Androgen Receptor Protein in the Androgen-dependent Dunning R-3327 Prostate Carcinoma

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ABSTRACT

The rat prostate carcinoma (Dunning R-3327) contains a relatively high concentration of androgen receptor (60 to 150 fmol/mg cytosol protein). We characterized this receptor for comparison with androgen receptors in normal organs of the rat. Binding of testosterone and dihydrotestosterone was of high affinity (Kd = 2 x 10^-9 M). Rates of dissociation were slow (t1/2 testosterone = 60 hr; t1/2 dihydrotestosterone = ~160 hr). At low ionic strength, the receptor was in an 8S form which dissociated to 4.5 to 5.0S in the presence of 0.4 M KCl. Fractionation of [3H]dihydrotestosterone cytosol by chromatography on phosphocellulose yielded a single peak of radioactivity eluting at 0.2 M Cl- in the presence of 0.4 M KCl. Determination of size at high ionic strength by gel filtration chromatography and sucrose gradient centrifugation indicated a Stokes radius of 53 Å and sedimentation coefficient of 55 (M.W. 115,000). Cytosols occasionally yielded a second peak of radioactivity eluting from phosphocellulose at 0.29 M Cl-. This fraction contained a smaller receptor (36 Å, 3.6S (M.W. 55,000)). Both receptor forms were observed in cytosols from the normal dorsal prostate. The larger high-salt form of the receptor is identical to native androgen receptor in normal tissues. Smaller receptor forms in the tumor and in normal androgen-responsive tissues have been shown previously to result from proteolytic cleavage of the native receptor during extraction.

INTRODUCTION

The tumor under investigation was discovered in 1961 by W. F. Dunning in an aged male Copenhagen rat and has been propagated since that time by s.c. transplantation in the Copenhagen-Fischer rat (1). Subsequent histochemical and biochemical studies have been consistent with a primary site of origin in the dorsal prostate (3, 8, 9, 12). Like the normal dorsal prostate, the tumor is androgen dependent and grows faster in male than in female rats (10). This growth in male rats is inhibited by castration or treatment with antiandrogens (8-10). The tumor metabolizes testosterone in a manner similar to prostate and other androgen-responsive tissues (10, 11). It has been shown to contain androgen-binding proteins (11) with sedimentation and binding properties similar to androgen receptors in other tissues (6).

We have shown that the androgen receptor in R-3327 tumor cytosol appears in 2 forms at high ionic strength. Characterization with respect to charge, size, and kinetics of binding indicates that the different forms of the androgen receptor in this tumor are identical to receptors in other androgen target organs (4). In studies reported elsewhere (14, 16), preparation and incubation of cytosols in the presence and absence of protease inhibitors have indicated that receptor forms smaller than 53 Å, 5.0S result from proteolytic cleavage of the native receptor.

MATERIALS AND METHODS

5α-[1,2,4,5,6,7-3H]Dihydrotestosterone (85 Ci/mmol) and [1,2,6,7-3H]testosterone (96 Ci/mmol) were purchased from New England Nuclear, Boston, Mass. Other materials were as noted previously (4). The Dunning dorsal prostate tumor (R-3327) was implanted s.c. into Copenhagen-Fischer rats at the Papanicolaou Cancer Research Institute, Inc., Miami, Fla. Tumors were allowed to grow until they reached a diameter of 2 to 3 cm, usually 4 to 6 months after implantation.

Rats were castrated 18 hr prior to removal of tissues and killed by decapitation. Initially, tumors and other organs were rinsed in ice-cold 0.9% NaCl solution, frozen on dry ice, and thawed prior to mincing and homogenization. However, in subsequent studies, organs for in vitro labeling were rinsed in cold 0.9% NaCl solution, blotted quickly on filter paper, and frozen in liquid nitrogen. They were stored for varying periods of time at -70°. Frozen tissues were pulverized with a mortar and pestle under liquid nitrogen and homogenized using an Ultraturrax at 0° in 4 volumes of 50 mM Tris (pH 8; 0°):1 mM EDTA:1 mM 2-mercaptoethanol:10% glycerol using 4 ml buffer per g tissue. Cytosols (105,000 x g supernatants) were prepared as described previously (13) by centrifuging 36,000 rpm for 75 min in a Beckman 40 rotor (Beckman Instruments, Palo Alto, Calif.). In some experiments, in vivo-labeled receptors were partially purified from cytosol by ammonium sulfate precipitation (13). Receptor concentration in unfractionated cytosols was estimated using a charcoal assay (13). In vitro labeling of receptors was carried out by incubation of 105,000 x g supernatants with 15 to 20 nM [3H]dihydrotestosterone or [3H]testosterone for 14 to 16 hr at 0°.

