Sex Steroid-Binding Protein in a Subline of Dunning R 3327 Prostatic Adenocarcinoma of the Rat

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Abstract

A transplantable tumor CUB-II, a subline derived from the Dunning R 3327 rat prostatic adenocarcinoma, contains a unique sex steroid-binding protein. The protein possesses binding sites for androgens as well as for estrogens, and the binding affinity to androgen is higher than that to estrogen. The sedimentation coefficient of the protein is 10S. Sodium thiocyanate inhibits the binding to both sex steroids. This type of binding is not present in the 0.4 M KCl extract of nuclei. These results suggest that the binding protein is not the receptor for steroid hormones in spite of its high affinity binding to androgens and estrogens. Since the original tumor does not contain such protein, production of this binding protein seems to take place during culture in vitro and/or serial transplantations of the tumor.

The Dunning R 3327 prostatic adenocarcinoma originated in the dorsolateral lobe of prostate of a Copenhagen rat (Dunning, 1963). The tumor is transplantable to Copenhagen rats or F1 hybrids of Copenhagen and Fischer rat, and shows androgen-dependent growth. An histological feature of the Dunning R 3327 tumor is a well differentiated adenocarcinoma (Smolev et al., 1977, Isaacs et al., 1978).

Since 1980, we have been maintaining the Dunning R 3327 tumor in the laboratory, and two sublines, CUA and CUB, were obtained (Minagawa et al., 1983). The CUB is characterized as a fast growing, androgen-independent tumor composed of spindle-shaped and large bizarre polygonal cells. The CUB has higher acid phosphatase activity than the original tumor. The cell line of this tumor was established, then retransplanted to Copenhagen rats and has been maintained by serial implantations (Igarashi et al., 1984). The transplanted tumor has similar histological features to those of the CUB and is designated as CUB-II.

It was reported that the Dunning R 3327 tumor contained androgen and estrogen receptors (Markland and Lee, 1979, Lea and French, 1981). In cytosols of the CUB there was observed no binding to androgens but binding to estrogens was noticed. However, in cytosols from the CUB-II, an unusual binding to androgens as well as to estrogens was observed. Therefore the present work was undertaken to clarify the
properties of the component which is responsible for this unusual binding.

Materials and Methods

Animals

Copenhagen rats were kindly donated by the National Cancer Institute (U.S.A.) and maintained by inbreeding. Male rats of this strain, 8–10 weeks old, weighing approximately 250 g, were used as recipients of the tumor. For the binding study on cytosols, animals were castrated 24 hr before sacrifice.

Tumor

The Dunning R 3327 tumor was donated by E. G. & G. Mason Research Institute (Worcester, Mass, U.S.A.). During successive transplantations of the original tumor, a subline, designated as CUB, was established. The 8th generation of the CUB was transferred to a cell culture, and Copenhagen rats were inoculated with the cultured cells of the 8th passage and tumors obtained were referred to as the CUB-II. The growth of the CUB-II was influenced neither by the sex of the recipient animals nor by any hormonal manipulations. The CUB-II was maintained by serial transplantations to Copenhagen rats, and tumors from the 11th to 25th generation were used in the present study. The doubling time of CUB-II was 3.0±0.3 days (M±S.E. in 32 tumors). In experiments on the CUB, tumors between from the 23rd to 35th generation were used.

Tissue preparations

Animals were sacrificed by decapitation, removed tumors were placed on ice, and necrotic tissues were separated. All procedures were carried out at 0–4°C. The tumor tissues were minced with scissors, homogenized in 5 volumes of TEDM buffer (0.01 M Tris-HCl, pH 7.4, 1 mM EDTA, 0.5 mM dithiothreitol, 10 mM molybdate) using a glass homogenizer and filtered through nylon cloth. Cytosols were obtained after centrifuging the homogenate at 105,000 x g for 60 min.

To prepare the nuclear extract, the tumor tissues from non-castrated rats were homogenized in 10 volumes of TSMK buffer (0.01 M Tris-HCl, pH 7.4, 0.25 M sucrose, 3 mM MgCl₂, 25 mM KCl) and spun at 700 x g for 10 min. The precipitate was washed with TSMK buffer, then the nuclear pellet was suspended in 0.05 M Tris-HCl buffer (pH 7.4) containing 0.4 M KCl, stirred at 4°C for 30 min. After centrifugation at 8,000 x g for 20 min, the resultant supernatant was saved as the nuclear extract.

