Properties of an Intermediate-Sized Androgen Receptor: Association with RNA†

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ABSTRACT: This study identifies an intermediate-sized androgen receptor and characterizes its relationship with the 9.1S and 4.4S receptor forms. Under low ionic conditions, at 2–4 °C, there exists a 9.1S (±0.17) (n = 30) oligomeric form which does not bind to DNA. Under high ionic conditions, this form dissociates to a 4.4S (±0.08) (n = 18) monomeric form. When the salt concentration is lowered, the 4.4S monomer converts to a species with an intermediate sedimentation coefficient of 7.7S (±0.15) (n = 17) which binds to DNA. Unlike the 9.1S oligomer the 7.7S form is not maintained by sodium molybdate under high ionic conditions but rather dissociates to the 4.4S monomer. To determine whether these forms were associated with RNA, the 7.7S form was incubated with RNase A and analyzed by density gradient centrifugation. The 7.7S form was digested fully by RNase to the 4.4S monomer. The 7.7S form demonstrated a buoyant density of 1.2459 ± 0.014 g/cm^3 (n = 6) in metrizamide gradients, suggesting a ribonucleoprotein component. The sedimentation coefficient of the 9.1S form was unaffected by RNase. These data suggest that the intermediate 7.7S receptor form is composed of 4.4S monomer associated with a ribonucleoprotein molecule(s).

We have reported previously the purification and physicochemical properties of androgen receptor proteins from several tissues (Chang et al., 1982, 1983, 1984; Chang & Tindall, 1984; Murthy et al., 1984, 1986; Rowley et al., 1984; Rowley & Tindall, 1986). The androgen receptor from Dunning prostatic tumor sedimented at 4.4 S with a corresponding Stoke's radius of 61 Å in high ionic strength buffer (400 mM KCl), indicating an M_r of approximately 120000 (Rowley et al., 1984). This form is likely to be the smallest intact polypeptide subunit (monomer) of the androgen receptor, since a similar M_r (118000) was detected by affinity labeling and SDS–gel electrophoresis under denaturing conditions. In contrast, in low ionic buffer the receptor, which was interpreted as an oligomeric form, sedimented at approximately 9 S with a Stoke's radius of 73 Å, suggesting an M_r of 275000–300000. The 9S receptor was nontransformed, since it did not bind to DNA–cellulose and was stabilized by sodium molybdate in high ionic buffer. This form of the receptor, which has been described for most classes of steroid receptors (Grody et al., 1982; Sherman et al., 1983), corresponded to the classical 8–10S form.

Of interest to our studies was a report describing a conversion of the monomeric androgen receptor to a larger sedimenting form of approximately 8S (Colvard & Wilson, 1981). We have designed our studies to understand better this conversion of the monomeric form to an 8S species and to address the question as to whether the newly formed 8S species is identical with the original 8–10S oligomeric species in its physicochemical properties.

In this paper we report the identification of an intermediate-sized (7.7S) androgen receptor which contains ribonucleoprotein. The 7.7S receptor is separate from, and exists in solution with, the oligomeric receptor in low ionic conditions (<50 mM KCl). This form of the receptor appears to be generated from the combination of monomer with an 8S "promoting factor" and/or proteolytic digestion of the oligomer by an endogenous protease.

Materials and Methods

Materials. The following materials were purchased: [^{3}H]dihydrotestosterone[1] and aqueous counting scintillant from Amersham; unlabeled dihydrotestosterone from Steraloids; Tes, [*N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid], monothioglycerol, 2-mercaptoethanol, sodium molybdate, sodium iodide, bacitracin, aprotinin, trypsin inhibitor, diisopropyl fluorophosphate, ribonuclease A (type X11-A), oxidized ribonuclease A (type X11-AO), and ribonuclease A attached to beaded agarose from Sigma; ammonium sulfate and potassium chloride from Fisher; calf thymus DNA and ribonuclease A from Worthington; ribonuclease A from Boehringer Mannheim; cellulose powder (Cellex-N-1) from Bio-Rad; charcoal (Norit A) from J. T. Baker, gelatin from Knox-Gel; [^{14}C]formaldehyde and methyl-[^{14}C]-labeled bovine serum albumin (BSA) from New England Nuclear; metrizamide from Nyegaard and Co.; cellulose phosphate (P11) from Whatman. All reagents were of analytical grade.

Preparation of Cytosol. Cytosol was prepared from rat Dunning R3327H prostatic tumors. Male Copenhagen-Fisher F1 rats bearing subcutaneous tumors were supplied by the Papanicolaou Cancer Research Institute (Miami, FL). Rats were castrated 24 h prior to harvesting of tumors. Tumors were removed, minced quickly, frozen in liquid nitrogen, and stored at −100 °C. Tumor tissue was thawed quickly (<1 min) in saline at room temperature and homogenized immediately in either TTES (10 mM Tes, 12 mM monothioglycerol, 1.5 mM EDTA, and 0.25 M sucrose, pH 7.4 at 22 °C) or TTESM (TTES plus 10 mM sodium molybdate) buffer (1:4 w/v at 0–2 °C). Homogenization was conducted by five, 10-s bursts at a setting of 5 with a Polytron PT10/30 homogenizer

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[1] Abbreviations: dihydrotestosterone, 17β-hydroxy-5α-androstan-3-one; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; BSA, bovine serum albumin; Tes, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; DIFP, diisopropyl fluorophosphate.

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(Brinkman, Inc.). Cooling (20–30 s) of homogenate was allowed between each burst. The homogenates were centriguted at 105000*g* for 60 min at 0–2 °C. The lipid layer was removed by passage of supernatant through cheesecloth into prechilled tubes. For all subsequent procedures, the temperature was maintained at 2 °C unless indicated otherwise.

