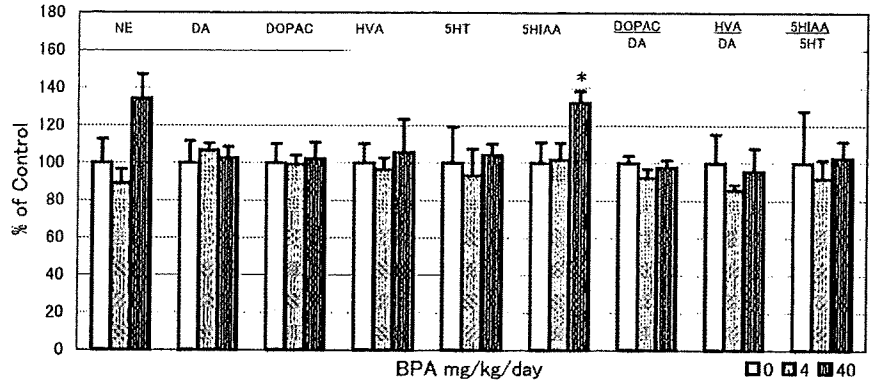

Fig. 4-4. Effects of perinatal administration of BPA on the neurotransmitter contents of striatum in maternal rats.

Results are shown as means ± SEM (%). Absolute values (nmoles/g tissue) for 100% were as follows: 0.669 for NE, 48.2 for DA, 7.16 for DOPAC, 3.75 for HVA, 1.39 for 5HT, and 2.05 for 5HIAA; absolute values of ratios for 100% were as follows: 0.150 for DOPAC/DA, 0.0819 for HVA/DA, and 1.85 for 5HIAA/5HT. N = 5–6. *: $p<0.05$ by Dunnett's multiple t-test.

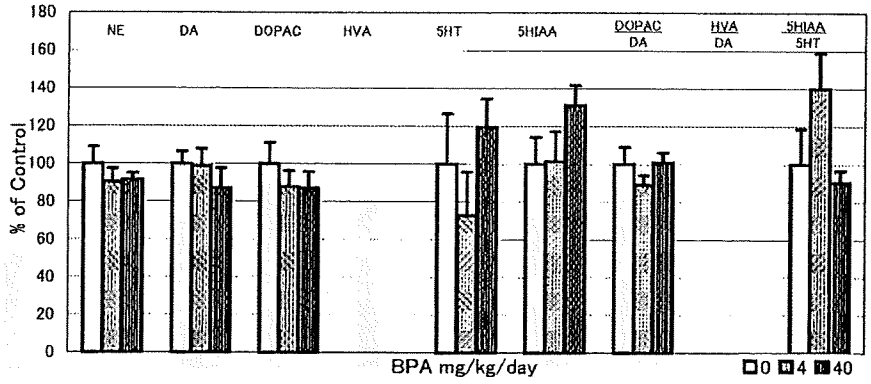

Fig. 4-5. Effects of perinatal administration of BPA on the neurotransmitter contents of midbrain in maternal rats.

Results are shown as means ± SEM (%). Absolute values (nmoles/g tissue) for 100% were as follows: 6.87 for NE, 4.75 for DA, 1.44 for DOPAC, 3.11 for 5HT, and 3.54 for 5HIAA; absolute values of ratios for 100% were as follows: 0.304 for DOPAC/DA, and 1.41 for 5HIAA/5HT. N = 4–6.

and extracellular substances were included. At present, it is not clear whether the changes in monoamine turnover observed in the dams in our experiments were due to the estrogenic activity of BPA. An effect of BPA on prolactin secretion has been reported[28, 29]. DA inhibits the secretion of prolactin in the anterior pituitary gland. Male rats were exposed to BPA from postnatal days 22 to 32[29]. During this period, BPA stimulated prolactin secretion in the same manner as pimozide (a dopamine antagonist) and 17β-estradiol. Steinmetz *et al.* reported that BPA induces hyperprolactinemia in F344 rats with an efficacy similar to that of estradiol[28]. On the assumption that such effects of BPA on prolactin secretion are via the inhibition of dopaminergic activity in the anterior pituitary gland, BPA would inhibit the activity of DA neurons. In our experiment, DA in the hippocampus significantly increased in the 4 mg/kg group. HVA levels and HVA/DA ratios in the occipital cortex of dams treated with BPA at 4 or 40 mg/kg were higher than the control, although without statistical significance. These results from female CD (SD) IGS rats are inconsistent with previous findings. This may be due to sex differences. Alternatively, the stimulation of prolactin secretion by BPA might be due to activities of BPA other than dopamine-mediated action. Changes in DA, 5HT, and their metabolites were observed in some brain regions of dams dosed with 4 or 40 mg/kg of BPA in our present study. Although the

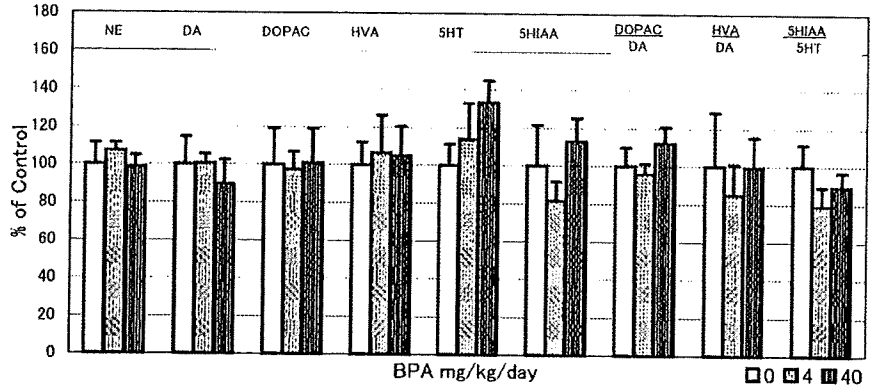

Fig. 4-6. Effects of perinatal administration of BPA on the neurotransmitter contents of hypothalamus in maternal rats.

