Vol. 130, No. 1, 1985 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 16, 1985 Pages 293-300

LOCALIZATION OF PROSTATIC BASIC PROTEIN ("PROBASIN") IN THE RAT PROSTATES BY USE OF MONOCLONAL ANTIBODY

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Received May 29, 1985

SUMMARY: Isolated nuclei of the rat prostates contain a unique androgen-dependent basic protein, "probasin". Despite that it was hardly detectable in the cytosol centrifugally prepared from the prostates, immunofluorescent histological analysis of whole tissues using monoclonal antibody, which was raised against probasin purified from the nuclei, revealed that probasin was abundantly localized in the lumen and acinal regions of the epithelium, but hardly in the nuclei. Previous extraction of secretory fluid from the prostates caused about 60% decrease in the probasin content of isolated nuclei. These suggest that probasin was originally a secretory component in the prostates, being redistributed from the secretory fluid and granule into nuclei during fractionation of subcellular components.

The male accessory sex organs of the rat consist of the prostate gland, coagulating gland and seminal vesicle. The prostate gland is composed of ventral prostate and dorsolateral prostate. These organs depend on androgen for maintenance of normal physiological function. We found that nuclei of the dorsolateral prostate contained a large amount of an androgen-dependent basic protein (mol. wt.≒20,000; pI≒11.5), "probasin" (1), which was already purified and characterized (2). We have studied its androgen dependency (1, 3, 4), changes during sexual maturation (5) and by dedifferentiation (6), its distribution in the male accessory sex organs (7) and its lobe-specific distribution in the dorsolateral prostate (8). The present study was undertaken to reveal localization of probasin in the rat prostates.

Abbreviations: PMSF, phenylmethlysulfonyl fluoride; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; CBB, Coomassie Brilliant Blue R-250; HAT medium, medium containing hypoxanthine, aminopterine and thymidine; ELISA, enzyme linked immunosorbent assay; FCS, fetal calf serum; PBS, 0.02 M sodium phosphate buffer (pH 7.2) containing 0.15 M NaCl; IgG, immunoglobulin.

0006-291X/85 $1.50

MATERIALS AND METHODS

Preparations of secretory fluid and subcellular components Male Sprague-Dawley rats (12-16 weeks of age) were used (1-3). Subcellular components were prepared at 0-4°C by the following two methods. Method A---The rat tissues were subjected to previous extraction of secretory fluid; the tissues were minced by scissors into 2-3 mm pieces in 3 volume (v/w) of isotonic medium, 10 mM Tris-HCl (pH 7.5) containing 0.25 M sucrose, 3 mM $CaCl_2$ and 1 mM PMSF, followed by centrifugation at 800 x g for 5 min. This pre-extraction was repeated twice, and the resulting extracts were used as secretory fluid. From the resulting precipitate, cytosol and nuclei were prepared by the method previously described (1, 3). Method B---Cytosol and nuclei were prepared from tissue homogenates without previous extraction of secretory fluid by the same method as those described previously (1, 3). Preparation of nuclei in the presence of citric acid was carried out according to the method of Higashi et al. (9).

SDS-PAGE and electroblotting SDS-PAGE was performed in 12.5% gels with a size of 176(height)x150(width)x2(thickness)mm (3). For immunological experiments, samples were run in duplicate gels with a size of 50(height)x85(width)x1(thickness)mm; one gel was subjected to staining with CBB, and the other was electroblotted onto nitrocellulose (7x10cm) at 8.5V (0.22A) overnight with 50 mM sodium phosphate buffer (pH 6.5). The blots were immunostained (10) by the direct immunoperoxidase method. Briefly the electrophoretic blots were soaked in 10% FCS in PBS containing 0.05% Triton X-100 for 30 min. They were incubated with monoclonal antibody diluted with 10% FCS in PBS containing 0.05% Triton X-100. After washed with PBS containing 0.5% Triton X-100, they were incubated with peroxidase-conjugated rabbit IgG to mouse IgG. For the color reaction, the blots were soaked in 0.01% o-dianisidine containing 0.01% hydrogen peroxide and 10 mM Tris-HCl (pH 7.5).

Preparation and definition of probasin Probasin (prostatic basic protein) was purified from the nuclei of the dorsolateral prostates of rats by the method described previously (2). Probasin was defined as an androgen-dependent protein with a molecular weight of about 20,000 and pI of about 11.5 in the rat prostates, or a protein having the same antigenicity as probasin.

