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Biochemical Characterization of the Protein Affinity Labeled by Dihydrotestosterone 17β-Bromoacetate: Comparison with the Human Androgen Receptor*

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ABSTRACT. Dihydrotestosterone 17β-bromoacetate covalently binds to a single protein of 58,000 mol wt from human genital skin fibroblast cytosol. Previous experiments have suggested that this protein is related to the human androgen receptor and may be a proteolytic fragment of the intact protein. In the present study the biochemical properties of the covalently radiolabeled protein were compared to those of the classically defined human androgen receptor radiolabeled noncovalently with [^{3}H]dihydrotestosterone. The radiolabeled proteins were indistinguishable by gel filtration chromatography, sucrose density gradient centrifugation analysis, chromatofocusing, and hydrophobic interaction chromatography. Both ligands labeled a protein with an apparent Stokes radius of 4.4 nm under high salt conditions. Analysis on sucrose density gradients showed peaks of 4.6S and 9.2S with either ligand. The protein radiolabeled with either ligand chromatofocused as two isoforms, a predominant form with a pI of about 5.4 and a minor isoform with a pI of about 4.5. Both radiolabeled proteins were found to have a high degree of hydrophobicity and eluted identically from a phenyl-Sepharose column. While the radiolabeled proteins were qualitatively indistinguishable, significantly more radiolabeled protein was quantitated using the affinity ligand. These data suggest that the affinity ligand may recognize precursor or degraded forms of the receptor that do not bind the natural ligand or that assays based on the use of noncovalent ligands could underestimate the receptor content of target cells. (*Endocrinology* **124:** 1270–1277, 1989)

BIOCHEMICAL studies of androgen receptors in human and animal tissues as well as attempts to purify these proteins have been impeded by the lability and low concentration of these receptors in target tissues. Such difficulties could conceivably be diminished by the use of an affinity ligand capable of efficiently forming covalent bonds with the steroid-binding region of the receptor molecule. Useful affinity ligands for steroid hormone receptors have been of two types: photoreactive ligands and electrophilic affinity labels (1, 2). A synthetic photoreactive ligand, [^{3}H]methyltrienolone, has been used in attempts to covalently label androgen receptors from a variety of tissues (3–8), but has been found to form covalent bonds with efficiencies estimated at only 0.4–6.8%. Chemically reactive affinity ligands, such as tamoxifen aziridine and dexamethasone 21-mesylate, have been shown to be capable of covalently labeling estrogen (9) and glucocorticoid (10) receptors, respectively, with high efficiency. Such a chemically reactive affinity ligand for the androgen receptor, dihydrotestosterone 17β-bromoacetate (DHT-BA), has been previously described (11, 12). We have recently found that his ligand covalently binds to a 58K protein from human genital skin fibroblasts. Nonradioactive DHT-BA competes with high relative affinity for the single specific [^{3}H]DHT-binding site in cytosol from these cells. Radioactive DHT-BA binds with high affinity and specificity to a saturable 58K protein. The affinity-labeled protein is not detectable in androgen receptor-negative cells (13). The present report describes studies of the biochemical characteristics of the affinity-labeled protein. These experiments demonstrate that under nondenaturing conditions the affinity-labeled complex is indistinguishable from androgen-receptor complexes formed with noncovalent ligands. Quantitation of the covalently and noncovalently labeled proteins reveals a 10- to 30-fold greater binding capacity for the affinity ligand.

