Immunochemical characterization of the androgen-dependent spermine-binding protein of the rat ventral prostate

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A solid-phase radioimmunoassay was developed to measure the level of the androgen-dependent spermine-binding protein (SBP) in the cytosol fraction of the rat ventral prostate during endocrine manipulation. The concentration of SBP and immunologically cross-reacting material (CRM) in the ventral prostate was at least 5000 times higher than the level of CRM detected in rat serum or cytosol from other rat tissues. Cytosol from the ventral prostate of intact rats was separated by DEAE-cellulose chromatography into three major fractions of CRM. One of these fractions corresponded to the elution position of SBP. Cytosol prepared from rats 48 h after castration lacked SBP and one of the two other fractions of CRM. This loss coincided with an increase in CRM in the remaining fraction. No significant difference was detected in the total level of CRM when intact and 48 h-castrated rats were compared. Injection of rats with 5 α -dihydrotestosterone (DHT) immediately after castration prevented these changes in the profile of CRM. Several proteins cross-reacting with antibodies to purified SBP were detected in cytosol by using an immunoblot procedure. The highest- M_r band corresponded to SBP. The effect of short- and long-term castration and subsequent DHT treatment on CRM was studied by using the immunoblot technique. Short-term castration (2 days) led to the disappearance of CRM coinciding with SBP (M_r 35 000–38 000) and an increase in smaller forms of CRM (M_r 24 000 and 22 000). Injection of rats with DHT 2 days after castration led to the reappearance of CRM corresponding to SBP, which returned to normal levels within 4 to 5 days of treatment. Long-term castration (up to 14 days) led to a gradual disappearance of all CRM; subsequent DHT treatment led to the reappearance of all forms of CRM and normal levels were attained within 5 days. We have identified SBP and the various forms of CRM as a secretory product of the rat ventral prostate by immunohistochemical staining and by DEAE-cellulose fractionation of prostatic fluid. Prostatic fluid is rich in proteolytic activity and these proteinases may be responsible for processing SBP to small forms of CRM.

SBP is an androgen-induced protein found in the rat ventral prostate (Mezzetti *et al.*, 1979). SBP binds various polyamines; however, among the natural polyamines, spermine has the highest

Abbreviations used: SDS, sodium dodecyl sulphate; SBP, spermine-binding protein; DHT, 5 α -dihydrotestosterone, 17 β -hydroxy-5 α -androstane-3-one; CRM, material cross-reacting with antibodies to purified spermine-binding protein; IgG, immunoglobulin G; poly(A)⁺, polyadenylated.

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affinity for SBP (Liang *et al.*, 1978). The function of SBP in the rat ventral prostate is unknown and the physiological importance of spermine-binding activity has not been determined, although the rat ventral prostate is rich in polyamines and in particular spermine (Pegg *et al.*, 1970).

Our previous work on SBP used a sucrose-gradient assay and [¹⁴C]spermine to measure the level of SBP in the rat ventral prostate. This method has many disadvantages; it lacks specificity, since other proteins and nucleic acids bind spermine, partial purification of samples is re-

quired for accurate measurement of activity, the assay is sensitive to salt concentration, and the assay procedure is complex and time-consuming. We have produced antibodies to SBP for use in a radioimmunoassay to measure the level of SBP. We have found that SBP is a secretory product of the rat ventral prostate. Several proteins, smaller than SBP and possibly derived from it, cross-react with antibodies to SBP and like SBP respond to castration and DHT treatment.