Results are shown as means ± SEM (%). Absolute values (nmoles/g tissue) for 100% were as follows: 9.56 for NE, 2.07 for DA, 0.498 for DOPAC, 0.192 for HVA, 4.91 for 5HT, and 2.55 for 5HIAA; absolute values of ratios for 100% were as follows: 0.240 for DOPAC/DA, 0.113 for HVA/DA, and 0.497 for 5HIAA/5HT. N = 4–6.

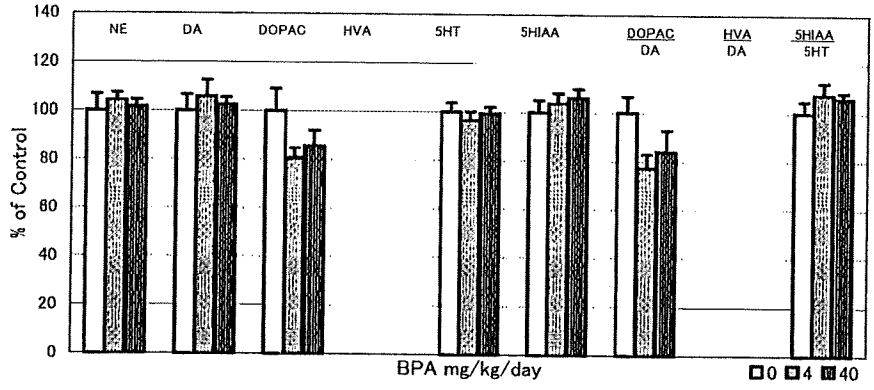

Fig. 4-7. Effects of perinatal administration of BPA on the neurotransmitter contents of medulla oblongata in maternal rats.

Results are shown as means ± SEM (%). Absolute values (nmoles/g tissue) for 100% were as follows: 2.64 for NE, 0.463 for DA, 0.232 for DOPAC, 2.36 for 5HT, and 2.62 for 5HIAA; absolute values of ratios for 100% were as follows: 0.502 for DOPAC/DA, and 1.11 for 5HIAA/5HT. N = 5–6.

effects of BPA on GABA (A) and nicotinic receptors have been reported[30, 31], those of BPA on dopaminergic and serotonergic neurons have not been described. Our results suggest that the metabolism of DA and 5HT was accelerated in BPA-treated female rats. We postulate that BPA may affect some DA- and 5HT-related brain functions.

The assay of brain substances of the male offspring sacrificed in the same series of experiments is now underway in our laboratory. Kubo *et al.* reported that sexual differentiation of the brain locus coeruleus is disrupted in rats perinatally exposed to BPA[32]. In that study, maternal rats received BPA at 1.5 mg/kg per day. We found that levels of NE in the forebrain of 9-wk-old offspring were dose-dependently increased and reached significance in the 40 mg/kg group; a similar increase was also observed in the hindbrain. Cell bodies of NE neurons are dense in the locus coeruleus and NE neurons might be altered by BPA in this disruption of sexual differentiation, although the size of the locus coeruleus is much smaller than the forebrain and hindbrain. According to Farabollini *et al.*, the maternal administration of BPA during the critical period of fetal brain organization produces different effects on the behavior of male and female offspring rats[33]. A comparison of data regarding neurotransmitters obtained from female and male offspring may explain such sexually differentiated behavior effects of BPA.

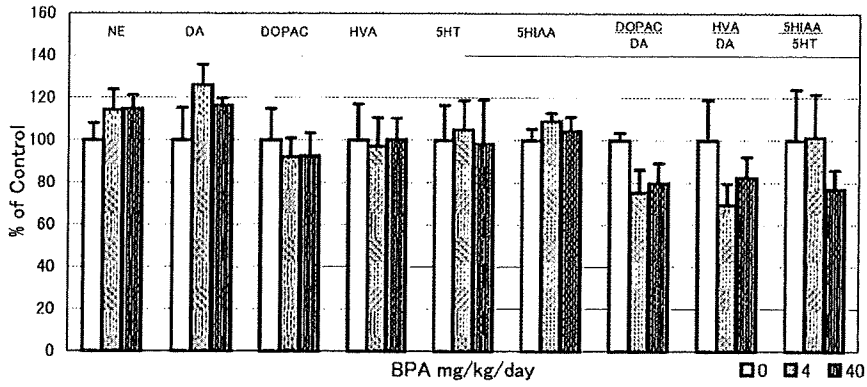

Fig. 4-8. Effects of perinatal administration of BPA on the neurotransmitter contents of cerebellum in maternal rats.

Results are shown as means ± SEM (%). Absolute values (nmoles/g tissue) for 100% were as follows: 0.785 for NE, 0.0527 for DA, 0.0520 for DOPAC, 0.0447 for HVA, 0.455 for 5HT, and 0.366 for 5HIAA; absolute values of ratios for 100% were as follows: 0.987 for DOPAC/DA, 0.896 for HVA/DA, and 0.939 for 5HIAA/5HT. N = 5–6.