Steroid Binding Assay. Samples were assayed for specific steroid binding activity with the dextran-coated charcoal adsorption method (Korenman, 1969). After the samples were labeled with [^{3}H]dihydrotestosterone under the indicated conditions, the samples were incubated with an equal volume of charcoal solution (1% charcoal, 0.1% dextran T-70, 0.1% gelatin, 1.5 mM EDTA, and 50 mM Tris-HCl, pH 7.4 at 22 °C) for 10 min at 0–2 °C. The samples were centrifuged at 1500*g* for 15 min, and an aliquot of the supernatant was added to scintillation cocktail and counted for radioactivity. Nonspecific steroid binding was determined by the addition of a 100-fold excess of unlabeled dihydrotestosterone. Nonspecific labeling was subtracted from total labeling to determine specific binding activity.

Density Gradient Analysis. Sucrose density gradients were prepared (linear, 6–20% w/v, 5 mL) in TTE buffer (10 mM Tes, 12 mM monothioglycerol, and 1.5 mM EDTA, pH 7.4 at 22 °C) or TTE buffer plus 400 mM KCl. For receptors prepared in the presence of sodium molybdate, 10 mM sodium molybdate was included in all gradients. Samples labeled previously with [^{3}H]dihydrotestosterone were treated with charcoal as described above, and 200-μL aliquots were layered onto the gradients. The following proteins were used as external standards for gradient calibration: myoglobin (2.0S); BSA (4.4S); bovine γ-globulin (6.9S); and bovine liver catalase (11.3S). These same proteins were used periodically as internal standards after they were labeled with [^{14}C] by reductive methylation as described previously (Rowley et al., 1984). Gradients were centrifuged at 65 000 rpm (400000*g*) in a VTi-65 vertical tube rotor (Beckman) for 90 min. Fractions (175 μL) were collected from the bottom of each gradient tube, and radioactivity in each fraction was determined by scintillation counting. Sedimentation coefficients of receptor forms were determined from the linear regression of standard proteins.

DNA–Cellulose Binding Assay. DNA–cellulose was synthesized by methods described previously (Albert & Herrick, 1971; Coty et al., 1979). DNA–cellulose columns were poured in multiple 3-mL syringe barrels to a bed volume of 0.5 mL each. Columns were equilibrated in TTES or TTESM buffer depending on whether sodium molybdate was present in the experimental sample. At the time of application, aliquots (200 μL) were removed from each sample and assayed for specific [^{3}H]dihydrotestosterone labeling by the charcoal method. Identical aliquots (200 μL) were diluted with TTES or TTESM buffer to lower the KCl concentrations to 30 mM and applied immediately to the columns. To triplicate columns were applied samples labeled with [^{3}H]dihydrotestosterone (total binding), and to triplicate columns were applied samples labeled with [^{3}H]dihydrotestosterone plus 100-fold excess of unlabeled dihydrotestosterone (nonspecific binding), in order to quantitate the degree of specific binding for each experimental manipulation. The columns were washed thoroughly with 12 bed volumes of TTES or TTESM buffer. The radioactivity of the final wash volume was in each case less than 100 cpm. The columns were eluted with 5 consecutive bed volumes of TTES or TTESM buffer containing 500 mM KCl, and the radioactivity in each bed volume was determined by scintillation counting. Specific binding activity eluted was compared to the specific binding activity applied and expressed as percent bound to DNA–cellulose.

Isopyknic Centrifugation. The buoyant density of the respective receptor forms was determined by using self-forming metrizamide gradients. The conditions for gradient self-formation (time and *g* force) were determined empirically for optimal resolution in the ribonucleoprotein–protein density regions (1.20–1.30 g/cm^3). In order to decrease the time to reach isodensity equilibrium, a vertical tube rotor (Beckman VTi-65) was used. Metrizamide solutions were made in TTE buffer (10 mM Tes, 12 mM monothioglycerol, and 1.5 mM EDTA, pH 7.4 at 22 °C) at 30%, 45%, and 50% w/v concentrations. Analyses were made in each of two ways. Initially, analyses were made according to modifications of procedures established earlier for density determinations of estrogen receptor by Baskevitch and Rochefort (1981). For these studies the receptor sample ($\sim$300 μL of cytosol) was diluted in TTE buffer to 1.7 mL, layered onto 3.7 mL of 45% metrizamide, and centrifuged at 45 000 rpm for 18 h at 2 °C. In addition, owing to our concern for time and potential receptor dissociation or degradation, we optimized conditions in order to reach the isodensity point with less time. For these experiments 300-μL aliquots of receptor were centrifuged at 65 000 rpm for 4 h at 2 °C through 4.53 mL of 30% metrizamide layered on top of 475 μL of a 50% metrizamide cusion. Fractions (150 μL) were collected from the bottom of each gradient tube. The density of metrizamide for each fraction was determined by reading the refractive index (RI) with a Bausch and Lomb refractometer. The RI was converted to density by the formula density (5 °C) = (RI (20 °C) × 3.453) − 3.601 (Rickwood & Birnie, 1975; Baskevitch & Rochefort, 1981). Control samples for each of these procedures were analyzed by sucrose density gradients to confirm the respective molecular form of the receptor. Statistical analyses of data were by Student's *t* test.

Phosphocellulose Chromatography. Receptor was partially purified by phosphocellulose column chromatography in order to separate the monomeric form (4.4S) from the previously described "8S promoting factor" following slight modifications of procedures reported previously (Colvard & Wilson, 1981). Briefly, cytosol prepared in TTES buffer was allowed to incubate with 15 nM [^{3}H]dihydrotestosterone for 12–20 h at 2 °C. This incubation served to transform the receptor to a phosphocellulose-binding state. Cytosol ($\sim$15 mL) was passed through a 4-mL phosphocellulose column, and the flow-through fraction (containing the 8S promoting factor activity) was saved. The column was washed extensively with 30 bed volumes of TTES buffer followed by 30 bed volumes each of TTES plus 50 mM KCl and TTES plus 100 mM KCl. The column was eluted with TTES buffer plus 300 mM KCl, and 0.5-mL fractions were collected. From each fraction, radioactivity was determined in 25-μL aliquots by scintillation counting. The peak of activity (4.4S monomeric form) was pooled ($\sim$5 fractions) and used for further experimentation as described in the text. In each case a 100–200-μL aliquot from the pooled fractions was tested for confirmation of form on low ionic strength sucrose density gradients.

