A Common Molecular Weight of the Androgen Receptor Monomer in Different Target Tissues†

M. P. Johnson,‡ C. Y. F. Young, D. R. Rowley, and D. J. Tindall*

Department of Cell Biology, Baylor College of Medicine, Houston, Texas 77030

Received December 3, 1986; Revised Manuscript Received January 14, 1987

ABSTRACT: Previously reported molecular weights for the monomeric steroid binding subunit of the androgen receptor protein have ranged from 25 000 to 167 000. The molecular weight appeared to vary among different species and target organs, as well as between different investigators. This study has examined androgen receptors from a diverse group of organs and species to determine whether these tissues share a common monomeric form. Gel filtration revealed peaks of specific [^{3}H]dihydrotestosterone binding activity corresponding to Stokes radii of 54, 33, and 20 Å in cytosols from several tissues. Phosphocellulose chromatography diminished the appearance of the smaller androgen receptor forms and facilitated the appearance of the larger 54-Å form. Mixing experiments suggested that phosphocellulose was stabilizing the 54-Å form by binding putative proteases which cleave this larger form. Methods were developed to generate homogeneous preparations of a given androgen receptor size for comparative study. Sucrose density gradient analysis showed sedimentation coefficients of 4.5–5.0, 3.5–4.0, and 2.5–3.0 S, respectively. The corresponding calculated molecular weights were 109 000–121 000, 52 000–59 000, and 22 000–27 000. Scatchard analysis of each of these androgen receptor forms demonstrated very similar affinity for [^{3}H]dihydrotestosterone ($K_d \sim 1$ nM), and each form possessed the ability to bind to DNA–cellulose. Extensively purified preparations of androgen receptor from R3327 tumor contained varying amounts of the three receptor forms even though molybdate and phosphocellulose were used to stabilize the androgen receptor protein during purification. It is concluded that androgen receptors from a variety of tissues share a common monomeric subunit (54 Å, 4.5–5.0 S; ~110 000–120 000) and that stabilization is necessary during analytical and purification procedures to prevent cleavage of the monomer by endogenous proteases.

We (Rowley et al., 1986) have reported recently that three forms of the androgen receptor can be identified on the basis of their sedimentation properties in low or high ionic strength buffers and their ability to bind to DNA–cellulose. When target tissue is homogenized in low ionic buffer, there exists a 9S oligomeric form that does not bind to DNA. Under high ionic conditions, this form dissociates to a 4–5S monomeric form. When the salt concentration is lowered, the 4–5S monomer associates with an unknown component containing RNA to form an intermediate 7–8S species that binds to DNA.

The monomeric form of the androgen receptor has been studied most. Its physicochemical and functional properties have been characterized in cytosol from several target tissues, and it appears that androgen receptor proteins from these various sources are very similar. [See Liao (1977) and Rowley and Tindall (1985) for reviews.] Despite the many similarities, there is a discrepancy regarding the molecular weight (M_r)[1] of the monomeric subunit.

For example, the size of the seminal vesicle androgen receptor varies from 25 000 in sheep (Foekens et al., 1982) to 60 000 in the steer (Chang et al., 1982) to 86 000 (Tindall et al., 1983) or 117 000 (Wilson & French, 1979) in the rat. In addition to these species differences, the molecular weight reported by different investigators for the androgen receptor of rat ventral prostate ranges from 28 000 to 117 000 (Mulder et al., 1983; Traish et al., 1984; Rennie et al., 1977; Chang et al., 1983; Mainwaring & Irving, 1973; Wilson & French, 1979). The molecular weights of androgen receptors from other tissues include 98 000 and 167 000 for rat uterus and calf uterus, respectively (Brinkman et al., 1985; Chang & Tindall, 1983); 106 000 for mouse brain (Attardi & Ohno, 1978); 120 000 for the Dunning R3327H rat prostatic tumor (Rowley et al., 1984); and 120 000 for human hyperplastic prostate (Murthy et al., 1984). It is also important to note that androgen receptors of different sizes have been identified in the same tissue preparation (Lea et al., 1979).

From this survey of published reports, it might be speculated that these discrepancies resulted, at least in part, from real differences in androgen receptor size between (1) various target organs, (2) different species, (3) normal and neoplastic tissues, or (4) male and female tissues. Alternatively, the discrepancies may be attributable to varying degrees of proteolytic breakdown of a common monomeric receptor subunit. In this case, the artifactual differences in receptor size might be the result of tissue-specific differences in protease activity and different laboratory procedures for collecting, storing, and processing tissue. Resolving this issue of receptor size is important for further progress in the area of androgen action. For example, in studies of receptor synthesis and metabolism or studies of

† This research was supported by NSF Grant DCB-8416979 and by NIH Grants CA-32387 and DK-37688 to D.J.T. and AM-35897 to D.R.R.

* Author to whom correspondence should be addressed. Recipient of Research Career Development Award HD-00318 from the National Institutes of Health.

‡ Recipient of Postdoctoral Fellowship HD-06717 from the National Institutes of Health.

[1] Abbreviations: BSA, bovine serum albumin; DFP, diisopropyl fluorophosphate; DHT, dihydrotestosterone; EDTA, ethylenediaminetetraacetate; K_{av}, distribution coefficient; K_d, apparent dissociation constant; M_r, molecular weight; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; Tes, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane.

0006-2960/87/0426-3174$01.50/0

receptor function, it will be necessary to understand the native structure of the receptor and to develop methods for preserving that structure during in vitro analysis and purification.

Therefore, the major objective of this study was to determine whether the monomeric steroid binding subunit of the androgen receptor was the same size in all target tissues and whether smaller forms resulted from in vitro degradation of the monomer by endogenous proteases. Another objective was to develop methods that would preserve the monomer's integrity during lengthy purification or to promote formation of a specific proteolytic form for detailed study.

Materials and Methods

The following materials were purchased: 17β-hydroxy-[1,2,4,5,6,7-^{3}H]-5α-androstan-3-one ([^{3}H]dihydrotestosterone; 141 Ci/mmol), nonradioactive dihydrotestosterone, and [^{3}H]lysine from Amersham; [^{14}C]formaldehyde, EN^{3}HANCE, and [^{14}C]methyl-labeled protein standards (γ-globulins, phosphorylase *b*, BSA, ovalbumin, and carbonic anhydrase) from New England Nuclear; Bio-Gel A-1.5m (100–200 mesh) and Bio-Gel HT (hydroxylapatite) from Bio-Rad; dithiothreitol from Boehringer Mannheim; calcium chloride, dimethylformamide, disodium ethylenediaminetetraacetate (EDTA), glycerol, potassium chloride, 1-propanol, sodium chloride, and sucrose from Fisher; charcoal (Norit A) from J. T. Baker; *N,N'*-methylenebis(acrylamide), acrylamide, and *N,N,N',N'*-tetramethylenediamine from Eastman; X-oMat AR film from Eastman Kodak; gelatin from Knox-gel; DNase (RNase free) from Miles Laboratories; dextran T70 and Sephadex G-75 from Pharmacia; Tris base from Schwarz/Mann; aprotinin, blue dextran, bacitracin, diisopropyl fluorophosphate (DFP), leupeptin, pepstatin, phenylmethanesulfonyl fluoride (PMSF), monothioglycerol, sodium iodide, sodium molybdate, Tes base, and unlabeled protein standards (BSA, catalase, ferritin, γ-globulin, myoglobin, thyroglobulin) from Sigma; 17β-[(bromoacetyl)oxy][1,2,4,5,6,7,16,17-^{3}H]-5α-androstan-3-one ([^{3}H]dihydrotestosterone 17β-bromoacetate) from Dr. Tom Lobl, Upjohn Co.; cellulose phosphate (P11) from Whatman; calf thymus DNA from Worthington.