We found that dams given BPA at 400 mg/kg weighed significantly less than controls[12]. The 40 mg/kg group weighed somewhat less than controls, but BPA at 4 mg/kg did not affect the body weight of dams. The weight of the 40 mg/kg group recovered to the control level during lactation. The body weight of female offspring did not statistically differ between control and BPA-treated groups at 1 to 9 wk of age. No differences were statistically significant in the weights of the liver and kidneys among groups. These results show that the neurochemical alterations in the brains of dams and offspring after BPA exposure were not caused by differences in somatic growth. Anogenital distances in female offspring were not significantly affected by BPA at 1, 3 or 9 wk of age as observed in the same rats in this study[12]. Though the anogenital distance is not always sensitive to the reproductive effects of chemicals, neurotransmitters in the brain might be more sensitive to BPA than reproductive organ sensitivity to the estrogenic action of BPA. At present we have no data to explain the reason why the changes in monoamines and metabolites occurred in pups as well as dams. These changes were observed in specific brain areas, except the increase in Ch. Levels of Ch were higher than control in all of the eight brain areas of 6-wk-old pups of the 4 and 40 mg/kg groups. Unfortunately, we have no data for Ch in pups at ages other than 6 wk.

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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), dorsolateral 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-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 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 (αERKO and βERKO) 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,

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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.* 1987*a*, 1987*b*). 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 cm^2 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, 1D isoelectric focusing with immobilized pH gradients was performed with Immobiline

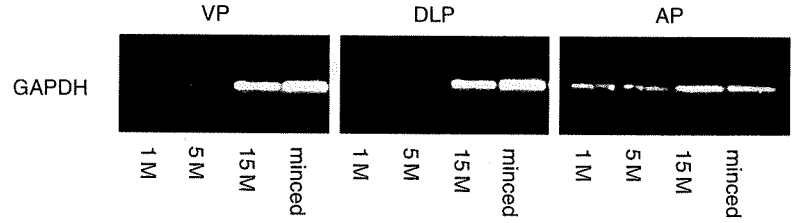

Figure 1 Intracellular contamination in secretion samples. Each prostatic lobe was cut into four or five pieces, and secretion out of the prostatic canals was allowed for 1, 5, and 15 min. Then the lobes were centrifuged and the supernatant was collected as the secretion sample. The samples were applied 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 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. 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 $Na_2S_2O_3$ 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 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 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 $K_3[Fe(CN)_6]$ and 50 mM $Na_2S_2O_3$. The gel pieces were incubated in distilled water for 1 h, 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 the digested peptides, 10 μl of 70% CH_3CN 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 Biotools 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 (NCBInr; 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	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
SPI-KT3	BC086887	AGAGGCTAGTTGCCATGATGC	GGACAGGCTCTATGCGTTTCC
SVS2	NM_009300	CAGAGCAGCTCCTCAGAGGG	TCTGGGTCATGTCACCACCA
ZnG	AF281658	CCCACAGGACATAGACCCCTT	CTCATGTCAGGCAGAGAGGGTA
β-Actin	X03765	CTGTCCCTGTATGCCTCTGGTC	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

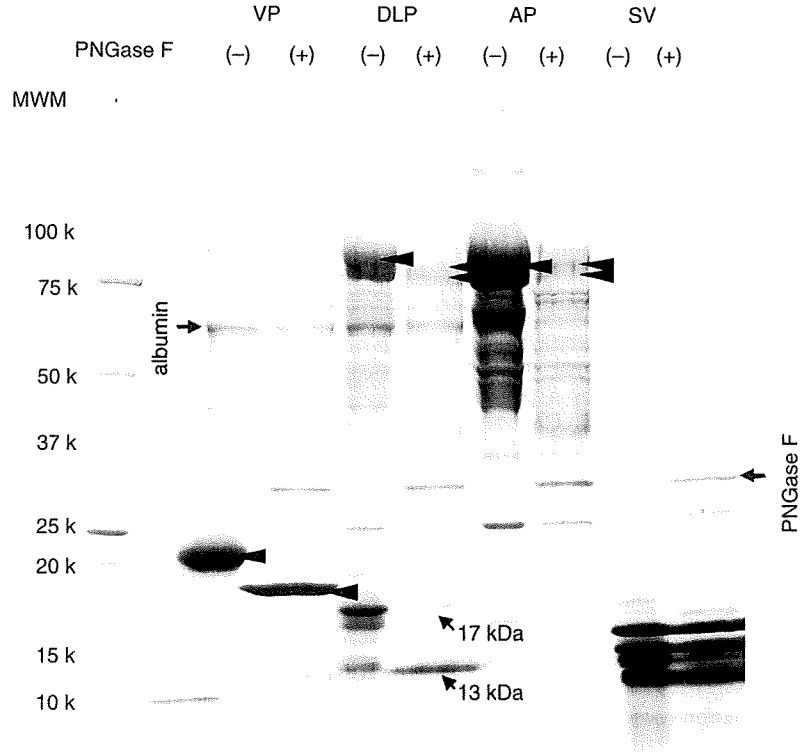

Figure 2 1D-SDS-PAGE analysis of mouse prostate secretory proteins. Secretion was prepared from the VP, DLP, and AP, as well as the SV. Each sample was incubated with (+) or without (−) PNGase F and applied to a 5–20% gradient SDS-PAGE gel. The gel was stained with Coomassie Brilliant Blue. Arrows indicate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

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-α2-glycoprotein (ZnG), a mammalian homologue of *Xenopus* anterior gradient 2 (AGR2), as well as PSP94 and probasin, were detected in the DLP secretion. Phospholipase Cα (PLCα), 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.