Phosphocellulose chromatography was performed as described previously (4). Phosphocellulose was packed in 9-mm diameter columns (Pharmacia K9/15) using approximately 0.5 ml sediment ion exchange agent for each ml of cytosol chromatographed. Before application of the sample, the column was washed with approximately 30 ml of buffer. After application of the sample, the column was again washed with TM buffer at a flow rate of approximately 20 ml/hr. Receptor protein was eluted in a 125-ml linear gradient of KCl (0 to 0.7 M) in TM buffer. Fractions of 1.3 ml were collected across the gradient, and 0.5-ml aliquots were counted in 5 ml Scintiverse.

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(Fisher Scientific Co.) toluene (1:1, v/v). Salt concentration across the gradients was monitored with a chloride ion electrode after complexing sulfhydryl groups of 2-mercaptoethanol by addition of a 1.2-fold molar excess of p-hydroxymercuribenzoate.

Gel filtration on Sephadex G-200 was carried out in columns with a 2.6-cm inside diameter, Pharmacia K26/40, packed to a height of 35 cm as described previously (9). The gel bed was equilibrated with TM buffer containing 0.5 M KCl. Samples of 4 to 5 ml were applied containing bovine γ-globulins (radius, 52Å) and ovalbumin (radius, 27Å); 15 mg of each were added together with 1.0 absorbance unit (280 nm) of blue dextran as internal markers. Flow rate was maintained at 15 ml/hr using a Pharmacia P-3 peristaltic pump. Fractions containing 50 drops (2.0 to 2.5 ml) were collected, and 1-ml aliquots were removed for liquid scintillation counting. The remaining solution was used for determination of marker proteins by absorption at 280 nm. The gel filtration radius (Einstein-Stokes radius) was estimated from a plot of (Ve/V0)1/3 versus radius, where Ve is the elution volume of protein, and V0 is the void volume measured with blue dextran.

Sucrose gradient centrifugation was carried out at 2° and 4.4-ml linear gradients of 5 to 20% weight per volume sucrose in TM buffer containing 10% glycerol with or without 0.4 M KCl. Samples of low-salt concentration were made to contain 0.4 M KCl before sedimenting in gradients containing 0.4 M KCl. Samples were run in triplicate containing either no marker protein or a combination of 2 of the following sedimentation markers: myoglobin, 2.1S; ovalbumin, 3.6S; bovine γ-globulin, 6.8S; and catalase, 1.3S. Centrifugation was for 18 to 24 hr at 48,000 rpm in a Beckman SW50.1 rotor. Gradients were fractionated from the bottom into 25 to 30 fractions (10 drops/fraction). A 25-μl aliquot was removed from each fraction for protein determination (5) to localize the marker proteins.

Identification of radioactive metabolites following incubation of labeled androgens and tumor cytosol was carried out by thin-layer chromatography (4).

RESULTS

Before investigating the in vitro binding of [3H]testosterone and [3H]dihydrotestosterone in tumor cytosol, it was established that both androgens could be recovered unmetabolized following incubation in tumor cytosol for 18 to 24 hr at 0°. This period of incubation was used to approach an equilibrium between receptor-bound and unbound steroid.

Specific binding of testosterone paralleled that of dihydrotestosterone and reached saturation at a low-steroid concentration (~20 nM), a characteristic of androgen as well as other steroid hormone receptors.

Dissociation rate constants at 0° (t1/2 testosterone = 60 hr; t1/2 dihydrotestosterone = ~160 hr), determined as the decrease in specific binding of [3H]testosterone and [3H]dihydrotestosterone with time after addition of an excess of unlabeled androgen, were slower than in cytosols of normal reproductive organs of the male rat (13). However, the relative rates of dissociation were the same as those obtained in normal tissues with testosterone dissociating 2.5 to 3.0 times faster than dihydrotestosterone (13).

Sucrose gradient analysis of cytosol incubated with [3H]dihydrotestosterone in the presence and absence of a 100-fold excess of unlabeled dihydrotestosterone showed specific binding to a macromolecule sedimenting at 8S (Chart 1). A small amount of receptor could be labeled in cytosol from intact rats;

\[
\text{M.W.} = \frac{6\eta N S}{1 - \bar{v} p}
\]

where \(\eta\) represents the viscosity of the medium (0.01 g/sec/cm), \(N\) is Avogadro’s number (6.02 x 10^23 g/mol), \(a\) is Stokes’ radius, \(S\) is Svedberg units (10^-13 sec), \(\bar{v}\) is the partial specific volume (0.725 ml/g), and \(p\) is the density (1.03 g/ml).

The charcoal adsorption assay was used for saturation analysis of androgen receptors and measurement of dissociation rate constants (13).