Saturation analysis of the binding

Cytosols were incubated at 4°C in 0.5 ml of TEDM buffer containing various concentrations (0.25–5 nM) of ³H-R 1881 ((17α-methyl-³H) 17β-hydroxy-17α-methyl-estra-4, 9, 11-trien-3-one, specific activity 87 Ci/mmol, New England Nuclear, Boston, U.S.A., for androgen binding) for 5 hr or ³H-estradiol ((2, 4, 6, 7-³H(N)) estradiol-17β, specific activity 93 Ci/mmol, New England Nuclear, Boston, U.S.A., for estrogen binding) for 4 hr. The ³H-ligands were dissolved in 0.01 ml of ethanol and added to the incubation tube. After incubation, 0.05% dextran-coated 0.5% charcoal was added, centrifuged, and the radioactivity in an aliquot of the resultant supernatant was counted with 15 ml of toluene containing 0.4% PPO and 0.01% POPOP using a liquid scintillation counter with automatic external standardization for quenching correction (LKB 1215 Rackbeta, Wallac OY, Finland). The counting efficiency was approximately 50% for ³H. In a parallel incubation, a 1000-fold molar excess of unlabeled ligand was added to the incubation tube and nonspecific binding which was not displaced under these conditions was subtracted in all instances from the total binding to calculate specific binding. The binding capacity and affinity were analyzed by the method of Scatchard (1949).

When the effect of additives on the binding was examined, R 5020 (17, 21-dimethyl-19-norpregna-4, 9-dien-3, 20-dione, New England Nuclear, Boston, U.S.A.) or other additives were dissolved in another 0.01 ml of ethanol and added to the incubation tube.

In the saturation analysis of the nuclear extract, ionic strength at incubation with the ligand and at separation of the bound ligand from unbound using the dextran-coated charcoal was adjusted with KCl to make the final concentration of 0.4 and 0.2 M, respectively.

Sucrose density gradient centrifugation

An aliquot of the cytosol was incubated with either 5 nM of ³H-R 1881 or the same concen-
concentration of $^3$H-estradiol under the conditions described in the former section. After incubation and the removal of unbound ligand with the dextran-coated charcoal, 0.3 ml of the resultant supernatant was applied on the top of a 5–20% sucrose gradient in the TEDM buffer (5 ml) containing 0.5 mM leupeptin and 10% glycerol. Centrifugation was performed with a RPS50-2 rotor in a Hitachi Ultracentrifuge (80P, Hitachi Co., Tokyo, Japan) at 216,700 $\times$ g for 16 hr. The gradient was fractionated into 0.2 ml fractions and the radioactivity in each fraction was counted. In a parallel incubation, a 1000-fold molar excess of the respective unlabeled ligand was added to observe the nonspecific binding. Bovine serum albumin (4.6S), human immunoglobulin G (7.0S) and catalase (11.3S) were used as references for calculation of the sedimentation coefficient.

**Protein determination**

Protein was measured by the biuret method (Gornall et al., 1949) with bovine serum albumin as the calibration standard.

**Results**

**Binding to $^3$H-R 1881 and $^3$H-estradiol of the CUB-II cytosol**

To examine the effect of incubation time on the binding, the cytosol was incubated with $^3$H-R 1881 or $^3$H-estradiol and the rate of binding after various time intervals was estimated (Fig. 1). The binding almost reached a plateau after 4 hr in $^3$H-R 1881 and after 3 hr in $^3$H-estradiol. Therefore, subsequent incubations were performed 5 hr and 4 hr in $^3$H-R 1881 and in $^3$H-estradiol, respectively.