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Materials and Methods

Affinity ligand synthesis

Nonradioactive DHT-BA was synthesized by the method described by Le Gaillard and Dautrevaux (14) with minor

modifications (13). DHT and bromoacetic acid were dissolved in tetrahydrofuran, and the sample was chilled to −20 C. Dicyclohexyl-carbodiimide and pyridine were added, and the sample was incubated on ice for 4.5 h. The reaction mixture was then filtered through glass wool to remove precipitated dicyclohexylurea, and the dissolved steroid was taken to dryness under a nitrogen stream. The residue was redissolved in chloroform and chromatographed on a silica gel column. The eluted peak of DHT-BA was again dried under a nitrogen stream. The structure of the purified product was confirmed with nuclear magnetic resonance spectroscopy, as previously described (13). Radioactive DHT-BA was synthesized by the method of Chang *et al.* (12). Bromoacetyl bromide was reacted with [1,2,4,5,6,7-^{3}H]DHT (140 Ci/mmol) in the presence of diisopropylethylamine at room temperature. After 4.5 h the solvent was evaporated, and fresh reactants were again added. After 18 h the solvent was evaporated, and the residue was dissolved in hexane-ether (6:4) and purified by chromatography on a silica gel column. Eluted radiolabel was further purified as necessary on TLC plates developed in chloroform-acetone (9:1). The resulting compound was greater than 95% pure as determined by TLC with purified nonradioactive DHT-BA as an internal marker.

Cell culture

Human genital skin fibroblast strains were established from explants of skin obtained from normal circumcisions. Explants were placed in 25-cm^2 flasks and bathed in 20% fetal calf serum in Minimum Essential Medium. When fibroblasts had grown out from the explant, the cells were harvested by trypsinization and sequentially reseeded in new flasks. Stocks of strains were frozen in liquid nitrogen at low transfer number. For experiments cells were grown in medium with 10% fetal calf serum; 24 h before cell harvest the medium was aspirated, the cells were washed with PBS, and serum-free Minimum Essential Medium was added to the plates (15). For some experiments serum-free medium with 500 μg/ml BSA was added for the last 24 h. No quantitative or qualitative differences in the subsequent experimental results were seen using the BSA-supplemented medium.

Preparation of radiolabeled cytosolic androgen receptors

Confluent cell monolayers were washed three times with Tris-saline (50 M Tris, pH 7.5, and 0.15 M NaCl) and harvested by scraping. The cell pellet was homogenized in a glass Dounce homogenizer (Kontes Co., Vineland, NJ) in an equal volume of buffer (20 mM Tris, pH 8.0; 1 mM EDTA; 10 mM Na_2MoO_4; and 100 μM leupeptin). The homogenates was centrifuged at 100,000 × *g* for 60 min at 4 C. The resulting supernatant (cytosol) was used in subsequent experiments. To prepare noncovalently radiolabeled human androgen receptors the genital fibroblast cytosol was incubated at 4 C for 4 h with 5 nM [1,2,4,5,6,7-^{3}H]DHT (140 Ci/mmol). This length of incubation had been previously determined to be sufficient to achieve saturation of the androgen receptor in human genital skin fibroblast cytosol. For covalent radiolabeling the cytosol was incubated at 4 C for 30 min with 5 nM [1,2,4,5,6,7-^{3}H]DHT-BA (also 140 Ci/mmol). This incubation duration had been previously shown to result in greater than 80% maximal incorporation of radiolabel into the 58K protein, with little radiolabeling of other proteins (13).

Gel filtration chromatography

Covalently or noncovalently radiolabeled androgen rec preparations were analyzed by gel filtration chromatog on Bio-Gel A 0.5 M (Bio-Rad, Richmond, CA). Aliquot ml) of radiolabeled cytosol were applied to a 180-ml bed vc (1.6 × 90 cm) column of Bio-Gel A 0.5 M equilibrated in l (20 mM Tris, pH 8.0, with 1 mM EDTA, 10 mM Na_2MoO_4 0.3 M NaCl). The column was eluted with the same buffer the column eluate was monitored for optical density at 28(Two-milliliter fractions of the column eluate were colle and aliquots were counted for radioactivity. The column calibrated with standard proteins of known Stokes radius vine γ-globulin, 5.2 nm; BSA, 3.5 nm; ovalbumin, 2.8 nm). void volume of the column was determined using blue Dex 2000. Stokes radii of receptor preparations were estimate linear interpolation on a plot of Stokes radius of the stanc as a function of $(-\log K_{av})^{1/2}$. $K_{av} = (V_e - V_o/V_t - V_o)$, w V_e is the elution volume of the given protein, V_o is the volume of the column, and V_t is the total column volume