Animals and Tissues. Steer seminal vesicles were obtained fresh from a local slaughterhouse. Porcine seminal vesicles were supplied by Dr. William L. Duax, Medical Foundation of Buffalo, NY. Ventral prostates and seminal vesicles were obtained from 24 h castrated young adult Sprague-Dawley rats. Uterine tissues were obtained from 7-week-old intact female Sprague-Dawley rats. Copenhagen male and Fisher female rats were purchased from Harlan Sprague-Dawley (Houston, TX) to establish a colony of Copenhagen–Fisher F_1 hybrid rats for propagation of the Dunning R3327 tumors. The R3327H subline was supplied by Dr. Norman Altman of the Papanicolaou Cancer Research Institute (Miami, FL). The R3327G subline was supplied by Dr. J. Isaacs of Johns Hopkins University. Tumors were grown subcutaneously until tumor weight reached 15–30 g. Castrations were performed via the scrotal route under ether anesthesia 24 h prior to sacrifice. Tissues were dissected, minced, frozen immediately in liquid nitrogen, and stored at −100 °C.

Cytosol Preparation. Tissues were thawed in saline for 1–2 min at 22 °C and homogenized in 4 volumes (w/v) of one of the following buffers: TTES (10 mM Tes base, 12 mM monothioglycerol, 1.5 mM EDTA, and 250 mM sucrose, pH 7.4 at 22 °C), TTEGK [TTE, 10% glycerol (v/v), and 400 mM KCl, pH 7.4 at 22 °C], TTEGKI (TTEGK and 5 mM serine protease inhibitor DFP), and TTESM (TTES and 20 mM sodium molybdate, pH 7.4 at 22 °C). The tissues were homogenized with a Polytron PT 10/30 homogenizer (Brinkman, Inc.) at a setting of 5 with five 10-s bursts with 30-s intervals for cooling. The homogenates were centrifuged in a Beckman 75 Ti rotor at 105000*g* for 1 h at 2 °C. The lipid layer of the supernatant fluid was aspirated and discarded. The remaining supernate (cytosol) was used immediately in all cases, except that G tumor cytosol for purification had been passed through phosphocellulose and then frozen and stored at −100 °C.

Steroid Binding Assays. The hydroxylapatite assay described by Peck and Clark (1977) was used to measure binding affinities of the androgen receptor for dihydrotestosterone as described previously (Chang et al., 1982), except that the buffer was TTES. Free steroid was removed from cytosol preparations by incubation of the sample with a pellet of dextran-coated charcoal, which had been prepared according to the method of Korenman (1969). The samples were treated at 0 °C for 5–10 min and then centrifuged at 1500*g* for 10 min.

Preparation of Nuclear Extract. Nuclear extracts of R3327H were prepared according to Barrack et al. (1983). Tissue was thawed and homogenized in 4 volumes of TEDGP buffer [10 mM Tris, 1.5 mM EDTA, 1 mM dithiothreitol, 10% glycerol (v/v), and 1 mM PMSF in 1-propanol, pH 7.4 at 0 °C] by using a Kontes Duall glass homogenizer. The homogenate was centrifuged at 800*g* for 20 min at 4 °C. The pellet was washed 3 times in TEDGP buffer, resuspended in 2 volumes of TDPM buffer (10 mM Tris, 1 mM dithiothreitol, 1 mM PMSF, and 5 mM $MgCl_2$, pH 7.4 at 0 °C) containing 0.1 mg (300 units) of DNase, and incubated 30 min at 0 °C. The treated pellet was recentrifuged, and the supernate was discarded before extracting the pellet with 3 volumes of TEDP buffer containing 0.6 M KCl for 30 min at 0 °C. The sample was centrifuged at 3000*g* for 20 min, and the supernate was labeled for 20 h at 0 °C with 10 nM [^{3}H]dihydrotestosterone. Free steroid was removed by treatment with a pellet of dextran-coated charcoal, and the treated nuclear extract was analyzed by gel filtration and density gradient sedimentation as described below.

Phosphocellulose Chromatography. Phosphocellulose resin was used in two ways. One method, termed "flow-through", was based on the observation of Wrange et al. (1979) and Birnbaumer et al. (1983) that passing nontransformed cytosol through a phosphocellulose column will reduce much of the protease activity. It required application of fresh unlabeled cytosol in low ionic strength buffer to a short column of phosphocellulose (2–4 volumes of cytosol/resin). The nontransformed receptor was passed through this column, and the undiluted flow-through fraction was labeled for 16 h at 0 °C with 12 nM [^{3}H]dihydrotestosterone. Free steroid was removed as described above, and the samples were analyzed to determine the molecular weight of the labeled androgen receptor protein. Another method, termed "batch" treatment, consisted of mixing 6 mL of cytosol in TTEGI buffer with a 3-mL bed volume of phosphocellulose resin washed in the same buffer. [^{3}H]Dihydrotestosterone (12 nM) was added, and the receptor was labeled for 16 h at 4 °C with gentle mixing. It should be noted that, initially, cytosolic receptor prepared in low ionic strength buffer shows little binding to polyanionic resins. However, overnight incubation at 4 °C in the presence of 12 nM [^{3}H]dihydrotestosterone promotes transformation and consequent binding to polyanionic resins such as phosphocellulose or DNA–cellulose (Rowley et al., 1986). This resin was then poured into a 12-mL syringe barrel and sequentially washed with at least 30 bed volumes each of TTEG

buffer, 50 mM KCl in TTEG buffer, and 100 mM KCl in TTEG buffer. The receptor was then eluted in TTEG buffer containing 400 mM KCl. One advantage of the extensive washing was that it reduced the nonspecific steroid binding to 5% or less.

Protease Inhibitor Cocktail. In some experiments, the serine protease inhibitor, DFP, was used alone. Other experiments utilized a complex mixture of inhibitors, similar to that used by Milgrom and colleagues (Logeat et al., 1985), which was designed to provide a broad spectrum of specificities. These inhibitors were added to TTEG or TTEGK buffer in the concentrations (specificities are indicated in parentheses) as follows: 1 mM PMSF (serine), 1 μg/mL pepstatin (carboxyl), 100 μg/mL bacitracin (serine, thiol), 0.1 mM leupeptin (serine, thiol), 77 μg/mL aprotinin (serine), and 1.5 mM EDTA (metallo).

Gel Filtration and Gradient Sedimentation. For gel filtration studies, columns of agarose A-1.5m (1.5 × 86 cm) were equilibrated with TTEGK buffer under a hydrostatic pressure of 40 cm. The columns had a flow rate of approximately 7.5 mL/h, and fractions of 2.25 or 2.0 mL were collected after application of 1.0-mL samples. Radioactivity in aliquots of these fractions was determined by scintillation spectroscopy. The columns were calibrated by using the external standard proteins, which were detected by measuring optical density at 280 nm, as follows: equine heart myoglobin (20.1 Å), bovine serum albumin (35.9 Å), bovine liver catalase (51.3 Å), bovine γ-globulin (52.0 Å), equine spleen ferritin (61.5 Å), and bovine thyroglobulin (86.1 Å). Stokes radii of receptor samples were estimated from linear regression of the known Stokes radii (Miller et al., 1975; Sherman et al., 1980) of external standard proteins vs. $(-\log K_{av})^{1/2}$, where $K_{av} = (V_e - V_0/V_t - V_0)$. V_e is the eluted volume, V_0 is the void volume determined with blue dextran, and V_t is the total volume determined with [^{3}H]lysine. The linear correlation coefficient for the column calibration was 0.98.

