Identification of prostatic-secreted proteins in mice by mass spectrometric analysis and evaluation of lobe-specific and androgen-dependent mRNA expression

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Abstract

Rats and guinea pigs have frequently been used to study the development of the prostate and the mechanism of androgen action, but the mouse prostate has also become an attractive model for prostate research, because an enormous range of genetically altered mice is now available. However, the secretion of proteins in the mouse prostate has not yet been thoroughly investigated. In the present study, major secreted proteins from the ventral prostate (VP), dorso-lateral prostate (DLP), and anterior prostate (AP) of mice were identified by means of 2D-gel electrophoresis followed by MALDI-TOF mass spectrometric analysis. A quantitative reverse transcriptase-PCR method was further employed to examine the androgen-dependent transcriptional regulation of the identified proteins. Proteome analysis revealed that the VP secretes spermine-binding protein, serine protease inhibitor

Kazal type-3, and a 91 kDa hypothetical scavenger receptor (AK035662). DLP and AP secrete a protein similar to immunoglobulin-binding protein, immunoglobulin-binding protein-like protein, and one of the experimental autoimmune prostatitis antigen proteins (EAPA2). Peroxiredoxin-6, glucose-regulated protein 78, zinc- α 2-glycoprotein, and phospholipase C α are also secreted. Castration of animals led to a decrease in the mRNAs of these secreted proteins, although the extents of changes varied greatly among different lobes. We present here an outlined view of mouse prostate secretion, which should contribute to an understanding of the biological functions of the prostate gland, as well as the androgen dependency of prostate secretion.

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Introduction

Rat models have been widely used to study prostate morphology, development, and pathology, as well as androgen-regulated gene expression, in order to understand the basic functions and pathology of this male accessory sex gland (Cunha *et al.* 1987). Mice have generally not been used because the small size of the gland makes morphological studies difficult, and because the mouse prostate is less susceptible to carcinogenesis (Shirai *et al.* 2000). Recently, however, a huge range of transgenic and knockout mice has become available with considerable potential for studies of the prostate (Abate-Shen & Shen 2002, Klein 2005). Transgenic adenocarcinoma of the mouse prostate (TRAMP) mice has been used to study the progression and chemoprevention of prostate cancer (Greenberg *et al.* 1995). Prolactin transgenic mice have been used to investigate the effect of prolactin on prostate growth (Wennbo *et al.* 1997). Estrogen receptor

knockout mice (α ERKO and β ERKO) have been used to examine the role of estrogen in prostate development (Weihua *et al.* 2001, Omoto *et al.* 2005). Aromatase knockout, prolactin receptor knockout, and conditional deletion of Rb mice have been used to study the involvement of those genes in prostate carcinogenesis (McPherson *et al.* 2001, Robertson *et al.* 2003, Maddison *et al.* 2004). However, despite these recent developments, the basic biological function of the prostate, prostatic secretion, is still poorly understood in the mouse. Identification of the secreted proteins will be helpful in understanding prostate development and pathology.

The rodent prostate consists of the ventral prostate (VP), lateral prostate (LP), dorsal prostate (DP), and anterior prostate (AP or coagulating gland). It is well known that rat prostatic secretory proteins, such as prostatein and cystatin-related protein, are mainly produced in the VP, and other proteins, such as prostatic secretory protein of 94 aa (PSP94), probasin,

and seminal vesicle secretion 2 (SVS2) are abundant in the LP and DP (Cunha *et al.* 1987). An early study revealed that spermine-binding protein (SBP) and serine protease inhibitor Kazal type-3 (SPI-KT3) are abundant in the mouse VP (Mills *et al.* 1987a, 1987b). Proteins secreted from the dorso-lateral prostate (DLP) and AP have not yet been identified, although Cunha's group developed a specific polyclonal antibody for major DLP protein(s) to be used as a differentiation marker (Donjacour *et al.* 1990).

In the present study, the major proteins secreted from the VP, DLP, and AP were identified by means of 2D-gel electrophoresis followed by MALDI-TOF mass spectrometric analysis. Further, a quantitative reverse transcriptase (RT)-PCR method was employed to examine the androgen dependence of the transcriptional regulation of the secretory proteins.

Materials and Methods

Animals

Animal experiments were conducted in accordance with *A Guide for the Care and Use of Laboratory Animals of Hiroshima University*. The male C57BL mice were purchased from Charles River Japan Co. (Kanagawa, Japan) and maintained with free access to basal diet and tap water. For proteome analysis, three of the 11-week-old mice were killed under ether anesthesia and the prostate and seminal vesicle (SV) were carefully dissected out. In addition, four animals were used for evaluating the sample preparation method. For the study of age-dependent mRNA expression, animals were killed at 1, 2, 4, 6, and 11 weeks (four animals per group), and each of the prostate lobes was dissected under a microscope and immediately fixed in RNA Later solution (Ambion, Inc., Austin, TX, USA). For the castration and hormone-replacement study, animals were divided into three groups, the castrated, castrated plus testosterone injected, and intact. Surgical castration was made at 10 weeks of age and animals were allowed to recover for 1 week. Testosterone propionate (Wako Junyaku KK, Osaka, Japan) was dissolved in the vehicle oil, Panacete 810 (Nippon Oils and Fats Co., Ltd, Tokyo, Japan), and the solution was

administered i.p. at a dose of 5 mg/kg body weight. Animals were killed under ether anesthesia 24 h after testosterone injection, and the prostate lobes were collected for RNA extraction.