Experimental

Materials

Staphylococcus aureus protein A, bovine serum albumin, chloramine-T and Tris were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Na^{125}I , carrier-free, and L-[4,5- ^3H]leucine, 190 Ci/mmol, were from Amersham International, Arlington Heights, IL, U.S.A. Calf serum was obtained from GIBCO, Grand Island, NY, U.S.A. DEAE-Sephacel, concanavalin A-Sepharose and CNBr-activated Sepharose 4B were from Pharmacia, Piscataway, NJ, U.S.A. Nitrocellulose (0.45 μm pore size) was from Millipore, Bedford, MA, U.S.A. Flexible, 96-well, poly(vinyl chloride) microtitre plates were from Dynatech, Alexandria, VA, U.S.A. Rabbit reticulocyte lysate treated with micrococcal nuclease was obtained from Bethesda Research Laboratories, Gaithersburg, MD, U.S.A. Oligo(dT)-cellulose was from P-L Biochemicals, Milwaukee, WI, U.S.A. Goat anti-rabbit IgG was from Miles Laboratories, Elkhart, IN, U.S.A. Hydroxyapatite, Bio-Gel HT, was obtained from Bio-Rad Laboratories, Richmond, CA, U.S.A. Other chemicals were of reagent grade or of the highest purity available.

Treatment of animals

Male rats (200–300 g) of the Sprague-Dawley strain (King Animals Laboratories, Oregon, WI, U.S.A.) were used. Rats were fed *ad libitum* a commercial pellet diet (Teklad, 4% fat; Winfield, IA, U.S.A.). Rats were castrated by the scrotal route while under diethyl ether anaesthesia. When indicated, rats were given daily subcutaneous injections containing 5 mg of DHT in 1 ml of sesame oil. DHT was dissolved in 0.1 vol. of ethanol before mixing with sesame oil. Controls received an oil and ethanol mixture only.

Preparation of cytosol

Rats were killed by cervical dislocation and the ventral prostates were dissected out free of their capsules. The following steps were performed at 0–5°C. Tissue was minced, homogenized in an all-glass Potter-Elvehjem homogenizer in 3 vol. of 1.5 mM-EDTA/20 mM-Tris/HCl, pH 7.5 (Buffer

ET), and centrifuged at 100 000 g for 1 h. The supernatant was used as 'cytosol'.

Fractionation of cytosol on DEAE-cellulose

Rat ventral prostate cytosol (4 ml, 72–75 mg of protein) was applied to a DEAE-cellulose column (0.9 cm $\times$ 10 cm) that had been equilibrated with Buffer ET. The column was washed with 10 ml of Buffer ET and then eluted with a 200 ml linear gradient of 0–0.45 M-NaCl in Buffer ET. Fractions (3 ml) were collected.

Purification of SBP and immunologically related proteins

SBP was purified by a modification of our previously published procedure (Mezzetti *et al.*, 1979). Preliminary studies had indicated a high level of SBP and other material cross-reacting with antibodies to purified SBP (CRM) in prostate fluid. A purification procedure was adopted which removed 80% of SBP and CRM from the rat ventral prostate. This procedure did not involve homogenization of tissue and therefore minimized contamination by intracellular proteins. Rats were killed by cervical dislocation and the ventral prostates were dissected out free of their capsules. The following steps were performed at 0–5°C. Tissue (30 g) was minced and suspended in 2 vol. of 320 mM-sucrose / 1.5 mM-EDTA / 20 mM-Tris / HCl, pH 7.5, and mixed continuously for 15 min. The tissue suspension was centrifuged at 1500 g for 10 min and the supernatant removed and saved. The tissue was washed again as above and the supernatants from the washes pooled. The pooled washes were centrifuged at 100 000 g for 1 h and the supernatant saved for isolation of SBP and CRM.

The supernatant was applied to a DEAE-cellulose column (2.6 cm $\times$ 20 cm) equilibrated with Buffer ET. The column was washed with 100 ml of Buffer ET and then eluted with a linear 1000 ml gradient of 0–0.45 M-NaCl in Buffer ET. Peak fractions containing SBP or other CRM, as indicated by radioimmunoassay or [^{14}C]spermine-binding activity (Mezzetti *et al.*, 1979), were pooled separately and dialysed against Buffer ET. Three major peaks of CRM were detected by radioimmunoassay. See Fig. 2(a) below for a typical elution profile, where fractions 24–35 contained Peak 1 of CRM, fractions 36–48 contained Peak 2 of CRM and fractions 49–60 contained Peak 3 of CRM. Peak 3 of CRM contained SBP. A peak of CRM was occasionally detected in the wash-through fractions of the column (see Fig. 2b; fractions 1–12), but the amount was variable.

