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 well investigated. In the present study, major secreted proteins from the ventral (VP), dorso-lateral (DLP) and anterior prostate (AP) of mice were identified by means of two-dimensional gel electrophoresis followed by MALDI-TOF mass spectrometric analysis. A quantitative RT-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). The DLP and the AP secrete a protein similar to immunoglobulin binding protein (IgBPLP) and one of the experimental autoimmune prostatitis antigen proteins (EAPA2). Peroxiredoxin-6, glucose-regulated protein 78, zinc-α2-glycoprotein and phospholipase Cα are also secreted. Castration of animals led to a decrease in the mRNAs of these secreted proteins, although the extents of change varied greatly among different lobes. We present here an overview 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.

Introduction

Rat models have been widely used to study prostate morphology, development, and pathology, as well

as androgen-regulated gene expression, in order to understanding 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 have 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 (alphaERKO and betaERKO) 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, conditional deletion of Rb mice have been used to study the involvement of those genes in prostate carcinogenesis (Robertson *et al.* 2003; McPherson *et al.* 2001; 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 for 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 PSP94 (prostatic secretory protein of 94 a.a.), probasin and 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; Mills *et al.* 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) for use 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 two-dimensional gel electrophoresis followed by MALDI-TOF mass spectrometric analysis. Further, a quantitative RT-PCR method was employed to examine the androgen-dependence of 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'. 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 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 done at 10 weeks of age, then the animals were allowed to recover for one 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 ip at 5 mg/kg body weight. Animals were sacrificed under ether anesthesia 24 hours 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 method previously reported (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 of saline containing 1% protease inhibitor mixture (Sigma P8340, St. Louis, Mo., U.S.A.). Each lobe was cut into 4 or 5 pieces, left to stand for 5 min, and transferred to a 1.5 ml microcentrifuge tube. After centrifugation at 10,000 xg for 5 min at room temperature, the supernatant was collected as the secretion sample. The incubation time of 5 min was chosen because the level of intracellular contamination (GAPDH, glyceraldehyde-3-phosphate dehydrogenase) was confirmed to be low at 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 Laboratories, Inc., Hercules, Ca., USA). For de-glycosidation, samples were incubated with PNGase F (50U/ µg

protein, New England Biolabs, Ipswich, MA, U.S.A.) at 37 ºC for 1.5 hours.

Electrophoresis (1D PAGE and 2D PAGE)

For SDS-PAGE gel electrophoresis, 15 µg total protein of each sample was mixed with the SDS-PAGE buffer containing 2-mercaptoethanol, and applied to a 5-20% gradient PAGE gel (10 cm x 10 cm SuperSep pre-cast gel, Wako Jyunyaku) 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, first-dimensional isoelectric focusing with immobilized pH gradients was performed with Immobiline DryStrip (Amersham Bio. Co., Piscataway, NJ, USA) and the Ettan IPGphor system (Amersham Bio. Co.) according to the manufacturer's protocol. For analytical 2D PAGE, 10 µg of de-glycosidated protein was applied to a 7 cm Immobiline DryStrip (pI=3-11, non-linear gradient). After rehydration, the strip was isoelectrofocused (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. It was then placed on the 2nd SDS-PAGE slab gel (5-20% gradient, SuperSep pre-cast gel, Wako Jyunyaku) and overlaid with hot agarose solution to connect the two gels. The 2nd 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 Corp., Carlsbad, CA,

USA), 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 of total protein was subjected to electrophoresis as described above and then stained with silver nitrate by incubation with 0.2 g/l $Na_2S_2O_3$ for 1 min followed by 1 g/l $AgNO_3$ for 20 min on ice, and washed with 20 g/l Na_2CO_3 containing 0.1 % HCHO. A 24 cm Immobiline DryStrip (pI=3-11) was also used for preparation. It was rehydrated with 200 µg of secreted protein and isoelectrofocused (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 above. Three sets of secretion samples from different animals were subjected to 2D electrophoresis and analyzed.