**Table 1. Binding of cytosols from CUB-II to $^3$H-R 1881 and $^3$H-estradiol.**

<table>
<thead>
<tr>
<th>No. of cases</th>
<th>Kd $\times 10^{-9}$M</th>
<th>Maximum binding sites fmol/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^3$H-R 1881</td>
<td>10 $0.7\pm0.4$</td>
<td>35±5</td>
</tr>
<tr>
<td>$^3$H-estradiol</td>
<td>12$^a$ $3.4\pm0.3$</td>
<td>10±3</td>
</tr>
</tbody>
</table>

$^a$: Seventeen determinations were performed independently using different tumors and in five cases the specific binding was not detected. Data are shown as M±S.E.

Cytosols were incubated with various concentrations of $^3$H-R 1881 or $^3$H-estradiol in the presence or absence of 1000-fold molar excess of the respective radioinert ligand. Binding parameters were calculated by the method of Scatchard (1949).

![Fig. 1. Time course of the binding of CUB-II cytosol to $^3$H-R 1881 and $^3$H-estradiol.](image-url)

Cytosols (4.2 and 4.0 mg as protein in (A) and (B)/tube, respectively) were incubated with 1 nM of $^3$H-R 1881 (A) or $^3$H-estradiol (B) in the presence or absence of 1 $\mu$M of the respective radioinert ligand for the time intervals indicated. The specific binding (closed circle) was obtained by subtracting the nonspecific binding (open triangle) from the total binding (open circle).
was detected in the nuclear extract of CUB-II, and no such high affinity binding to either ligand was observed in blood plasma of the CUB-II-bearing rats (data not shown).

**Sucrose density gradient centrifugation of the binding in the cytosols**

The cytosol prelabeled with either $^3$H-R 1881 or $^3$H-estradiol was fractionated in a sucrose density gradient centrifugation (Fig. 3). A radioactive peak of the $^3$H-R 1881 was observed at the site of 10S. In the cytosol incubated with $^3$H-estradiol, only a small radioactive peak was obtained at the same site as that observed with $^3$H-R 1881.

**Dissociation of $^3$H-R 1881 and $^3$H-estradiol bound to the cytosols**

The time course of dissociation of the

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Fig. 2. Binding of CUB-II cytosol to $^3$H-R 1881 and $^3$H-estradiol.

Cytosols (4.8 mg as protein/tube) were incubated with various concentrations of $^3$H-R 1881 (A) or $^3$H-estradiol (B) at 4°C for 5 hr (A) or 4 hr (B). Kd's and maximum binding sites calculated from the Fig. were $0.3 \times 10^{-9}$M and 30 fmol/mg protein ($^3$H-R 1881), and $2.6 \times 10^{-9}$M and 7 fmol/mg protein ($^3$H-estradiol), respectively.

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Fig. 3. Sucrose density gradient centrifugation of the CUB-II cytosol prelabeled with $^3$H-R 1881 and $^3$H-estradiol.

Cytosols were incubated at 4°C with 5 nM of either $^3$H-R 1881 for 5 hr or $^3$H-estradiol for 4 hr. After separating unbound steroid, an aliquot (0.3 ml, 2 mg as protein) was applied on the top of a 5–20% sucrose gradient containing 0.5 mM leupeptin and 10% glycerol, and centrifuged at 216,700×g for 16 hr. 0.2 ml of each fraction was collected and the radioactivity was counted (open circle: $^3$H-R 1881; open triangle: $^3$H-estradiol). In a parallel incubation, 1000-fold molar excess of the respective unlabeled ligand was added to observe the nonspecific binding (closed circle: $^3$H-R 1881; open triangle: $^3$H-estradiol). The site of the binding component is indicated by an empty arrow. Black arrows indicate the location of markers (BSA; bovine serum albumin 4.6S, h-IgG; human immunoglobulin G 7.0S, catalase; 11.3S).
bound $^3$H-R 1881 and $^3$H-estradiol at 4°C was examined (Fig. 4). The bound $^3$H-R 1881 was relatively stable, while a rapid dissociation was noticed in the bound $^3$H-estradiol. This also indicated that the binding affinity to $^3$H-estradiol seems to be lower than that to $^3$H-R 1881.

Inhibition of the binding to $^3$H-R 1881 and $^3$H-estradiol by other steroids

The effect of various steroids on the

Fig. 4. Time course of dissociation of the bound $^3$H-R 1881 and $^3$H-estradiol.