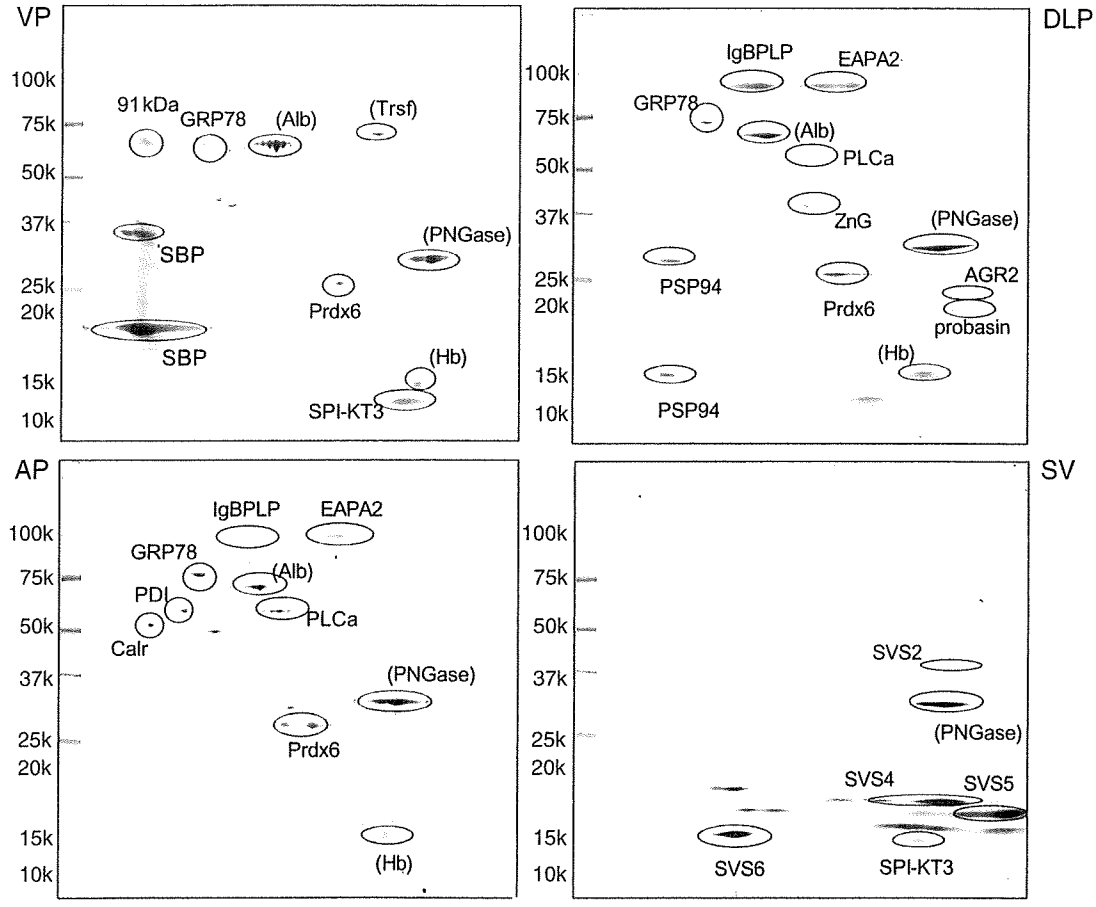

Figure 3 2D-PAGE analysis of mouse prostate secretory proteins. Each sample from the VP, DLP, AP, and SV was treated with PNGase F and applied to an immobilized pH gradient gel, followed by a second SDS-PAGE. Gels were stained with Sypro Ruby. The identified spots are indicated in the figure. Serum albumin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

Table 2 Identified mouse prostatic secretory proteins

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
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	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	Homolog 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
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	SPIKT3	91 kDa	PSP94	ZnG	GRP78	AGR2
VP							
Cast	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
Cast+T	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)
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)

[a]Mean±S.E.M. (n=5). Values are mRNA levels divided by β-actin mRNA levels (mol/mol β-actin).
[b]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 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 PLCα were also found in the prostatic secretion for the first time, in addition to previously reported prostatic proteins, including SBP (Mills *et al.* 1987*b*), SPI-KT3 (Mills *et al.* 1987*a*), 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±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†

Means±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 *et al.* 1987*b*)
SPI-KT3	VP, (SV)	+ + +	Known prostatic protein (Mills *et al.* 1987*a*)
91 kDa	VP	+ +	Scavenger receptor cys-rich (SRCR) domains
PSP94	VP, DLP	+ + +	Known prostatic protein (Xuan *et al.* 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 *et al.* 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.* 1987*a*,*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 cystine-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 IgBPLP 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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RESEARCH ARTICLE

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Imposex in marine gastropods may be caused by binding of organotins to retinoid X receptor

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Abstract Organotin compounds have been widely used as antifouling paints for ships and fishing nets since the 1960s and have thus been released into marine environments. Aquatic invertebrates, particularly marine gastropods, are extremely sensitive to organotin compounds such as tributyltin (TBT) and triphenyltin (TPT) and undergo changes in sexual identity in response to exposure. This worldwide phenomenon is one of the worst consequences of pollution by man-made chemicals and has led to the ban of such compounds in antifouling paints in a number of countries, although organotin compounds still exist in the environment. So far, very low-concentrations of TBT or TPT have been shown to induce imposex (superimposition of male genitalia on female) in marine gastropods. Although the imposex induction mechanism has been controversial for many years, it was recently reported that TBT and TPT are potent and efficacious activators of retinoid X receptor (RXR), a member of the nuclear receptor superfamily. In this review, I discuss the involvement of RXR in the development of gastropod imposex.

Introduction

In their book "Our Stolen Future", Colborn et al. (1996) pointed out that a number of environmental chemicals affect hormonal systems and have adverse health effects on wildlife and probably on humans. Such chemicals are referred to as endocrine disruptors, and their effects have emerged as a major environmental issue. The nuclear receptors of intrinsic hormone systems are likely to be targets of endocrine disruptors, because their intrinsic ligands are fat-soluble and low-molecular-weight agents, as are the environmental pollutants. Many synthetic compounds, including the drug diethylstilbestrol (DES), dichlorodiphenyltrichloroethane (DDT), polychlorinated biphenyls (PCB), and alkylphenols, have been shown to bind nuclear receptors (Sohoni and Sumpter 1998; Blair et al. 2000; Nishihara et al. 2000; Gray et al. 2001). The effects of synthetic chemicals on sex hormone receptors such as the estrogen receptor (ER) and androgen receptor (AR) have attracted much attention, focusing on the reproductive failures observed in wildlife.

