A role for estrogen receptor β in the regulation of growth of the ventral prostate

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In normal rats and mice, immunostaining with specific antibodies revealed that nuclei of most prostatic epithelial cells harbor estrogen receptor β (ERβ). In rat ventral prostate, 530- and 549-aa isoforms of the receptor were identified. These sediment in the 4S region of low-salt sucrose gradients, indicating that prostatic ERβ does not contain the same protein chaperones that are associated with ERα. Estradiol (E_2) binding and ERβ immunoreactivity coincide on the gradient, with no indication of ERα. In prostates from mice in which the ERβ gene has been inactivated (BERKO), androgen receptor (AR) levels are elevated, and the tissue contains multiple hyperplastic foci. Most epithelial cells express the proliferation antigen *Ki-67*. In contrast, prostatic epithelium from wild-type littermates is single layered with no hyperplasia, and very few cells express *Ki-67*. Rat ventral prostate contains an estrogenic component, which comigrates on HPLC with the testosterone metabolite 5α-androstane-3β,17β-diol (3βAdiol). This compound, which competes with E_2 for binding to ERβ and elicits an estrogenic response in the aorta but not in the pituitary, decreases the AR content in prostates of wild-type mice but does not affect the elevated levels seen in ERβ knockout (BERKO) mice. Thus ERβ, probably as a complex with 3βAdiol, is involved in regulating the AR content of the rodent prostate and in restraining epithelial growth. These findings suggest that ligands specific for ERβ may be useful in the prevention and/or clinical management of prostatic hyperplasia and neoplasia.

Epidemiological and experimental studies indicate that estrogenic hormones are involved in both the induction and prevention of prostatic cancer (1–7), but their precise role is not well understood. Excessive exposure to estrogens during critical stages of development or long-term treatment of adult animals with estrogens or androgens leads to prostatic neoplasia (8, 9). In apparent contrast, diets rich in phytoestrogens, particularly soy products, are associated with a low risk of prostate cancer (10–12) and have chemopreventive properties in experimental tumor models (12, 13). Some of these conflicting observations may be explained by the fact that there are two distinct estrogen receptors, ERα and ERβ, which have unique and sometimes opposing roles (14). For example, recent studies have demonstrated that, in the rodent uterus, ERβ acts to restrain the stimulatory action of ERα (15).

Early studies, using both ligand-binding and immunochemical techniques, detected two types of estrogen-binding substances in human prostate (16), one of which is the classical estrogen receptor now known as ERα. Low levels of this receptor are present in the stroma of rodent prostates, but none is detectable in the epithelium (17, 18). Because of this difference in the levels of this receptor, it was proposed that the effects of estrogen on the epithelium are indirect via an initial interaction with the stroma (18). But after the discovery of ERβ in rat prostatic epithelium (19) it appeared that at least some of the epithelial effects might involve direct interaction with ERβ. The developmental and hormonal regulation of ERβ mRNA suggests that ERβ in the epithelium may be important in regulating prostatic growth (20). The recent availability of BERKO mice in which the ERβ gene has been inactivated (21) provides an approach for elucidating a role for ERβ in cellular processes by determining how these are changed when this receptor is absent.

The aims of the present study are to demonstrate, localize, and characterize the ERβ in the rodent prostate and to identify the aspects of prostate physiology that are mediated by this receptor by comparing BERKO with wild-type mice. Moreover, it is important to learn the identity of the natural ligand that activates ERβ in these processes. Because exogenous estrogen can disrupt the hypothalamic—pituitary–gonadal system, direct actions on prostatic estrogen receptors, which have been observed in organ cultures (22, 23), can be masked *in vivo* by effects on the central nervous system–gonadal axis. Our attention has centered on 5α-androstane-3β,17β-diol (3βAdiol), a metabolite of testosterone via 5α-dihydrotestosterone, which is reported to act as an estrogen in the prostate (24). It binds to both ERα and ERβ, with a slightly higher affinity for the latter (25), but it does not bind to the androgen receptor (AR) (26).

Materials and Methods

Animals. Male Sprague–Dawley rats (6–12 weeks old) were purchased from Mollegaard (Ejby, Denmark). BERKO mice (21) from our colony and their wild-type littermates were housed in the transgenic facility at Huddinge Hospital. Monthly checks were made for standard mouse pathogens by the Swedish Veterinary Association. Our mice did have pasteurella, which is endemic in most mouse colonies in this country. All animals were housed in a controlled environment on an illumination schedule of 12 h light/12 h dark and fed a standard pellet diet (containing soy meal, except where soy-free diet is indicated), with water provided ad libitum.

Chemicals, Antibodies, and Receptor Proteins. 3βAdiol, 5α-dihydrotestosterone, and estradiol (E_2) were purchased from Sigma; 6,7-tritiated E_2 (48 Ci/mmol) was from New England Nuclear; and 1,2-tritiated 3βAdiol (52.2 Ci/mmol) was from Amersham Pharmacia. Polyclonal anti-AR (PA1–111A, rabbit) was from Affinity BioReagents (Neshanic Station, NJ); anti-progesterone (anti-PR) (C-19, rabbit) and anti-*Ki-67* (M-19, goat) were from Santa Cruz Biotechnology, and anti-heat shock protein 27 (anti-Hsp27) (SPA-801, rabbit) was from StressGen (Victoria, Canada). Peroxidase-conjugated anti-chicken IgG (rabbit) was from Sigma. Anti-lysozyme antibody was a gift from Giannis Spyrou (Department of Biosciences, Karolinska Insti-

Abbreviations: ERα and ERβ, estrogen receptors α and β; BERKO, ERβ knockout; LBD, ligand-binding domain; AR, androgen receptor; PR, progesterone receptor; Hsp27, heat shock protein 27; E_2, estradiol-17β; 3βAdiol, 5α-androstane-3β,17β-diol.