Linear sucrose density gradients (6–20% w/v, 5 mL) were prepared in TTEGK buffer. Samples of 300 μL were layered onto gradients and centrifuged in a Beckman SW50.1 rotor for 20 h at 190000g. Fractions of 175 μL were collected from the botton of each tube, and the radioactivity was determined by liquid scintillation counting. Sedimentation coefficients of receptor samples were estimated from linear regression of the external standard proteins as follows: equine heart myoblobin (2.0 S), bovine serum albumin (4.4 S), bovine γ-globulin (6.9 S), and bovine liver catalase (11.3 S).

Molecular weights were calculated according to Siegel and Monty (1966), using the formula: $M_r = 6\pi\eta NaS/(1 - \bar{v}\rho)$, where η is the viscosity of the medium (0.01 g s^{-1} cm^{-1}, N is Avogadro's number [6.02 × 10^{23} (g·mol)$^{-1}$], a is the Stokes radius × 10^{-3} cm, S is Svedberg units × 10^{-13}, $\bar{v}$ is an estimation of the partial specific volume (0.725 cm^3/g, and ρ is the density (1.03 g/cm^3).

Receptor Purification. Androgen receptor was partially purified from R3327G tumor by incorporating methods designed to optimize receptor stability. The affinity resin dihydrotestosterone 17β-hemisuccinyl-3,3′-diaminodipropylamine–Sepharose 4B was synthesized according to the method described by Chang et al. (1982). Cytosol was prepared in TTESM buffer and was applied to a phosphocellulose column. The undiluted flow-through fraction containing molybdate-stabilized, nontransformed receptor was applied to the affinity column, which was washed sequentially with 10 bed volumes of TTES, 10 bed volumes of 0.7 M KCl in TTES, 5 bed volumes of TTES, 5 bed volumes of 30% glycerol in TTES, and 5 bed volumes of TTES. Receptor was eluted by incubation of the resin with 0.1 μM [^{3}H]dihydrotestosterone ± 10 μM nonradioactive dihydrotestosterone in TTES buffer containing 0.3 M NaI and 10% dimethylformamide (v/v) for 20 h at 0 °C. Salt and free steroid of this affinity eluate were removed from the labeled receptor by chromatography on Sephadex G-75. The concentration of KCl was adjusted to 400 mM, and the sample of partially purified receptor was analyzed by gel filtration. The binding affinity (K_d) of the eluted receptor for dihydrotestosterone was determined by Scatchard analysis (Scatchard, 1949). Receptor was also partially purified from the R3327H tumor subline by a combination of differential DNA chromatography and steroid affinity chromatography as published previously (Rowley et al., 1984). Quantitative parameters of the purifications, such as recoveries and total receptor yields at each step, were similar to those reported.

Affinity Labeling. Androgen receptor purified by the above procedure (Rowley et al., 1984) was labeled covalently with 10 nM [^{3}H]dihydrotestosterone 17β-bromoacetate with or without a 1000-fold excess of unlabeled dihydrotestosterone 17β-bromoacetate, following procedures we have described previously (Chang et al., 1982). The samples were labeled for 3 h at 4 °C and then treated for 30 min at 22 °C to effect exchange of ligands and covalent attachment. The samples were then precipitated with 10% trichloroacetate. The pellets were dissolved in application buffer and electrophoresed through sodium dodecyl sulfate (SDS)–polyacrylamide gels. The gels were dried and applied to X-oMat film for fluorography.

DNA–Cellulose Binding Assays. DNA–cellulose was prepared according to methods described previously (Alberts & Herrick, 1971; Coty et al., 1979). Binding of the androgen receptor to DNA was quantitated by using minicolumns of DNA–cellulose as detailed in recent publications (Rowley et al., 1984, 1986). Briefly, the assay required application of labeled cytosol onto columns that were subsequently washed in TTES and then eluted with TTES containing 500 mM KCl. The amount of specifically bound [^{3}H]DHT eluted was compared to the amount applied and was expressed as the percentage bound to DNA–cellulose.

RESULTS

Gel Filtration and Density Gradients. When cytosolic androgen receptor from the androgen-responsive Dunning R3327G tumor was labeled and analyzed by gel filtration on agarose A-1.5 m in high ionic strength buffer, the majority of the [^{3}H]dihydrotestosterone binding activity eluted in a peak corresponding to a Stokes radius of 54 Å (Figure 1A). The data in Figure 1A also suggest that a mixture of smaller receptor forms may have been present that eluted at larger volumes. When an aliquot of the 54-Å peak fraction was subsequently analyzed on 6–20% sucrose gradients in the same buffer, a single peak of bound radioactivity was detected whose sedimentation coefficient was 4.8 S (Figure 1B). This value can be combined with the estimated Stokes radius of 54 Å to calculate a molecular weight for the androgen receptor of 116 000, using the formula of Siegel and Monty (1966).

Since proteolysis was considered as a possible cause for the discrepancy in molecular weight of the androgen receptor, we made use of reports by Wrange et al. (1979) and Birnbaumer et al., (1983) that described how phosphocellulose chromatography reduced or removed protease activity from cytosol. When nontransformed G tumor cytosol was treated by the phosphocellulose flow-through procedure as detailed under Materials and Methods, a single, symmetrical peak of an-