Preparation of secretion samples

Preparation of secretion samples was performed based on the previously reported method (Donjacour *et al.* 1990). Each dissected prostate lobe from an 11-week-old mouse was rinsed well in saline and placed on a 35 mm culture dish with 100 µl saline containing 1% protease inhibitor mixture (Sigma). Each lobe was cut into four or five pieces, left to stand for 5 min, and transferred to a 1.5 ml microcentrifuge tube. After centrifugation at 10 000 *g* for 5 min at room temperature, the supernatant was collected as the secretion sample. The incubation time of 5 min was chosen because inner cellular contamination (glyceraldehyde-3-phosphate dehydrogenase; GAPDH) was confirmed to below by 5 min (Fig. 1). For the SV secretion sample, the content of the vesicle was collected and suspended in saline with protease inhibitors. The protein concentration of each sample solution was determined with a Protein Assay kit (Bio-Rad Lab.). For de-glycosidation, samples were incubated with PNGase F (50 U/µg protein; New England Biolabs, Ipswich, MA, USA) at 37 °C for 1.5 h.

Electrophoresis (1D and 2D-PAGE)

For SDS-PAGE gel electrophoresis, 15 µg total protein of each sample were mixed with the SDS-PAGE buffer containing 2-mercaptoethanol, and applied to a 5–20% gradient PAGE gel (10×10 cm² SuperSep pre-cast gel; Wako Junyaku) with molecular weight markers, Precision Plus (Bio-Rad Lab.). The electrophoresis was carried out at a constant current of 20 mA. The gel was fixed and stained with 45% methanol and 10% acetic acid containing 0.2% Coomassie Brilliant Blue, followed by de-staining with 7% methanol and 7% acetic acid.

For 2D-gel electrophoresis, 1D isoelectric focusing with immobilized pH gradients was performed with Immobiline

Figure 1 Intracellular contamination in secretion samples. Each prostatic lobe was cut into four or five pieces, and secretion out of the prostatic canals was allowed for 1, 5, and 15 min. Then the lobes were centrifuged and the supernatant was collected as the secretion sample. The samples were applied to the SDS-PAGE (5–20%) at 0.3 µg protein/lane, transferred to a piece of PVDF membrane and immunostained with antibody to GAPDH, an inner cellular marker (36 kDa band). The intracellular contamination was lesser in the samples incubated for 1 and 5 min (1, 5 M) than in those incubated for 15 min (15 M) or prepared from minced tissues (minced). The incubation time of 5 min was chosen for sample preparation in the present study.

DryStrip (Amersham) and the Ettan IPGphor system (Amersham) according to the manufacturer's protocol. For analytical 2D-PAGE, 10 µg de-glycosidated protein was applied in a 7 cm Immobiline DryStrip (pI 3–11, nonlinear gradient). After rehydration, the strip was isoelectrofocussed (15 kVh). The Immobiline gel was then treated with SDS equilibration buffer (50 mM Tris–HCl, pH 8.8, 6 M urea, 30% glycerol, and 2% SDS) containing 10 mg/ml DTT for 15 min, followed by the same buffer containing 25 mg/ml iodoacetamide. The Immobiline gel was then placed on the second SDS-PAGE slab gel with 5–20% gradient (SuperSep pre-cast gel, Wako Jyunyaku) and overlaid with hot agarose solution to connect the two gels. The second electrophoresis was run at a constant current of 20 mA. The gel was fixed with 50% methanol and 7% acetic acid, stained overnight in Sypro Ruby (Invitrogen), and de-stained with 10% methanol and 7% acetic acid. Stained gels were scanned with a Molecular Imager FX Pro (Bio-Rad Lab.), with excitation at 532 nm. In the case of preparative 2D-PAGE for mass spectrometric analysis, 60 µg total protein were subjected to electrophoresis as described previously and then stained with silver nitrate by incubation with 0.2 g/l Na₂S₂O₃ for 1 min followed by 1 g/l AgNO₃ for 20 min on ice, and washed with 20 g/l Na₂CO₃ containing 0.1% HCHO. A 24 cm Immobiline DryStrip (pI, 3–11) was also used for preparation. It was rehydrated with 200 µg secreted protein and isoelectrofocussed (25 kVh), then placed on 12.5% SDS-PAGE gel and overlaid with hot agarose solution. The electrophoresis was performed at a constant current of 400 mA. The gel was stained with silver nitrate as described earlier. Three sets of secretion samples from different animals were applied to 2D-electrophoresis and analyzed.

Western blotting

Total proteins, 0.3 µg of each preparation of prostate secretion, were applied to SDS-PAGE (5–20% gel). Proteins were then transferred to a piece of Hybond-P polyvinylidene difluoride (PVDF) membrane (Amersham). The membrane was incubated with a monoclonal antibody to GAPDH (Ambion) at 1 µg/ml followed by a peroxidase-conjugated antibody to mouse IgG (MBL Co., Nagoya, Japan) at a dilution of 1:1000. Protein bands were detected using the ECL system (Amersham).