Western blotting

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

Mass spectrometry (MS)

Protein spots were excised from the polyacrylamide gel and silver nitrate was removed with 15 mM

$K_3[Fe(CN)_6]$ and 50 mM $Na_2S_2O_3$. The gel pieces were incubated in distilled water for 1 hour several times, then incubated with CH_3CN 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 digested peptides, 10 µl of 70% CH_3CN containing 0.1% trifluoroacetic acid was added to each gel piece. A 0.5 µl aliquot 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 Biotools software (Bruker Daltonics) in combination with a peptide mass fingerprinting analysis system, MASCOT Ver.2.1 (Matrix Science, London, UK). The peptide mass fingerprinting was performed based on MSDB and NCBInr databases 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 a RNA Isolation kit (Promega Co., Madison, WI, U.S.A.), and 2 µg of total RNA was reverse-transcribed as described previously (Fujimoto *et al*. 2004). An ABI Prism 7700 (PerkinElmer Life Sciences, Boston, MA) was employed

for quantitative measurement of cDNA using a QuantiTect Sybr Green PCR kit (Qiagen, Valencia, CA, USA). Specific primer sets with a Tm 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 (PerkinElmer 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 sec at 94 ºC, and 30 sec at 58 ºC and 40 sec at 72 ºC. All mRNA contents were normalized with reference to β-actin mRNA.

Serum T Levels

Serum T levels were measured with an ELISA kit, purchased from Neogen Corp. (Lexington, , KY, U.S.A.).

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 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 kDa and 90kDa, which showed lower staining intensities. The mobility of other bands was not changed much by PNGase F treatment, though some smear-like 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 was present in the molecular weight range below 25 kDa. A 68 kDa band of albumin, 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. Due to the limitation of pore size of the immobilized pH gradient gel for isoelectric focusing, proteins with a molecular weight 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). Three sets of prostate secretions from independent control mice were analyzed and provided identical patterns. The spots were successfully identified and results are 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 (IgBPLP). Zn-α2-glycoprotein (ZnG), a mammalian homologue of *Xenopus* anterior gradient 2 (AGR2), as well as PSP94 and probasin, was detected in the DLP secretion. Phospholipase Cα (PLCα), calreticulin (Calr) and protein disulfide isomerase were also identified in both DLP and AP secretions. SVS 2, 4, 5 and 6 were identified in the seminal vesicle fluid.

Lobe-specific mRNA expression of identified secreted proteins

Expression of identified proteins in the prostate was further confirmed by examining 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 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

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 level reached 38 ng/ml one hour after a testosterone injection. The mRNA levels of identified secreted proteins were decreased one 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 varied between lobes as well. GRP78 mRNA, for instance, was decreased to 1/11 of the control by castration in the DLP 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-treated and the 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

Expression of identified secreted protein mRNAs was examined in each lobe of the prostate at the ages of 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. IgBPLP and EAPA2 were major proteins in the DLP/AP. A 91 kDa protein predicted from a mouse urinary bladder cDNA (AK035662), Prdx6 and PLCα 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 found to be 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 is the main physiological function of the prostate gland. Prostatic secretory proteins have been studied in rats as well as 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 (PAP) (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, PSP94 and SVS2 (seminal vesicle protein 2) (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 chains, 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 40-100 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 nature of the 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; Mills *et al.* 1987b). 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. In the 2D electrophoresis, there were two spots for SBP (18, 35 kDa). A spot with higher molecular weight is probably an aggregated dimmer, since amount of this protein seems to be extremely high. The 91 kDa protein is expected to consist of 841 a.a. with two predicted extracellular (CUB) domains and three scavenger receptor cystine-rich (SRCR) domains, and is expressed preferentially in the VP. The size of the protein, however, seems to be less