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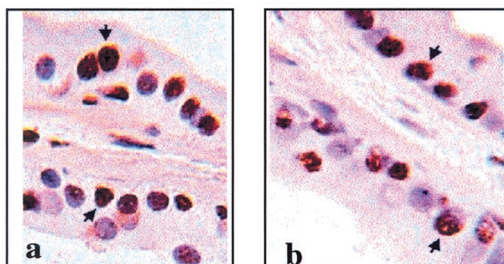

Fig. 1. Immunohistochemical demonstration of ERβ in ventral prostates of rats and mice. Frozen sections of ventral prostates from mice (*a*) and rats (*b*) stained for ERβ with anti-ERβ, 503-IgY. Positive immunoreaction is indicated by a brown color, and typical examples are marked by arrows. Stromal cell nuclei and some epithelial cell nuclei that are negative for ERβ are made evident by the blue counterstain.

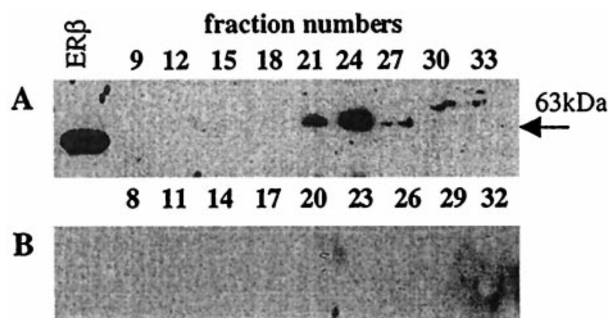

Fig. 3. Western blot detection of ERβ in fractions from gradient sedimentation of rat prostatic cytosol. (*A*) Resolved proteins from every fourth fraction from the gradient depicted in Fig. 2*c* were probed with ERβ LBD antibody. (*B*) A duplicate of the blot in *A*, except that the primary antibody was preadsorbed with ERβ 503 before Western blotting.

tute, Huddinge, Sweden). Two isoforms of ERβ protein (530 aa and 485 aa) were purchased from Panvera (Madison, WI), and one isoform (503 aa), as well as human ERα, ERβ 485, and ERβ ligand-binding domain (LBD), were expressed in SF9 cells and were supplied by KaroBio.

ERβ Antibody Preparation and Testing. Two polyclonal antibodies, which recognize ERβ and not ERα, were prepared in this laboratory (27). LBD IgG was raised in rabbits, with the use of the ERβ LBD as an antigen. IgG was isolated from the antiserum with protein A coupled to Sepharose. The other antibody (503 IgY) was raised in chickens and purified from egg yolks, with the use of the 503-aa receptor as an antigen. This IgY is useful in immunohistochemistry but does not give as strong signals on Western blots as does the LBD antibody. Both antibodies recognize the two ERβ proteins from Panvera. Incubation of LBD IgG with ERβ LBD protein for 12 h at 4°C eliminated its signals on Western blots, as did incubation of 503 IgY with any of the ERβ proteins coupled to activated Sepharose. Preadsorption with the freely soluble antigens was much less effective. In these preadsorption experiments, BSA coupled to Sepharose was used as a control.

Immunohistochemistry. For immunostaining of ERβ, frozen 8-μm sections were mounted on organosilane-coated slides, air-dried for 30 min, fixed with ice-cold methanol (3 min) and acetone (3 min), air-dried for 30 min, and stored at −20°C. After thawing, sections were fixed in 4% paraformaldehyde for 10 min and rinsed with PBS. Slides were incubated with 0.5% H_2O_2 to quench endogenous peroxidase and with 10% rabbit serum to block unspecific binding. Sections were incubated overnight at 4°C with the primary antibody (503 IgY) and diluted by 1:1,000 in PBS with 3% BSA. Negative controls were incubated with 3% BSA in PBS or with the antibody preadsorbed with Sepharose-coupled ERβ. Slides were washed with PBS and incubated for 1 h at room temperature with secondary antibody (peroxidase-conjugated anti-chicken IgG) diluted by 1:1,000 in PBS. After thorough washing in PBS, sections were developed with diaminobenzidine (Zymed), lightly counterstained with Mayer's hematoxylin, dehydrated, and mounted with permount. *Ki-67* staining with M-19 goat antibody was done according to protocols described in ref. 15.

Histology. For histological evaluation, ventral prostates were dissected under a microscope. They were either fixed overnight in 4% paraformaldehyde and processed routinely for paraffin embedding or were frozen in liquid nitrogen. Both paraffin and frozen sections (50 sequential sections of each sample) were stained with hematoxylin and eosin and evaluated by light microscopy.

Sucrose Gradient Sedimentation. Tissues, frozen in liquid nitrogen, were pulverized in a dismembrator (Braun, Melsungen, Germany) for 45 s at 1,800 rpm and added to a buffer composed of 10 mM Tris·HCl (pH 7.5), 1.5 mM EDTA, and 5 mM sodium molybdate. For MCF-7 cells the suspension was 100 μg, and for rat prostate it was 1 g of tissue/ml buffer. Cytosol was obtained by centrifugation of the homogenate for 1 h at 4°C at 204,000 × *g* in a 70Ti rotor.