CRM of Peak 3, containing SBP, was applied to another DEAE-cellulose column (0.9 cm $\times$ 10 cm) equilibrated with Buffer ET. The column was

washed with 10 ml of Buffer ET containing 0.1 M-NaCl and then eluted with a linear 400 ml gradient of 0.1–0.4 M-NaCl in Buffer ET. The last peak of protein to be eluted from the column contained SBP as determined by [^{14}C]spermine-binding activity. Fractions containing SBP that were more than 90% pure, as determined by SDS/polyacrylamide-gel electrophoresis, were pooled and used for further studies.

Peaks 1 and 2 of CRM were applied to separate DEAE-Sephacel columns (2.6 cm $\times$ 10 cm) equilibrated with Buffer ET. The columns were eluted with linear 400 ml gradients of 0.1–0.35 M-NaCl in Buffer ET. Fractions containing CRM were identified by radioimmunoassay, pooled, and dialysed against 0.01 M- KH_2PO_4 , pH 7.5. The dialysed fractions were placed on separate hydroxyapatite columns (1.6 cm $\times$ 10 cm) equilibrated with 0.01 M- KH_2PO_4 , pH 7.5. The columns were eluted with linear 500 ml gradients of 0.01–0.3 M- KH_2PO_4 , pH 7.5. CRM was identified by radioimmunoassay and peak fractions were pooled, dialysed against water, and freeze-dried.

Immunochemical procedures

Antisera to purified SBP were produced in female New Zealand White rabbits by the procedure of Vaitukaitis *et al.* (1971). An antibody fraction was partially purified from sera by precipitation with $(\text{NH}_4)_2\text{SO}_4$ (Livingston, 1974). Affinity-purified antibodies to SBP were prepared by using purified CRM of Peak 2 (see above and Fig. 4b below) coupled to CNBr-activated Sepharose 4B by the procedure described by Ostrowski *et al.* (1979). Purified SBP and *S. aureus* protein A were iodinated with Na^{125}I by using chloramine-T (Greenwood *et al.*, 1963). Specific radioactivities were in the range of $(2\text{--}4) \times 10^7$ c.p.m./ μg of protein.

Immunochemical detection of proteins cross-reacting with antibody to SBP after SDS/polyacrylamide-gel electrophoresis (Laemmli, 1970) on 12.5% (w/v) acrylamide/0.625% (w/v) NN' -methylenebisacrylamide slab gels and transfer to nitrocellulose ('immunoblot') was performed with ^{125}I -labelled *S. aureus* protein A as described by Howe & Hershey (1981).

A solid-phase immunoassay for SBP was developed which was similar to the procedure of Mariotti *et al.* (1980) for IgG. The final assay mixture contained 30 000 c.p.m. of ^{125}I -SBP, 1% (w/v) bovine serum albumin, 5% (v/v) calf serum, 50 mM-Tris/HCl, pH 7.5, 0.3 M-NaCl, 3 mM- NaN_3 , and 0.45–30 ng of SBP (standards or samples) in a volume of 150 μl .

Two-dimensional immunodiffusion was performed as described by Ouchterlony & Nilsson (1978).

RNA translation and immunoprecipitation

Total RNA was isolated from the rat ventral prostate with guanidium thiocyanate (Chirgwin *et al.*, 1979) and centrifugation through CsCl (Chirgwin *et al.*, 1979; Glisin *et al.*, 1974). Poly(A) $^+$ mRNA was isolated by using oligo(dT)-cellulose (Aviv & Leder, 1972) and translated *in vitro* by using a message-dependent rabbit reticulocyte lysate (Pelham & Jackson, 1976). Immunoprecipitations were carried out as described by Ostrowski *et al.* (1979) by using affinity-purified antibodies to SBP. Labelled translation products were detected after SDS/polyacrylamide-gel electrophoresis by fluorography (Bonner & Laskey, 1974).