Results

Transformation Properties and Molecular Forms of the Receptor. It has been well established that there is a correlation between steroid receptor form and DNA-binding characteristics. In studies of this nature sodium molybdate has proven useful since it can stabilize the oligomeric (9S) form of the steroid receptor in high ionic environments and therefore maintain the receptor in a nontransformed state in which it

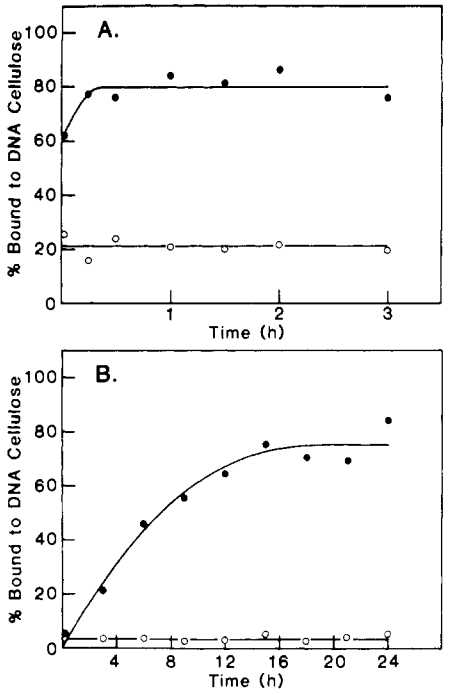

FIGURE 1: Time course of receptor transformation to a DNA-cellulose binding state in the absence (A) or the presence (B) of molybdate. Cytosol was prepared in either TTES (A) or TTESM (B) buffer and labeled with 16 nM [^{3}H]dihydrotestosterone ± a 100-fold excess of unlabeled dihydrotestosterone for 1 h at 0 °C. Samples were adjusted to 500 mM KCl and incubated for 0–24 h at 0 °C. Negative control samples received TTES or TTESM buffer only. Specific [^{3}H]dihydrotestosterone binding was determined for experimental and negative control samples separately at each time point by the charcoal adsorption assay. At each time point, aliquots were diluted to <30 mM KCl with either TTES or TTESM buffer and applied to DNA-cellulose columns (0.5-mL bed volume) equilibrated in either TTES or TTESM buffer as described in Materials and Methods. The total time for the sample to pass through the column was <3 min. The eluted specific [^{3}H]dihydrotestosterone binding activity is expressed as a percentage of applied specific [^{3}H]dihydrotestosterone binding activity and is indicated as the percent bound to DNA–cellulose on the ordinate (filled circles); negative controls (open circles).

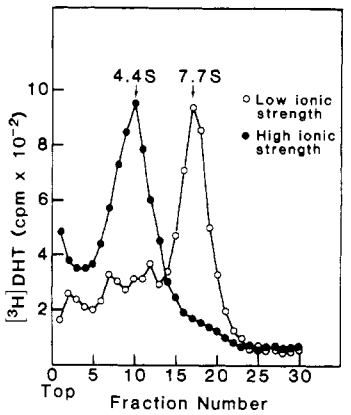

FIGURE 2: Density gradient analysis of transformed androgen receptor. Sodium molybdate (10 mM) was included in all sucrose gradients. Samples attaining maximal levels of transformation (16-h incubation with 500 mM KCl) were diluted to <30 mM KCl as indicated in Figure 1 and applied to low ionic sucrose gradients (open circles). For high ionic gradients (filled circles), to each sample an identical volume of high-salt buffer (TTESM plus 500 mM KCl) was added in order to maintain each sample in a high ionic environment. Gradients were centrifuged in a vertical tube rotor for 90 min, fractionated, and analyzed as described in Materials and Methods.

will not bind DNA–cellulose (Rowley et al., 1984; Carroll et al., 1984). Accordingly, we have used both DNA–cellulose and sodium molybdate to assess differences between various forms of the androgen receptor.

Figure 1 illustrates the time course of receptor transformation in the presence of 500 mM KCl either without or with sodium molybdate (10 mM), as measured by the percent of total receptor activity which bound to DNA–cellulose columns. In the absence of molybdate (Figure 1A), the receptor transformed rapidly to a DNA-binding state. Brief (~5-s) exposure to KCl (500 mM) and immediate dilution to <30 mM KCl was sufficient to transform approximately 60% of the receptor population (Figure 1A, 0 time point). Only 15 min of incubation with KCl (500 mM) was required to reach maximal levels (approximately 80% bound to DNA–cellulose). In addition, only 200 mM KCl was required for full transformation with a 3-h incubation (data not shown). Aproximately 20% of control samples not treated with salt bound to DNA–cellulose at each time point throughout the 3-h time course. Although transformation of receptor to a DNA-binding state was possible in the presence of molybdate, approximately 16 h of incubation was required before full transformation was achieved (Figure 1B). In contrast to control values observed in Figure 1A, only 3% of negative control samples that did not receive salt treatment contained DNA-binding activity throughout the time course.

When receptor from the above experiments was analyzed on high ionic strength sucrose density gradients, we found a correlation between the appearance of the 4.4S form and acquired DNA–cellulose binding. Figure 2 shows the gradient profiles of molybdate-stablized receptor incubated for 16 h with 500 mM KCl (transforming conditions). Under high ionic conditions, the receptor sedimented as a 4.4S (±0.08) (n = 18) species. In contrast, when an identical preparation was dialyzed to lower the KCl concentration to approximately 30 mM (conditions required before application to the DNA columns) and then centrifuged through low ionic gradients, the receptor was changed to a form of intermediate size, 7.7S (±0.15) (n = 17). Receptor transformed in the absence of molybdate (500 mM KCl for 3 h) and dialyzed gave the same results, a 4.4S species in high ionic strength gradients and a 7.7S form in low ionic strength gradients.