Mass spectrometry (MS)

The protein spots were excised from the polyacrylamide gel and silver nitrate was removed with 15 mM K₃[Fe(CN)₆] and 50 mM Na₂S₂O₃. The gel pieces were incubated in distilled water for 1 h, incubated with CH₃CN for 10 min, and dried in a centrifuge–vacuum concentration system. Each gel piece was incubated with a 20 µl aliquot of 10 µg/ml trypsin solution (sequence grade, Sigma) for 30 min on ice. Excess trypsin solution was removed, and the gel piece was incubated overnight at 35 °C. To extract the digested peptides, 10 µl of 70% CH₃CN containing 0.1% trifluoroacetic acid were added

to each gel piece. An aliquot of 0.5 µl of the extract solution was spotted onto a target plate for an UltraFlex mass spectrometer (Bruker Daltonics, Bremen, Germany) along with 0.5 µl of 10 mg/ml α -cyano-4-hydroxycinnamic acid (MS grade, Nacalai tesque Co., Kyoto, Japan). MS was performed using an accelerating voltage of 20 kV, with data acquisition between 1000 and 4000 Da. Some of the fragment peaks were further analyzed by MS/MS. The MS and MS/MS data were evaluated with Biotoools software (Bruker Daltonics) in combination with a peptide mass fingerprinting analysis system, MASCOT version 2.1 (Matrix Science, London, UK). The peptide mass fingerprinting was performed based on mass spectroscopy protein sequence database (MSDB; Imperial College London, UK) and the nr database at the National Centre for Biotechnology Information (NCBI; Bethesda, MD, USA) with terminal modifications of peptides set as fixed carbamidomethyl and flexible oxidation ends. The peptide mass tolerance was set to 0.3%.

Quantification of mRNAs by real-time RT-PCR

Total RNA was prepared from each lobe of the prostate with an RNA Isolation kit (Promega), and 2 µg total RNA were reverse transcribed as described previously (Fujimoto *et al.* 2004). An ABI Prism 7700 (Perkin–Elmer Life Sciences, Boston, MA, USA) was employed for quantitative measurement of cDNA using a QuantiTect Sybr Green PCR kit (Qiagen). Specific primer sets with a *T_m* of about 59 °C were designed for each mRNA (Table 1). Prior to quantitative analysis, PCR products were prepared separately and purified by gel electrophoresis. The DNA sequences were confirmed with a capillary DNA sequencer, ABI 310 (Perkin–Elmer Life Sciences). Extracted fragments were used as standards for quantification. The PCR conditions were 15 min of initial activation followed by 45 cycles of 20 s at 94 °C, 30 s at 58 °C, and 40 s at 72 °C. All mRNA contents were normalized with reference to β -actin mRNA.

Serum testosterone levels

Serum testosterone levels were measured with an ELISA kit, purchased from Neogen Corp. (Lexington, KY, USA).

Statistical analysis

Statistical comparisons were made using Student's *t*-test.

Results

1D-PAGE analysis

The secretory proteins from the VP, DLP, AP, and SV were treated with a de-glycosidation enzyme, PNGase F, and analyzed with SDS-PAGE (Fig. 2). In the VP, a broad band at 20–25 kDa was evidently the major band, and a 10 kDa band

Table 1 Quantitative PCR primers for mouse genes

	GenBank accession #	5'-Primer (5'→3')	3'-Primer (5'→3')
Genes			
91 kDa protein	AK035662	GGACCTTCCACAAGCGAACAT	GCACTCCTCCAGGTGTTCTCTC
AGR2	M_011783	TTCATCACTTGGACGAATGCC	ACGTACTGGCCATCAGGAGAA
Calr	NM_007591	ACCGTGAAGCATGAGCAGAAT	TGTTGATCAGCACATTCTTGCC
EAPA2	AY528666	CCAGACAGGCAGAATTGGGTT	CTCCTCGGAATCTATATTGGCG
GRP78	NM_022310	TCTTGCCATTCAAGGTGGTTG	TTCTTTCCCAATACGCCTCAG
IgBPLP	XM_620455	CTGTGAGTTGCCCGAGCCT	CACAATGGAGAACGCCTCCT
PDI	MUSPDIA	CGCAACAACTTTGAGGGTGA	TTGGGCAGGAACAGCAGAAT
PLCα	M73329	ATTGCACTGCCAACACAAACA	AACTGAAGCTGGTCCTGCTTG
Prdx6	BC013489	AGGACGCTAACACATGCCTG	GTGCCTGTCAGCTGGAGAGAG
Probasin	AF005204	ACACTGCATGTGCTAGGCGT	TCCCACACAAATGTGACGG
PSP94	U89840	CCAACGCTACTAGGCCTTTGA	GCCCACACGAAGCACATTTAC
SBP	NM_011321	TGGAACCCGGTCAGATAACTTT	TCGACCCCTTCTAACACCAAA
SPI-KT3	BC086887	AGAGGCTAGTTGCCATGATGC	GGACAGGCTCTATGCGTTTCC
SVS2	NM_009300	CAGAGCAGCTCCTCAGAGGG	TCTGGGTCTATGTACCACCA
ZnG	AF281658	CCCACAGGACATAGACCCCTT	CTCATGTGAGGCAGAGAGGGTA
β-Actin	X03765	CTGTCCCCTGTATGCCTCTGGTC	TGAGGTAGTCCGTCAGGTCCC

seemed to be secondary. PNGase F treatment shifted the major band to a sharper 19 kDa band, while other bands were unaffected. The main band in the DLP appeared to be a broad band at 80–100 kDa, together with bands at 17 and 13 kDa.