than 91kDa in the gel. Since the sequence coverage of peptide mass fingerprinting is only 13%, the actual reading frame may be shorter than predicted. 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 intracellular 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 (Wang *et al.* 2004; Jones *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 kDa 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, and this was confirmed by the present study. The predicted IgGBPLP sequence derived from the cDNA sequence (XM_620455), however, is calculated to contain 1866 a.a. with a molecular weight 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 homologue exists (Wilhelm *et al.* 2002). The predicted cDNA sequence corresponding to this protein (BC099756), which recently

became available, encodes a 206 kDa protein (1914 a.a.) instead of 115 kDa. There is a 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 a.a. 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 (Johnson *et al*. 2000; Xuan *et al.* 1999). The other identified DLP proteins include GRP78, Prdx6, ZnG, AGR2, Calr and PDI. Prostatic secretion of ZnG has been reported in humans, and ZnG is widely distributed in body fluids and in various epithelia (Hale *et al*. 2001; Lei *et al.* 1998). AGR2 is a mammalian homologue of *Xenopus* anterior gradient 2, which was recently reported to be secreted from human prostate under androgen regulation (Zhang *et al*. 2005). It is over-expressed 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., SVS 2, 4, 5 and 6, as well as SPI-KT3 (Lundwall *et al.* 1997; Lai *et al.* 1991). Except SPI-KT3, SV proteins are specifically expressed in the SV and not in the prostate gland, which is different from the case in rats, where SVS2, for instance, is highly expressed in DLP/AP (Cunha *et al.* 1987; Kwong *et al.* 2000).

Quantitative determination of mRNA expression revealed a clear transcriptional differentiation of secreted proteins among 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 were overall correlated with the intensity of protein staining in the gel, although spots of protein with larger molecular weight such as EAPA2 and IgBPLP showed lesser intensity in the 2D gel, since the 1D gel used in the present study is only able to hold proteins with molecular weights of less than 80-100 kDa. Since all the identified secreted proteins was significantly decreased a week after castration of the animal, these protein transcripts are androgen-dependent either 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 to 50% (Imasato *et al.* 2001). In the mouse, however, large decreases in mRNAs were evident in all of the lobes. We examined the effect of single injection of testosterone on the mRNAs in castrated animals to confirm the androgen dependency of transcription. The serum testosterone level clearly exceeded the control level after one hour and remained high at 24 hours after the injection. Although most of the mRNA levels increased significantly after the injection,

which support their androgen-inducibility, most of them did not subsequently revert to the intact control level. This may suggest that activity of androgen dependent genes in the prostate is determined by both short-term and long-term transcriptional regulation mechanisms controlled by androgen.

Although rodent prostate models have been used for investigating the mechanism of prostate carcinogenesis, anatomical differences between rodent and human prostates have led to concerns about the validity of rodents as model for human prostate cancer (Shirai *et al.* 2000). Although mice are resistant to induction of prostate tumors by chemical carcinogens, a number of transgenic or knockout mouse lines have become available in which prostate carcinomas preferentially occur (Abate-Shen & Shen 2002). 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 lungs by 30 weeks. In addition, the androgen-depletion by castration results in decreased tumor incidence. There features are similar to those seen in humans, although metastasis to bone, a characteristic feature of human prostate cancer, is rare in TRAMP mice. The expression pattern of secretory proteins may be related to the development of prostate carcinogenesis. Since the present study has characterized the mouse prostate secretion pattern, it should now be possible to examine the relationship of secreted proteins 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 at 2 weeks after birth,

which is consistent with the fact that branching morphogenesis of the mouse prostate is completed in the first 15 days after birth (Sugimura *et al*. 1986).

The present study has provided an overview of the major secretory function of the mouse prostate, and has 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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Figure Legends

Figure 1. Intracellular contamination in secretion samples. Each prostatic lobe was cut into 4 or 5 pieces, and left for 1, 5, 15min to allow secretion from the prostatic canals. Then the lobes were centrifuged and the supernatant was collected as the secretion sample. The samples were subjected 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 intracellular marker (36 kDa band). The intracellular contamination was less in the samples incubated for 1 and 5 min (1M, 5M) than in those incubated for 15 min (15M) or prepared from minced tissues (minced). The incubation time of 5 min was chosen for sample preparation in the present study.