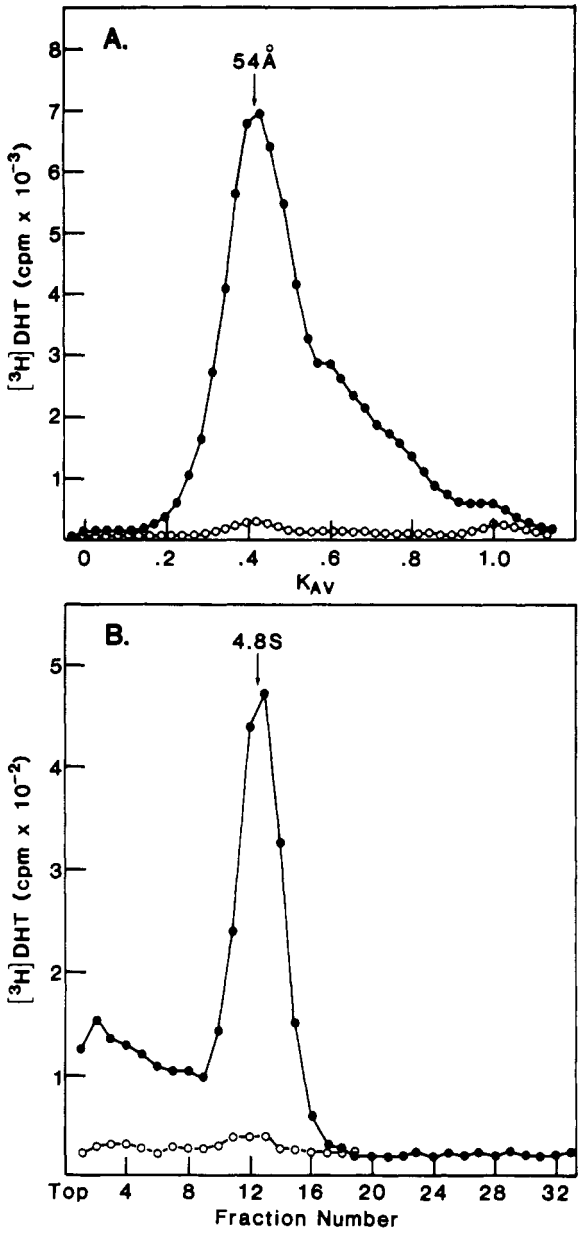

FIGURE 1: Hydrodynamic properties of R3327G rat tumor cytosol androgen receptor. Panel A: Gel filtration chromatography of bound [^{3}H]dihydrotestosterone (DHT) from G tumor that was homogenized in TTES buffer and labeled with 6 nM [^{3}H]DHT in the presence (open circles) or absence (closed circles) of 600 nM nonradioactive DHT during centrifugation for 1 h at 105000g and 2 °C to prepare cytosol. Cytosol was treated with a pellet of dextran-coated charcoal to remove free steroid, and aliquots of 1.0 mL were applied to parallel columns of agarose A-1.5m that were equilibrated and eluted in TTEGK buffer. The columns were calibrated with external standards as described under Materials and Methods. The void volume is indicated by a distribution coefficient (K_{av}) of 0.0, whereas a K_{av} of 1.0 refers to the total column volume. Panel B: Sucrose density gradient sedimentation of androgen receptor from an aliquot of the peak fraction from the gel filtration analysis shown in panel A. The 300-μL sample was layered onto 6–20% sucrose gradients (5 mL) in TTEGK buffer and centrifuged for 20 h in a Beckman SW50.1 rotor at 190000g and 2 °C. Gradients were calibrated with parallel external standard proteins as described under Materials and Methods.

drogen binding activity was detected at 54 Å, with no evidence of smaller binding components (Figure 2). Recovery of androgen receptor in the flow-through fraction of phosphocellulose columns was estimated to be 80% of the activity applied.

Androgen receptors in cytosols from several other androgen target organs and species were labeled with [^{3}H]dihydrotestosterone and analyzed by agarose gel filtration, with or without prior treatment by the phosphocellulose flow-through method. For example, analysis of phosphocellulose-treated cytosol from rat seminal vesicle clearly showed peaks of androgen binding at 54 and 33 Å (Figure 3). Without phosphocellulose treatment, the 54-Å peak was rarely observed; instead the 33-Å form was most often seen. Similar results were obtained in studies of cytosols from steer seminal vesicle, porcine seminal vesicle, and rat uterus. Indeed, the 54-Å receptor was not observed at all in steer seminal vesicle, unless phosphocellulose treatment was employed (data not shown). Therefore, it appeared that phosphocellulose treatment facilitated the appearance of the 54-Å receptor, possibly by stabilizing this larger form.

The phosphocellulose flow-through treatment did not influence the apparent forms of the androgen receptor observed in cytosol from rat ventral prostate. Despite use of this method, the predominant form of the androgen receptor in ventral

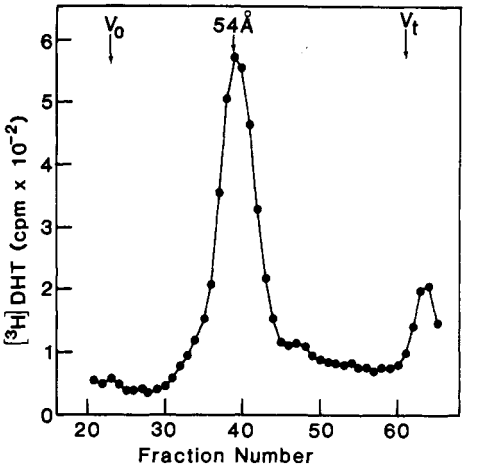

FIGURE 2: Gel filtration chromatography of R3327G tumor cytosol androgen receptor treated by the phosphocellulose flow-through method. Four volumes of nontransformed G tumor cytosol in TTES buffer were applied to a phosphocellulose column (2-mL bed volume) and allowed to drop through. The flow-through fraction was labeled with 6 nM [^{3}H]dihydrotestosterone for 16 h at 0 °C. Free steroid was removed by charcoal treatment, and a sample was analyzed on agarose A-1.5m in TTEGK. V_0 denotes the void volume, and V_t indicates the total column volume.

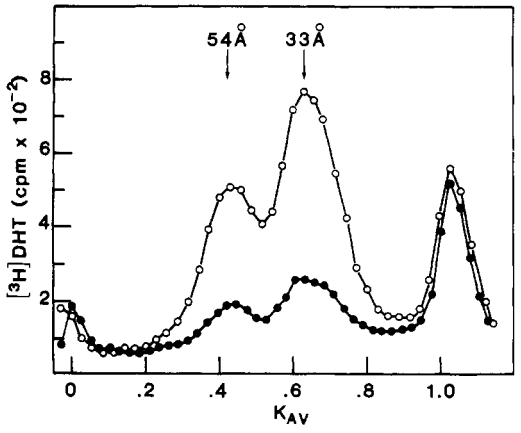

FIGURE 3: Gel filtration chromatography of rat seminal vesicle cytosol androgen receptor treated by the phosphocellulose flow-through method. Cytosol was prepared in TTES buffer and treated with phosphocellulose as described under Materials and Methods and in Figure 2. The flow-through fraction was labeled with 6 nM [^{3}H]-dihydrotestosterone in the absence (open circles) or presence (closed circles) of 600 nM nonradioactive dihydrotestosterone for 3 h at 0 °C. Samples were charcoal treated and analyzed on agarose A-1.5m in TTEGK.

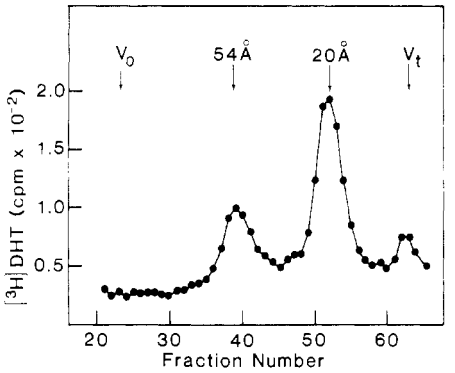

FIGURE 4: Gel filtration chromatography of rat ventral prostate androgen receptor partially purified by the phosphocellulose batch procedure. Prostate cytosol was prepared in TTEGI buffer. Two volumes of the cytosol were mixed with phosphocellulose resin equilibrated in the same buffer. [^{3}H]Dihydrotestosterone (12 nM) was added to the sample prior to incubation for 16 h at 4 °C with gentle mixing to promote binding of receptor to the resin. The resin was poured into a short column and sequentially washed with 30 bed volumes each of TTEG buffer, 50 mM KCl in TTEG buffer, and 100 mM KCl in TTEG buffer over a period of 3–4 h. The resin was eluted in TTEG buffer containing 400 mM KCl, and the peak of [^{3}H]dihydrotestosterone binding activity (1.0 mL) was applied to an agarose gel filtration column (A-1.5m), which was equilibrated and eluted in the same buffer.

prostate cytosol was a 33-Å [^{3}H]dihydrotestosterone binding component, with a variable amount of a 20-Å form seen also in some experiments (data not shown). However, a batch method of phosphocellulose chromatography was used, as described under Materials and Methods, to facilitate detection of the 54-Å androgen receptor in ventral prostate cytosol. Figure 4 shows peaks of prostatic androgen binding activity eluting from the agarose gel filtration column at 54 and 20 Å. Recovery of receptor from the batch procedure was 30% or less.

For comparison to the cytosolic androgen receptor, nuclear androgen receptor was extracted from R3327H tumor of an intact rat, as described under Materials and Methods, and labeled for 20 h at 0 °C prior to analysis. The elution profile from gel filtration chromatography demonstrated a peak of androgen binding activity at 54 Å with a shoulder at 33 Å (data not shown).