Cytosols (4.4 mg as protein/tube) were prelabeled with 2.5 nM of either $^3$H-R 1881 for 5 hr (open circle) or with $^3$H-estradiol for 4 hr (closed circle) at 4°C. After separating unbound steroid with the dextran-coated charcoal, the radioinert ligand was added to each cytosol to make the final concentration of 2.5 nM, and incubated at 4°C for time intervals indicated to estimate the rate of dissociation. After separating unbound steroids, bound radioactivity in the incubation tube was counted. Nonspecific binding which was obtained from cytosols prelabeled in the presence of the additional 1000-fold molar excess of the respective ligand, treated and counted in the same manner, was subtracted. Bound ligands at the start of incubation were 28 and 4 fmol/mg protein in $^3$H-R 1881 and $^3$H-estradiol, respectively (Ordinate). Half lives of dissociation in bound $^3$H-R 1881 and $^3$H-estradiol are calculated from the Fig. as 12.8 hr, and 2.65 hr, respectively.

Fig. 5. Inhibition of binding of the CUB-II cytosol to $^3$H-R 1881 and $^3$H-estradiol by various steroids.

Cytosols (4.2 mg and 3.9 mg as protein in (A) and (B)/tube, respectively) were incubated with 1 nM of either $^3$H-R 1881 (A) or $^3$H-estradiol (B) in the presence of various steroids. The results are expressed as percentage binding of the binding incubated in the absence of additives (A: 41 fmol/mg protein, B: 6 fmol/mg protein). Open circle: R 1881; closed circle: estradiol; open square: dihydrotestosterone; open triangle: R 5020; closed triangle: diethylstilbestrol.
added to the incubation, suggesting that the same binding sites were shared with estrogen and androgen and the binding of this protein was more favorable to androgens than to estrogens.

**Effect of sodium thiocyanate on the binding**

It was reported that sodium thiocyanate caused a dissociation of bound ligand from the androgen-receptor complex (Kaufman et al., 1982). However, this was not the case in the estrogen receptor (Sica et al., 1976, Sato et al., 1981). The addition of sodium thiocyanate to the incubation of the CUB-II cytosol and $^3$H-R 1881 induced a complete loss of the binding (Data not shown). Therefore, the effect of this salt on the binding of the CUB-II cytosol to $^3$H-R 1881 seemed to be analogous to that of the androgen receptor. A previous report from this laboratory (Minagawa et al., 1983) showed the presence of the estrogen receptor in cytosols from tissues of Dunning R 3327 and its subline, CUB, from which CUB-II had branched. The cytosol of CUB showed the binding to $^3$H-estradiol irrespective of the presence or absence of sodium thiocyanate (Fig. 6). On the other hand, binding to $^3$H-estradiol of the CUB-II cytosol disappeared in the incubation which included sodium thiocyanate. This may indicate that the properties of the binder in the CUB-II cytosol which is responsible for the binding to $^3$H-estradiol are not identical to those of the estrogen receptor, but similar to those of the binding protein which binds to $^3$H-R 1881.

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**Fig. 6. Effect of sodium thiocyanate on the binding of cytosols from CUB-II and CUB to $^3$H-estradiol.**

Cytosols prepared from CUB-II (A, 3.2 mg as protein/tube) and CUB (B, 2.0 mg as protein/tube) were preincubated in the presence (open triangle) or absence (open circle) of 0.4 M NaSCN at 4°C for 1 hr, then the incubations were continued further with various concentrations of $^3$H-estradiol. Kd's ($\times 10^{-9}$ M) and maximum binding sites (fmol/mg protein) calculated from the Fig. are 3.2 and 9 (CUB-II without NaSCN), 0.1 and 27 (CUB without NaSCN), and 0.1 and 20 (CUB with NaSCN), respectively.
Discussion