Sedimentation studies were carried out as described (28). Prostate cytosols were incubated for 3 h at 0°C with 10 nM tritiated E_2 or with 3βAdiol in the presence or absence of excess radioinert E_2, and the bound and unbound steroids were sepa-

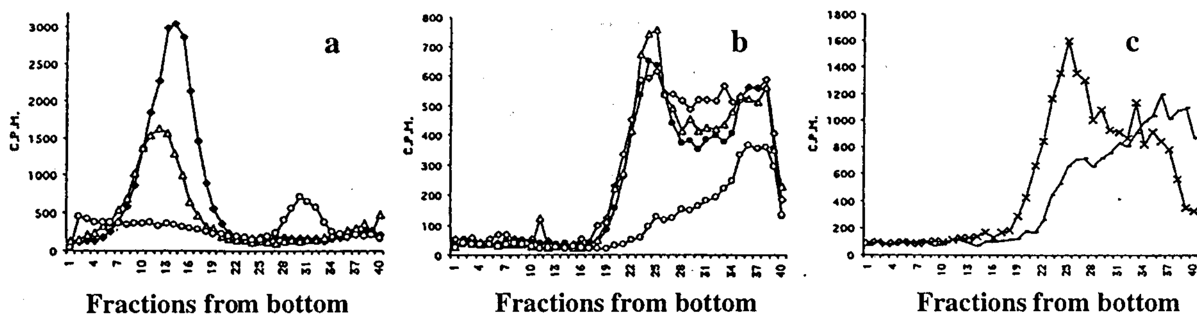

Fig. 2. Sucrose density gradient sedimentation profiles of ERα and ERβ. (*a*) ERα was from the cytosol of MCF7 cells (◆) and uteri of ovariectomized rats (△). ERβ (○) was 503 aa from extracts of SF9 cells expressing this protein. (*b*) Prostate cytosols were from rats that were 6 (○), 8 (◇), 10 (●), and 12 (△) weeks of age. (*c*) Prostate cytosol was from 9-week-old rats. Tritiated ligands: *a* and *b*, 10 nM E_2; *c*, 5 nM tritiated 3βAdiol with (−) and without (×) 5 nM radioinert E_2.

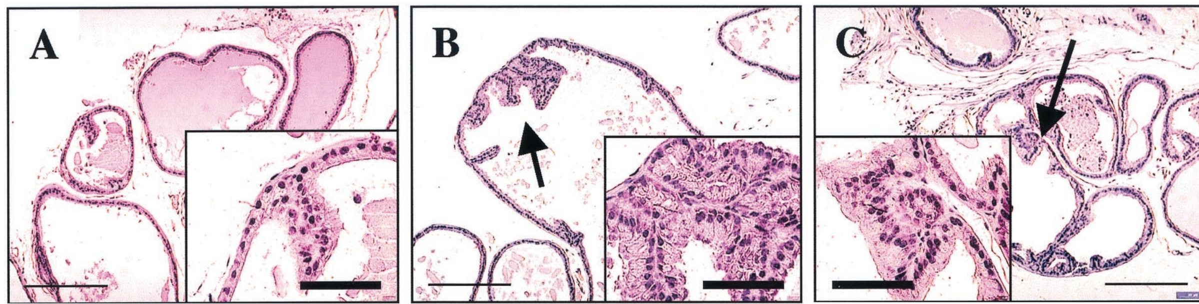

Fig. 4. Histology of ventral prostates from 5-month-old BERKO vs. wild-type mice. (*A–C*) Hematoxylin/eosin staining of a fixed section from the peripheral zone of a wild-type mouse prostate (*A*) and similar staining of fixed sections from the peripheral (*B*) and central (*C*) zones of a BERKO mouse prostate. Hyperplastic foci in the BERKO prostate are indicated with arrows. (Higher magnification of each section is depicted in a lower corner of each picture.) (Thick horizontal bars = 50 μm; thin bars = 200 μm.)

rated with dextran-coated charcoal. Sucrose density gradients [10–30% (wt/vol) sucrose] were prepared in buffer containing 10 mM Tris·HCl, 1.5 mM EDTA, 1 mM α-monothioglycerol (Sigma), and 10 mM KCl. Samples of 200 μl were layered on 3.5-ml gradients and centrifuged at 4°C for 16 h at 300,000 $\times g$ in a Beckman L-70K ultracentrifuge with an SW-60Ti rotor. Successive 100-μl fractions were collected from the bottom by paraffin oil displacement, with a collector of our own design, and assayed for radioactivity by liquid scintillation counting.

Protein Resolution and Western Blotting. All tissue handling was done at 4°C. Tissues were homogenized with a Polytron for a few seconds in buffer containing 600 mM Tris·HCl and 1 mM EDTA (pH 7.4), with two Boehringer protease inhibitor mixture tablets added per 50 ml. Tissue extracts were obtained by centrifugation for 1 h at 105,000 $\times g$. The protein contents of the tissue extracts were measured by Bio-Rad protein assay, with BSA as a standard. This extraction procedure was used to compare the protein profiles in prostates from BERKO and wild-type mice and from 3βAdiol-treated rats and mice.

For ERβ detection, samples were precipitated with trichloroacetic acid, and the precipitate was washed with methanol. Pellets were dissolved in SDS sample buffer, and the proteins were resolved by SDS/PAGE with 4–20% gradient gels (NOVEX, San Diego). For detection of AR, PR, lysozyme, and Hsp27, proteins were resolved on 9% gels. Transfer to polyvinylidene difluoride membranes was either by semidry blotting or in a Tris-glycine buffer. Blots were probed with specific primary antibodies, followed by appropriate secondary antibodies conjugated with horseradish peroxidase. Detection was by enhanced chemiluminescence.