It was apparent from these results that the largest form of the androgen receptor that could be observed under conditions of high ionic strength in all androgen target tissues was 54 Å. It was postulated that smaller molecular weight forms of the receptor resulted from in vitro degradation of the 54-Å monomer by endogenous protease(s). Therefore, the following mixing experiment was conducted in order to demonstrate that target organs such as rat ventral prostate contained a putative protease capable of degrading the 54-Å receptor and that this proteolytic activity could be at least partially removed by phosphocellulose.

The mixing experiment required two cytosolic extracts, each prepared separately. The R3327H tumor served as a source of stabilized 54-Å monomer. The R3327H cytosol was prepared in low ionic strength buffer (TTES) and passed through a short column of phosphocellulose resin as described under Materials and Methods to stabilize the 54-Å form. The flow-through fraction containing stabilized receptor was then labeled by 6 nM [^{3}H]dihydrotestosterone in the presence or absence of excess nonradioactive dihydrotestosterone and incubated for 4 h at 0 °C. Free steroid was removed by treatment with a pellet of dextran-coated charcoal. Rat ventral prostate cytosol, prepared in TTES, served as a source of the putative receptor-degrading protease. Prostate cytosol was applied to a similar phosphocellulose column, and the flow-through fraction containing nontransformed receptor was discarded. The phosphocellulose resin was washed with 5 bed volumes (10 mL) of TTES buffer and then eluted with 8 mL of TTEG containing 400 mM KCl. This eluate, containing the partially purified enzyme activity, was incubated in the presence of 800 nM nonradioactive dihydrotestosterone during dialysis for 4 h at 0 °C against TTEG. This step was required in order to decrease the ionic strength of the eluate, and to "mask" any androgen receptor that might be present in the eluate. This eluate was charcoal treated, and a small aliquot was incubated with an equal volume of the H tumor receptor fraction for 4 h at 0 °C. For control, an aliquot of the stabilized H tumor receptor extract was diluted in TTES buffer and incubated in parallel to the mixed sample. Molecular properties of androgen receptors from these diluted and mixed samples were determined by gel filtration and gradient sedimentation analyses. The results shown in Figure 5 demonstrated that the diluted control sample contained mostly the stabilized 54-Å, 4.8S (M_r ~116 000) monomeric androgen receptor with a small amount of the 33-Å form. In contrast, mixing with the phosphocellulose eluate, which was derived from ventral prostate cytosol, resulted in a single peak of binding activity corresponding to 33-Å, 3.9S (M_r ~58 000). The same 33-Å form was observed when equal masses of H tumor and ventral prostate were homogenized together in TTES buffer, labeled 20 h at 0 °C, and analyzed by gel filtration under high ionic conditions (data not shown).

Comparison of Three Androgen Receptor Forms. The above studies revealed primarily three sizes of androgen receptors from the various target organs as determined by gel filtration analysis: 54, 33, and 20 Å. In order to study and compare other properties of these forms, such as their affinity for DHT and the ability to bind DNA–cellulose, it was desirable to find conditions that would generate a relatively homogeneous preparation of a given receptor form. As suggested by Figure 2, the 54-Å form could be uniformly prepared in a stabilized form if R3327 tumor cytosol were treated by the rapid and easy phosphocellulose flow-through method and labeled for 20 h at 0 °C. Moreover, the mixing experiments demonstrated that the 33-Å form could be generated reproducibly by homogenizing equal amounts of R3327 tumor and rat ventral prostate in low ionic strength buffer and labeling for 20 h at 0 °C.

During the planning of experiments designed to generate the 20-Å form, it was noted that several investigators had reported the use of calcium to stimulate proteolysis of the progesterone (Sherman et al., 1974; Vedeckis et al., 1980) and glucocorticoid (Naray et al., 1981; Kalimi et al., 1983; Sherman et al., 1983a,b) receptor proteins to form a small 20-Å "meroreceptor". Similiar conditions were used in several unsuccessful attempts to generate a 20-Å form of the androgen receptor from R3327 tumor and rat ventral prostate by calcium-activated proteolysis. Concentrations of calcium ranging from 100 to 400 mM had no effect. However, it was determined empirically that uniform preparations of a 20-Å, 3.0S form could be generated reproducibly by homogenizing rat ventral prostate in high ionic strength buffer (TTEGK) and labeling for 20 h at 0 °C (see Figure 6). It is important to note that, for the experiment depicted in Figure 6, the buffer contained a mixture of protease inhibitors (see Protease Inhibitor Cocktail under Materials and Methods). The 20-Å form could also be generated in the absence of any protease inhibitors (data not shown), but the appearance of this small

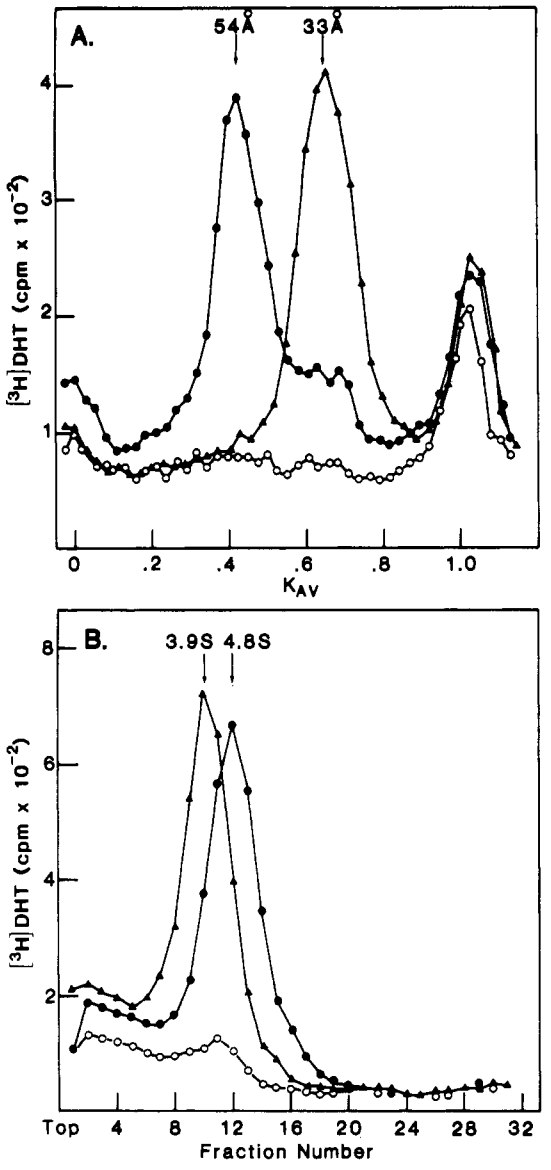

FIGURE 5: Hydrodynamic properties of stabilized and proteolyzed cytosol androgen receptor from R3327H tumor. Tumor cytosol was treated by the phosphocellulose flow-through procedure described under Materials and Methods and labeled with 6 nM [^{3}H]dihydrotestosterone in the presence (open circles) or absence (closed circles and triangles) of 600 nM nonradioactive dihydrotestosterone for 4 h at 0 °C. Free steroid was removed by charcoal treatment. Nontransformed cytosol from rat ventral prostate was applied to a second phosphocellulose column, and the flow-through fraction was discarded. The phosphocellulose column was then washed in TTES and eluted with TTEGK. This eluate was dialyzed to low ionic strength in the presence of 800 nM nonradioactive dihydrotestosterone. The eluate was then charcoal treated, and an aliquot was mixed with the phosphocellulose-treated tumor cytosol and incubated at 0 °C for 4 h (closed triangles). A parallel sample of the tumor cytosol was diluted with TTES for control (closed circles). Samples were analyzed by gel filtration (panel A) and sucrose gradients (panel B).