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 following PNGase treatment.

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 2nd 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 represent serum contamination.

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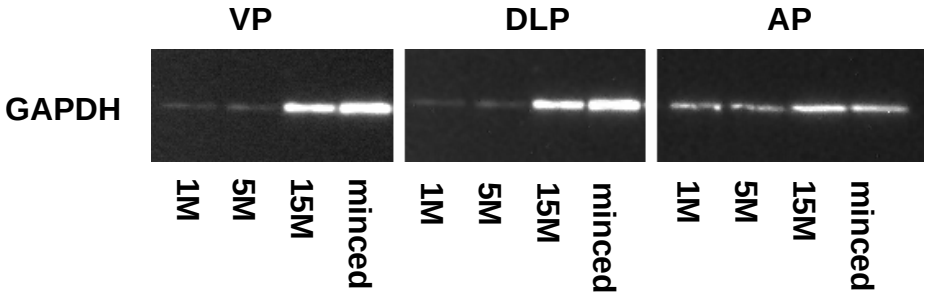
Figure 1.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AP
GAPDH
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5M
15M
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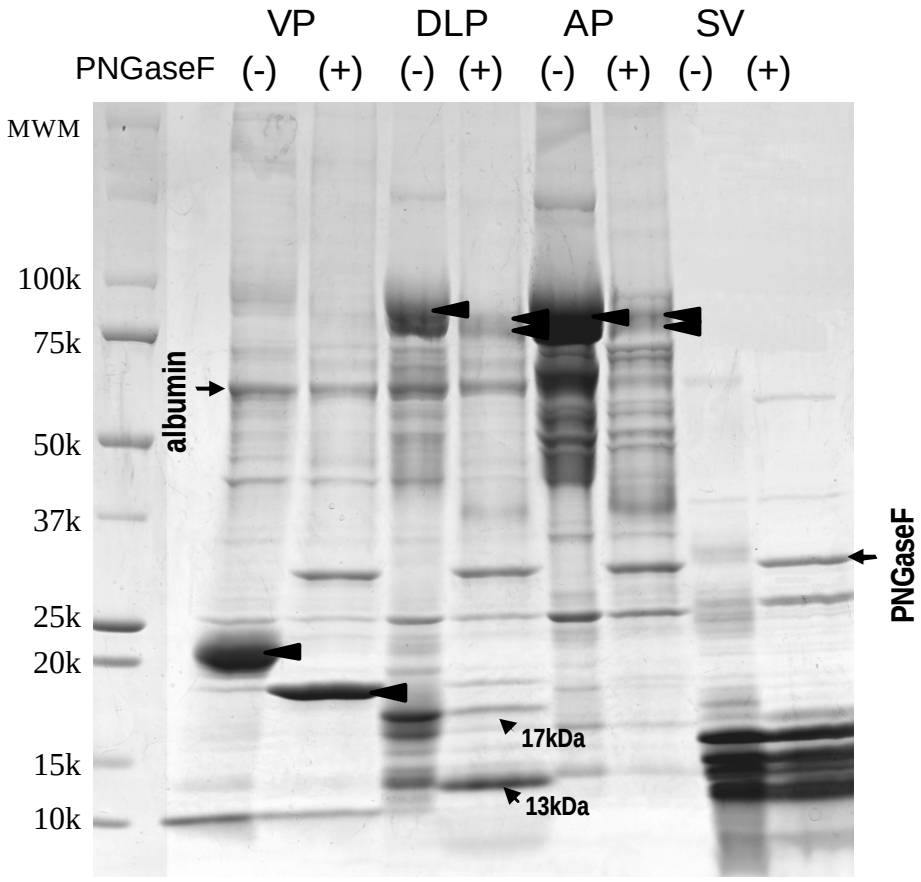
1M
5M
15M
minced
1M
5M
15M
minced