N-Terminal Sequencing of Proteins. To obtain sufficient ERβ for N-terminal amino acid sequencing, cytosol from 13 g of rat ventral prostate was prepared in 50 ml of the Tris-EDTA buffer described above. This buffer was diluted 10-fold with 20 mM sodium phosphate buffer (pH 7.4) to reduce the ionic concentration. Heparin-Sepharose (1 ml) was added, and the mixture was gently rotated for 1 h at 5°C. Heparin-Sepharose was recovered by centrifugation and washed five times with 20 mM sodium phosphate buffer. Proteins were eluted with 1 M NaCl, precipitated with 10% trichloroacetic acid, washed with methanol, and resolved on SDS gels in six lanes. Proteins were transferred to polyvinylidene difluoride membranes, a strip was cut from one lane for detection of ERβ by Western blotting, and the rest of the membrane was stained with Coomassie brilliant blue. Protein bands corresponding to those reacting with the LBD antibody were cut from the membrane, and N-terminal sequencing was performed with the use of an Applied Biosystems 473A protein sequencer.

Estrogenic Components of Rat Prostate. Prostates from 5-month-old rats, raised on a soy-free diet from birth, were homogenized in saline, and the homogenates were extracted three times with ethyl acetate. The extract was dried under nitrogen, and the residue was dissolved in ethanol. The ethanol-soluble extract was resolved by HPLC on a C18 column (4.6 $\times$ 150 mm; Agilent Technologies, Palo Alto, CA) with fractions taken every 30 s from 11.5 to 12.5 and 16.5 to 18.5 min. The solvent system of acetonitrile/water had the following acetonitrile concentrations: 1–2 min, 0%; 2–3 min, 0–40%; 3–33 min, 40–60%; 33–34 min, 60–100%. Reference standards (3βAdiol, E_2, and testosterone) were located by photoabsorption at 254 nm. Dried fractions from the HPLC were dissolved in 50 μl of 70% ethanol and analyzed for estrogen/antiestrogen activity with the use of a cell-based estrogen receptor-dependent transcription assay (29). Briefly, 2.5×10^4 293/hERβ_{485} reporter cells per well were seeded in 96-well culture plates in 100 μl of Coon's/F12 medium (w/o phenol red) supplemented with 10% FCS (stripped twice with dextran-coated charcoal) and 2 mM L-glutamine. After 24 h, conditioned medium was replaced with 100 μl of Coon's/F12 supplemented with 1% FCS (estrogen depleted), 2 mM L-glutamine, gentamicin (50 μg/ml), and 1 μl of the HPLC fraction in the presence or absence of 0.5 nM E_2. The measure of estrogenicity is the amount of alkaline phosphatase protein expressed from the ΔERE2-ALP reporter and determined by chemiluminescence. In all experiments, cells were exposed to 0.1% ethanol, extracts, and/or E_2 for 72 h before harvest and analysis of reporter gene expression. Each extract was tested in triplicate.

Biological Effects of 3βAdiol vs. E_2. 3βAdiol (3 mg/kg/day) or E_2 (0.15 mg/kg per day) dissolved in Intralipid (Amersham Pharmacia) was given daily by s.c. injection to both rats and mice. Control animals received equal volumes of vehicle. After 7 days of treatment, the animals were asphyxiated by CO_2, and the ventral prostates, hearts, aortas, and pituitaries were collected and either placed in appropriate fixatives or immediately frozen in liquid nitrogen for separation of extracted proteins by PAGE and identification by Western blotting.

To determine the effect of 3βAdiol on AR and PR levels, 100 μg of protein from pooled prostatic cytosol from four 5-month-old mice or 80 μg of cytosolic protein from 9-week-old rats was loaded in each lane of the gel. Control samples were obtained by pooling prostates from three animals. That similar amounts of protein were loaded into each lane was confirmed by Coomassie blue staining of the membrane after Western blotting. For comparison of the estrogenicity of 3βAdiol in different tissues, lysozyme was used as a marker for the aorta and PR and Hsp27 for the pituitary. These were identified by gel electrophoresis