Although the original Dunning R 3327 tumor contains both androgen and estrogen receptors (Markland and Lee, 1979; Lea and French, 1981) and binding to $^3$H-R 1881 and $^3$H-estradiol is also observed in cytosols from CUB-II in the present study, the binding protein in CUB-II does not seem to be a steroid receptor (King and Mainwaring, 1974), since the high affinity bindings to both androgens and estrogens in the CUB-II take place at the same binding sites and no binding is detected in the nuclear extract. In blood plasma of rats some sex steroid-binding proteins have been reported. Suzuki et al. (1981) found a protein, with a molecular weight of 65,000, which bound to dihydrotestosterone with Kd of $1.2 \times 10^{-8} \text{M}$ but did not bind to estrogen. On the other hand, the rat plasma contains another binding protein which binds to estrone as well as estradiol and sediments at approximately 4S but this protein does not bind to androgen (Soloff et al., 1972). Although contamination of the blood plasma might take place in the tumor cytosols used for the binding experiments in the present study, binding affinity and specificity to androgens and estrogens discriminate the binding component in the CUB-II cytosol from the sex steroid-binding proteins in the rat plasma.

There have been reports concerning proteins in target tissues of androgens which are capable of binding to sex steroids but are different from receptors in many respects. The rat prostate produces a protein designated as prostatic binding protein as a secretory product (Heyns and DeMoor, 1977). This protein has also been studied by other groups and called by alternative names; prostatein by Lea et al. (1979), estramustine-binding protein by Forsgren et al. (1979), or $\alpha$-protein by Chen et al. (1979). Kd's for dihydrotestosterone and estradiol are the order of $10^{-7} \text{M}$ and the sedimentation coefficient in 3.5S in sucrose density gradient centrifugation. The other binding protein, 9S binding protein reported by Wilson et al. (1977), which sedimented at 9S and bound to dihydrotestosterone with an equilibrium binding constant of approximately $10^{-5} \text{M}$, was distributed in androgen-target tissues of rats. Androgen binding protein produced by Sertoli cells of testis (Hansson et al., 1975) is another sex steroid-binding protein, whose molecular weight is about 90,000 (Larrea et al., 1981). This protein shows a Kd of $10^{-9} \text{M}$ for dihydrotestosterone and does not bind to estradiol. In cytosols of the rat liver, an estrogen-binding protein was observed (Dickson et al., 1978). This can bind to estradiol and also to metabolites of dihydrotestosterone with Kd of the order of $10^{-8} \text{M}$. Physiochemical properties of the binding protein of the CUB-II such as high binding affinity and large molecular weight estimated from the sedimentation coefficient are different from any of the above-mentioned proteins, so that the binder in the CUB-II seems to be a novel type of binding protein which has not been referred to in the literatures.

Except for secretory proteins, the proteins capable of binding to sex steroids may influence the hormone action in target tissues, but the exact roles of these binding proteins are still obscure. The growth of CUB-II is completely independent of sex hormones and the binding protein is not present in nuclei, so that it may contribute, at least partly, to the independent growth of the tumor, but the physiological significance of this binding protein is also not yet clear.

A marked inhibitory effect of androgen on the binding with $^3$H-estradiol seems to be a characteristic property of the binding protein of the CUB-II. In this context, the human testosterone-binding globulin resembles the binding protein of CUB-II, since the human testosterone-binding globulin
shows Kd's for dihydrotestosterone and estradiol as $10^{-10}$ M and $10^{-9}$ M, respectively, and the binding of this globulin to estradiol is inhibited by the addition of dihydrotestosterone (Rosner and Smith, 1975, Petra, 1979, Cheng et al., 1983).

Since the sex steroid-binding protein observed in the present study is detected only in the CUB-II but not in the R 3327 and CUB which are the original tumors of that subline, production of this protein seems to take place suddenly during culture in vitro and/or serial transplantations of the tumor. It was reported that under culturing conditions, especially under conditions of deficient nutrition, chromosome aberrations are easily evoked in the cells (Freed and Schatz, 1969). Moreover, Isaacs et al. (1982) and Wake et al. (1982) claimed that the Dunning R 3327 tumor showed a tendency to change its chromosomal constitution frequently during serial transplantations. This may explain the erratic induction of the unique binding protein in a newly established subline, CUB-II. Alternatively, progression to the CUB-II might be induced by clonal selection where clusters of cells capable of producing this unique binding protein predominate (Rennie, 1982). Therefore, studying the binding proteins in a series of Dunning R 3327 tumors may throw light on the progression of the tumors.

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