Sucrose density gradient centrifugation analysis

Linear gradients of 5–20% sucrose in buffer (20 mM pH 8.0; 1 mM EDTA; 10 mM Na_2MoO_4; and 0.3 M NaCl) prepared in 5-ml polyallomer tubes using a Beckman der gradient former (Beckman Instruments, Fullerton, CA) some experiments radiolabeled androgen receptors in c cytosol were used. Bound ligand was separated from free lig by treatment of the cytosol with a slurry of dextran-co charcoal (50 mg/ml charcoal and 5 mg/ml Dextran T-7 Tris-EDTA buffer. The charcoal slurry (250 μl/ml cytosol) added directly to the sample, and the tubes were stirred bri and then centrifuged at 2,000 × *g* for 10 min at 4 C. supernatant containing protein-bound radioactivity was t for analysis. The charcoal treatment step was omitted in periments using fractions from gel filtration columns, since peak fractions containing only protein-bound radioacti were used. The samples (200 μl) were applied to the top of sucrose gradients and centrifuged for 18 h at 150,000 × *g* (40, rpm in a Beckman SW 50.1 rotor). The tubes were then pierc and four-drop fractions were collected from the bottom of e gradient. These fractions were counted for radioactivity. external standard proteins used as markers were BSA (4.6 bovine γ-globulin (6.6S), and aldolase (7.9S). The sedimen tion positions of the standard proteins were determined measuring the optical density (at 280 nm) of each gradi fraction.

Chromatofocusing

Radiolabeled cytosol preparations or column fractions w prepared for chromatofocusing by passage through Sephe G-25 columns (PD-10 columns; bed volume, 9 ml; 1.5 × 5 c Pharmacia, Piscataway, NJ) equilibrated in 25 mM imidaz buffer, pH 7.4. The protein-bound radioactivity was applied 1-ml (bed volume) columns of polybuffer exchanger (PBE Pharmacia) equilibrated in the same imidazole buffer.

samples were washed into the columns with 0.3 ml imidazole buffer and eluted with Polybuffer 74 (pH 3). Fractions (0.5 ml) were collected. The pH of each fraction was measured, and an aliquot was counted for radioactivity.

Hydrophobic interaction chromatography

Samples were prepared for chromatography on phenyl-Sepharose by adjustment to 1 M in ammonium sulfate. The high salt solution was then applied to a phenyl-Sepharose column equilibrated in the same buffer. The column was washed until a constant background of radioactivity and protein concentration (as estimated by optical density at 280 nm) was achieved in the eluate. The column was eluted by washing with 50 mM Tris buffer and, finally, water. Collected fractions were monitored for absorbance at 280 nm and for radioactivity.

Gel electrophoresis and fluorography

Affinity-labeled proteins were electrophoretically analyzed on 7.5% polyacrylamide slab gels under reducing conditions using the Laemmli buffer system (17). The gels were fluorographed using diphenyloxazole in acetic acid, as previously described (18).

Quantitation of receptor content of intact cells

Standard whole cell monolayer binding assays (13, 19) were used to quantitate receptor content using either DHT or the affinity ligand DHT-BA. Cells for monolayer binding assays were grown as described above, and the medium was changed to serum-free Minimum Essential Medium 18–24 h before experiments. In some experiments the medium was supplemented with 500 μg/ml BSA for the last 24 h without any discernible effect on subsequent assay results. On the day of the experiment the wells were rinsed with medium without serum and incubated at 37 C for 60 min with medium containing various concentrations (0.05–10.0 nM) of radioactive DHT or DHT-BA. Parallel wells were incubated with radiolabel plus a 500-fold excess of nonradioactive ligand. At the end of the incubation the cell monolayers were washed five times with 50 mM Tris, pH 7.4, and 150 mM NaCl (Tris-saline) with 2 g/liter BSA (fraction V). The cells were washed twice more using Tris-saline and then harvested by trypsinization. The cells were pelleted by centrifugation for 5 min at 800 × *g* and washed twice more in Tris-saline. The final pellet was resuspended in 1 ml water, and the cells were broken by sonication. Aliquots of the sonicate were taken for determination of protein concentration by the method of Lowry *et al.* (20) and for quantitation of bound radioactivity by liquid scintillation counting. Androgen receptor content was estimated by the method of Scatchard (21) in the case of experiments in which full saturation curves were performed or by estimates of specific binding (total binding minus nonspecific binding) in the case of some experiments done in duplicate at a single saturating concentration of ligand. Receptor content was expressed as femtomoles of specifically bound radioactivity per mg cellular protein.