Organotin compounds such as tributyltin (TBT) and triphenyltin (TPT) have been used worldwide in antifouling paints for ships and fishing nets since the mid-1960s. Their release into the marine environment has resulted in pollution worldwide. Most marine gastropods in organotin-polluted areas have shown reproductive failure due to oviduct blockage by vas deferens formation, resulting in population decline or mass extinction (Bryan et al. 1986; ten Hallers-Tjabbes et al. 1994). This phenomenon is called "imposex" as an abbreviation of "imposed sexual organs", because male genital organs, such as the penis and vas deferens, are imposed upon female organs (Smith 1971). Approximately 150 species of imposex-affected gastropods have been found in the world (Fent 1996; Matthiessen et al. 1999). Gastropod imposex is reportedly induced by very low concentrations of TBT or TPT and is thought to be one of the mechanisms of endocrine disruption in wildlife (Smith 1971; Bryan et al. 1986, 1987, 1988; Gibbs and Bryan 1986; Gibbs et al. 1987; Axiak et al. 1995; Horiguchi et al. 1997b). Despite several hypotheses on the cause of imposex induction, such as aromatase inhibition, testosterone excretion-inhibition, functional disorder of the female cerebropleural ganglia, and involvement of amidated tetrapeptide Ala-Pro-Gly-Trp-NH2 (APGWamide) (Bettin et al. 1996; Ronis and

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Mason 1996; Oberdörster and McClellan-Green 2000, 2002), the detailed biochemical mechanism behind this phenomenon remains obscure.

It is well known that steroidal sex hormones such as 17β-estradiol (E_2) and 5α-dihydrotestosterone (DHT) exert important roles in physiological processes, including sexual development and reproduction in vertebrates. However, homologues of ER and AR have not been found in invertebrates (Escriva et al. 1997). Because gastropods are mollusks, they may not have functional receptors for androgen, suggesting that vertebrate-type sex hormones may not be involved in male sexual development in the gastropods. Recently, it was reported that TBT and TPT are high-affinity ligands for human retinoid X receptor (RXR) and peroxisome proliferator-activated receptor (PPAR) γ (Kanayama et al. 2005). In addition, a functional homologue of RXR has been cloned from the rock shell (*Thais clavigera*) and the natural ligand of RXR, 9-*cis* retinoic acid, induces imposex in this species (Nishikawa et al. 2004). These reports suggest that the induction of imposex by organotin compounds may be mediated by RXR.

Differences in nuclear receptors between invertebrates and vertebrates

Nuclear receptors are structurally related proteins classified into a large superfamily that includes receptors for hydrophobic molecules such as steroid hormones (e.g., estrogens, androgens, progesterone, glucocorticoids, mineralocorticoids), retinoic acids (all-*trans* and 9-*cis* isomers), thyroid hormone, 1,25 $(OH)_2$ vitamin D_3, fatty acids. In addition to these receptors, the superfamily also contains a large number of so-called orphan nuclear receptors whose ligands do not exist or have not been identified (Giguére 1999). Nuclear receptors share a common structural organization with a highly conserved DNA-binding domain and a moderately well-conserved ligand-binding domain (LBD) (Fig. 1). Phylogenetic study and extensive polymerase chain reaction (PCR) surveys have revealed that nuclear receptor genes appeared very early on during metazoan evolution, but could not be found in fungi, plants, or unicellular eukaryotes (Escriva et al. 1997, 2000). By virtue of genome projects, we now know that *Homo sapiens*, *Drosophila melanogaster*, and *Caenorhabditis elegans*, respectively, have 48, 21, and 220 kinds of nuclear receptor genes (Maglich et al. 2001). There is a striking difference between vertebrates and invertebrates with respect to their nuclear receptor sets. For instance, receptors for sex and adrenal steroid hormones have not been found in any fully sequenced invertebrate genomes. Although ER-like cDNA was reportedly isolated from the mollusk *Aplysia californica*, it could not bind to estrogens and was a constitutive activated transcription factor like the orphan nuclear receptors (Thornton et al. 2003). So far, functional steroid hormone receptors including AR, ER, progesterone receptor (PR), glucocorticoid receptor (GR), and mineralocorticoid receptor (MR), have not been found in any invertebrate species (Escriva et al. 1997; Laudet 1997).

Reproductive abnormalities in wildlife can be associated with exposure to environmental pollutants capable of mimicking the action of sex hormones. In fact, there are many synthetic chemicals that have been shown to possess estrogenic activity by in vitro binding assay, reporter gene assay, or uterotrophic assay. The typical characteristic of chemicals having estrogenic activity is a phenol with a hydrophobic moiety at the para-position and without bulky groups at the ortho-position (Blair et al. 2000; Nishihara et al. 2000). Although these compounds may have adverse health effects in vertebrates (Colborn et al. 1996), they may not alter the function of the reproductive system through the medium of ER in invertebrates.