Because of previous published results (Colvard & Wilson, 1981) we postulated that the generation of this intermediate-sized form was due to the association of monomer (4.4S) with the "8S promoting factor" when the salt concentration (ionic strength) was lowered. It appeared therefore that under conditions used to test for binding to DNA–cellulose the receptor population was in the intermediate-sized (7.7S) form when applied to DNA–cellulose columns. In order to assess the binding of the 4.4S monomer to DNA–cellulose we prepared cytosol in TTES buffer and separated the 4.4S monomer from the 8S promoting factor with phosphocellulose chromatography as described in Materials and Methods. After this chromatographic step, the receptor was maintained in the monomeric (4.4S) form after the salt concentration was lowered. Under these conditions the monomeric form demonstrated maximal levels (>80%) of binding to DNA–cellulose (data not shown).

Since the above studies demonstrated that the 7.7S species binds to DNA–cellulose, we wondered whether the fraction of the receptor population which bound to DNA–cellulose (negative control samples of Figure 1A) might be in a 7.7S form. One way to test this possibility was to analyze these samples in high ionic density gradients with 10 mM molybdate included in the gradients. Under these conditions the 9.1S receptor is stabilized (Rowley et al., 1984) and the 7.7S receptor converts back (dissociates) to the 4.4S form normally

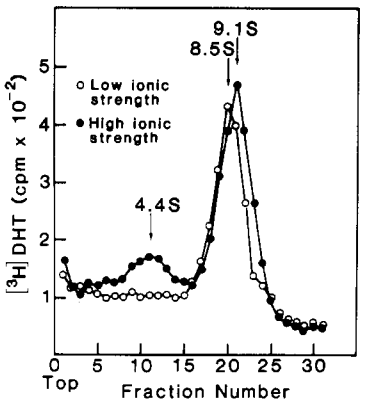

FIGURE 3: Density gradient analysis of nontransformed receptor prepared in the absence of molybdate. Negative control samples from Figure 1A were used. Samples were applied to either low ionic gradients (open circles) or high ionic gradients (filled circles). All gradients contained 10 mM sodium molybdate.

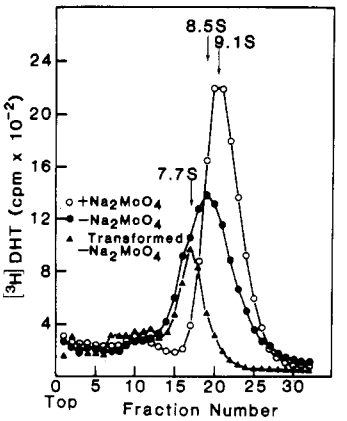

FIGURE 4: Density gradient analysis of transformed and nontransformed receptor. A tissue homogenate was divided three ways, and cytosol was prepared from each. To the homogenate of fraction one (open circles) was added sodium molybdate to a final concentration of 10 mM. To the cytosol of fraction two (filled triangles) was added KCl to a final concentration of 500 mM. Fraction three (filled circles) received buffer only. All three cytosol fractions were labeled with [^{3}H]dihydrotestosterone (3 nM) for 3 h. All fractions were dialyzed in order to lower the salt to <30 mM KCl and analyzed on low ionic sucrose gradients.

detected in high salt (see Figure 2). Hence, in the presence of molybdate and high ionic strength the population of receptor which is 9.1S should remain as such and the population which is 7.7S should dissociate to 4.4S, thus resolving better these two species. Figure 3 shows that when negative control sample froms Figure 1A (samples withough molybdate and without salt) were analyzed in low salt gradients, a single peak of activity was detectable with a sedimentation coefficient of 8.5S (±0.21) (n = 5). When identical aliquots were applied to high ionic density gradients containing 10 mM molybdate, two peaks of activity were resolved. A major peak was observed at 9.1 S, and a minor peak was observed at 4.4 S. The relative amount of activity in the 4.4S peak represented approximately 20% of the total binding activity. These data therefore support the hypothesis that the 8.5S peak in low salt was composed of approximately 20% 7.7S and 80% 9.1S and is consistent with the DNA–cellulose binding characteristics of these samples as well. In contrast, the negative control samples taken from the experiments described in Figure 1B (samples with molybdate but without salt) sedimented at 9.1S (±0.17) (n = 30) (data not shown).

To substantiate further this hypothesis we tested whether we could generate the composite 8.5S peak made up of both the 7.7S form and the 9.1S form by varying the experimental manipulation of a common cytosol sample. We divided a tissue homogenate into three fractions. To one fraction molybdate was added (10 mM final concentration, pH monitored). To fractions two and three buffer was added as a volume control, and cytosol was prepared from each fraction. Fractions were labeled with [^{3}H]5α-dihydrotestosterone for 1 h, and to fraction two KCl (500 mM) was added to transform fully the receptor. Fractions one and three received volume controls. All fractions were allowed to incubate for an additional 3 h, dialyzed to lower the salt concentration, and analyzed on low ionic strength density gradients. The results of this experiment are shown in Figure 4. Fraction one (to which molybdate was added) migrated as 9.1S. Fraction two (which received KCl) sedimented at 7.7 S. Fraction three (which received neither molybdate nor salt and was analogous to the negative controls in Figure 1A) sedimented at 8.5 S. These results agree with the concept that the 8.5S peak represents a combination of 7.7S transformed receptor and 9.1S nontransformed receptor.