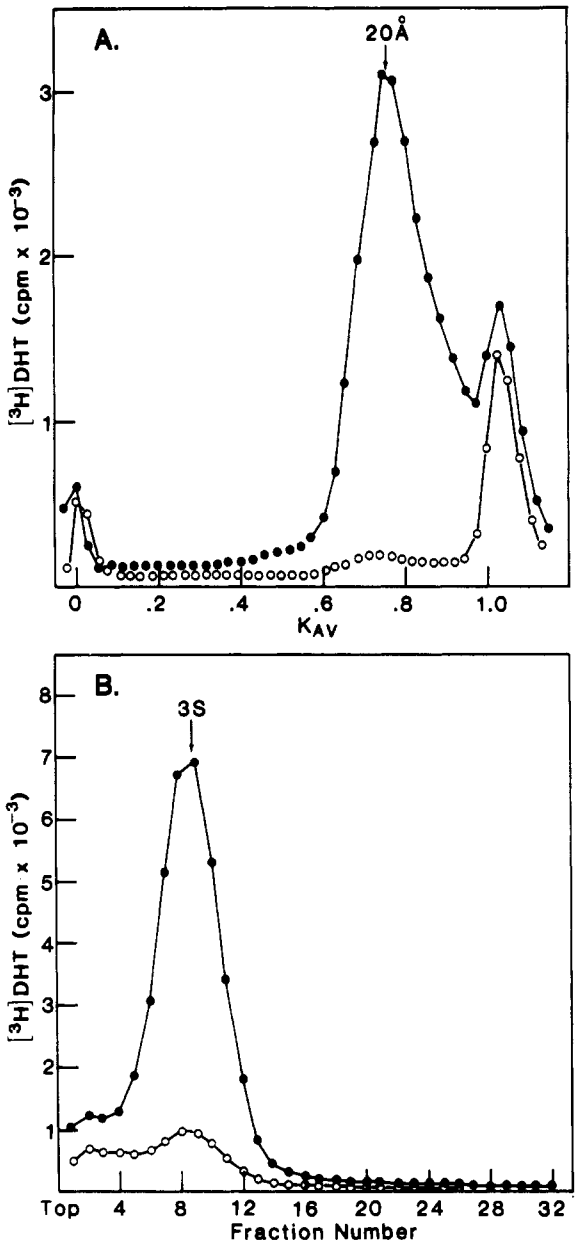

FIGURE 6: Hydrodynamic properties of rat ventral prostate cytosol androgen receptor prepared by homogenizing tissue in high ionic strength TTEGK buffer containing a mixture of inhibitors of serine protease, thiol protease, carboxyl protease, and metalloprotease as described under Materials and Methods. The cytosol extract was labeled for 20 h at 0 °C with 9 nM [^{3}H]dihydrotestosterone in the presence (open circles) or absence (closed circles) of 900 nM nonradioactive dihydrotestosterone. Free steroid was removed by charcoal treatment, and aliquots were analyzed by agarose gel filtration (panel A) and sucrose density gradients (panel B).

form in their presence indicated that the receptor-degrading activity of ventral prostate was uniquely resistant to many protease inhibitors. The serine protease inhibitor, diisopropyl fluorophosphate (DFP), was also ineffective at a concentration of 5 mM. It is also interesting to note that the binding activity of these 20-Å receptor preparations was 10-fold higher if the protease inhibitors were included in the homogenizing buffer and column buffer.

Once these protocols to generate a specific form of the receptor were available, it was then possible to examine and compare other properties. For example, sucrose density gradient sedimentation analysis of the 54-, 33-, and 20-Å receptor forms yielded sedimentation coefficients of 4.5–5.0, 3.5–4.0, and 2.5–3.0 S, respectively. The corresponding calculated molecular weights were 109 000–121 000, 52 000–59 000, and 22 000–27 000. To measure the affinity of these forms for dihydrotestosterone, the respective receptor forms were generated in parallel. During the 20-h incubation at 0 °C, aliquots of each form were incubated with 0.25–10 nM [^{3}H]DHT in the presence or absence of a 100-fold molar excess of nonradioactive DHT. After incubation, androgen binding activity was assayed by using the hydroxylapatite assay as described under Materials and Methods. Aliquots of labeled cytosol for each form were analyzed by gel filtration to confirm that the desired size was generated. Comparisons of the quantitative

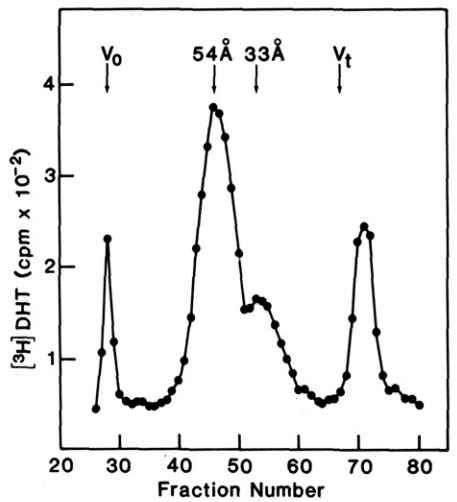

FIGURE 7: Gel filtration chromatography of R3327G tumor androgen receptor purified 2000-fold. Cytosol was prepared in molybdate-containing buffer and immediately passed through a column of phosphocellulose. The undiluted flow-through fraction was applied to an affinity column of dihydrotestosterone 17β-hemisuccinyl-3,3′-diaminodipropylamine–Sepharose 4B. The resin was washed extensively as described under Materials and Methods, and the receptor was simultaneously eluted and labeled with [^{3}H]dihydrotestosterone as outlined under Materials and Methods. Salt and free steroid were removed by chromatography on Sephadex G-75 before analysis on an agarose A-1.5m column. V_0 indicates the void volume, and V_t denotes the total column volume.

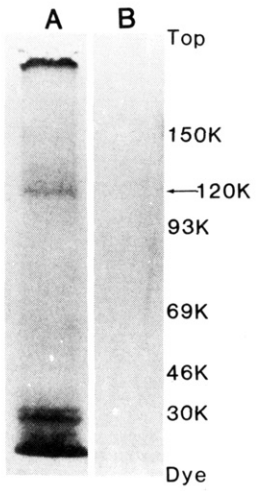

FIGURE 8: SDS–polyacrylamide gel electrophoresis of affinity-labeled androgen receptor. R3327H tumor receptor was partially purified by using a combination of differential DNA chromatography and steroid affinity chromatography as described in Rowley et al. (1984). Equal volumes of samples were precipitated by incubation with 10% trichloroacetate for 15 min. The precipitates were dissolved in 100 μL of sample buffer containing SDS and applied to a polyacrylamide gel with a final acrylamide concentration of 10%. Gel electrophoresis was performed according to the Laemmli (1970) procedure. The gels were calibrated with the following ^{14}C-labeled proteins: human γ-globulin (150 000), phosphorylase A, (97 000), BSA (69 000), ovalbumin (43 000), and carbonic anhydrase (30 000). The gels were treated with EN^{3}HANCE solution and dried. The dried gel was exposed to Kodak X-oMat AR film for fluorographic demonstration of radioactivity. Lane A represents the sample labeled with [^{3}H]-dihydrotestosterone 17β-bromoacetate, and lane B represents sample labeled additionally with a 1000-fold excess of unlabeled dihydrotestosterone 17β-bromoacetate.

binding data were made by using Scatchard analysis to determine the dissociation constant (K_d) for each receptor form. Three such experiments were conducted, and the results did not reveal any differences in binding affinity of these three forms for dihydrotestosterone. The K_d was 0.97 ± 0.76 nM (mean ± standard deviation, n = 9). Another property of all steroid receptor proteins, including the androgen receptor, is the ability of the transformed receptor to bind to DNA. This property has been examined in vitro for the 54-, 33-, and 20-Å receptor forms by using minicolumns of DNA–cellulose as described under Materials and Methods. Results of these experiments showed that all three receptor forms bound to DNA–cellulose, as 50–70% of the applied specific [^{3}H]dihydrotestosterone binding activity was retained and eluted from the resin in all assays.