Results

The hydrodynamic properties of the proteins radiolabeled with either the noncovalent or the covalent ligand were evaluated by gel filtration chromatography and sucrose density gradient centrifugation analysis. Gel filtration chromatography (Fig. 1) of cytosolic proteins radiolabeled with either [^{3}H]DHT or [^{3}H]DHT-BA revealed two peaks of bound radioactivity. The first, a minor peak, eluted in the void volume of the column. The major peak of radiolabeled protein eluted with an apparent Stokes radius of 4.4 nm. Major peaks of protein (as detected by absorbance at 280 nm) eluted in the void volume of the column and several fractions after the 4.4-nm peak. No major peak of absorbance coincided with the radiolabeled receptor peak (data not shown). The apparent mol wt of the protein radiolabeled with the covalent ligand as well as that of the noncovalently radiolabeled human androgen receptor was estimated from a plot of the distribution coefficients (K_{av}) of standard proteins as a function of the logarithm of their mol wt (which assumes globularity of the protein). Both proteins had an apparent mol wt of 106,000 by this method of estimation.

Sucrose density gradient centrifugation (Fig. 2) of either the covalently or noncovalently radiolabeled preparations under conditions of ionic strength identical to the gel filtration experiments showed the predominant species sedimenting at approximately 9S. An additional 4–5S moiety was well resolved on the gradient of the affinity-labeled protein, but was not clearly resolved on the gradient of the noncovalently labeled sample because of dissociation of the ligand.

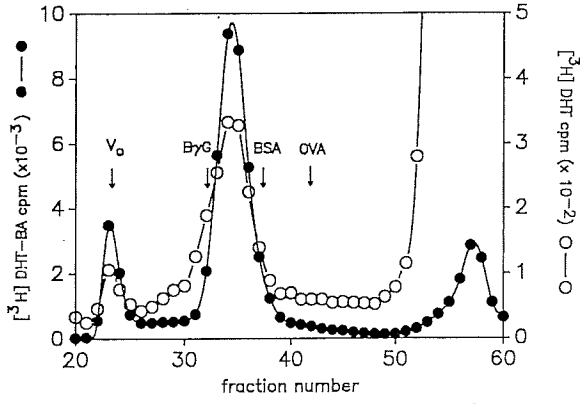

FIG. 1. Gel filtration chromatography of human androgen receptors using covalent and noncovalent ligands. Human genital skin fibroblast cytosol was labeled with [^{3}H]DHT-BA (●) or [^{3}H]DHT (O) and chromatographed on a column of Bio-Gel A (0.5 M). Aliquots of eluted fractions were counted for radioactivity. The column void volume (V_o) was determined using blue Dextran 2000, and the column was calibrated using standard proteins bovine γ-globulin (BγG), BSA, and ovalbumin (OVA). The figure shown is representative of 11 experiments with DHT-BA and 3 experiments with DHT.