Other methods

Protein concentrations were determined by the dye-binding assay of Bradford (1976), with bovine serum albumin as standard. Cleavage of SBP with CNBr in 70% (v/v) formic acid was as described by Steers *et al.* (1965). Amino acid analysis was performed as described by Chen *et al.* (1982). Chemical deglycosylation of SBP with trifluoromethanesulphonic acid was carried out as described by Edge *et al.* (1981). SBP was analysed for the presence of carbohydrates by using the periodate/Schiff reagent (Segrest & Jackson, 1972), the phenol/ H_2SO_4 acid assay for sugars (Ashwell, 1966), g.l.c. of trifluoroacetate derivatives after methanolysis (Zanetta *et al.*, 1972) and the thiobarbituric assay for sialic acid (Warren, 1963).

Results

Purification of SBP by the present method yielded a protein preparation which was a diffuse band when analysed by SDS/polyacrylamide-gel electrophoresis with an M_r range of 35 000–38 000 (Fig. 1, lane 2). Two major bands were discernible in many preparations. SBP is a glycoprotein; it was stained by the periodate/Schiff reagent for carbohydrates; there was 15% carbohydrate present on SBP as measured by the phenol/ H_2SO_4 assay for sugars; fucose, galactose, mannose, and N -acetylglucosamine were present on SBP as determined by g.l.c. No sialic acid was detected on SBP by g.l.c. or by the thiobarbituric assay for sialic acid. The heterogeneity of our preparation, therefore, could be the result of variable carbohydrate composition. Deglycosylation of purified SBP using trifluoromethanesulphonic acid and anisole increased the mobility and decreased the apparent heterogeneity of purified SBP on SDS/polyacrylamide-gel electrophoresis (Fig. 1a, lane 3). Consistent with the presence of a single protein was the observation that CNBr cleavage of SBP yielded

Fig. 1. Analysis of purified SBP and SBP immunoprecipitated from labelled 'in-vitro' translation products of rat ventral prostate poly(A)⁺ mRNA by SDS/polyacrylamide-gel electrophoresis

(a) Analysis of purified SBP by SDS/polyacrylamide-gel electrophoresis: 1, M_r standards (top to bottom): rabbit muscle phosphorylase *b*, bovine serum albumin, egg-white ovalbumin, bovine erythrocyte carbonic anhydrase, soya-bean trypsin inhibitor and bovine α -lactalbumin; 2, 50 μ g of purified SBP; 3, 50 μ g of deglycosylated SBP; 4, 50 μ g of purified SBP cleaved with CNBr. Proteins were detected by Coomassie Brilliant Blue staining. (b) Analysis of [³H]leucine-labelled 'in-vitro' translation products of rat ventral-prostate poly(A)⁺ mRNA. Total labelled translation products (1) and labelled translation products immunoprecipitated with affinity-purified antibodies to SBP (2) were analysed by SDS/polyacrylamide-gel electrophoresis and fluorography.

only two peptides, which gave sharp bands on SDS/polyacrylamide-gel electrophoresis (Fig. 1a, lane 4). Only one methionine residue was detected by amino acid analysis of SBP, an M_r of 35 000 for SBP being assumed.

Antibodies to purified SBP were produced in rabbits. An immunoglobulin fraction, isolated by (NH₄)₂SO₄ precipitation of whole sera and an affinity-purified antibody, prepared by using a purified form of CRM attached to Sepharose (Peak 2 of CRM; see the Experimental section), were used in many of the experimental procedures (radioimmunoassay and immunoblot) with similar

Fig. 2. Elution profile of cytosol from intact and castrated rats on DEAE-cellulose

(a) Rat ventral-prostate cytosol (4 ml, 72 mg of protein) prepared from four intact rats was fractionated on DEAE-cellulose. Fractions (3 ml) were collected. The amounts of CRM as determined by radioimmunoassay (▲) and A_{280} (●) are given for each fraction. (b) As (a), except ventral-prostate cytosol (4 ml, 80 mg of protein) was prepared from rats 48 h after castration.

results. Antibody specificity was analysed in view of the apparent heterogeneity of the purified SBP. A single radioactive protein (M_r 34 000) was detected by immunoprecipitation of labelled products after translation *in vitro* of rat ventral prostate poly(A)⁺ mRNA (Fig. 1b, lane 2). Addition of unlabelled purified SBP to the translation mixture prevented immunoprecipitation of this protein. When control (preimmune) IgG was used for the immunoprecipitation, this protein was not precipitated. The antibody preparation was specific when tested by immunoelectrophoresis (multiple, fused precipitin lines) or double diffusion (single precipitin line, see below).