Preparation of antibody and immunological methods A monoclonal antibody against probasin was prepared by the method of Köhler and Milstein (11) with some modifications (12). Spleen cells prepared from a female BALB/c mouse immunized with probasin purified from nuclei of the dorsolateral prostate were fused with P3U1 myeloma cells in 50% (w/v) polyethylene glycol 1540, and selected in HAT medium. Positive hybridoma cultures were detected by ELISA and subjected to two successive cloning by limited dilution. One stable clone which secreted antibody against probasin was obtained. The hybridoma cells ($5x10^6$) were intraperitoneally injected into a female BALB/c mouse that had received an intraperitoneal injection of 0.5 ml of 2,6,10,14-tetramethylpentadecane 10 days previously. IgG was purified from ascites fluid by ammonium sulfate precipitation and ion-exchange chromatography on a DEAE-Sepharose CL-6B column. Monoclonal antibody isotype was determined to be IgG_{2a} containing the kappa light chain using a screening kit from Zymed Laboratories Inc. Indirect immunofluorescence was carried out at 20-25°C as follows: Paraffin sections (5 μm) were prepared by fixing tissues in Perfix (Fisher Scientific Co.) for 5 days, followed by embedding the fixed tissues in a paraffin. Frozen sections were prepared by embedding fresh tissues in TISSUE-TEK II (Miles Laboratories, Inc.) at -20°C, and used for immunological experiments within 2 h. Nuclei were suspended in PBS. Paraffin sections, frozen sections and isolated nuclei were adhered on glass slides, and the glass slides were incubated with 10% ovalbumin for 30 min to minimize non-specific binding, followed by 3 times washing with PBS for 5 min. The glass slides were then incubated with monoclonal antibody for 30 min, followed by 3 times washing with PBS, and then incubated with FITC (fluorescein isothiocyanate)-labeled anti-mouse IgG for 30 min. After washing, they were mounted in PBS-glycerol (1:9). Fluorescence was observed with Leitz microscope.

Determination of protein content The protein content was determined by the method of Lowry et al.(13), using bovine serum albumin as standard.

demonstrated (6). If the transverse relaxation time in the bound state (T_{2B}) of the small molecule is short compared to the preexchange lifetime (τ_B), then the process is slow on the NMR time scale and the bound state is usually not detected due to line broadening. The determination of the observed spectral intensity then yields the partition coefficient directly.

MATERIALS AND METHODS

Materials: The DPPC was obtained from Sigma Chemical Co., St. Louis, MO. Gas chromatographic analysis (7) revealed the DPPC to be more than 99.5% pure. Ethanol-d_6 (density = 0.918/ml) was obtained from Cambridge Isotope Laboratories, Woburn, MA. Liposomes were prepared from the DPPC as described elsewhere (8) in phosphate buffered saline, pH = 7.6 at 22°C. Spectra were obtained at 46°C and thus the lipid was in the liquid-crystalline state.

Methods: The ^2H NMR spectra were obtained on a Nicolet NMC 300 FT-NMR spectrometer. The lock coil of 5 mm ^1H proton probe was used as the observe coil. The magnetic field strength was 7.05 Tesla with a frequency of 46.066 MHz for ^2H. The temperature was maintained at 46°C using a NTC temperature control unit. The spectrometer was run in the unlocked mode. The spectra were time averaged for 128 to 512 scans depending on the ethanol-d_6 concentration. Peak intensities were obtained using minimum Chi square residual fit of each resonance to a Lorentzian line, and peak area was taken as height times linewidth.

RESULTS AND DISCUSSION

The partition coefficient of ethanol-d_6 to the DPPC liposomes can be determined by ^2H NMR spectroscopy in the following manner. The addition of ethanol-d_6 to H_2O will result in the exchange of the hydroxyl deuteron with a proton to yield stoichiometric amounts of HOD and CD_3CD_2OH molecules. The exchanging ethanol species between the free and bound states will, therefore, be CD_3CD_2OH. The observed ^2H spectrum will consist of three resonances, namely, the HOD, CD_2 and CD_3 resonances. The intensity of the HOD will be the sum of the natural deuterium concentration (i.e. $[HOD]_{nat} = 0.01776$ m) and the concentration of added ethanol $[EtOH]_T$. Because exchange between the free and the bound states is slow on the NMR time scale and the bound state is broadened beyond detection, the intensity of the methylene group of the ethanol would be twice the added ethanol concentration minus twice the bound concentration $[EtOH]_B$. The ratio of the methylene to HOD integrated intensities is given by

$$R = \frac{2([EtOH]_T - [EtOH]_B)}{[HOD]_{nat} + [EtOH]_T} \quad (1)$$

which can be rearranged to

epithelium (Fig. 1C). The fluorescence was absent when the antibody was depleted (Fig. 1B). Either in the normal prostates or in the prostates of castrated rats, none of immunofluorescence was detected in nuclear regions. Essentially the similar results were obtained with frozen sections of the fresh prostates. On the other hand, probasin was definitely detected by immunofluorescent analysis of nuclei isolated from the dorsolateral and ventral prostates (Fig. 1D and 1E). These results suggest that probasin in the rat prostates was mainly localized as a secretory component in the lumen of the normal prostates, and that probasin in isolated nuclei resulted from redistribution during fractionation of subcellular components.