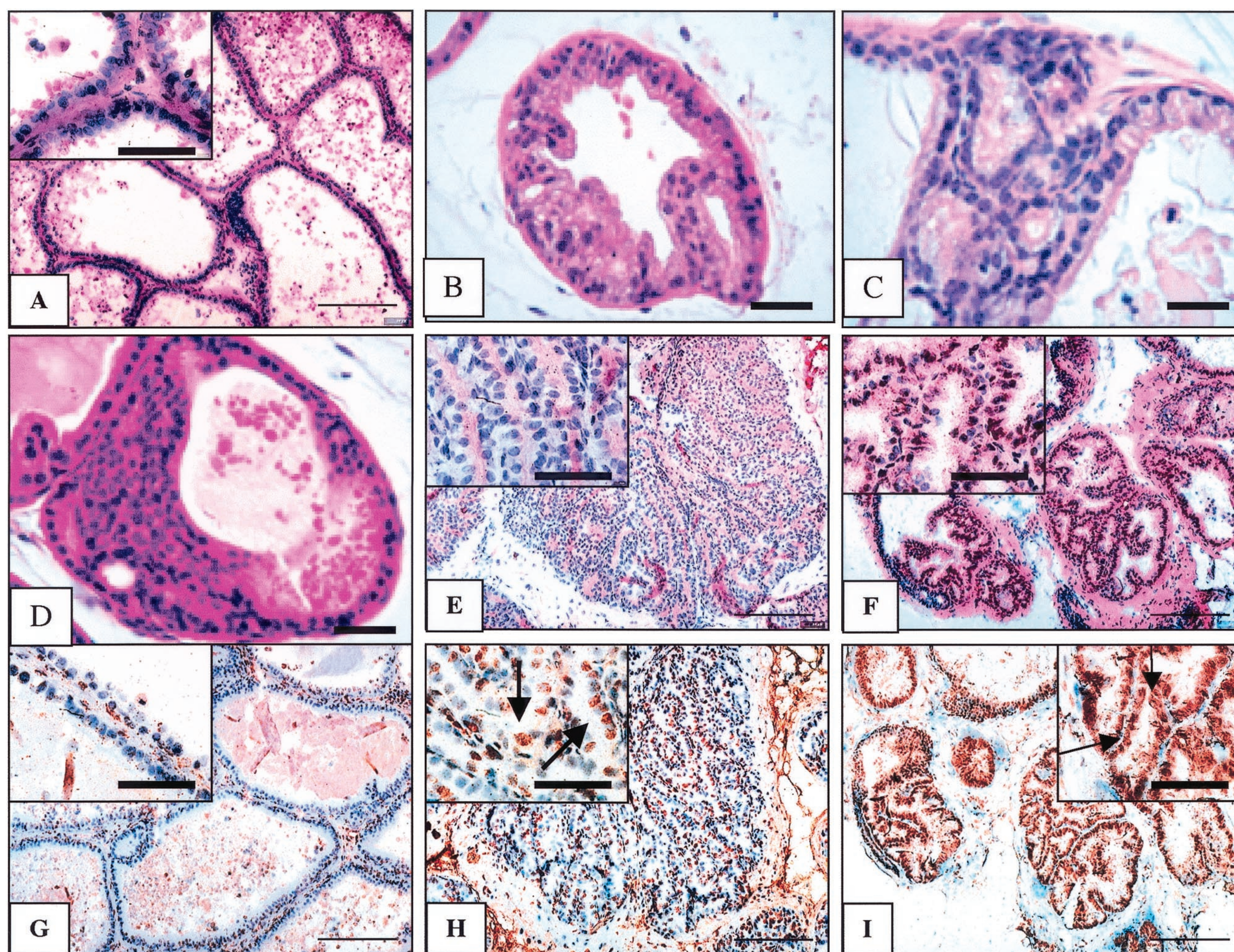

Fig. 5. Histology and Ki-67 staining of ventral prostates from 1-year-old BERKO and wild-type mice. Hematoxylin/eosin staining of frozen sections of ventral prostates from wild-type (*A*) and BERKO (*B–F*) mice, and immunostaining for Ki-67 in similar sections from wild-type (*G*) as compared with BERKO (*H* and *I*) mice. Higher magnification is depicted in an upper corner. (Thick horizontal bars = 50 μm; thin bars = 200 μm.)

followed by Western blotting, with specific antibodies as described above.

Results

Demonstration of ERβ in the Mouse and Rat Ventral Prostate. In the ventral prostate of adult mice and rats, nuclei in the majority of the epithelial cells (90%) stain positively for ERβ (Fig. 1). There is no detectable cytoplasmic staining in epithelial cells. Stromal cells are mostly negative.

Characterization of ERβ in Rat and Mouse Prostate by Sucrose Gradient Sedimentation. As expected (28), in low-salt extracts of rat uterus or MCF7 breast cancer cells, ERα binds tritiated E_2 and sediments as an 8S peak (Fig. 2*a*). Cytosol of SF9 cells overexpressing ERβ 503 shows a sedimentation peak at 4S. In rat ventral prostate cytosols, binding of either tritiated E_2 or 3βAdiol is observed only in the 4S region. Comparison of cytosols from 6-, 8-, 10-, and 12-week-old rats (Fig. 2*b*) revealed that this binding of E_2 is almost undetectable at 6 weeks of age, but by 8 weeks it increases to adult levels, $\approx$60 fmol/mg protein. Unlabeled E_2 (5 nM) competes with 5 nM tritiated 3βAdiol for binding in the 4S peak (Fig. 2*c*), indicating that binding of the latter is to an estrogen receptor. No competition was observed between 5α-dihydrotestosterone and 3βAdiol (data not shown).

To determine whether the binding substance in the 4S peak actually is ERβ, fractions from the sucrose gradient were analyzed by Western blotting, and the E_2-binding peak of the gradient was found to coincide with the ERβ band of molecular mass 63 kDa (Fig. 3*a*). The specificity of the band was confirmed by preadsorption of the primary antibody with ERβ 503 (Fig. 3*b*).

Identification of Prostatic ERβ by N-Terminal Sequencing. DNA-binding proteins were extracted from ventral prostates of 12-week-old rats with the use of heparin-Sepharose. When this extract was probed with LBD antibody on Western blots, a doublet of molecular mass 62–63 kDa was observed (data not shown). Protein bands corresponding to this doublet were excised from the membrane, and their N-terminal regions were sequenced by automated Edman degradation. The amino acid sequences MEIKNSPSSLSSPA and MSIXXASSHKEFSQLR were read from protein bands of molecular mass 62–63 kDa. These two sequences correspond to ERβ containing 530 and 549 aa, respectively. There was no immunoreactive band at 55 kDa, which is the size of the short form of the receptor containing 485 aa (19).