PNGase F digested the major band into two sharper bands of approximately 80 and 90 kDa, which showed lower staining intensities. The mobility of other bands was not changed much by PNGase F treatment, although some smear-like

Figure 2 1D-SDS-PAGE analysis of mouse prostate secretory proteins. Secretion was prepared from the VP, DLP, and AP, as well as the SV. Each sample was incubated with (+) or without (–) PNGase F and applied to a 5–20% gradient SDS-PAGE gel. The gel was stained with Coomassie Brilliant Blue. Arrows indicate major shifted bands by PNGase F.

staining disappeared. When the AP was compared with the DLP, the patterns of bands larger than 25 kDa were similar, as was the effect of PNGase digestion. However, several DLP-specific bands were present in the molecular weight range below 25 kDa. A band of albumin, 68 kDa, representing contamination from serum, was present in the preparations of prostatic secretion, especially in the VP and DLP. The pattern of SV protein bands was completely different from those of prostatic proteins. The major SV bands were observed between 10 and 16 kDa.

2D-PAGE and identified proteins

Secretory proteins from all the lobes were treated with PNGase F and subjected to 2D-PAGE analysis. Owing to the limitation in the pore size of the immobilized pH gradient gel for isoelectric focusing, proteins with a molecular mass of over 100 kDa could not be analyzed in the 2D-PAGE. Gels

were stained and the major spots were picked up for MS analysis (Fig. 3). The analysis of three sets of prostate secretions from independent control mice provided identical patterns. The spots were successfully identified and the results were summarized in Table 2. SBP and SPI-KT3 were major proteins in the VP. In addition, a 91 kDa protein, predicted from urinary bladder cDNA data (AK035662) was identified in the VP, along with glucose-regulated protein 78 (GRP78 or heat-shock 70 kDa protein 5) and peroxiredoxin 6 (Prdx6). Two higher molecular weight proteins in the DLP and AP were identified as experimental autoimmune prostatitis antigen 2 (EAPA2) and a predicted protein similar to immunoglobulin-binding protein (immunoglobulin binding protein-like protein; IgBPLP). Zn- α 2-glycoprotein (ZnG), a mammalian homologue of *Xenopus* anterior gradient 2 (AGR2), as well as PSP94 and probasin, were detected in the DLP secretion. Phospholipase C α (PLC α), calreticulin (Calr), and protein disulfide isomerase were also identified in both DLP and AP secretions. SVS2, 4, 5, and 6 were identified in the SV fluid.

Figure 3 2D-PAGE analysis of mouse prostate secretory proteins. Each sample from the VP, DLP, AP, and SV was treated with PNGase F and applied to an immobilized pH gradient gel, followed by a second SDS-PAGE. Gels were stained with Sypro Ruby. The identified spots are indicated in the figure. Serum albumin (Alb), transferrin (Trsf), and hemoglobin (Hb) were considered to be due to serum contamination.

Table 2 Identified mouse prostatic secretory proteins

	Protein name	Accession #	Observed MW/pi	Theoretical MW/pi	P	Sequence coverage (%)
Abbreviation						
SBP	Spermine-binding protein	NP_035451	18,35/4.5	22/4.6	0.999	46
91 kDa	Protein predicted from cDNA AK035662	Q8BZE1	80/4.5	93/4.8	0.973	13
GRP78	Glucose-regulated protein 78 kDa	A37048	70/5.0	72/5.0	1.000	39
Prdx6	Peroxisiredoxin 6	O08709	25/6.0, 6.5	25/5.6	1.000	57
SPI-KT3	Serine protease inhibitor, KT3	NP_033284	10/7.5	9/8.0	1.000	40
PSP94	Prostatic-secretory protein 94	NP_065622	13,28/5.0	13/5.5	1.000	76
IgBPLP	IgG-binding protein-like protein	XP_620455	100/ 5.5	210/5.5	1.000	28
EAPA2	Experimental auto-immune prostatitis antigen 2	NP_98193	100/6.5	103/6.2	1.000	42
PLCα	Phospholipase Cα	AAA39944	55/6.0	57/6.0	1.000	29
ZnG	Zinc-α2-glyco-protein	Q64726	35/6.0	34/5.8	0.992	28
AGR2	Homolog of <i>Xenopus</i> anterior gradient 2	BAB25181	20/9.5	20/9.5	1.000	56
Probasin	Probasin	AAC01954	22/9.5	21/10.1	1.000	56
Calr	Calreticulin	NP_031617	50/4.5	48/4.2	1.000	35
PDI	Protein disulfide isomerase	AAA39906	60/5.0	57/4.6	0.972	35

Lobe-specific mRNA expression of identified secreted proteins

Expression of identified proteins in the prostate was further confirmed by examining the mRNA levels; the results are summarized in Table 3. Lobe-specific expression of secreted proteins was evident. Both SBP and SPI-KT3 mRNAs were extremely abundant in the VP but virtually undetectable in the other lobes. The mRNA expression of the 91 kDa protein was also VP-specific. Abundant expression of IgGBPLP and EAPA2 mRNA was detected in the DLP and AP. Probasin expression was specific to the DLP/AP, while PSP94 mRNA was specific

to the VP/DLP. The level of ZnG mRNA was highest in the DLP. The other protein mRNAs were expressed uniformly among the prostatic lobes.