These antibodies were used to develop a solid-phase radioimmunoassay for SBP in which CRM was measured by using a standard curve with a range of 2–200 ng of SBP/ml. Radioimmunoassay of serial dilutions of rat ventral, prostate cytosol resulted in a displacement curve which was

Fig. 3. Kinetics of the effect of castration and DHT treatment on the level of CRM

Rat ventral-prostate cytosol was prepared (five animals per group) from (a) intact rats, from rats 12, 24, 30, 36, 42 and 48 h after castration, and (b) from rats which 48 h after castration were given DHT (5 mg/day) for 1, 2, and 5 days. Cytosol (4 ml, 75 mg of protein) was fractionated on DEAE-cellulose and the amount of CRM in each fraction was determined by radioimmunoassay. The amount of cross-reacting material in fractions 24–35 (Peak 1) (▲), 36–48 (Peak 2) (■) and 49–60 (Peak 3) (●), representing the three major peaks of CRM, was summed for each column. The amount of CRM in each of these peaks is expressed as a percentage of the total CRM in fractions 11–60.

parallel to the radioimmunoassay standard curve, indicating immunological similarity between the standard (purified SBP) and CRM in rat ventral-prostate cytosol. Rat serum and cytosol prepared from the rat dorsal/lateral prostate, coagulating gland (anterior prostate), seminal vesicle, epididymis, testis, brain, heart, lung, kidney, and spleen were analysed by using the radioimmunoassay for SBP. All of these tissues had CRM equivalent to less than 10 ng of SBP/mg of protein; whereas cytosol from the rat ventral prostate had CRM equivalent to 50 μ g of SBP/mg of protein.

Previous studies had indicated that the level of SBP in the rat ventral prostate decreased upon castration (Mezzetti *et al.*, 1979). We measured the level of CRM in rat ventral-prostate cytosol from intact rats and from rats 2 days after castration, by using the radioimmunoassay for SBP. The level of CRM in intact rats was equivalent to 52.7 ± 2.7 (mean $\pm$ S.E.M., $n =$ six animals) μ g of SBP/mg of protein. The total level of CRM did not change 2 days after castration ($67.9 \pm 8.2 \mu$ g of SBP/mg of protein; Student's t test, $P > 0.1$). These results differ significantly from previously published

results (Mezzetti *et al.*, 1979), where the level of [^{14} C]spermine-binding activity of a partially purified fraction of rat ventral-prostate cytosol decreased by 50% 2 days after castration.

The high levels of CRM and the absence of an early effect of castration on the level of CRM could be due to the presence of immunologically similar proteins in rat ventral-prostate cytosol. Therefore we fractionated, by DEAE-cellulose chromatography, ventral-prostate cytosol from intact rats and from rats castrated 48 h previously and we determined the amount of CRM in each fraction by radioimmunoassay. Three major peaks of CRM were found when cytosol from intact rats was fractionated on DEAE-cellulose (Fig. 2a). Peaks of CRM were eluted in fractions 24–35 (Peak 1), 36–48 (Peak 2) and 49–60 (Peak 3). Other fractions contained lower, but readily detectable, levels of CRM. A similar profile (three major peaks of CRM) was observed when cytosol was prepared from individual rats. We tested the possibility that these three peaks of CRM were artefacts generated by the degradation of SBP during the preparation of cytosol by adding ^{125} I-SBP to tissue before homogenization. We recovered more than 95% of the radioactivity in the cytosol fraction and more than 70% of this radioactivity was eluted in fractions containing SBP (Peak 3). The remaining 30% of the radioactivity was not localized in either of the two other peaks of CRM but was distributed among all other fractions.