Imposex in marine gastropods

Among the variety of endocrine-disrupting events in marine invertebrates, imposex is one of the most documented. Imposex is induced by TBT at concentrations as low as 1 ng/L of tin (Sn) (Gibbs et al. 1987; Axiak et al. 1995) and is used extensively all over the world as a biomarker to monitor TBT pollution (Gibbs et al. 1987; ten Hallers-Tjabbes et al. 1994; Horiguchi et al. 1997a; Terlizzi et al. 1998, 2004). Not only TBT but also TPT has been shown to have a strong effect on the development of imposex in *T. clavigera* (Horiguchi et al. 1997b). So far, several hypotheses have been proposed to explain imposex induction. The first is that TBT increases androgen levels by inhibiting the enzyme activity that metabolizes testosterone. An aromatase enzyme complex is responsible for converting androgenic to estrogenic steroids. This enzyme complex consists of the microsomal CYP19 enzyme and the flavoprotein nicotinamide adenine dinucleotide phosphate reduced-form reductase. The latter is responsible for transferring reducing equivalents to CYP19 within the membrane of the endoplasmic reticulum. Bettin et al. (1996) reported that TBT increases androgen levels through inhibition of aromatase activity in marine neogastropods at relatively high doses. The TBT also inhibits the catalytic activity of human aromatase from transfected cells or a granulosa cell-like tumor cell line (Cooke 2002; Heidrich et al. 2001; Saitoh et al. 2001). However, it is doubtful whether the inhibitory effect of TBT on aromatase activity is a cause of the imposex, because the role of vertebrate sex steroids is unclear in invertebrates (LeBlanc et al. 1999). The second hypothesis is that TBT acts as a neurotoxin to abnormally release the peptide hormone termed penis morphogenic factor (PMF) (Féral and Le Gall 1983). The peptide hormone APGWamide has been proposed as the putative PMF, because injection of APGWamide significantly induces imposex in the mud snail *Ilyanassa obsoleta* (Oberdörster and McClellan-Green 2000,

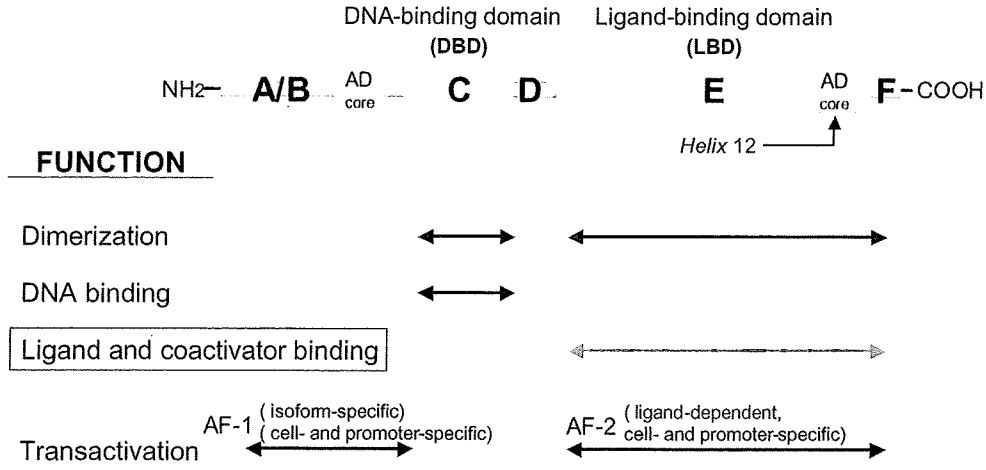

Fig. 1 Typical structure and functional domain of a nuclear receptor. Nuclear receptors are highly structurally related and share a common structural organization with a variable amino-terminal domain (**a**/**b**); a central, well-conserved DNA-binding domain (**c**); a non-conserved hinge domain (**d**); and a carboxyl-terminal, moderately conserved ligand binding domain (**e**). The ligand-independent transactivation function (**af**-1) is contained within the **a**/**b** region, and the ligand-dependent transactivation function (**af**-2) is within the **e** region