Histology of Normal and BERKO Prostates. At 5 months of age, ventral prostates of wild-type mice have a well differentiated

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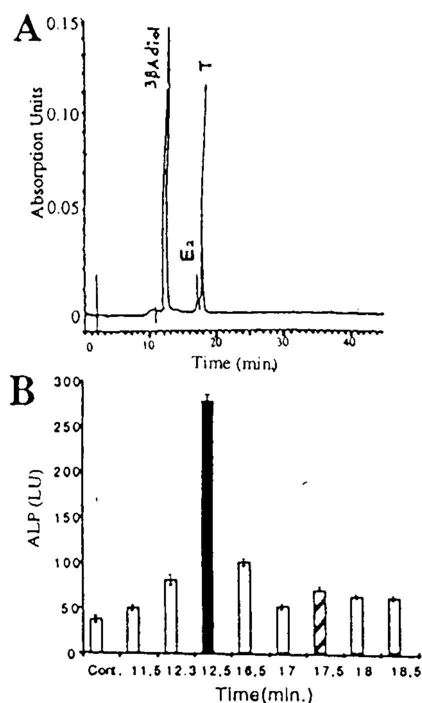

Fig. 6. Identification of 3βAdiol as an endogenous estrogen in rat ventral prostate. (*A*) HPLC separation of standards: 3βAdiol, E_2, and testosterone (T). 3βAdiol elutes at 12 min, E_2 at 17 min, and testosterone after 18 min. (*B*) Estrogenic activity in HPLC fractions from extracts of rat ventral prostates, as indicated by the production of alkaline phosphatase in the reporter cell system described. Vertical bars are mean values from triplicate determinations, with the error range shown for each value. The solid bar coincides with the elution of 3βAdiol (12.5 min), and the striped bar, with that of E_2 (17.5 min).

secretory appearance with single-layered columnar or cuboidal epithelium and large lumina but no hyperplastic foci (Fig. 4*A*). In BERKO mice, there are increased infoldings of the epithelium and foci of epithelial hyperplasia in some but not all acinae (Fig. 4 *B* and *C*). At 1 year of age, 8 of 10 BERKO mouse prostates contained multiple hyperplastic lesions (Fig. 5 *B–F*), whereas corresponding wild-type prostates show no indication of hyperplasia (Fig. 5*A*).

Ki-67 in the Mouse Prostate. At 1 year of age, *Ki-67*-containing cells are rare in the prostatic epithelium of wild-type mice (Fig. 5*G*), whereas in BERKO mouse prostates most of the epithelial cells stain positively for this proliferation antigen (Fig. 5 *H* and *I*).

Identification of 3βAdiol as an Endogenous Estrogen in Rat Prostate. Prostate extracts from 5-month-old rats, raised on a soy-free diet, activated ERβ in a reporter cell assay. Upon resolution of the components of the extract by HPLC, two peaks of estrogenicity were found (Fig. 6*B*). The major peak of estrogenicity coelutes from the C18 column with 3βAdiol (Fig. 6*A*), with very little activity in the fraction where E_2 elutes.

Tissue Specificity of 3βAdiol as an Estrogen in Rats. Western blots with total cellular extracts revealed that both E_2 and 3βAdiol induce lysozyme in the aorta (Fig. 7*A*). In the pituitary gland, E_2 but not 3βAdiol induces PR (Fig. 7*B*) and Hsp27 (Fig. 7*C*). The estrogenic response in the aorta confirms that the steroid was delivered.

AR Content of Wild-Type and BERKO Prostates. With prostatic cytosols of normal rats and mice, AR is easily detected on

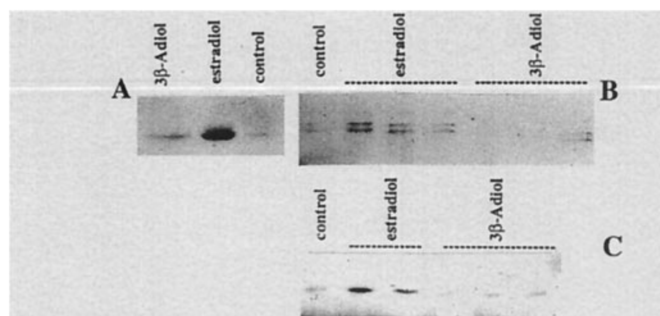

Fig. 7. Effect of 3βAdiol vs. E_2 on the expression of estrogen-regulated genes. Lysozyme (*A*) was measured in the aorta, and PR (*B*) and Hsp27 (*C*), in the pituitary of male rats. The control lanes of *A* and *B* represent data obtained by pooling of tissue from four animals; in the treatment groups, aortas and pituitaries from three individual rats were used. Lysozyme and Hsp27 were measured in high-salt, and PR, in low-salt cell extracts.

Western blots as a 114-kDa protein. The content of AR is higher in BERKO prostates than in those of wild-type littermates (Fig. 8*A*). In normal mice (Fig. 8*B*) but not in BERKO mice (Fig. 8*C*), 3βAdiol treatment causes a significant decrease in AR levels. When rats are treated with 3βAdiol, there is a similar reduction in the intensity of the AR but not the PR signal (Fig. 8*D*).