Androgen dependency in mRNA expression of identified secreted proteins

Transcriptional regulation of identified proteins by androgen was examined by comparing mRNA levels among castrated, castrated plus testosterone-treated, and intact animals (Table 4). Serum

Table 3 mRNA levels of identified proteins in each prostatic lobe in 11-week-old mice

	SBP	SPIKT3	91 kDa	PSP94	ZnG	GRP78	AGR2
VP	511 ± 58.9	184 ± 36.0	3.6 ± 0.8	2.6 ± 0.6	0.9 ± 0.1	1.7 ± 0.5	0.14 ± 0.03
DLP	0	0	0	5.8 ± 1.5	12.3 ± 1.1	6.1 ± 1.7	0.38 ± 0.04
AP	0	0	0	0	1.4 ± 0.3	2.7 ± 0.2	0.54 ± 0.04
	PLCa	Calr	PDI	Prdx6	Probasin	EAPA2	IgBPLP
VP	0.17 ± 0.021	0.7 ± 0.1	15.0 ± 2.1	2.5 ± 0.3	0.06 ± 0.02	0	0.0 ± 0.0
DLP	0.36 ± 0.085	1.2 ± 0.3	8.3 ± 2.1	21.7 ± 2.6	2.15 ± 0.23	18.9 ± 1.5	67.9 ± 10.3
AP	0.60 ± 0.088	1.1 ± 0.3	4.0 ± 0.8	29.0 ± 5.6	0.78 ± 0.05	5.5 ± 0.8	53.0 ± 0.3

Means ± S.E.M. (n = 5). Values are mRNA levels divided by β-actin mRNA levels (mol/mol β-actin). 11-week-old male C57BL mice were killed and total RNA was isolated from each prostate lobe. mRNA levels were measured by real-time RT-PCR.

Table 4 Androgen regulation of mRNA levels of identified proteins

	SBP	SPIK3	91 kDa	PSP94	ZnG	GRP78	AGR2
<i>VP</i>							
<i>Cast</i>	8.2±0.91 ^a	0.24±0.08	0.09±0.02	0.011±0.004	0.38±0.032	0.57±0.36	0.03±0.004
<i>Cast+T</i>	48±7.9 (5.9) ^b	9.3±3.2 (39)	0.8±0.23 (8.4)	0.4±0.008 (38)	0.56±0.10 (1.5)	1.02±0.14 (1.8)	0.14±0.03 (4.9)
<i>Intact</i>	511±58.9 (62)	184±36 (769)	3.6±0.79 (40)	2.6±0.55 (236)	0.92±0.09 (2.4)	1.7±0.47 (3.0)	0.12±0.02 (4.3)
<i>DLP</i>							
<i>Cast</i>	—	—	—	0.002±0.001	0.64±0.11	0.55±0.11	0.01±0.001
<i>Cast+T</i>	—	—	—	0.08±0.046 (38)	2.0±0.48 (3.1)	3.4±0.73 (1.8)	0.06±0.02 (6.9)
<i>Intact</i>	—	—	—	5.8±1.48 (2633)	12.3±1.1 (19)	6.1±1.7 (11)	0.38±0.04 (45)
<i>AP</i>							
<i>Cast</i>	—	—	—	—	0.06±0.01	0.33±0.59	0.01±0.002
<i>Cast+T</i>	—	—	—	—	0.21±0.05 (3.6)	1.33±0.19 (4.1)	0.08±0.013 (9.5)
<i>Intact</i>	—	—	—	—	1.4±0.3 (24)	2.7±0.22 (8.3)	0.54±0.04 (64)
	PLCa	Calr	PDI	Prdx6	Probasin	EAPA2	IgBPLP
<i>VP</i>							
<i>Cast</i>	0.14±0.081	0.2±0.03	5.6±1.2	3.7±0.73	—	—	—
<i>Cast+T</i>	0.23±0.03 (1.6)	0.7±0.1 (3.3)	11.7±2.1 (2.1)	3.8±1.41 (1.0)	—	—	—
<i>Intact</i>	0.17±0.021 (1.2)	0.7±0.09 (3.5)	15.0±2.1 (2.7)	2.5±0.3 (0.7)	—	—	—
<i>DLP</i>							
<i>Cast</i>	0.09±0.014	0.2±0.05	1.7±0.32	1.5±0.41	0.02±0.003	0.19±0.072	1.2±0.29
<i>Cast+T</i>	0.15±0.02 (1.7)	0.3±0.07 (1.5)	4.5±1.0 (2.7)	2.1±0.22 (1.4)	0.05±0.002 (2.3)	2.4±0.55 (13)	1.6±0.6 (1.4)
<i>Intact</i>	0.36±0.085 (4.0)	1.2±0.3 (6.4)	8.3±2.1 (5.0)	21.7±2.6 (14)	2.2±0.23 (124)	18.9±1.5 (102)	67.9±10.3 (58)
<i>AP</i>							
<i>Cast</i>	0.20±0.03	0.4±0.06	1.4±0.3	1.2±0.15	0.02±0.004	0.17±0.52	0.25±0.18
<i>Cast+T</i>	0.34±0.085 (1.7)	0.7±0.05 (1.8)	2.8±0.5 (2.0)	4.7±0.93 (4.0)	0.04±0.008 (2.6)	4.8±0.9 (29)	1.1±0.8 (4.4)
<i>Intact</i>	0.60±0.088 (3.0)	1.1±0.3 (2.8)	4.0±0.8 (2.8)	29.0±5.6 (25)	0.78±0.051 (49)	5.5±0.8 (33)	53.0±0.31 (215)

^aMean±S.E.M. (n=5). Values are mRNA levels divided by β-actin mRNA levels (mol/mol β-actin).