Molecular Weight of Purified Androgen Receptor. One of the best tissue sources of androgen receptor for purification appears to be the Dunning R3327 rat prostate tumor. This tissue has high concentrations of receptor (150 fmol/mg of protein) with high affinity for dihydrotestosterone (Pollack et al., 1982). In light of the apparent instability of androgen receptors from several target tissues, it was important to examine the molecular weight of the partially purified product to determine if the 54-Å monomeric species could be preserved during lengthy purification.

The purification protocol described under Materials and Methods differed from those published previously (Chang et al., 1982, 1983; Rowley et al., 1984) in that the use of molybdate and the phosphocellulose flow-through step were incorporated to optimize receptor stability. Accordingly, the R3327G tumor androgen receptor was subjected to a purification procedure requiring 2 days for an enrichment of 2000-fold. Other quantitative parameters of the purification, such as recoveries and yields of receptor protein at each step, were similar to our previous studies (Chang et al., 1982). Receptor prepared in this manner was analyzed by gel filtration in high ionic strength buffer as shown in Figure 7. The majority of the binding activity eluted at 54 Å, but a smaller peak corresponding to 33 Å was also observed. The affinity (K_d) of this partially purified receptor for dihydrotestosterone was determined to be 1 nM by Scatchard analysis.

It is interesting to note that when androgen receptor was purified from Dunning tumor by our previous protocol, which did not include molybdate or the phosphocellulose flow-through step, analysis of receptor size by photoaffinity labeling and SDS–polyacrylamide gel electrophoresis showed a band of specific activity at M_r 120 000 and also revealed a band (or doublet) at approximately M_r 25 000–30 000 (see Figure 8).

Discussion

These studies demonstrated that an androgen receptor protein with a common Stokes radius of 54 Å was present in all tissue sources examined; including different species, different target organs, male and female genders, normal and neoplastic tissue, and cytosolic and nuclear extracts. These observations of the 54-Å form, as the largest receptor form seen in all target tissues studied, suggested that the 54-Å molecule might be a common monomeric steroid binding subunit of the androgen receptor. This estimate of the Stokes radius is consistent with our previous study of the androgen receptor from the R3327H tumor subline (Rowley et al., 1984; 61 Å), as well as reports by Attardi and Ohno (1978; 53 Å), Lea et al., (1979; 53 Å), and Wilson and French (1979; 58 Å), in which various rat organs were studied. The molecular weights listed in Table I demonstrate that the results of the present study form the nucleus for a consensus that the molecular weight of the androgen receptor protein monomer of mammalian tissues is approximately 110 000–120 000. Moreover, this estimate of molecular weight was supported by analysis of purified nondenatured receptor (Figure 7) and of affinity-labeled denatured androgen receptor (Figure 8).

Table I: Molecular Weights of Androgen Receptors

tissue source	$M_r \times 10^{-3}$
rat ventral prostate[a,b]	109–121
rat seminal vesicle[a–c]	109–121
rat Dunning H tumor[a,b,d]	109–121
rat Dunning G tumor[a]	109–121
rat testis[b,c]	113–117
rat epididymis[b,c]	113–117
rat uterus[a]	109–121
mouse brain[e]	106
porcine seminal vesicle[a]	109–121
bovine seminal vesicle[a]	109–121
human BPH[f]	120

[a] Results in this paper. [b] Wilson & French, 1979. [c] Lea et al., 1979. [d] Rowley et al., 1984. [e] Attardi & Ohno, 1978. [f] Murthy et al., 1984.

Observations of smaller receptor forms in the gel filtration and density gradient studies were informative also. Peaks of binding activity at 33 and 20 Å appeared in several tissues to varying degrees. The corresponding molecular weights were 52 000–59 000 (3.5–4.0 S) and 22 000–29 000 (2.5–3.0 S), respectively. Other investigators have described androgen receptor forms of 35–38 Å (Chang et al., 1982; Lea et al., 1979; Traish et al., 1984; Wilson & French, 1979) and 20–24 Å (Lea et al., 1979; Rennie et al., 1977; Wilson & French, 1979). The most promising hypothesis to explain the variable presence of these three sizes of androgen receptor in androgen target organs is that the monomeric receptor is being observed in various stages of proteolysis, resulting from tissue-specific differences in the content of endogenous proteases. This hypothesis is supported by the data from the mixing experiments, which suggest a precursor–product relationship between the 54- and 33-Å receptor molecules. Similarly, Wilson and French (1979) reported the presence in rat ventral prostate of a DFP-insensitive protease that converted a 58-Å androgen receptor to 37 Å. The mixing experiment in the present study also demonstrated that phosphocellulose can bind a factor (the putative protease) that is capable of cleaving the 54-Å monomeric androgen receptor. However, phosphocellulose treatments were clearly not 100% effective, especially with target organs such as ventral prostate and seminal vesicle. The variable effectiveness of the procedure may reflect tissue-specific differences in the content of proteases and/or cofactors.

In this study, none of the commonly used protease inhibitors was effective in preventing formation of the 33- or 20-Å receptor form in cytosol from rat ventral prostate (see Figure 6). The complex protease inhibitor cocktail did not stabilize the 54-Å monomer of ventral prostate. Since the inhibitors did not prevent cleavage of the androgen receptor, it was not possible to identify a given class of proteases or to determine whether the 54-Å → 33-Å → 20-Å conversions required progressive cleavage by a single protease or multiple proteases. The observation that the protease inhibitor cocktail substantially increased the yield of androgen binding activity is consistent with the results of a comprehensive study by Prins and Lee (1982) of the effects of various classes of protease inhibitors on androgen binding activity. The mixing experiment in Figure 5, showing quantitative conversion from 54 to 33 Å, implies that proteolytic conversion of receptor size is not necessarily accompanied by loss of steroid binding activity. Moreover, Scatchard analyses of binding data for each of the three androgen receptor forms suggested that the affinity of the steroid binding site for androgens was not adversely affected by the loss of other regions of the molecule. It is reasonable to consider that proteolysis at the steroid binding site might alter its three-dimensional configuration, and thereby could account for loss of binding activity, in a process that might be independent of proteolytic conversions of receptor size.

Table II: Molecular Forms of the Androgen Receptor[a]

	s (S)	R_s (Å)	$M_r \times 10^{-3}$	affinity (K_d) (nM)	DNA binding
oligomer	9	73	280	~1	no
RNA bound	7–8			~1	yes
monomer	4.5–5.0	54	109–121	~1	yes
fragment I	3.5–4.0	33	52–59	~1	yes
fragment II	2.5–3.0	20	22–27	~1	yes

[a] s, sedimentation coefficient; R_s, Stokes molecular radius; M_r, molecular weight.