Discussion

This study demonstrates that ERβ protein is abundant in the nuclei of epithelial cells in the ventral prostates of rats and mice, and its absence results in hyperplastic foci in prostates of young adult mice. These become more pronounced with age, and by 1 year 80% of the BERKO mice show hyperplastic lesions. Whether they result from an actual increase in proliferation rate or from some other action, such as a decrease in apoptosis, is not certain. However, it does appear that epithelial cells in the ventral prostate of BERKO mice are not in G_0 but are always in the cell cycle, as indicated by their expression of the proliferation

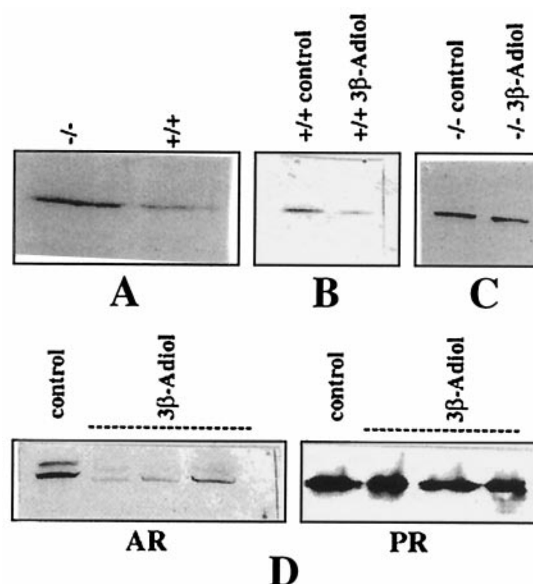

Fig. 8. Effect of 3βAdiol on the androgen and progesterone receptor content in mouse and rat ventral prostates. (*A*) AR levels in BERKO (−/−) vs. wild-type (+/+) mice. (*B*) Down-regulation of AR by 3βAdiol in wild-type mice. (*C*) Lack of down-regulation of AR in BERKO mice. (D) AR and PR levels in rats ± 3βADiol. Analyses of pooled tissue from treated animals were made in triplicate.

antigen *Ki-67* (30). Thus ERβ seems to be a regulator of some checkpoint in the cell cycle. A clue to whether the hyperplasia eventually leads to actual cancer must await detection of prostatic intraepithelial neoplasia in embedded sections of tissue from older BERKO mice.

The distribution patterns of ERα and ERβ in the ventral prostate suggest that the two receptors have different roles in this organ. ERα is located in stromal tissue and is not detectable in the epithelium. ERβ is present in the epithelium, where it is coexpressed with AR. The two estrogen receptors have distinct physical characteristics. In these studies, ERβ sediments as a broad 4S peak regardless of salt concentration, whereas ERα sediments as a sharp 4S peak in high-salt (400 mM) and as an 8S peak in low-salt (10 mM) gradients (28). Because the 8S ERα complex results from its association with heat shock proteins and other chaperones (31), it appears that ERβ does not form the same type of complexes as ERα.

AR appears to be one of the genes that are regulated by ERβ. There is elevated expression of this receptor in BERKO mouse prostates, and 3βAdiol (an ER ligand) decreases AR levels in wild-type but not in BERKO mice. Regulation of AR could be one mechanism through which ERβ reduces or limits prostatic growth and induces a more differentiated prostate phenotype. Such a differentiating role for ERβ has been suggested on the basis of developmental studies of ERβ mRNA in the prostate (20). The extent to which an increase in AR levels contributes to the BERKO prostate phenotype is still unclear. Elevation of AR has been considered to be a possible causative factor in the development of prostate cancer (32), but the precise relationship between prostatic androgen receptors, circulating androgen levels, and prostate cancer remains poorly understood (33–35).

If ERβ regulates prostatic growth, the physiological ligand for this receptor has to be identified. The concentration of estradiol in the prostate is low, $\approx$0.1 pmol/g tissue, whereas the level of 3βAdiol is 100-fold higher (10 pmol/g), and this compound has been reported to act as an estrogen in the prostate (24). The present study demonstrates that E_2 and 3βAdiol compete for binding to a component of prostate cytosol that sediments in a low-salt gradient as a 4S peak. This component is identified as ERβ on the basis of its absence in BERKO mouse prostates, as well as by Western blotting of sucrose gradient fractions, where the E_2-binding peak coincides with ERβ immunoreactivity. Rat prostatic tissue contains estrogenic activity, the majority of which migrates on HPLC with 3βAdiol and not E_2. If 3βAdiol is the endogenous ERβ ligand involved in limiting androgen-induced prostatic growth, one could predict that inhibitors of 5α-reductase, such as Finasteride, would not be effective in controlling prostatic growth because, by blocking 5α-dihydrotestosterone formation, they remove the biosynthetic precursor of the antiproliferative ligand, 3βAdiol.

From the foregoing results, ERβ appears to be a physiological regulator of prostatic epithelial growth and differentiation. This phenomenon shows an interesting analogy to the previously described antiproliferative action of ERβ in the immature rat uterus, where it appears to counteract the stimulatory effect of ERα (15). These combined observations suggest that activating ligands specific for ERβ could be important modulators of both prostatic and uterine growth and may have clinical utility in the prevention and/or treatment of neoplastic diseases of these organs.