The observation of the composite 8.5S peak of activity in sucrose gradients was dependent on sample preparation and handling. In the absence of molybdate, we noted a gradual shift of receptor population from the oligomeric 9.1S form to

Table I: Interrelationship between Three Molecular Forms of the Androgen Receptor and Observed Physicochemical Properties

sedimentation coefficient[a] (S)	ability to bind to DNA–cellulose[b]	stabilization by molybdate[c]	sensitivity to RNase A[d]	buoyant density[e] (g/cm^3)
9.1 ± 0.17 (n = 30)	−	+	−	1.2848 ± 0.032 (n = 6)
4.4 ± 0.08 (n = 18)	+	NA	−	1.2779 ± 0.034 (n = 4)
7.7 ± 0.15 (n = 17)	+	−	+	1.2459 ± 0.014 (n = 6)

[a] Sedimentation coefficients were determined by vertical tube gradients with 90-min centrifugation. Values presented are the mean of n separate determinations plus or minus the standard error. All values were statistically different at the p <0.01 level as determined by the Student's t test. [b] Ability to bind to DNA–cellulose was determined by DNA–cellulose chromatography. Positive designation (+) refers to >80% binding to DNA–cellulose. Negative designation (−) refers to <3% binding to DNA–cellulose. [c] Stabilization by molybdate refers to the effects of 10 mM sodium molybdate on molecular form. Positive designation (+) refers to the ability of molybdate to stabilize the respective molecular form of the receptor in high ionic density gradients. Negative designation (−) refers to the inability of molybdate to prevent dissociation of the respective molecular form of the receptor to the 4.4S form in high ionic gradients. NA = not applicable. [d] Sensitivity to RNase A refers to the effects of 5 units/mL RNase A at 2 °C for 1 h. Positive designation (+) refers to the ability of RNase A to digest the respective molecular form of the receptor to the 4.4S form. Negative designation (−) indicates no effect of RNase A on the respective molecular form of the receptor. [e] Determined by isopyknic centrifugation through metrizamide gradients.

the 7.7S-sized form with time. Overnight incubation of oligomeric receptor at 2 °C converted all of the receptor population to the 7.7S-sized form with a concurrent increase in binding to DNA–cellulose (data not shown). The conversion was protease sensitive, since a protease inhibitor cocktail (DIFP, 5 mM; bacitracin, 0.1 mM; aprotinin 1 IU/mL; and trypsin inhibitor, 0.1 mM) inhibited the conversion to approximately 50% (data not shown). In addition, increased temperature (20 °C, 15 min) converted all of the oligomeric form to the 7.7S-sized form. As contrasted to the oligomeric form, the 7.7S-sized species appeared to remain stable after brief elevated temperatures and overnight incubation at 2 °C (data not shown). Other studies from our laboratory have shown that the 4.4S monomeric form is quite unstable and

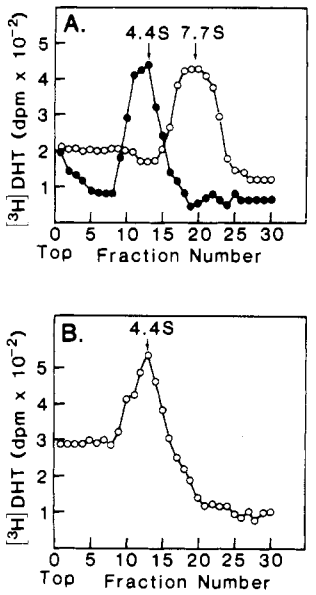

FIGURE 5: Density gradient analysis of intermediate-sized receptor generated from monomer plus 8S promoting factor with and without RNase A digestion. The 4.4S monomeric receptor form was separated from the 8S promoting factor by phosphocellulose chromatography as described in Materials and Methods. (A) The 4.4S monomeric receptor was incubated either with (open circles, 4:1 v/v ratio) or without (filled circles) the 8S promoting factor for 10 min at 2 °C. Samples were analyzed with low ionic strength sucrose density gradients according to procedures described in Materials and Methods. (B) The 4.4S monomeric receptor was incubated (10 min, 2 °C) with 8S promoting factor which had previously been treated with RNaseA (5 units/mL) for 1.5 h at 2 °C. The sample was analyzed with low ionic strength sucrose gradients.

highly susceptible to proteolysis (Johnson et al., 1986). However, the conversion of the 4.4S monomer to the 7.7S intermediate-sized form upon desalting was highly reproducible (Table I). This conversion occurs whether desalting was accomplished either with a 3–5-h dialysis or by immediate dilution. Accordingly, it appears that proteolysis had little if any effect on the sedimentation coefficient value for the 7.7S intermediate-sized receptor form. In addition the salt-induced conversion of the oligomeric species to the monomeric form was not specific for chloride ion. We tested several salts including sodium iodide, potassium chloride, and ammonium sulfate. All agents tested, which increased the ionic strength, resulted in the dissociation of the oligomeric form (data not shown).

In order to study more directly the 7.7S form, we isolated the 4.4S monomer by two different methods and tested the conversion to 7.7S. The 4.4S monomer was isolated in a partially purified state by phosphocellulose chromatography. As shown in Figure 5A, when the phosphocellulose column flow-through (fractions containing the 8S promoting factor) was added to the 4.4S receptor, a peak of activity was observed at 7.7 S. The flow-through fractions alone contained no androgen receptor binding activity.

To further support this concept, we isolated 4.4S receptor fractions directly from high ionic density gradients. To these fractions was added the phosphocellulose flow-through (1:1, v/v). The preparation was then dialyzed to lower the salt concentration and analyzed on low ionic density gradients. The receptor migrated as a peak of activity at 7.7 S identical with those data shown in Figure 5A. These data support the hypothesis that the 7.7S intermediate receptor is generated directly by association of 4.4S monomer with a putative molecular component(s) described previously as the 8S promoting factor.