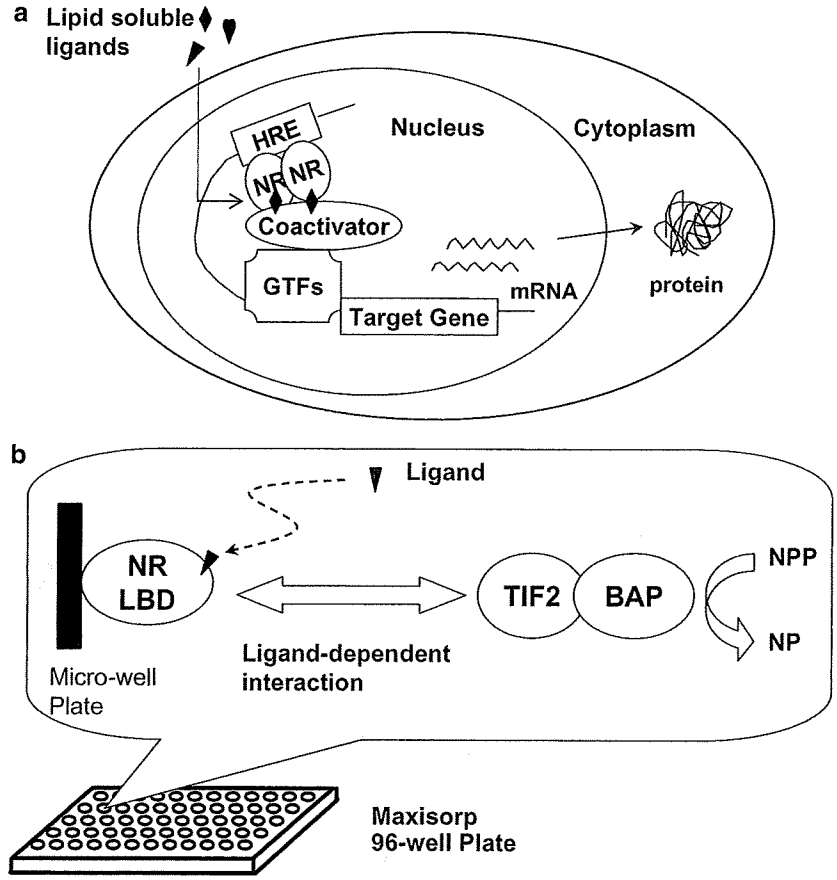

Fig. 2 **a** Nuclear receptors act as ligand-activated transcription factors by directly interacting with DNA-response elements of target genes as homodimers, heterodimers, or monomers. The effects of nuclear receptors on transcription are mediated through recruitment of co-regulators. Upon ligand binding, the receptors undergo a conformational change that allows the recruitment of coactivator complex. Recruitment of coactivator complex to the target promoter causes chromatin decomposition and transcriptional activation through interaction with general transcription factors (*GTFs*). **b** Principle of the screening method for nuclear receptor ligand. Nuclear receptor ligand-binding domain (*NRLBD*) is immobilized on the surface of a 96-well microplate. Coactivator TIF2 is prepared as a fusion protein with bacterial alkaline phosphatase (*BAP*). Test chemicals are added to the well with TIF2-BAP fusion protein. If the test chemical works as a ligand, it induces conformational change in NRLBD and recruits the TIF2-BAP on the plate surface. *p*-Nitrophenyl phosphoric acid (*NPP*) is used as a substrate for BAP. The BAP converts NPP to *p*-nitrophenol (*NP*), which appears *yellow*

2002). They proposed that PMF causes the development of male sex characteristics following an external stimulus such as TBT exposure. However, PMF cannot be the primary factor in the induction of imposex symptoms by TBT. There must be something other factor that directly interacts with TBT in the initial step of imposex induction.

Tributyltin and Triphenyltin as high-affinity ligands for nuclear receptors

Recently, Kanayama et al. (2005), by comprehensive screening of suspected endocrine disruptors versus human nuclear receptors, reported that TBT and TPT are high-affinity ligands for RXR and PPARγ. Their screening method was based on the ligand-dependent interaction between nuclear receptors and coactivators (Kanayama et al. 2003). In the initiation step of transcriptional activation, cognate ligands change the three-dimensional conformations of nuclear receptors (Bourguet et al. 1995; Renaud et al. 1995; Brzozowski et al. 1997; Agostini et al. 1998; Nolte et al. 1998). Next, a coactivator is exclusively recruited to its ligand-bound form of the receptor, but not to the ligand-free form (Fig. 2a). Kanayama et al. developed an in vitro detection method for ligand-dependent interaction between coactivator and nuclear receptors and applied it to the high-throughput screening (Fig. 2b). Using this system, they found that several suspected endocrine disruptors affected multiple nuclear receptors simultaneously. Among them, the effects of organotin compounds on RXR and PPARγ were most obvious. The agonistic effect of TBT on RXR was as strong as that of its endogenous ligand, 9-*cis* retinoic acid, and the effect of TPT on PPARγ was as strong as that of its well-known ligand, rosiglitazone. They also showed that TBT and TPT induced the transactivation function of RXR and PPARγ in mammalian culture cells (Kanayama et al. 2005). The dose range of TBT or TPT that induced transcriptional activation was 10–100 nM; this is almost pharmacologically relevant to the range reported to induce imposex in gastropods.

In mammals, PPARγ binds to DNA as a heterodimer with RXR and plays a central role in adipocyte gene expression and differentiation (Tontonoz et al. 1994). The PPARγ is abundantly expressed in adipocytes, and its ligands induce the efficient conversion of fibroblastic cells to adipocytes, as measured by induction of adipocyte-specific genes and lipid accumulation (Lehmann et al. 1995). In fact, TBT or TPT promotes differentiation of mouse preadipocyte 3T3-L1 cells to adipocytes (Kanayama et al. 2005). Therefore, organotin compounds may have adverse health effects on mammals by disturbing the endocrine processes mediated by the PPARγ/RXR pathway.

However, as I mentioned before, the composition of members of the nuclear receptor superfamily is quite different between vertebrates and invertebrates. The subgroup members of thyroid hormone receptor (TR), retinoic acid receptor (RAR), vitamin D receptor (VDR), and PPAR appear to have been late acquisitions during the evolution of the nuclear receptor superfamily (Escriva et al. 1997; Laudet 1997). Therefore, PPARγ might not be present in marine gastropods (Fig. 3). In contrast, RXR is special among the nuclear receptor superfamily. It is widely conserved in the evolutionary tree and its homologue, called ultraspiracle (USP), is found even in arthropods (Laudet 1997).

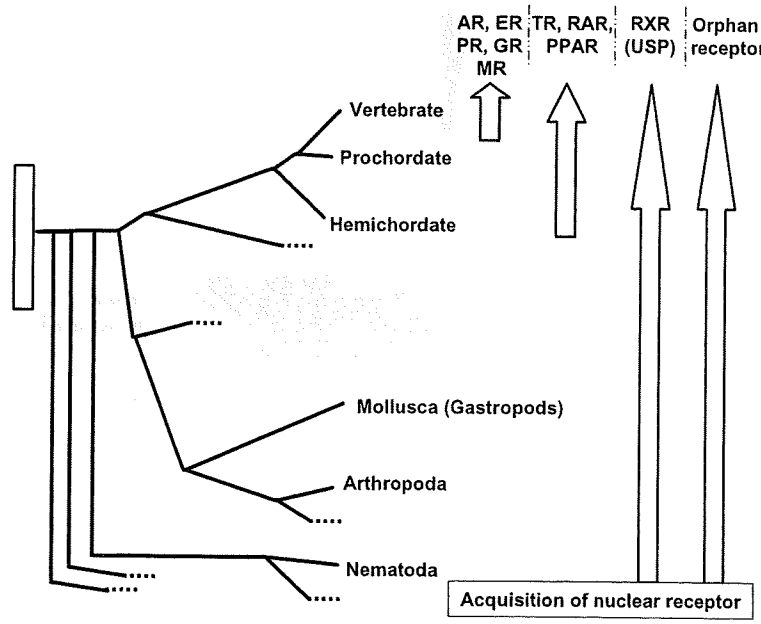

Fig. 3 Evolutionary tree and acquisition of nuclear receptors. Steroid hormone receptors (*AR* androgen receptor, *ER* estrogen receptor, *PR* progesterone receptor, *GR* glucocorticoid receptor, *MR* mineralocorticoid receptor) exist only in vertebrates. The subfamily of TR, RAR, and PPAR are present in vertebrates to hemichordates. In contrast, RXR or its homologue USP exist even in insects and nematodes