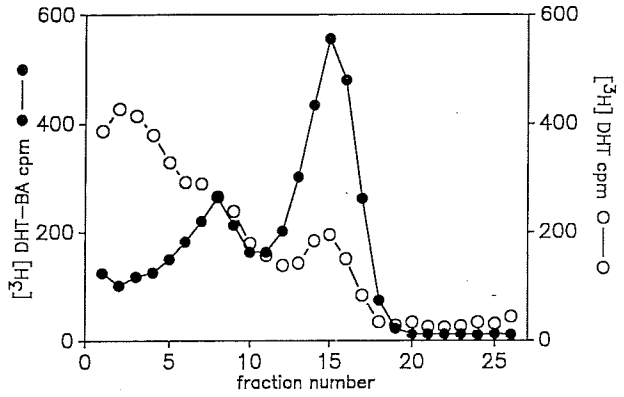

FIG. 2. Sucrose density gradient centrifugation of human androgen receptors using covalent and noncovalent ligands. An aliquot (200 μl) from the peak fraction (no. 34) of affinity-labeled androgen receptor from the gel filtration column (●) was applied to a gradient of 5–20% sucrose in Tris-EDTA-molybdate buffer with 0.3 M NaCl. Noncovalently labeled receptors (O; DHT) were prepared in crude cytosol, charcoal treated to separate bound from free ligand, and applied to an identical gradient. The samples were centrifuged for 18 h at 150,000 × *g*, fractionated into 4-drop fractions, and counted for radioactivity. Fraction O marks the top of each gradient. The experiment shown is representative of 5 experiments with DHT-BA and 10 experiments with DHT.

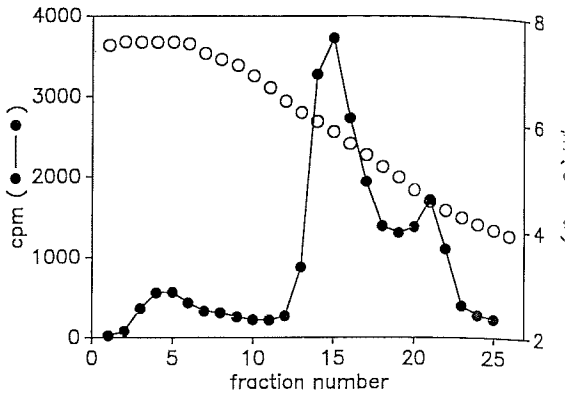

FIG. 3. Chromatofocusing of noncovalently labeled human and receptors. Human androgen receptors (labeled with [3H]DHT) prepared in imidazole buffer (pH 7.4) by passage through a PD- filtration column. The sample was applied to a 1-ml column of P (Pharmacia) equilibrated in the same buffer and eluted with poly (Pharmacia) at pH 3. Eluted fractions (1 ml) were monitored f (O) and counted for radioactivity (●). The experiment shown i resentative of four such experiments with DHT.

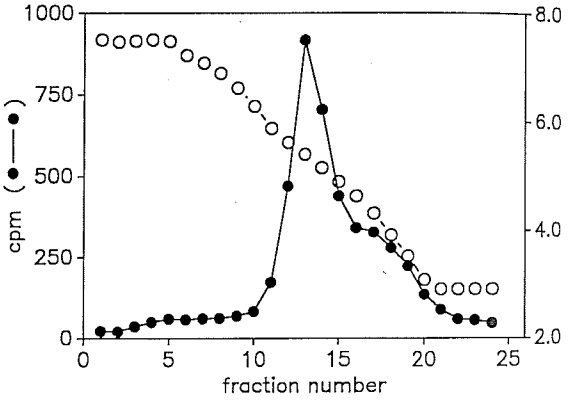

FIG. 4. Chromatofocusing of covalently labeled human androg ceptors. Human androgen receptors (labeled with [3H]DHT-BA prepared in imidazole buffer (pH 7.4) by passage through a PD- filtration column. The sample was chromatofocused as describ Fig. 3. The data shown are representative of five experiments DHT-BA.

