (37). Residues most likely to be acetylated include serine, alanine, or methionine. Aspartic or glutamic acid residues stimulate acetylation, whereas proline inhibits acetylation, and positively charged lysine and arginine usually, but not always, inhibit acetylation (37). The biological significance of these differences remains to be determined. However, they could be utilized to design experiments that begin to address the role of α-N-terminal acetylation in cell function.

In this study, we were not able to correctly quantify CypA expression variations between the different mouse prostate development time points and 12T-7f prostate tumors based on the mass spectrometry measurements and had to rely on more traditional but extremely powerful immunohistochemical and Western blot approaches. From the IMS results, both isoforms of CypA were found to co-localize to the different prostate lobes. However, there is insufficient resolution in the protein images to determine which prostate cell type(s) expresses CypA. Although it is possible to perform IMS at resolutions as small as 10 μm (38), for this study we chose to print matrix droplets on the tissue sections in an array format with a center-to-center distance of 150 μm, the smallest current possible distance to avoid cross-contamination across spots. This mode of matrix deposition, however, also allows the recovery of excellent quality MS data in terms of signal intensity and resolution and therefore the detection of a high number of distinct protein signals (4, 33). The immunohistochemical images for CypA were found to correlate very well with the IMS images in that predominant expression was detected in all of the prostate lobes. However, no distinction could be made between the two CypA isoforms. CypA was also found to be uniquely expressed in epithelial and stromal...

**Fig. 11.** Western blot analyses of total CypA expression. A, Western blots of total CypA and β-actin. Prostate tissue extracts (30 μg of protein) from 3- and 15-week CD-1 normal mouse prostates and 15-week 12T7-f prostate tumors were separated by 12% SDS-PAGE. Immunoblotting was performed using primary antibodies specific to CypA and β-actin as described under “Materials and Methods.” UB, upper band; LB, lower band. B, densitometric analysis of CypA expression. Densitometry was performed using ImageJ software (Wayne Rasband, Research Services Branch, National Institute of Mental Health, Bethesda, MD). CypA expression was normalized to the β-actin levels. UB, upper band in A; LB, lower band in A.

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cells, and subcellular distributions between nucleus and cytoplasm could even be determined. CypA staining intensity was found to be increased in 12T-7f prostate tumors with respect to the normal prostate suggesting that CypA expression was elevated in the tumors. More precise semiquantitative CypA expression measurements were then performed by Western blot analyses. In this case, a factor of ~2–5 in CypA relative abundance was detected between the fully mature prostate and 12T-7f prostate tumors. To reliably detect a 2–5-factor increase in CypA protein expression from MALDI MS tissue profiles or images, the number of necessary samples in each group would have needed to be as high as 20.

CypA is known to be overexpressed in solid tumors including colon cancer, melanoma, pancreatic carcinoma, and non-small cell lung carcinomas in addition to the hematologic malignancies of T-cell acute lymphocytic leukemia, B-cell immunoblastic sarcoma, and Hodgkin and non-Hodgkin lymphoma (27, 29). Thus, CypA may play a role in oncogenesis. Knockdown of CypA expression in non-small cell lung cancer xenografts using stable RNA interference results in decreased proliferation and increased apoptosis (28), implying that CypA regulates cell cycle progression. The concept that chronic inflammation in or surrounding neoplastic lesions promotes and regulates tumor development has gained momentum. It was originally proposed that chronic inflammation induced the formation of PIN followed by the development of prostate cancer (39). Inflammation has also been correlated with gastric cancer (40, 41) and colon cancer (42). The role of CypA in prostate cancer remains to be established.

In summary, comparison of temporal variations in protein expression and localization of numerous proteins throughout the development of the mouse prostate with 12T-7f prostate tumors were made possible by profiling and imaging MALDI MS. The expression profiles of hundreds of protein signals were followed from the first weeks of life (weeks 1–4) through sexual maturation (weeks 5–6) and old age (week 40). Several signals were found to be uniquely expressed during the early time points, whereas others progressively increased with sexual maturation. For the protein signal identified as cyclophilin A, a clear change in the amount of α-N-acetylation and non-acetylation was observed between the normal developing mouse prostate and 12T-7f prostate tumors. Complementary information for CypA including the precise cellular and subcellular localization and relative abundance for different stages of the developing prostate and 12T-7f prostate tumors was obtained by immunohistochemistry and Western blot analyses. The combined utilization of these three analytical strategies has allowed us to further advance our knowledge of normal prostate and prostate cancer development.

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