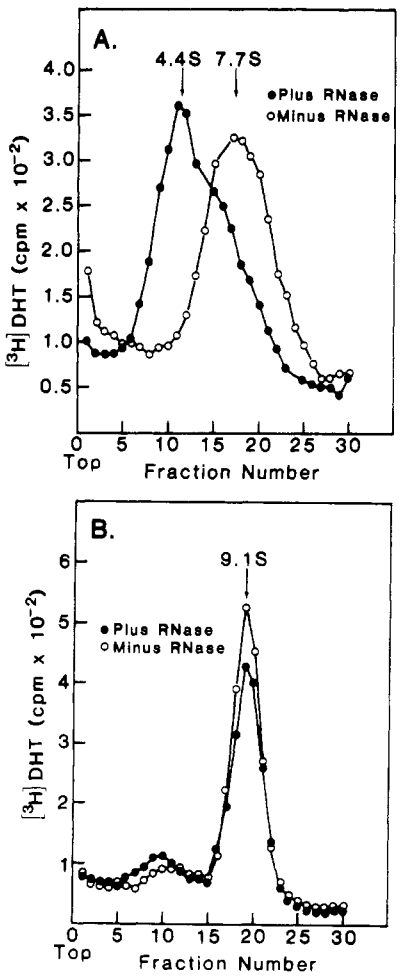

FIGURE 6: Density gradient centrifugation of intermediate-sized (A) and oligomeric (B) receptor with and without RNase A digestion. (A) Cytosol was prepared in TTES buffer, labeled with [^{3}H]dihydrotestosterone (3 nM) for 1 h, and incubated with KCl (500 mM) for an additional 3 h. The sample was diluted with TTES buffer in order to lower the salt to <30 mM KCl and incubated either with (filled circles) or without (open circles) 5 units/mL RNase A (previously boiled for 10 min) for 15 min at 20 °C. Samples were analyzed by verticle tube sucrose density gradient centrifugation as described in Materials and Methods. (B) Density gradient analysis of oligomeric receptor with and without RNase digestion. Cytosol was prepared in TTES buffer and labeled with [^{3}H]dihydrotestosterone (3 nM) during centrifugation. Samples were incubated either with (filled circles) or without (open circles) 5 units/mL RNase A for 1 h at 2 °C.

Ribonucleoprotein Characteristics of the 7.7S-Sized Receptor. Since the 7.7S-sized receptor appeared to result from the association of receptor monomer (4.4S) with the 8S promoting factor under low ionic conditions, we next were interested in the nature of the 8S promoting factor and the resulting 7.7S-sized form. Several reports (Liao et al., 1980; Tymoczko et al., 1982; Hutchens et al., 1982; Sherman et al., 1983; Tymoczko & Phillips, 1983) have previously suggested that larger forms (5–8S) of steroid receptors may contain RNA or ribonucleoprotein as evidenced by ribonuclease digestion studies. Accordingly, we tested ribonuclease digestion of both the oligomeric form and the 7.7S-sized form of the androgen receptor. Receptor was prepared in the 7.7S form and incubated either with or without 5 units/mL of RNase A for 15 min at 20 °C or for 1 h at 2 °C and then analyzed in low ionic strength gradients. Figure 6A shows that treatment with RNase A converted the majority of the 7.7S receptor to 4.4S. The degree of digestion with RNase A was somewhat variable. In some experiments there remained a

shoulder of activity on the 4.4S peak as evidenced in Figure 6A. In other experiments the 4.4S peak of activity displayed a narrower base. Both elevated and lower temperature conditions showed identical patterns of digestion. To test whether the RNA-containing factor might be associated only with the 7.7S form or whether it exists as a separate entity under high ionic conditions, we prepared 4.4S form in high ionic buffer and incubated this preparation either with or without RNase A prior to dialysis. Under these conditions the RNase digestion prevented the 4.4S to 7.7S conversion (data not shown), indicating the RNA-containing factor exists free in solution under high ionic conditions and does not associate with the 4.4S receptor until the ionic strength is lowered.

We wondered if the factor that interacted with 4.4S receptor might be free RNA. We therefore tested whether 4.4S receptor would interact with total RNA extracted from Dunning tumor tissue to form a 7.7S species. The 4.4S receptor was prepared in a partially purified state by phosphocellulose chromatography as described earlier. Receptor prepared in this manner was incubated with increasing concentrations of total RNA extracted from Dunning tumor (20 ng–400 μg/0.2 mL) by the method of Chirgwin et al. (1979) for 1 h at 2 °C. In the absence of RNA the receptor migrated as a 4.4S species. The lowest concentration of RNA tested was a 10:1 w/w ratio of RNA to receptor (on the basis of a receptor M_r of 120000). Both this ratio and a 100:1 ratio produced no differences in sedimentation as compared to control. In contrast, a ratio of 1000:1 produced a smear of activity throughout the middle of the gradient without a discernable peak of activity (data not shown). With concentration ratios of 10000:1, a plateau of receptor activity was observed toward the bottom third of the gradients with a distinct peak observable in the bottom fractions. With concentrations higher than 10000:1, a single distinct peak was observed in the bottom fractions of the gradient beyond our molecular weight standards (i.e., greater than 11.3S, data not shown). Labeled steroid incubated with RNA alone sedimented in the free steroid region of the gradient. These data demonstrate that the 4.4S receptor will combine directly with RNA to generate a heterogeneous population of larger sedimenting forms. However, they suggest that the RNA species, which combines with the 4.4S form to generate the 7.7S form, is not free RNA since no single 7.7S form was generated by any of the concentrations of RNA tested.

Since RNase digested the 7.7S receptor, we tested whether RNase might also digest the 9.1S receptor in a similar fashion. These experiments could not be performed at the elevated temperatures, since warming the 9.1S receptor to 15 °C for 20 min converted most of it to the 7.7S form. Hence, only the lower temperature incubation conditions were used. In addition, the receptor was labeled with [^{3}H]dihydrotestosterone during the centrifugation step to decrease the time of preparation in order to maintain the receptor in the 9.1S form. The total time from homogenization to gradient analysis was under 2 h for these studies. Figure 6B shows 9.1S receptor incubated either with or without RNase A (5 units/mL) for 1 h at 2–4 °C. RNase did not affect the receptor form. Elevated concentrations of RNase A (up to 25 units/mL) and increased incubation periods (2 h) were tested and similarly did not change the sedimentation profile. The sensitivity of the receptor forms to RNase digestion and a summary of the sedimentation properties, DNA–cellulose binding properties, and stabilization by molybdate are presented in Table I.