The mol wt of the human fibroblast androgen receptor was calculated from the hydrodynamic data described above by the method of Siegel and Monty (22) with the assumption of a partial specific volume of the protein of 0.725 cm^3/g. Based on such data the apparent mol wt of the molybdate- and leupeptin-stabilized nondenatured androgen receptor under high salt (0.3 M NaCl) conditions was calculated to be 170,000 for the 9.2S species and 85,000 for the smaller (4.6S) species. The affinity-labeled protein had an identical calculated mol wt. Under denaturing conditions [*i.e.* on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)] the apparent mol wt of the affinity-labeled protein from either the void volume or the 4.4-nm gel filtration peak was 58,000.

The isoelectric point of the human androgen receptor was determined under nondenaturing conditions by chromatofocusing of receptor preparations labeled with DHT. The noncovalently labeled receptor chromatographed as two isoforms with pI 5.6 ± 0.3 and 4.6 ± 0.3 (n = 4). A typical chromatofocusing experiment using the noncovalent ligand is shown in Fig. 3. The covalently labeled protein also chromatofocused as two isoforms with pI values of 5.3 ± 0.1 and 4.4 ± 0.1 (n = 5). Figure 4 shows one such experiment using the affinity label. While the affinity-labeled protein always chromatofocused as a more abundant approximately pI 5.4 species, the relative amounts of the two isoforms labeled with the noncovalent ligand were more variable. In some experiments with the noncovalent ligand the quantity of receptor in the more acidic isoform exceeded the quantity in the basic species. Denaturing SDS-PAGE of affinity-lal samples from chromatofocusing peaks containing e of the isoforms showed a single radiolabeled prote 58,000 mol wt.

The hydrophobicity of the proteins radiolable DHT and DHT-BA was examined by chromatogr of the respective preparations on columns of ph Sepharose. The affinity-labeled protein was indi guishable from the noncovalently labeled human ar gen receptor by this technique (Figs. 5 and 6). Whil majority of applied protein eluted in the high salt v both covalently and noncovalently labeled pro bound tightly to the hydrophobic interaction columr eluted with water. Buffers at ionic strengths as lo

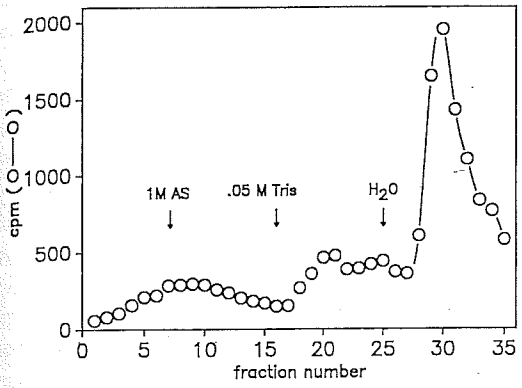

FIG. 5. Hydrophobic interaction chromatography of noncovalently labeled human androgen receptors. Noncovalently labeled human androgen receptors were prepared by incubating human genital fibroblast cytosol with [^{3}H]DHT as described. Bound hormone was separated from free hormone by treating the cytosol with dextran-coated charcoal. The sample was then made 1 M in ammonium sulfate and applied to a phenyl-Sepharose column equilibrated in the same solution. The column was eluted with successive washes of ammonium sulfate (1M AS), 50 mM Tris, and water (H_2O). Column fractions were monitored for radioactivity. The data shown are representative of two experiments.

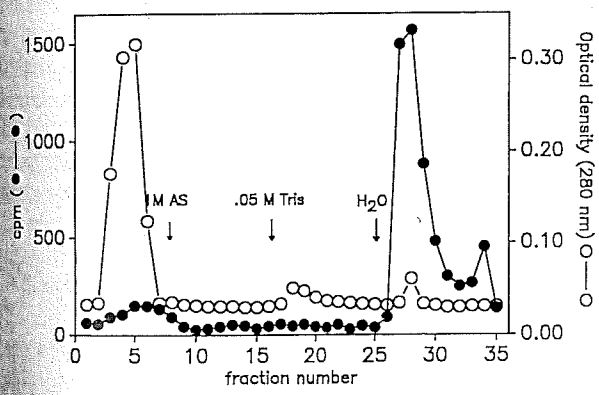

FIG. 6. Hydrophobic interaction chromatography of covalently labeled human androgen receptors. Peak [^{3}H]DHT-BA-labeled fractions (no. 33–36) from a Bio-Gel A (0.5 M) column were made 1 **m** in ammonium sulfate, applied to a phenyl-Sepharose column, and eluted as described in Fig. 5. Column fractions were monitored for optical density at 280 nm (O) and for radioactivity (●). The data shown are representative of five experiments.

that of 10 mM Tris could not elute the receptor from the column (data not shown). The covalently labeled species, analyzed by electrophoresis under denaturing conditions, had an apparent mol wt of 58,000.