Figure 2.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DLP
AP
SV
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albumin
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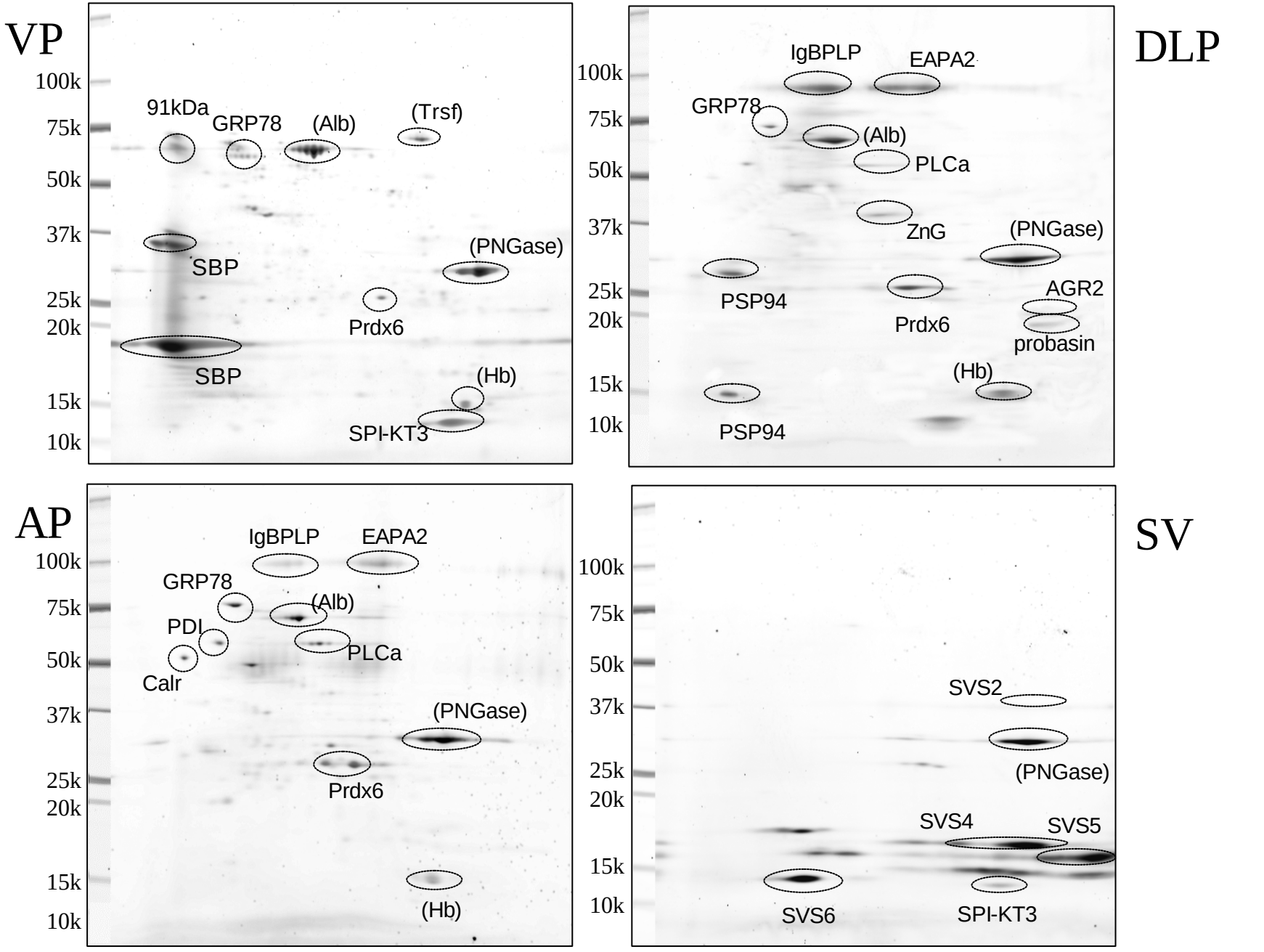
PNGaseF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50k
37k
25k
20k
15k
10k
91kDa
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(Alb)
(Trsf)
SBP
(PNGase)
Prdx6
SBP
(Hb)
SPI-KT3
DLP
IgBPLP
EAPA2
GRP78
(Alb)
PLCa
ZnG
(PNGase)
PSP94
AGR2
Prdx6
probasin
(Hb)
PSP94
AP
IgBPLP
EAPA2
GRP78
(Alb)
PDI
PLCa
Calr
(PNGase)
Prdx6
(Hb)
SV
SVS2
(PNGase)
SVS4
SVS5
SVS6
SPI-KT3