The digestion of the 7.7S-sized receptor form with RNase A appears to affect principally the 8S promoting factor component of this molecule. Ribonuclease A digestion was effective under high ionic conditions (400 mM KCl), since when the salt concentration was lowered there was not a conversion of 4.4S to 7.7S observed in the ribonuclease A treated sample. In addition, as part of those experiments discussed previously in Figure 5, when 8S promoting factor was isolated by phosphocellulose chromatography and incubated with ribonuclease A (5 units/mL, 1 h at 2 °C), the ability to convert 4.4S monomer to the 7.7S-sized form was abolished (Figure 5B).

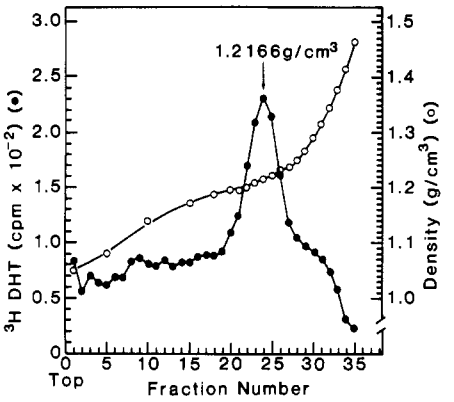

FIGURE 7: Buoyant density gradient analysis of intermediate-sized receptor with metrizamide. Cytosol was prepared in TTES buffer, labeled with [^{3}H]dihydrotestosterone (3 nM) for 1 h, and incubated with KCl (500 mM) for an additional 3 h. The sample was dialyzed against TTE buffer to lower the ionic strength and remove sucrose. Samples were analyzed with centrifugation through metrizamide gradients as described in Materials and Methods. Aliquots were removed from each sample and analyzed additionally with sucrose gradients in order to assess the molecular form of the receptor.

In order to control potential impurities in the ribonuclease A preparations and/or potential protease activity, we tested several parameters. All three sources of ribonuclease A were electrophoretically pure and were boiled for 10 min to destroy any possible residual proteases before being used. As a control to test for the possibility that RNase might bind to receptor directly and thus alter its sedimentation, we used ribonuclease A affixed to Sepharose beads in a column chromatography step. The 7.7S-sized receptor was applied, and only monomeric form (4.4S) was observed in the column flow-through (data not shown). In addition, ribonuclease A void of enzymatic activity (through oxidation) was incubated under identical conditions and was shown not to affect the sedimentation of the 7.7S-sized receptor.

To further confirm the hypothesis that RNA was associated with the 7.7S-sized receptor, we analyzed receptor preparations with isopyknic centrifugation through metrizamide gradients to determine buoyant densities. According to published results, ribonucleoproteins demonstrate a buoyant density of 1.20–1.25 g/cm^3 in metrizamide (Rickwood & Birnie, 1975). Free soluble nucleic acids range from 1.11 to 1.15 g/cm^3, and fully hydrated polypeptides are more dense in the region of 1.25–1.30 g/cm^3. As shown representatively in Figure 7 and summarized in Table I, the 7.7S-sized receptor sedimented with an average density of 1.2459 ± 0.014 g/cm^3 (n = 6) in metrizamide gradients. As summarized in Table I, the monomeric (4.4S) form and the oligomeric (9.1S) form demonstrated a slightly higher density of 1.2779 ± 0.034 (n = 4) and 1.2848 ± 0.032 g/cm^3 (n = 6), respectively (p = 0.1 and 0.02). The value for the 7.7S-sized receptor falls in the dense side of the region typically referred to as ribonucleoprotein (Rickwood & Birnie, 1975).

In order to control the possibility that the 7.7S receptor might be dissociating or unstable in metrizamide, we also tested

preparations of 7.7S receptor that were cross-linked with formaldehyde according to procedures described by Foekens et al. (1985). These preparations gave distinct peaks of binding activity with a sedimentation coefficient of 7.7 S in both high and low ionic sucrose gradients, indicating an effective cross-linking and a resistance to dissociation. When these samples were analyzed with metrizamide gradients, the buoyant densities were identical with our previous results. These results are consistent with and confirm the hypothesis that the 7.7S-sized receptor contains RNA and shows characteristics of a ribonucleoprotein. Table I provides a summary of these results and contrasts the properties of the three forms of the androgen receptor.

Discussion

The observations reported here describe an intermediate-sized 7.7S form of the androgen receptor which exists as a separate entity and with distinct properties different from the 4.4S monomer and the 9.1S oligomeric species reported previously. It is clear that the intermediate-sized receptor results from the association of receptor momoner with an as yet unidentified moiety, referred to previously as "8S promoting factor" (Colvard & Wilson, 1981). Our results here suggest that the 7.7S-sized receptor is a ribonucleoprotein. Accordingly, the intermediate-sized receptor demonstrated a buoyant density within the ribonucleoprotein range. However, the density is suggestive of a higher density ribonucleoprotein. This density is consistent with a hypothesis that the amount of RNA in this form is low, relative to the amount of polypeptide. Of interest to our studies was a previous report by Baskevitch and Rochefort (1981) characterizing the buoyant densities of various forms of uterine estrogen receptor using metrizamide. They reported the "8S cytosol" estrogen receptor to band at a density of 1.238 ± 0.002 g/cm^3 and concluded that this corresponded to a "low density protein or a high density nucleoprotein". The heat-transformed 5S estrogen receptor had a significantly higher density of 1.257 ± 0.002 g/cm^3. These results are consistent with our observations of a lower density for the 7.7S form as compared to the 4.4S monomer.