We compared the three major peaks of CRM for their ability to bind [^{14} C]spermine by using a sucrose-gradient assay (Mezzetti *et al.*, 1979). Only CRM in Peak 3 had spermine-binding activity. However, when CRM in Peak 2 was partially purified, a low level (10–20% of Peak 3) of spermine-binding activity was detected. No spermine-binding activity was detected in partially purified CRM of Peak 1. All three major peaks of CRM bound to concanavalin A-Sepharose and were eluted with α -methyl mannoside; therefore all appeared to be glycoproteins. Measurements of CRM by radioimmunoassay on serial dilutions of the three major peaks of CRM resulted in displacement curves parallel with the radioimmunoassay standard curve, indicating immunological similarity between the standard (purified SBP) and the three major fractions of CRM. More than 90% of the ^{125} I-SBP in the radioimmunoassay could be displaced by the CRM in each of these peaks.

A single major peak of CRM was found in fractions 24–35 (Peak 1) when cytosol from 48-h-castrated rats was separated on DEAE-cellulose (Fig. 2b). A small but sharp peak of CRM was also detected in the wash-through fractions of the column. This material was similar in size to CRM

Fig. 4. Identification of CRM by SDS/polyacrylamide-gel electrophoresis and immunoblot analysis

(a) Immunoblot analysis of cytosol and CRM separated by DEAE-cellulose chromatography. Rat ventral-prostate cytosol (100 µg of protein) from intact (1) and 48 h-castrated (2) rats, 10 µg of Peak 3 CRM (3), 50 µg of Peak 2 CRM (4), and 50 µg of Peak 1 CRM (5) from Fig. 2(a) were subjected to SDS/polyacrylamide-gel electrophoresis. Proteins were electrophoretically transferred to nitrocellulose, incubated with affinity-purified antibodies to SBP, washed, incubated with ^{125}I -labelled *S. aureus* protein A, washed, dried and exposed to Kodak XAR film for 2 h at room temperature. (b) Analysis of partially purified Peak 1 CRM (1) and Peak 2 CRM (2) by SDS/polyacrylamide-gel electrophoresis. Proteins were detected by Coomassie Brilliant Blue staining.

of Peak 1 as determined by SDS/polyacrylamide-gel electrophoresis and immunochemical detection (immunoblot). Serial dilutions of this material also resulted in displacement curves parallel with the radioimmunoassay standard curve. The CRM in Peak 2 and Peak 3 (SBP) was decreased compared with the profile from intact rats. Recovery of ^{125}I -SBP, added to tissue from castrated rats before homogenization, was similar to the results from intact rats (see above). Injection of rats with DHT immediately after castration and daily thereafter prevented any change in the profile of CRM (i.e. the profile of CRM was typical of that of an intact rat).

The average recovery of CRM from these two columns was equivalent to 3.7 mg of SBP, whereas the amount of CRM in the cytosol applied to the column was equivalent to 3.03 mg of SBP or a recovery of 122%.

In Fig. 3 we present a kinetic analysis of the effect of castration and subsequent DHT treatment on the relative levels of CRM in the three major peaks. Castration caused a decrease in CRM in Peak 2 and Peak 3 which began 24 h after castration. CRM in Peak 3 (SBP) decreased to a minimum (15% of intact value) by 48 h after castration. CRM in Peak 2 decreased by 50% in this same interval. A 50% increase in the level of CRM in Peak 1 was observed 48 h after castration. If rats were given DHT 48 h after castration, there was an increase in CRM in Peak 2 and Peak 3 and a decrease in CRM in Peak 1, such that after 5 days of treatment the relative levels of CRM were near normal values.

The three major peaks of CRM were compared by two-dimensional immunodiffusion. All three peaks of CRM and cytosol gave single fused precipitin arcs; therefore all three peaks of CRM are immunologically very similar.