Characteristics of gastropod retinoid X receptor

Retinoid X receptor homologue has been cloned from *T. clavigera* (Nishikawa et al. 2004). Gastropod RXR has a DNA binding domain (DBD) composed of two C_2C_2-type zinc finger motifs and a putative LBD in the C-terminal region (Fig. 4a). The highest similarity with other species is in the DBD, where 85–90% of the amino acids residues are identical (Fig. 4b). The LBD of gastropod RXR also shows considerable similarity with that of vertebrate RXRs but has much less similarity with USP, the RXR homologue first found in *D. melanogaster*. Although RXR binds 9-*cis* retinoic acids in organisms ranging from cnidarians (*Tripedalia cystophora*) to vertebrates, USP from arthropods is unable to do so (Heyman et al. 1992; Mangelsdorf et al. 1992; Henrich and Brown 1995; Kostrouch et al. 1998). As expected by the similarity of a gastropod homologue to vertebrate RXR, the binding of gastropod RXR to 9-*cis* retinoic acid has been confirmed experimentally (Nishikawa et al. 2004). The dissociation constant in the binding of 9-*cis* retinoic acid to gastropod RXR is 15.2 nM, which is similar to the values reported for vertebrate RXRs (1–10 nM)

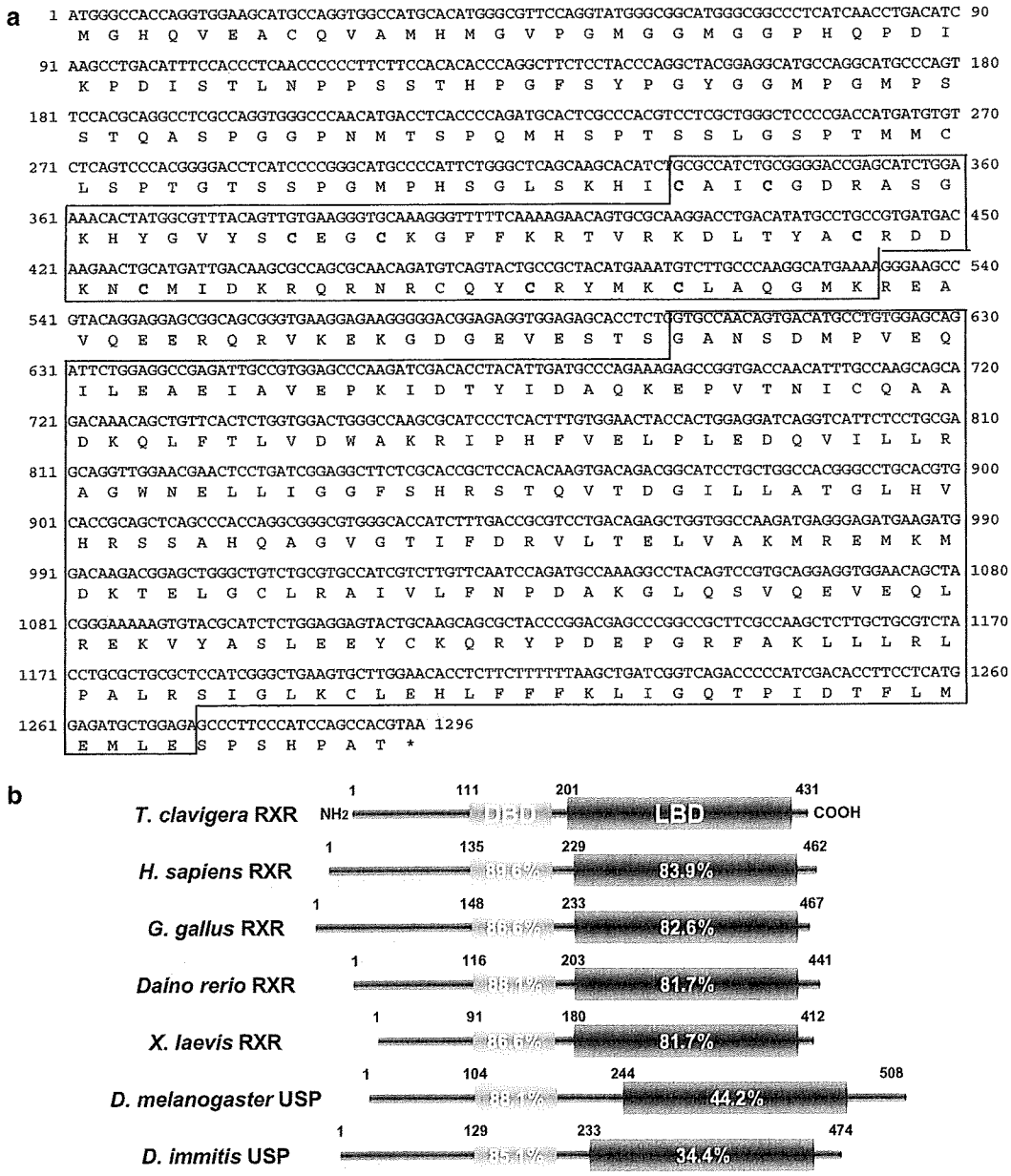

Fig. 4 **a** The entire coding sequence of gastropod RXR. The DNA and LBDs are *boxed*. The database accession number for the sequence is AY704160. **b** A schematic representation of RXR from various species is shown, along with the percentage of identical amino acid residues shared with those of gastropod RXR