It appeared from the mixing experiments that the 33-Å receptor fragment was relatively stable at 0 °C in low ionic strength buffer for at least 24 h. Indeed, it was difficult to reproducibly generate the 20-Å fragment until it was empirically determined that homogenization of ventral prostate in high ionic strength buffer (TTEGK) was effective. Although the 20-Å form had been observed to varying degrees in low ionic strength buffers, homogenization under hypertonic conditions was optimal. The benefit of salt extraction during homogenization suggests that the putative protease, which degrades androgen receptor to a 20-Å fragment, might be present in higher concentrations in the nucleus than in the cytosol. Several investigators have reported the use of calcium to induce proteolytic cleavage of the progesterone receptor (Sherman et al., 1974; Vedeckis et al., 1980) and the glucocorticoid receptor (Naray et al., 1981; Kalimi et al., 1983; Sherman et al., 1983a,b) to a meroreceptor of approximately 20 Å that retained the steroid binding site but lacked the property of DNA binding. In contrast, it appears that ventral prostate and the R3327 tumors did not contain a similar calcium-activatable protease. Furthermore, the 20-Å fragment of the androgen receptor differs from meroreceptors of other steroid receptors because it clearly retains the ability to bind to DNA–cellulose. Lea et al. (1979) have also reported the ability of a 22-Å, 3.0S androgen receptor fragment to bind DNA–cellulose. These data imply that the steroid binding domain and DNA binding domain may be localized within a relatively short distance on the receptor molecule. However, these data must be interpreted with caution, since in vitro binding of receptor to nonspecific calf thymus DNA–cellulose may not be related to the ability of androgen receptor to bind within regulatory regions of specific genes in vivo.

In summary, the results of this study resolve the previous discrepancy concerning the molecular weight of the androgen receptor by providing evidence that the native androgen receptor monomer has a molecular weight of approximately 110 000–120 000 in all androgen target tissues. The 33- and 20-Å receptor fragments probably result from proteolysis of the monomer by endogenous enzymes. Procedures were developed to reproducibly generate uniform preparations of the monomer or its two fragments for comparative studies. Table II lists their properties in relationship to the other two androgen receptor forms which have been characterized in this laboratory [the oligomeric form (Rowley et al., 1984, 1986) and the intermediate, RNA-bound form (Rowley et al., 1986)]. It is concluded that the monomeric steroid binding subunit of the androgen receptor is the same size in all target tissues and that stabilization is necessary during analytical and purification procedures.

ACKNOWLEDGMENTS

We thank W. Lyle Harris, Lata Murthy, and James Prescott for technical assistance, David Scarf for artwork,

Debbie Delmore for photography, and Toni Noble for preparation of the manuscript.

REFERENCES

Alberts, B., & Herrick, G. (1971) *Methods Enzymol. 11*, 198–217.

Attardi, B., & Ohno, S. (1978) *Endocrinology (Philadelphia) 103*, 760–770.

Barrack, E. R., Bujnovsky, P., & Walsh, P. C. (1983) *Cancer Res. 43*, 1107–1116.

Birnbaumer, M., Schrader, W. T., & O'Malley, B. W. (1983) *J. Biol. Chem. 255*, 1637–1644.

Brinkman, A. O., Kuiper, G. G. J. M., deBoer, W., Mulder, E., & van der Molen, H. J. (1985) *Biochem. Biophys. Res. Commun. 126*, 163–169.

Chang, C. H., & Tindall, D. J. (1983) *Endocrinology (Baltimore) 113*, 1486–1493.

Chang, C. H., Rowley, D. R., Lobl, T. J., & Tindall, D. J. (1982) *Biochemistry 21*, 4102–4109.

Chang, C. H., Rowley, D. R., & Tindall, D. J. (1983) *Biochemistry 22*, 6170–6175.

Coty, W. A., Schrader, W. T., & O'Malley, B. W. (1979) *J. Steroid Biochem. 10*, 1–12.

Foekens, J. A., Mulder, E., Vrij, L., & van der Molen, H. J. (1982) *Biochem. Biophys. Res. Commun. 104*, 1279–1286.

Kalimi, M., Hubbard, J., & Ray, A. (1983) *J. Steroid Biochem. 18*, 665–671.

Korenman, S. G. (1969) *Steroids 13*, 163–177.

Lea, O. A., Wilson, E. M., & French, F. S. (1979) *Endocrinology (Philadelphia) 105*, 1350–1360.

Liao, S. (1977) *Biochem. Actions Horm. 5*, 351–406.

Logeat, F., Pamphile, R., Loosfelt, H., Jolivet, A., Fournier, A., & Milgrom, E. (1985) *Biochemistry 24*, 1029–1035.

Mainwaring, W. I. P., & Irving, R. (1973) *Biochem. J. 134*, 113–127.

Miller, L. K., Diaz, S. C., & Sherman, M. R. (1975) *Biochemistry 14*, 4433–4443.

Mulder, E., Vrij, A. A., & Brinkman, A. O. (1983) *Biochem. Biophys. Res. Commun. 114*, 1147–1153.

Murthy, L. R., Chang, C. H., Rowley, D. R., Scardino, P. T., & Tindall, D. J. (1984) *Prostate (N.Y.) 5*, 567–579.

Naray, A. (1981) *J. Steroid Biochem. 14*, 71–76.

Peck, E. J., & Clark, J. H. (1977) *Endocrinology (Philadelphia) 101*, 1034–1043.

Pollack, A., Irving, G. L., Block, N. L., Lipton, R. M., Stover, B. J., & Claflin, A. J. (1982) *Cancer Res. 42*, 2184–2190.

Rennie, P. S., VanDoorn, E., & Bruchovsky, N. (1977) *Mol. Cell. Endocrinol. 9*, 145–157.

Rowley, D. R., & Tindall, D. J. (1985) *Biochem. Actions Horm. 13*, 305–324.

Rowley, D. R., Chang, C. H., & Tindall, D. J. (1984) *Endocrinology (Baltimore) 114*, 1776–1783.

Rowley, D. R., Premont, R. T., Johnson, M. P., Young, C. Y. F., & Tindall, D. J. (1986) *Biochemistry 25*, 6988–6995.

Scatchard, G. (1949) *Ann. N.Y. Acad. Sci. 511*, 660–672.

Sherman, M. R., Atienza, S. B. P., Shansky, J. R., & Hoffman, L. M. (1974) *J. Biol. Chem. 249*, 5351–5363.

Sherman, M. R., Tuazon, F. B., & Miller, L. K. (1980) *Endocrinology (Baltimore) 106*, 1715–1727.

Sherman, M. R., Moran, M. C., Tuazon, F. B., & Stevens, Y. W. (1983a) *J. Biol. Chem. 258*, 10366–10377.

Sherman, M. R., Tuazon, F. B., Stevens, Y. W., & Niu, E. M. (1983b) in *Steroid Hormone Receptors: Structure and Function* (Eriksson, H., & Gustafsson, J. A., Eds.) pp 3–24, Elsevier, Amsterdam.

Siegel, L. M., & Monty, K. J. (1966) *Biochim. Biophys. Acta 112*, 346–362.

Tindall, D. J., Chang, C. H., Lobl, T. J., & Rowley, D. R. (1983) *Ann. N.Y. Acad. Sci. 438*, 39–53.

Traish, A. M., Muller, R. E., & Wotiz, H. H. (1984) *Endocrinology (Baltimore) 114*, 1761–1769.

Vedeckis, W., Freeman, M. R., Schrader, W. T., & O'Malley, B. W. (1980) *Biochemistry 19*, 335–343.

Wilson, E. M., & French, F. S. (1979) *J. Biol. Chem. 254*, 6310–6319.

Wrange, O., Carlstedt-Duke, J., & Gustafsson, J.-A. (1979) *J. Biol. Chem. 254*, 9284–9290.