Table 1 Quantitative PCR primers for mouse genes

Genes	GenBank Accession#	5'-Primer (5'->3')	3'-Primer (5'->3')
91kDa protein	AK035662	GGACCTTCCACAAGCGAACAT	GCACTCCTCCAGGTGTTCCTC
AGR2	M_011783	TTCATCACTTGGACGAATGCC	ACGTACTGGCCATCAGGAGAA
calr	NM_007591	ACCGTGAAGCATGAGCAGAAT	TGTTGATCAGCACATTCTTGCC
EAPA2	AY528666	CCAGACAGGCAGAATTGGGTT	CTCCTCGGAATCTATATTGGCG
GRP78	NM_022310	TCTTGCCATTCAAGGTGGTTG	TTCTTTCCCAAATACGCCTCAG
IgBPLP	XM_620455	CTGTGAGTTGCCCGAGCCT	CACAATGGAGAACGCCTCCT
PDI	MUSPDIA	CGCAACAACTTTGAGGGTGA	TTGGGCAGGAACAGCAGAAT
PLCα	M73329	ATTGCACTGCCAACACAAACA	AACTGAAGCTGGTCCTGCTTG
Prdx6	BC013489	AGGACGCTAACAACATGCCTG	GTGCCTGTCAGCTGGAGAGAG
probasin	AF005204	ACACTGCATGTGCTAGGCGT	TCCCACACAAAATGTGACGG
PSP94	U89840	CCAACGCTACTAGGCCTTTGA	GCCCACACGAAGCACATTTAC
SBP	NM_011321	TGGAACCCGGTCAGATAACTTT	TCGACCCCTTCTAACACCAAA
SPIKT3	BC086887	AGAGGCTAGTTGCCATGATGC	GGACAGGCTCTATGCGTTTCC
SVS2	NM_009300	CAGAGCAGCTCCTCAGAGGG	TCTGGGTCATGTCACCACCA
ZnG	AF281658	CCCACAGGACATAGACCCCTT	CTCATGTCAGGCAGAGAGGGTA
βActin	X03765	CTGTCCCTGTATGCCTCTGGTC	TGAGGTAGTCCGTCAGGTCCC

Table 2 Identifed mouse prostatic secretory proteins

Abbreviation	Protein Name	Accession #	Observed MW/ pI	Theoretial MW/pI	P	Sequence coverage (%)
SBP	spermine binding protein	NP_035451	18,35/ 4.5	22/ 4.6	0.999	46
91kDa	protein predicted from cDNA AK035662	Q8BZE1	80/ 4.5	93/ 4.8	0.973	13
GRP78	glucose regulated protein 78kD	A37048	70/ 5.0	72/ 5.0	1.000	39
Prdx6	peroxiredoxin 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 autoimmune 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-glycoprotein	Q64726	35/ 6.0	34/ 5.8	0.992	28
AGR2	homologue of *Xenopus* 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
Carl	carleticulin	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

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**

Mean±SEM (n=5). Values are mRNA levels divided by β-actin mRNA levels (mol/mol β-actin)

11-week-old male C57BL mice were sacrificed. Total RNA was isolated from each prostate lobe and mRNA levels were measured by real-time RT-PCT.