Experiments to demonstrate redistribution of "probasin" into nuclear fraction from secretory fluid in the rat prostates As reported previously (1), probasin was recognizable as a protein with a molecular weight of about 20,000 (20Kd-protein or 20K-NHP) on SDS-PAGE patterns of proteins in the rat prostates. In order to examine redistribution of probasin from secretory fluid into nuclear fraction, secretory fluid was removed from the dorsolateral prostate by three times extraction with isotonic medium prior to homogenization of the tissue, and then subcellular components were prepared. SDS-PAGE patterns of proteins in secretory fluid, cytosol and nuclei indicate that 20Kd-protein was detectable both in the secretory fluid (Sec-1, -2 and -3 in Fig. 2) and in the nuclei, but not in the cytosol (Fig. 2). The content of 20Kd-protein in the nuclei was decreased by about 60% by the previous extraction of secretory fluid (Nuc(A) vs. Nuc(B) in Fig. 2). In addition, the 20Kd-protein content in nuclei prepared in the presence of 2.5% citric acid was as low as about 1/5 of that in nuclei prepared from the prostates in the absence of citric acid (data not shown). As shown in Fig 3, all the 20Kd-protein in secretory fluid and nuclei from the dorsolateral and ventral prostates were immunologically identical to probasin. This experiment also indicates that probasin was fractionated in the cytosol of the ventral prostate, but not in the cytosol of the dorsolateral prostate, and that 21Kd-protein, which is detectable in the secretory fluid and nuclei of the dorso-

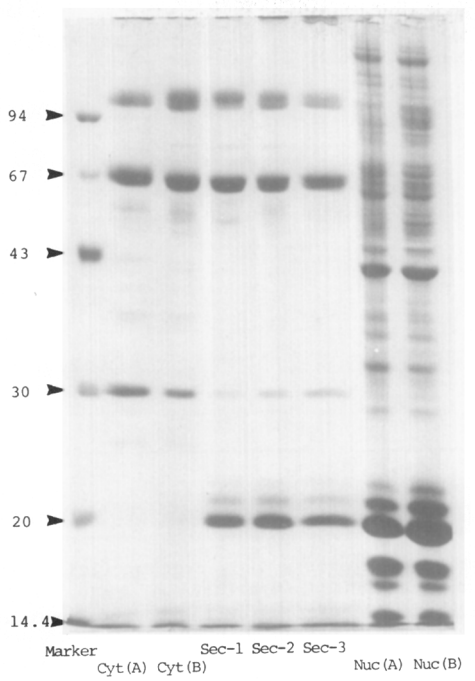

Fig. 2. SDS-PAGE patterns of proteins in secretory fluid, cytosols and nuclei from the dorsolateral prostate of rats. Cytosols (100 μg protein), secretory fluids (100 μg protein) and nuclei (50 μg DNA) were subjected to SDS-PAGE. (A): After extraction of secretory components (Sec-1, -2 and -3) according to the method A, cytosol (Cyt(A)) and nuclei (Nuc(A)) were prepared. Relative values of protein contents were about 50% for Sec-1, about 15% for Sec-2, about 11% for Sec-3, and about 24% for Cyt (A). (B): Cytosols (Cyt(B)) and nuclei (Nuc(B)) were prepared without pre-extraction of secretory component according to the method B. Standard proteins are shown in the left-hand; numbers refer to the molecular weight (x 10^{-3}).

lateral prostate (Fig. 3), has the same antigenicity as probasin. The content ratios of 20Kd-protein/21Kd-protein in nuclei were not influenced by the isolation method of nuclei. It was obvious, therefore, that probasin was distributed in the secretory fluid in addition to the nuclei. Together with the fact that probasin has an extremely basic pI (about 11.5), these results suggest that most of probasin was redistributed into nuclear fraction from secretory fluid in the lumen and secretory granule in the epithelial cells during fractionation of subcellular components.

DISCUSSION

A unique prostatic basic protein (mol.wt.≒20,000; pI≒11.5), "probasin", whose content was dependent on androgen level, was hardly detectable in cytosol, but abundant in isolated nuclei of the dorsolateral prostate of rats.