In addition, our results suggest that the RNA component is important for the structural integrity of the intermediate-sized form, since ribonuclease A digestion results in dissociation to the monomeric form. The 8S promoting factor may itself be a ribonucleoprotein. It is also possible that RNA may act separately, as a bridge between the 4.4S receptor monomer and the 8S promoting factor. Although the oligomeric form (9.1S) of the receptor was not digestable with ribonuclease, we cannot rule out the presence of RNA in this species, since ribonuclease digestion may have been prevented by conformational characteristics and/or steric hinderance. Although the isodensity points of both the monomeric and oligomeric species in metrizamide were in the range to be considered low-density proteins, it should be noted that there existed a variation as reflected by the standard error of the mean values. Hence, a minor composition of RNA cannot be ruled out. Related to this discussion are the studies of Tymoczko and associates (Tymoczko & Lee, 1985; Anderson & Tymoczko, 1985). They have shown that a ribonuclease-sensitive 7–8S form is generated from the 9–10S oligomeric form of the rat liver glucocorticoid receptor. Moreover, they have shown that a small molecular weight factor ($M_r < 500$), which is neither protein or nucleotide, will convert the 7–8S form to the 9–10S form. These data suggest that RNA is a component of both the 7–8S form and the 9–10S form and that loss of a low molecular weight factor from the 9–10S form converts it to the 7–8S ribonuclease-sensitive DNA-binding form. It is not known whether conversion of the oligomeric form of the androgen receptor in low ionic buffer with time is the result of direct conversion of 9S to 7.7S or proceeds through a complete dissociation of oligomer to monomer followed by monomer binding to another molecule (8S promoting factor) to create the 7.7S-sized form. Currently, without having both species purified or monoclonal antibodies to the purified species, it is difficult to address this question in greater detail.

The nontransformed oligomeric species of steroid receptors has been reported to have a sedimentation coefficient ranging from 8S to 10S (Grody et al., 1982; Sherman et al., 1983; Tindall et al., 1984). In addition, there has existed in the literature a certain degree of variation regarding other properties of the putative oligomeric receptor such as size, molecular weight, isoelectric point, DNA-binding properties, etc. Our data would suggest that one possible reason for this variation of values is that the "cytosolic receptor" in low ionic strength buffer may exist as a population made up of two different receptor forms, oligomeric form and intermediate-sized form, each with its own set of characteristics. Moreover, our data suggests that the conversion of oligomer to intermediate-sized form is a continual process and probably results from proteolytic activity. Indeed, the care of receptor preparation, centrifugation time and temperature, the length and temperature of labeling with steroid, and the length of time for analysis, could all contribute to the degree (percentage) of conversion from oligomeric form to intermediate-sized form. We have also observed considerable differences in degree and rate of conversion among different tissues. This was expected, given the apparent proteolytic nature of the conversion and the variable milieu of proteases exhibited between different tissues. Hence, depending on the tissue, the conditions and care of cytosol preparation, and the time of labeling, the observed peak of binding activity in low ionic sucrose density gradients could range from a higher sedimentation coefficient of the oligomeric form to a lower sedimentation coefficient of the intermediate-sized form.

The possible relevance of the 9S, 7.7S, and 4.4S androgen receptor species described in this report is not yet understood. Further studies are in progress to define more fully the nature of the interaction of androgen receptor with RNA and the potential physiological significance.

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Assessment of Mechanistic Proposals for the Binding of Agonists to Cardiac Muscarinic Receptors†

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ABSTRACT: *N*-[^{3}H]Methylscopolamine has been used to characterize muscarinic receptors in crude homogenates prepared from hearts of Syrian golden hamsters. The Hill coefficient is one for specific binding of the radioligand itself and for its inhibition by muscarinic antagonists; markedly lower values are obtained for its inhibition by muscarinic agonists. The binding patterns of agonists have been analyzed in terms of a mixture of sites differing in affinity for the drug and reveal the following. (1) All agonists discern at least two classes of receptor in atrial and ventricular homogenates. (2) The number of classes and the relative size of each differ for different agonists in the same region and for the same agonist in different regions. (3) Atrial and ventricular affinities are in good agreement for some agonists but differ for others. (4) Guanylyl imidodiphosphate (GMP-PNP) is without effect on the specific binding of the radioligand but alters the binding of carbachol via an apparent redistribution of receptors from one class to another; the apparent affinity at either class remains unchanged. (5) Carbachol reveals two classes of sites in ventricular preparations, and the nucleotide mediates an interconversion from higher to lower affinity; three classes are revealed in atrial preparations, and the nucleotide eliminates the sites of highest affinity with a concomitant increase in the number of sites of lowest affinity. Taken together, the data are incompatible with the notion of different, noninterconverting sites; rather, there appear to be several possible states of affinity such that the equilibrium distribution of receptors among the various states is determined by the tissue, by the agonist, and by neurohumoral modulators such as guanylyl nucleotides. The effects of agonists and GMP-PNP cannot be rationalized in terms of a ternary complex model in which the low Hill coefficients arise from a spontaneous equilibrium between receptor (R) and G protein (G) and in which agonists bind preferentially to the RG complex.

Hill coefficients for the specific binding of drugs in equilibrium with membrane-bound, muscarinic receptors are near or equal to 1 for antagonists and significantly lower for agonists [for reviews, see Birdsall et al. (1979, 1980a), Ehlert et al. (1981), and Sokolovsky et al. (1984)]. In the absence of evidence for negative, homotropic cooperativity (Birdsall et al., 1978; Ellis & Hoss, 1980), the data are well described by a scheme in which a population of receptors is homogeneous with respect to antagonists and heterogeneous with respect to agonists. The paradox inherent in this model is clarified somewhat by reports that the binding of agonists is sensitive in a noncompetitive fashion to sulfhydryl-specific reagents,

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