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1. Kozak, I., Bartsch, W., Krieg, M. & Voigt, K. D. (1982) *Prostate* **3,** 433–438.
2. Shao, T. C., Tindall, D. J. & Cunningham, G. R. (1986) *Prostate* **8,** 349–362.
3. Martikainen, P. M., Mäkelä, S. I., Santti, R. S., Harkonen, P. L. & Suominen, J. J. (1987) *Prostate* **11,** 291–303.
4. Kawamura, H., Kimura, M. & Ichihara, I. (1993) *Anat. Anz.* **175,** 569–575.
5. Krieg, M., Weisser, H. & Tunn, S. (1993) *Verh. Dtsch. Ges. Pathol.* **77,** 19–24.
6. Pylkkanen, L., Mäkelä, S. & Santti, R. (1996) *Eur. J. Urol.* **30,** 243–248.
7. Olbina, G., Miljkovic, D., Hoffman, R. M. & Geller, J. (1998) *Prostate* **37,** 126–129.
8. Messina, M. J., Persky, V., Setchell, K. D. & Barnes, S. (1994) *Nutr. Cancer* **21,** 113–131.
9. Bosland, M. C., Ford, H. & Horton, L. (1995) *Carcinogenesis* **16,** 1311–1317.
10. Barnes, S., Peterson, T. G. & Coward, L. (1995) *J. Cell. Biochem.* **22,** 181–177.
11. Goodman, M. T., Wilkens, L. R., Hankin, J. H., Lyu, L. C., Wu, A .H. & Kolonel, L. N. (1997) *Am. J. Epidemiol.* **146,** 294–306.
12. Landstrom, M., Zhang, J. X., Hallmans, G., Aman, P., Bergh, A., Damber, J. E., Mazur, W., Wahala, K. & Adlercreutz, H. (1998) *Prostate* **36,** 151–161.
13. Hempstock, J., Kavanagh, J. P. & George, N. J. (1998) *Br. J. Urol.* **82,** 560–563.
14. Paech, K., Webb, P., Kuiper, G. G., Nilsson, S., Gustafsson, J.-Å., Kushner, P. J. & Scanlan, T. S. (1997) *Science* **277,** 1508–1510.
15. Weihua, Z., Saji, S., Mäkinen, S., Cheng, G., Jensen, E. V., Warner, M. & Gustafsson, J.-Å. (2000) *Proc. Natl. Acad. Sci. USA* **97,** 5936–5941.
16. Ekman, P., Barrack, E. R., Greene, G. L., Jensen, E. V. & Walsh, P. C. (1983) *J. Clin. Endocrinol. Metab.* **57,** 166–176.
17. Bodker, A., Andersson, K. E., Batra, S., Juhl, B. R. & Meyhoff, H. H. (1993) *Scand. J. Urol. Nephrol.* **27,** 169–174.
18. Cooke, P. S., Buchanan, D. L., Young, P., Setiawan, T., Brody, J., Korach, K. S., Taylor, J., Lubahn, D. B. & Cunha, G. R. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 6535–6540.
19. Kuiper, G. G., Enmark, E., Pelto-Huikko, M., Nilsson, S. & Gustafsson, J.-Å. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 5925–5930.
20. Prins, G. S., Marmer, M., Woodham, C., Chang, W., Kuiper, G. G., Gustafsson, J.-Å. & Birch, L. (1998) *Endocrinology* **139,** 874–883.
21. Krege, J. H., Hodgin, J. B., Couse, J. F., Warner, M., Mahler, J. F., Sar, M., Korach, K. S., Gustafsson, J.-Å. & Smithies, O. (1998) *Proc. Natl. Acad. Sci. USA* **95,** 15677–15682.
22. Schneider, M. R., Hartmann, R. W., Sinowatz, F. & Amselgruber, W. (1986) *J. Cancer Res. Clin. Oncol.* **112,** 258–265.
23. Lamartiniere, C. A., Zhang, J. X. & Cotroneo, M. S. (1998) *Am. J. Clin. Nutr.* **68,** 1400.S–1405S.
24. Voigt, K.-D. & Bartsch, W. (1986) *J. Steroid Biochem.* **25,** 749–757.
25. Kuiper, G. G., Carlsson, B., Grandien, K., Enmark, E., Haggblad, J., Nilsson, S. & Gustafsson, J.-Å. (1998) *Endocrinology* **138,** 863–870.
26. Turcotte, G., Chapdelaine, A., Roberts, K. D. & Chevalier, S. (1988) *J. Steroid Biochem.* **29,** 69–76.
27. Saji, S., Jensen, E. V., Nilsson, S., Rylander, T., Warner, M. & Gustafsson, J-Å. (2000) *Proc. Natl. Acad. Sci. USA* **97,** 337–342.
28. Jensen, E. V., Suzuki, T., Kawashima, T., Stumpf, W. E., Jungblut, P. W. & DeSombre, E. R. (1968) *Proc. Natl. Acad. Sci. USA* **59,** 632–638.
29. Barkhem, T., Carlsson, B., Nilsson, Y., Enmark, E., Gustafsson, J.-Å. & Nilsson, S. (1998) *Mol. Pharmacol.* **54,** 105–112.
30. Gerdes, J., Lemke, H., Baisch, H., Wacker, H. H., Schwab, U. & Stein, H. (1984) *J. Immunol.* **133,** 1710–1715.
31. Pratt, W. B. & Toft, D. O. (1997) *Endocr. Rev.* **18,** 306–360.
32. Sultan, C., Terouanne, B., Tahiri, B., Lumbroso, S., Avances, C. & Orio, F., Jr. (1999) *Bull. Cancer* **86,** 618–621.
33. Gann, P. H., Hennekens, C. H., Ma, J., Longcope, C. & Stampfer, M. J. (1996) *J. Natl. Cancer Inst.* **88,** 1118–1126.
34. Gustafsson, O., Norming, U., Gustafsson, S., Eneroth, P., Astrom, G. & Nyman, C. R. (1996) *Br. J. Urol.* **77,** 433–440.
35. Vatten, L. J., Ursin, G., Ross, R. K., Stanczyk, F. Z., Lobo, R. A., Harvei, S. & Jellum, E. (1997) *Cancer Epidemiol. Biomarkers Prevention* **6,** 967–969.