Table 4 Androgen regulation of mRNA levels of identified proteins

		SBP	SPIKT3	91 kDa	PSP94	ZnG	GRP78	AGR2
VP	Cast	8.2 ± 0.91*1	0.24 ± 0.08	0.09 ± 0.02	0.011 ± 0.004	0.38 ± 0.032	0.57 ± 0.36	0.03 ± 0.004
	Cast+T	48 ± 7.9 **(5.9)***2	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)**
	Intact	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)**
DLP	Cast				0.002 ± 0.001	0.64 ± 0.11	0.55 ± 0.11	0.01 ± 0.001
	Cast+T				0.08 ± 0.046 **(38)**	2.0 ± 0.48 **(3.1)**	3.4 ± 0.73 **(1.8)**	0.06 ± 0.02 **(6.9)**
	Intact				5.8 ± 1.48 **(2633)**	12.3 ± 1.1 **(19)**	6.1 ± 1.7 **(11)**	0.38 ± 0.04 **(45)**
AP	Cast					0.06 ± 0.01	0.33 ± 0.59	0.01 ± 0.002
	Cast+T					0.21 ± 0.05 **(3.6)**	1.33 ± 0.19 **(4.1)**	0.08 ± 0.013 **(9.5)**
	Intact					1.4 ± 0.3 **(24)**	2.7 ± 0.22 **(8.3)**	0.54 ± 0.04 **(64)**

		PLCa	Calr	PDI	Prdx6	probasin	EAPA2	IgBPLP
VP	Cast	0.14 ± 0.081	0.2 ± 0.03	5.6 ± 1.2	3.7 ± 0.73			
	Cast+T	0.23 ± 0.03 **(1.6)**	0.7 ± 0.1 **(3.3)**	11.7 ± 2.1 **(2.1)**	3.8 ± 1.41 **(1.0)**			
	Intact	0.17 ± 0.021 **(1.2)**	0.7 ± 0.09 **(3.5)**	15.0 ± 2.1 **(2.7)**	2.5 ± 0.3 **(0.7)**			
DLP	Cast	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
	Cast+T	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)**
	Intact	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)**
AP	Cast	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
	Cast+T	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)**
	Intact	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)**

*1 Mean±SEM (n=5). Values are mRNA levels divided by β-actin mRNA levels (mol/mol β-actin)

*2 Values 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 hours 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.

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

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

Mean±SEM (n=4). Values are mRNA levels divided by β-actin mRNA levels (mol/mol β-actin)

1, 2, 4, 6 and 11-week-old male C57BL mice were sacrificed. Total RNA was isolated from each prostate lobe and amounts of mRNA were measured by real-time RT-PCT.

* $p<0.05$ and ** $p<0.01$ vs. 1W

Table 6 Summary: identifed mouse prostatic secretory proteins

Abbreviation	Lobe Specificity	mRNA decrease by castration	Description
SBP	VP	++	*known prostatic protein (Ref. 13)*
SPI-KT3	VP, (SV)	+++	*known prostatic protein (Ref. 12)*
91kDa	VP	++	Scavenger receptor cys-rich (SRCR) domains
PSP94	VP, DLP	+++	*known prostatic protein (Ref. 16)*
ZnG	DLP > VP, AP	+	Ribonuclease activity?
GRP78	VP, DLP, AP	+	heat shock protein 70 family
AGR2	VP, DLP, AP	+	human homologue expressed in prostatic cancer cell lines
PLCα	VP, DLP, AP	+	enzyme envolved in phosphatidylinositol metabolism
Carl	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 (Ref. 17)*
EAPA2	DLP, AP	+++	no homology with any known protein
IgBPLP	DLP, AP	+++	IgG binding? Willebrand factor D domains, Trypsin inhibitor like