We also analysed the three major peaks of CRM, as well as cytosol from intact and 48 h-castrated rats by using an immunoblot procedure (Fig. 4a). Proteins were separated by SDS/polyacrylamide-gel electrophoresis, transferred to nitrocellulose, exposed to antibodies to SBP and then exposed to ^{125}I -protein A, which bound to antibodies attached to various cross-reacting proteins. The immunoblot technique detected several cross-reacting proteins in cytosol from intact rats (Fig. 4a, lane 1). Cytosol from castrated rats (Fig. 4a, lane 2) lacked a major diffuse band (M_r 35000–38000) when compared with cytosol from intact rats. This diffuse band corresponded to CRM from Peak 3 of Fig. 2(a), which contained SBP (Fig. 4a, lane 3). After castration, there was also an increase in two lower- M_r bands (24000 and 20000), which corresponded to CRM from Peak 1 of Fig. 2b (Fig. 4a, lane 5). Although the amount of CRM in Peak 2 of Fig. 2(a) decreased upon castration, a decrease in protein bands (M_r 27000, 25000, 23000 and 21000) corresponding to this fraction (Fig. 4a, lane 4) was difficult to assess with the immunoblot technique owing to the overlapping mobilities of CRM in Peaks 1 and 2.

The proteins detected with the immunoblot technique were specific for antibodies to SBP. No CRM was detected when control (pre-immune) IgG was used or when affinity-purified antibodies to SBP were incubated with purified SBP before use in the immunoblot.

CRM in Peaks 1 and 2 were partially purified (see the Experimental section) and the proteins in

these fractions analysed by SDS/polyacrylamide-gel electrophoresis (Fig. 4b). The material purified from Peak 1 (Fig. 4b, lane 1) contained five major bands. Two of these bands (M_r 24000 and 20000) had mobilities corresponding with proteins detected by the immunoblot method. Proteins of higher mobility appear to be contaminating components of the highly abundant secretory protein, α -protein (Chen *et al.*, 1979). The material purified from Peak 2 (Fig. 4b, lane 2) contained four major bands (M_r 27000, 25000, 23000 and 21000), whose mobilities also corresponded with proteins detected by the immunoblot method.

The effect of short- and long-term castration and subsequent DHT treatment on CRM was studied by using the immunoblot technique (Fig. 5). Short-term castration (2 days) led to the disappearance of the CRM coinciding with SBP (M_r 35000–38000) and an increase in smaller forms of CRM. Injection of rats with DHT, 2 days after castration, led to the reappearance of the CRM corresponding to SBP, which returned to normal levels after 4–5 days of treatment. Long-term castration (up to 14

days) led to a gradual disappearance of all CRM; subsequent DHT treatment led to the reappearance of all forms of CRM and normal levels were attained within 5 days.

The large amount (1–5% of cytosol protein) of CRM present in cytosol from rat ventral prostate prompted us to check whether these proteins were part of prostate fluid or an intracellular component. Prostate fluid and cytosol prepared from prostate tissue depleted of fluid were fractionated on DEAE-cellulose and fractions were analysed by using the radioimmunoassay for SBP. Most CRM (84%) was present in the prostate-fluid fraction and the elution profile was similar to the profile in Fig. 2(a). Cytosol from fluid-depleted tissue contained relatively little CRM (16%) and what was present probably represents residual fluid not removed by the washing step. Immunohistochemical detection (Petrusz *et al.*, 1975) of CRM in fixed, embedded and sectioned ventral-prostate tissue localized CRM in the prostate lumen where prostate fluid is stored. This staining was specific for antibodies to SBP.

Fig. 5. Effect of short- and long-term castration and subsequent DHT treatment on CRM detected by immunoblot analysis. Rat ventral-prostate cytosol was prepared from rats after castration for the indicated number of days (C) and then given DHT for the indicated number of days (D). Equal amounts (50 μ g) of protein were applied to each lane and separated by SDS/polyacrylamide-gel electrophoresis. Immunoblot analysis was performed as described in Fig. 4.

Discussion

In the present study we have used antibodies raised against purified SBP to identify several proteins immunologically similar to SBP. All of these proteins are smaller than SBP when analysed by SDS/polyacrylamide-gel electrophoresis. These proteins, and changes in their relative proportions upon castration, were not due to artefacts, such as proteolysis of SBP during the preparation of cytosol. Therefore what may be occurring is the conversion *in vivo* of SBP and other immunologically similar proteins to smaller proteins (which are resolved by chromatography on DEAE-cellulose or by SDS/polyacrylamide-gel electrophoresis) by some processing step, such as proteolysis. How these proteins are related will be clearer when the sequences of these proteins have been determined or when peptide maps of purified material have been analysed. Preliminary studies comparing limiting trypsin cleavage products of SBP with CRM have indicated similarities when compared by SDS/polyacrylamide-gel electrophoresis.