^bValues in parenthesis are fold change in mRNA over the castrated. 10-week-old male C57BL mice were castrated and maintained for a week (cast). They were killed 24 h after testosterone administration at 5 mg/kg bw, ip (Cast+T). Total RNA was isolated from each prostate lobe and amounts of mRNA were measured by real-time RT-PCR.

testosterone levels were 0, 5.2 ± 0.15 , and 1.5 ± 0.15 ng/ml in castrated, castrated plus testosterone injected, and intact groups respectively. Serum testosterone levels reached 38 ng/ml, 1 h after a testosterone injection. The mRNA levels of identified secreted proteins decreased 1 week after castration, although the extent of the decrease differed among protein species. For instance, SPI-KT3 mRNA in the VP was greatly decreased in castrated animals to only 1/769 of the intact control level, while castration reduced SBP expression to 1/62 of the control. The extent of change in mRNA expression also varied between lobes. GRP78 mRNA in the DLP, for instance, was decreased to 1/11 of the control by castration but only to about 1/3 of the control in the VP. The mRNA levels were normalized by β -actin levels, which were not affected by castration and testosterone treatment. The β -actin levels in the VP were 3.5 ± 0.36 , 3.8 ± 0.30 , and 3.5 ± 0.34 fg/ng total RNA in the castrated, the castrated plus testosterone and intact groups respectively. The values were 4.8 ± 0.74 , 5.3 ± 0.99 , and 4.3 ± 0.40 for the DLP, and 3.9 ± 0.24 , 4.2 ± 0.60 , and 4.7 ± 0.26 for the AP.

Ontogeny in mRNA expression of identified secreted proteins

The expression of identified secreted protein mRNAs was examined in each lobe of the prostate at ages 1, 2, 4, 6, and 11 weeks (Table 5). Low levels of mRNA expression were noted at 1 week. Significant increases of SBP and EAPA2 mRNAs began at 2 weeks and continued thereafter. Increases in other secretory protein mRNAs, including 91 kDa protein, PSP94 and IgGBPLP mRNAs, were apparent at 4 weeks.

Discussion

In the present study, the major secretory proteins of the mouse VP, DLP, and AP were identified by mass spectrometric analysis after 2D-gel electrophoresis (Table 6). IgBPLP and EAPA2 were major proteins in the DLP/AP. A 91 kDa protein predicted from a mouse urinary bladder cDNA (AK035662), Prdx6 and PLC α were also found in the prostatic secretion for the first time, in addition to previously reported prostatic proteins, including SBP (Mills *et al.* 1987b), SPI-KT3 (Mills *et al.* 1987a), PSP94 (Xuan *et al.* 1999), and probasin (Johnson *et al.* 2000). The mRNAs for these proteins were expressed in a lobe-specific manner and were regulated by androgen. Our study has delineated the main mouse prostatic secretion pattern for the first time. The data will be useful for studying androgen-dependent gene regulation in the prostate, and may also provide markers for studying functional differentiation of prostate tissue.

Production and secretion of prostatic proteins are the main physiological functions of the prostate gland. Prostatic secretory proteins have been studied in rats as well as in humans, especially from the viewpoint of androgen-dependent regulation of expression and to identify possible markers of prostate cancer. The major human prostatic-secreted proteins are PSA (prostate-specific antigen), PSP94, and prostatic acid phosphatase (Lee *et al.* 1986). In rat, the composition of prostatic proteins is different; only PSP94 is common with the human case, and the production of each protein varies among lobes. In the VP, prostatic-binding protein or prostatein is the major secreted protein, while cystatin-related protein and kallikreins are also produced abundantly (Heyns 1990). The LP and DP secrete probasin,

Table 5 Ontogeny of mRNA levels of identified proteins in the prostate

	SBP	91 K	PSP94	EAPA2	IgBPLP
VP					
1W	0.1 $\pm$ 0.04	0.1 $\pm$ 0.02	0.1 $\pm$ 0.05	–	–
2W	17.9 $\pm$ 3.8*	0.1 $\pm$ 0.01	0.2 $\pm$ 0.08	–	–
4W	78.5 $\pm$ 15.3*	0.8 $\pm$ 0.07 [†]	2.6 $\pm$ 0.23 [†]	–	–
6W	503.0 $\pm$ 106*	3.6 $\pm$ 0.23 [†]	16.0 $\pm$ 3.9 [†]	–	–
11W	474.9 $\pm$ 77.3*	4.4 $\pm$ 0.50 [†]	4.2 $\pm$ 0.6 [†]	–	–
DLP					
1W	–	–	0.0 $\pm$ 0.03	0.18 $\pm$ 0.06	0.05 $\pm$ 0.02
2W	–	–	0.0 $\pm$ 0.01	0.42 $\pm$ 0.06*	0.09 $\pm$ 0.02
4W	–	–	1.1 $\pm$ 0.32*	0.88 $\pm$ 0.27	0.68 $\pm$ 0.21
6W	–	–	12.7 $\pm$ 1.5 [†]	3.95 $\pm$ 0.79*	7.43 $\pm$ 1.5*
11W	–	–	4.0 $\pm$ 0.2 [†]	15.3 $\pm$ 2.36 [†]	102 $\pm$ 22.0 [†]
AP					
1W	–	–	–	0.18 $\pm$ 0.04	0.04 $\pm$ 0.01
2W	–	–	–	0.74 $\pm$ 0.02*	0.1 $\pm$ 0.02
4W	–	–	–	1.10 $\pm$ 0.4	1.9 $\pm$ 0.26 [†]
6W	–	–	–	3.55 $\pm$ 0.53 [†]	30.1 $\pm$ 3.6 [†]
11W	–	–	–	5.50 $\pm$ 0.80 [†]	53.2 $\pm$ 8.1 [†]