Identification of radioactive metabolites following incubation of labeled androgens and tumor cytosol was carried out by thin-layer chromatography (4).

Chart 1. Sucrose density gradient centrifugation of tumor cytosol from intact or castrated male rats. Cytosols were incubated for 18 hr with 15 nM [3H]-dihydrotestosterone in the absence (D) or presence (C) of a 100-fold excess of unlabeled steroid. Samples of 0.4 ml were layered onto 5 to 20% sucrose gradients and centrifuged for 18 hr at 47,000 rpm (200,000 x g average) in a Beckman SW 50.1 rotor at 2°. Gradients were fractionated from the bottom, and each fraction was analyzed for radioactivity and proteins. Note the logarithmic scale of cpm. Right, calibration plot of sedimentation coefficients obtained using internal protein standards (catalase, 11.3S; γ-globulin, 6.8S; ovalbumin, 3.6S; and myoglobin, 2.0S).
However, binding increased severalfold in tumor cytosol from rats castrated to reduce the level of endogenous androgens. The addition of salt caused a shift in specific binding to a slower sedimenting form (Chart 2). In the presence of 0.4 M KCl, a single peak of radioactivity sedimented at 4.5S. Fractionation of labeled cytosol by (NH₄)₂SO₄ precipitation at 33% saturation yielded a mixture of high- and low-salt forms of the receptor (8S to 5S) due to the presence of (NH₄)₂SO₄ in the sample. (NH₄)₂SO₄ fractionation removes most of the nonspecifically bound [³H]dihydrotestosterone, while 60% of the receptor-binding activity is recovered.

Fractionation of [³H]dihydrotestosterone-labeled cytosol by chromatography on a phosphocellulose column yielded 2 peaks of radioactivity eluting at 0.2 and 0.29 M Cl⁻, respectively (Chart 3). Determination of size at high ionic strength by gel filtration on Sephadex G-200 and sedimentation in sucrose gradients revealed the leading edge of Peak I to contain a 53 Å, 5S receptor form (M.W. 115,000) (Chart 4). Peak II contained a smaller amount of the same form due to overlap with Peak I but was predominately a smaller form, 36 Å, 3.6S (M.W. 55,000). Small peaks of aggregated receptor appeared in the void volume of the Sephadex G-200 columns as noted previously (4). This material pelleted in sucrose gradients and was not counted.

Dorsal prostate from Copenhagen-Fischer rats also contained both receptor forms; however, the 36 Å, 3.6S form predominated. Aggregated material was noted also in the void volume of the Sephadex G-200 column.

In subsequent studies, the smaller receptor form was rarely observed when tumors were frozen in liquid nitrogen and stored at -70° prior to homogenization. The labeled 53 Å, 5S form of the receptor in these cytosols could be heated to 25° for 30 min without loss of binding activity or cleavage to a smaller form (Chart 5).

**DISCUSSION**

Our studies have shown that the androgen-dependent dorsal prostate tumor (R-3327) contains an androgen receptor protein with properties similar to the androgen receptor in other tissues. Receptor-binding sites are saturable with low concentrations of testosterone or dihydrotestosterone. The concentration of androgen receptor varies from 80 to 150 fmol/mg cytosol protein. Binding is of high affinity (Kᵦ = 2 × 10⁹ M), and the bound androgen exhibits a slow rate of dissociation from the receptor at 0°. Like androgen receptors in other tissues (4, 13-16), the receptor in tumor cytosol has a sedimentation coefficient of 8S at low ionic strength. Markland and Lee (7) have recently reported an 8S receptor in R-3327 using the synthetic androgen, methyltrienolone. In our studies, sedimentation of the receptor was observed to shift at high ionic strength to a smaller form which in most cytosols sedimented at 5.0S. The receptor in tumor cytosol could be partially purified on phosphocellulose, a procedure which removes nonspecifically bound androgen. Two high-salt forms of the partially purified receptor differed in charge, as indicated by salt elution from phosphocellulose and separation according to size (53 Å, 5.0S; 36 Å, 3.6S) by gel filtration and sucrose gradient centrifugation. Similar forms were observed in dorsal prostate from the Copenhagen-Fischer rat; however, the smaller form (36 Å, 3.6S) predominated.