We have found that prostate fluid contains high levels (1–5% of fluid protein) of an androgen-induced serine proteinase and it will cleave SBP. However, the cleavage products were smaller than any of the CRM components found in this study. Proteolysis of SBP was inhibited *in vitro* by spermine, Ca^{2+} and a basic secretory glycoprotein (M_r 20000), all of which are found in prostate fluid (Pegg *et al.*, 1970; Mann, 1964; Chamberlin *et al.*, 1983). Spermine, Ca^{2+} and the basic secretory protein did not affect hydrolysis of synthetic proteinase substrates such as tosylarginine methyl ester; therefore we think that these substances act by forming a complex with SBP, thus altering potential proteolytic-cleavage sites. The proper combination and concentration of these or other fluid components may allow selective cleavage of SBP to the CRM identified in the present study.

The physiological role of SBP and CRM in the rat ventral prostate is unknown. Its identification as a component of prostate fluid may indicate a role in the physiology of rat semen. The rat ventral prostate may be analogous to the mouse submaxillary gland, where an androgen-induced proteinase cleaves other androgen-induced proteins (Barka, 1980).

In an earlier study on SBP (Mezzetti *et al.*, 1979) it was reported that induction of spermine-binding activity in castrated rats was detectable 30 min after injection of DHT and activity returned to control levels after 2 h of treatment. Also the level of this binding activity decreased to only 50% of control levels 40 h after castration. In the present study an increase in the level of SBP after

castration, as measured by radioimmunoassay, was not detected until 2 days after treatment with DHT and control levels were reached only after 5 days of treatment. SBP decreased to 15% of control levels 48 h after castration.

At present we are unable to explain the earlier observations in relation to those of the present study. In the earlier studies a [^{14}C]spermine-binding assay involving sucrose-gradient centrifugation was used. This assay method required 5–10 μg of purified protein or 50–100 μg of partially purified material. The radioimmunoassay used in the present study easily measures 5–10 ng of SBP, a 1000-fold higher sensitivity. The sucrose-gradient assay is sensitive to salt concentration, cations and interference by other spermine-binding proteins and nucleic acids, whereas the radioimmunoassay is generally insensitive to these factors. It is possible (but has not been proved) that spermine-binding activity may be dependent on minor structural alterations of SBP. Such a process may involve, for example, phosphorylation of SBP (Liang *et al.*, 1978). We have found that SBP can be phosphorylated by an androgen-sensitive cyclic AMP-independent protein kinase of the rat ventral prostate (Ahmed *et al.*, 1978), but not by cyclic AMP-dependent (Reddi *et al.*, 1971) or phospholipid-sensitive (Qi *et al.*, 1983) protein kinases (K. Ahmed, J. F. Kuo & S. Liao, unpublished work).

From the results of the present study we believe that the decrease in SBP on castration is due to at least two separate processes. On castration, synthesis of SBP stops and SBP present in prostate fluid is converted into smaller forms by a proteinase in prostate fluid. These smaller forms do not bind spermine as well as SBP, but are immunologically detectable with antibodies raised against SBP. The decrease in SBP upon castration is relatively rapid for a secretory protein (15% remains 2 days after castration). The levels of other androgen-dependent secretory proteins in the rat ventral prostate, such as prostatic binding protein (Heyns *et al.*, 1978) and the serine proteinase discussed above (R. A. Hiipakka & S. Liao, unpublished work) do not change significantly 2 days after castration. Selective proteolytic processing of SBP may be responsible for this difference. The effect of castration and DHT treatment on the level of SBP and CRM may reflect earlier changes in the synthesis of SBP due to alterations in the mRNA levels for this protein. These possibilities require investigation. SBP should be a good marker for studying the molecular mechanisms regulating androgen-dependent processes in the rat ventral prostate.

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