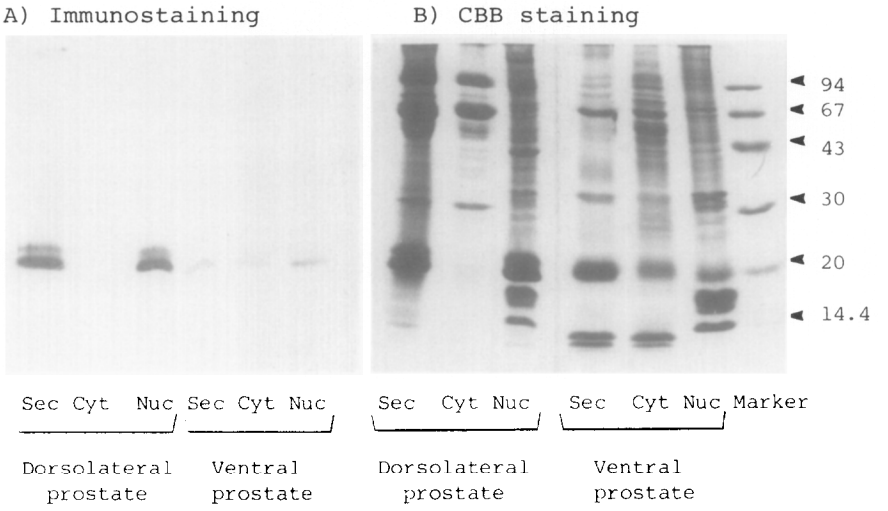

Fig. 3. Immunoblotting of probasin in secretory fluids, cytosols and nuclei from the dorsolateral and ventral prostates of rats. A) 1.25 μg proteins (Sec and Cyt) and 0.25 μg DNA (Nuc) from the dorsolateral prostate, and 10 μg proteins (Sec and Cyt) and 4 μg DNA (Nuc) from the ventral prostate were subjected to SDS-PAGE, followed by immunoblotting. Two bands seen in Sec and Nuc prepared from the dorsolateral prostate were 21Kd-protein (upper band) and 20Kd-protein (lower band), respectively. B) 25 μg proteins (Sec and Cyt) and 10 μg DNA (Nuc) from the dorsolateral and ventral prostates were subjected to SDS-PAGE, followed by staining with CBB.

Probasin in the nuclei was not solubilized with 1% Triton X-100 (2), but effectively solubilized with 0.35 M NaCl from the nuclei previously washed with 1% Triton X-100. We reported, therefore, that probasin was not a cytosol protein but a nuclear protein (1-3). However, immunofluorescent analysis by use of monoclonal antibody, which was raised against 20Kd-protein purified from nuclei of the dorsolateral prostate, revealed that probasin was localized in lumen and in acinal regions of the dorsolateral and ventral prostates, but hardly in the nuclei. Although the monoclonal antibody was also reactive to 21Kd-protein in addition to 20Kd-protein, the following reasons reveal that fluorescence of 20Kd-protein, but not 21Kd-protein, was mainly analyzed by immunofluoresecent experiments; 1) density ratios of immunostaining of 20Kd-protein/21Kd-protein are parallel to the content ratios, 2) the content ratios of 20Kd-protein/21Kd-protein were not changed by the isolation method of nuclei. Direct immunostaining experiments indicated that a protein with the same antigenicity as probasin in the nuclei was found in secretory fluid of the dorsolateral and ventral prostates. In addition, the probasin content in

the nuclei was markedly decreased either by pre-extraction with isotonic medium or by preparation of nuclei in the presence of citric acid. These results suggest that probasin was originally a secretory component in the prostates, being redistributed from the sectretory fluid and granule into nuclear fraction during fractionation of subcellular components under non-denaturing conditions. Although the presence of probasin in nuclei can not be excluded, it is difficult to discuss its amount in nuclei in vivo; the present study indicates that the probasin content in nuclei of the dorsolateral prostate is maximumly about 20% of the total. Recently, Venkatraman et al. (14) reported that an androgen-dependent nuclear protein (mol.wt≒19,000) in the rat ventral prostate was localized on the nuclear envelope. As pointed out by them, this protein appears to be identical to an androgen-dependent 21,000-daltons protein (21-kDa protein) in the ventral prostate reported by Kishimoto et al.(15). We also found that an androgen-dependent protein with a molecular weight of about 20,000 (20Kd-protein or 20K-NHP) was localized in nuclei of the rat prostates (1) and other male accessory sex organs (7); the relative contents per DNA were 100% for the dorsolateral prostate, 16% for the coagulating gland, 5.5% for the ventral prostate and 2% for the seminal vesicle. There are several lines of evidence to show that these nuclear proteins are identical to probasin; androgen dependency, molecular weight, localization in nuclei, and extractability from nuclei. We found that 20Kd-protein in nuclei of the ventral prostate had the same antigenicity as probasin in the dorsolateral prostate. It is clear, therefore, that most of probasin in the nuclei either from the ventral prostate or from the dorsolateral prostate is redistributed from secretory components in the prostates, and that probasin can be characterized as a major secretory component in the dorsolateral prostate and as a minor one in the ventral prostate and other male accessory sex organs. Adhesive nature of probasin to nuclei suggests high affinity to spermatozoa.

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