We have observed previously 3 high-salt forms of the androgen receptor (53 Å, 5.0S; 36 Å, 3.6S; 23 Å, 3.0S) in reproductive organs of the male rat after partial purification by

![Chart 2. Sedimentation analysis of androgen receptor binding in tumor cytosol. [³H]Dihydrotestosterone, 15 nM, was incubated in the absence (○) or presence (□) of a 100-fold excess of unlabeled dihydrotestosterone. Samples of 0.4 ml were extracted with charcoal to remove free steroid and were analyzed in 5 to 20% sucrose gradients as in Chart 1. Left, sedimentation of labeled binding proteins in buffer of low ionic strength (TM-buffer containing 10% glycerol); middle, sedimentation in TM-buffer containing 10% glycerol and 0.4 M KCl; right, labeled receptor precipitated in 33% saturated ammonium sulfate dissolved in TM-buffer containing 10% glycerol and sedimented in the same buffer at low ionic strength. M, myoglobin; C, catalase; yG, γ-globulin; Ov, ovalbumin.](image-url)
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phosphocellulose chromatography (4). In these studies, it was shown that the 36 Å, 3.6S receptor in ventral prostate cytosol could be converted to a 23 Å, 3.0S form by heating to 37°C for 15 min. This conversion was blocked in the presence of soybean trypsin inhibitor. Forms smaller than 4.5 to 5.0S were not present in cytosols prepared from male reproductive organs in the presence of the potent serine protease inhibitor DFP (14). An exception was ventral prostate which was shown to contain a protease insensitive to DFP. When this protease was inhibited by the addition of rabbit bile to ventral prostate cytosol, a 4.5 to 5.0S receptor was recovered as the predominant form (14). The DFP-insensitive protease of ventral prostate is secreted into seminal fluid and may have contaminated the dorsal prostate cytosols analyzed in this study, thus accounting for the

Chart 3. Phosphocellulose column chromatography of androgen receptor Intumor cytosol. Cytosol samples of 5 ml were incubated with 15 nM [3H]dihydrotestosterone for 18 hr in the absence (——) or presence (— — — — ) of a 100-fold excess of unlabeled dihydrotestosterone. Labeled cytosols were applied to phosphocellulose columns as described in "Materials and Methods." The columns were washed with 50 mM Tris-HCl (pH 7.4); 10 mM 2-mercaptoethanol and eluted on a 0.0 to 0.7 M KCl gradient (125 ml) in the same buffer.

Chart 4. Gel filtration and sucrose density gradient analyses of [3H]dihydrotestosterone-bound receptor recovered in Peaks I and II following phosphocellulose chromatography of tumor cytosol as shown in Chart 3. Fractions 43 to 50 containing the leading portion of Peak I (Chart 3) were pooled as were Fractions 54 to 60 from Peak II. KCl concentrations of the pooled fractions were adjusted to 0.5 M. Aliquots of each pool were analyzed on Sephadex G-200 columns and sucrose gradients (5 to 20%) as described in "Materials and Methods." Arrows, elution positions of blue dextran (V0); bovine γ-globulin (γG) 53 Å; and ovalbumin (Ov) 27 Å, 3.6S.

Chart 5. Effect of heating on the 53 Å, 5S form of tumor cytosol receptor. Cytosol was incubated with [3H]dihydrotestosterone at 0°C for 16 hr and divided into equal aliquots, one of which was heated at 25°C for 30 min. Heated and unheated (control) aliquots were fractionated on phosphocellulose columns (left). Peak fractions of radioactivity recovered from phosphocellulose were analyzed by gel filtration on Sephadex G-200 (right). Arrows, elution positions of blue dextran (V0); bovine γ-globulin (γG); and ovalbumin (Ov).
predominance of the 36 Å, 3.6S receptor fragment. With care in dissecting the dorsal prostate and preparation of cytosol in the presence of DFP, receptor in dorsal prostate cytosol is predominantly 53 Å, 5.0S. Preparation of R-3327 cytosol receptor in the presence of DFP indicates that the tumor also lacks the DFP-insensitive protease. The androgen receptor in R-3327 cytosol appears more stable than does the androgen receptor in cytosols of other tissues (4) since heating to 25–37°C for short periods did not result in loss of binding activity or conversion of the 53 Å, 5.0S receptor to a smaller form. The occasional generation of 3.6S fragment during preparation of cytosol may have resulted from exposure to a membrane-bound protease. This proteolytic activity is readily inhibited when cytosols are prepared in buffer containing DFP (14).

Thus, by several criteria, the androgen receptor in R-3327 appears identical to receptors in normal tissues of the rat. Studies with protease inhibitors indicate that the smaller androgen receptor forms which have been reported previously result from proteolytic breakdown of the native 53 Å, 5.0S form (14, 16). Receptor in its native form can be recovered in relative abundance from this well-differentiated androgen-dependent tumor.

Recent reports have clearly demonstrated the presence of specific receptor for estradiol as well as androgen in R-3327 (2, 7). Competition assays have shown negligible binding of androgen to the estrogen receptor. Estradiol binds to the androgen receptor (2, 7); however, its affinity is only ~7% of the androgen receptor affinity for dihydrotestosterone.

REFERENCES

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