Means $\pm$ S.E.M. (n=4). Values are mRNA levels divided by β -actin mRNA levels (mol/mol β -actin) 1, 2, 4, 6 and 11-week-old (W) male C57BL mice were killed. Total RNA was isolated from each prostate lobe and amounts of mRNA were measured by real-time RT-PCR. *P<0.05 and [†]P<0.01 vs. 1W.

Table 6 Summary: identified mouse prostatic secretory proteins

	Lobe specificity	mRNA decrease by castration	Description
Abbreviation			
SBP	VP	++	Known prostatic protein (Mills <i>et al.</i> 1987b)
SPI-KT3	VP, (SV)	+++	Known prostatic protein (Mills <i>et al.</i> 1987a)
91 kDa	VP	++	Scavenger receptor cys-rich (SRCR) domains
PSP94	VP, DLP	+++	Known prostatic protein (Xuan <i>et al.</i> 1999)
ZnG	DLP > VP, AP	+	Ribonuclease activity?
GRP78	VP, DLP, AP	+	Heat-shock protein 70 family
AGR2	VP, DLP, AP	+	Human homolog expressed in prostatic cancer cell lines
PLCa	VP, DLP, AP	+	Enzyme involved in phosphatidylinositol metabolism
Calr	VP, DLP, AP	+	Calcium-binding protein
PDI	VP, DLP, AP	+	Enzyme involved in protein folding
Prdx6	DLP, AP > VP	++	Antioxidant protein
Probasin	DLP, AP	+++	Known prostatic protein (Johnson <i>et al.</i> 2002)
EAPA2	DLP, AP	+++	No homology with any known protein
IgBPLP	DLP, AP	+++	IgG binding? Willebrand factor D domains, trypsin inhibitor like

PSP94, and SVS2 (Imasato *et al.* 2001). A kinesin heavy chain-like protein and an IgG-binding protein were recently reported in the secretion of the AP (Esposito *et al.* 2001, Wilhelm *et al.* 2002).

In spite of the morphological similarity of the prostate in mouse and rat, previous studies have suggested a substantial difference in prostatic secretion between the two species (Donjacour *et al.* 1990). Since mouse prostatic proteins are known to be highly glycosylated, we first examined the effects of glycosidase digestion on prostatic proteins. Endo H glycosidase, which cleaves mainly within the chitobiose core of high mannose, did not change the SDS-PAGE pattern (data not shown). On the other hand, PNGase F, which removes all types of N-linked glycosylation, changed the pattern. A broad band of SBP in the VP was converted to a sharp band with smaller molecular weight, and smear-like bands between 40 and 100 kDa in the DLP/AP were also converted to sharper bands, indicating that proteins were de-glycosylated by the enzyme. In spite of highly glycosylated characteristics of mouse prostate proteins, the biological role of glycosylation is not yet understood.

In the mouse prostate, only VP-secreted proteins have been investigated, and two major proteins, SBP and SPI-KT3, were identified (Mills *et al.* 1987a,b). The present study confirmed the secretion of these two proteins and also revealed the presence of other proteins, including 91 kDa protein, Prdx6 and GRP78. The 91 kDa protein is expected to consist of 841 aa with two predicted extracellular (CUB) domains and three scavenger receptor cysteine-rich (SRCR) domains, and is expressed preferentially in the VP. The size of the protein, however, seems to be less than 91 kDa in the gel. Since the sequence coverage of peptide mass fingerprinting is only 13%, the actual reading frame may be shorter than the predicted one. Prdx6 is another new component of the prostatic secretion found in the present study. Since it is an antioxidant enzyme that reduces peroxide and alkyl hydroperoxide to water and

alcohol respectively (Wang *et al.* 2003), it may provide seminal plasma antioxidant capability. GRP78 belongs to the heat-shock protein 70 family, which had been considered as intercellular proteins. However, a recent proteomic analysis of human prostasomes revealed the presence of heat shock proteins in prostatic secretion (Utleg *et al.* 2003). In addition, heat-shock protein 70 has been reported to be secreted from a variety of prostatic cell lines, and to show growth-inhibitory activity (Jones *et al.* 2004, Wang *et al.* 2004). Secreted mouse GRP78 may have a similar activity.

Although mouse DLP proteins had not been biochemically identified, Cunha's group has recognized 110 and 55 kDa bands in SDS-PAGE as major DLP/AP proteins (Donjacour *et al.* 1990). They reported that DLP/AP proteins are highly glycosylated, which was confirmed by the present study. The predicted IgGBPLP sequence derived from the cDNA sequence (XM_620455), however, is calculated to contain 1866 aa with a molecular mass of 201 kDa. Because peptide sequencing by the peptide-mass fingerprinting method covered the whole predicted sequence (27% coverage), the 100 kDa spot probably contains a mixture of cleaved fragments derived from the 201 kDa protein, although this remains to be confirmed. Recently, an IgG-binding protein of 115 kDa was reported to be secreted also from the rat AP, suggesting that a rat homolog exists (Wilhelm *et al.* 2002). The predicted cDNA sequence corresponding to this rat protein (XM_620455), which became available more recently, encodes 206 kDa protein (1914 aa) instead of 115 kDa. There is 84% similarity between the mouse and the rat sequences. Secretion of EAPA2, which is one of the antigens found in experimental autoimmune prostatitis, is also a noteworthy finding in the present study. This protein of 914 aa contains no known domain structure and has no homology with any known functional protein. Secretion of both PSP94 and probasin was detected in the DLP, as expected, since both proteins are well characterized in rats and have been reported in mice (Xuan

et al. 1999, Johnson *et al.* 2000). The other identified DLP proteins include GRP78, Prdx6, ZnG, AGR2, Calr, and protein disulfide isomerase (PDI). Prostatic secretion of ZnG has been reported in humans, and ZnG is widely distributed in body fluids and in various epithelia (Lei *et al.* 1998, Hale *et al.* 2001). AGR2 is a mammalian homolog of *Xenopus* AGR2, which was recently reported to be secreted from human prostate under androgen regulation (Zhang *et al.* 2005). It is overexpressed in prostate cancer and the expression level is correlated with pathological grade. Calr is a highly conserved calcium-binding protein involved in a wide variety of cellular processes (Krause & Michalak 1997). Interestingly, the Calr gene was identified as an androgen-inducible gene in the rat VP (Zhu *et al.* 1998). PDI is involved in the maintenance of folding of synthesized proteins. Specific expression of PDI in the prostate was recently reported in humans (Lexander *et al.* 2005). Since both Calr and PDI are considered to be localized in the lumen of endoplasmic reticulum, they may represent contaminants introduced during preparation of the secretion sample. Secretion from the AP is similar to that from the DLP, i.e. the major secretory proteins are IgGBPLP and EAPA2, but little ZnG and no PSP94 are found in the secretion. The results of mass spectrometric identification of SV proteins were generally in agreement with previous reports, i.e. SVS2, 4, 5, and 6, as well as SPI-KT3 (Lai *et al.* 1991, Lundwall *et al.* 1997). Except SPI-KT3, SV proteins are specifically expressed in the SV and not in the prostate gland, which differs from the rat case, where SVS2, for instance, is highly expressed in DLP/AP.

Quantitative determination of mRNA expression revealed a clear transcriptional differentiation of secreted proteins among the lobes. The levels of secretory protein mRNAs were very high, ranging from 1 to 500 times that of the housekeeping gene β -actin, used as an internal control in the present study. The mRNA levels overall correlated with the intensity of protein staining in the gel, although spots of protein with larger molecular weight, such as EAPA2 and IgGBPLP show lesser intensity in the 2D-gel, since the 1D-gel used in the present study is only able to hold proteins with molecular weights less than 80–100 kDa. Since all the identified secreted proteins decreased significantly a week after castration of the animal, these protein transcripts are androgen-dependent directly or indirectly through involution of the gland. In rats, various studies have shown a faster response of the VP to androgen action, as compared with other lobes. For instance, castration decreased probasin mRNA expression to 1% of the control level after a week, while the decrease in the DLP was only 50% (Imasato *et al.* 2001). In the mouse, however, large decreases in mRNAs were evident in all the lobes. We examined the effect of a single injection of testosterone on the mRNAs in castrated animals to confirm the androgen dependency of transcription. The serum testosterone level well exceeded the control level within 1 h and was still high 24 h after an injection. Although most mRNA levels increased significantly after an injection,

which clearly demonstrates their androgen inducibility, most of them were not restored to the intact control level. It may suggest that the full activity of androgen-dependent genes in the prostate is involved in both short- and long-term transcriptional regulation mechanisms by androgen.

Although rodent prostate models have been used for investigating the mechanism of prostate carcinogenesis, anatomical differences between rodent and human prostate have led to concerns about the validity of rodents as suitable models for human prostate cancer. Besides, mice are resistant to induction of prostate tumors by chemical carcinogens. However, a number of transgenic or knockout mouse lines have become available in which prostate carcinomas preferentially occur. For instance, the TRAMP transgenic line expresses the SV40 antigen under the control of the rat probasin promoter. The TRAMP mice develop high-grade prostatic intraepithelial neoplasia and prostate cancer within 12 weeks of birth, and ultimately develop metastases to the regional lymph nodes and lung by 30 weeks. In addition, androgen depletion by castration results in decreased tumor incidence. Their futures are similar to the human case, although metastasis to bone, a characteristic feature of human prostate cancer, is rare. The expression pattern of secretion proteins may be related to development of prostate carcinogenesis. Since the present study has revealed mouse prostate secretion, these can now be examined in relation to the development of prostate carcinogenesis as well as androgen-dependent differentiation of the gland. The ontogeny of mRNA expression of secreted proteins indicated that significant expression started 2 weeks after birth, which is consistent with the fact that branching morphogenesis of the mouse prostate is completed in the first 15 days of birth.

The present study has provided an understanding of the major secretory function of the mouse prostate, and identified common aspects of secretory functionality between mouse and human, e.g. for heat-shock proteins, ZnG and peroxiredoxin. The identified secretory proteins should be available as models of androgen-dependent gene regulation and are candidates as markers for prostatic differentiation. Like human PSA or PSP94, some of the identified proteins may be useful as pathological markers associated with prostate disorders; this would facilitate prostate research in mouse models.

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