A number of experiments were carried out to attempt to determine whether the 58K protein is a proteolytic fragment of a larger intact steroid-binding subunit of the androgen receptor. When intact cells were labeled *in vivo* with the ^{3}H-labeled affinity ligand in monolayer cultures and the cells harvested and broken in the presence of a variety of protease inhibitors (phenymethylsulfonylfluoride, leupeptin, bacitracin, benzamidine, and aprotinin), no change in the apparent mol wt of the radiolabeled protein was seen when the samples were analyzed electrophoretically (Fig. 7). Similarly, when cells were broken in the absence of these protease inhibitors and the cytosolic extract radiolabeled *in vitro* with the affinity ligand, only the 58K protein was observed (data not shown).

Since other electrophilic affinity ligands for steroid hormone receptors effectively label 100% of the receptor

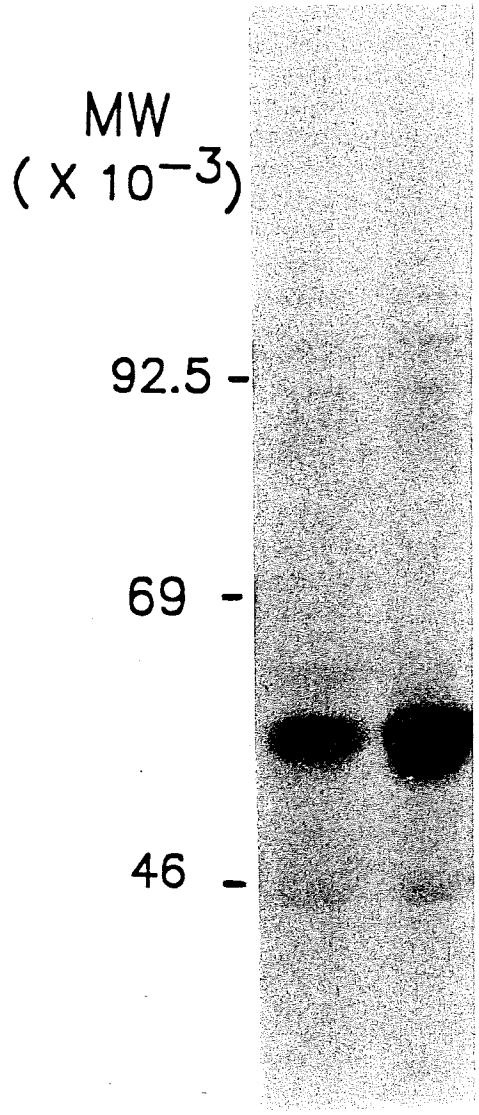

FIG. 7. Effects of protease inhibitors on the apparent mol wt of the affinity-labeled human androgen receptor. Intact monolayers of cells (15-cm dishes) were incubated for 1 h at 37 C with 5 nM [^{3}H]DHT-BA. The cells were washed in ice-cold Tris-buffered saline and harvested. Cells were broken in homogenization buffer (20 mM Tris, pH 8.0; 1 mM EDTA; and 10 mM Na_2MoO_4) in the absence (*left lane*) or presence (*right lane*) of protease inhibitors. MW, Mol wt. The protease inhibitors used were phenylmethylsulfonylfluoride (1 mM), leupeptin (0.15 mM), bacitracin (0.1 mM), benzamidine (1 mM), and aprotinin (1 trypsin inhibitory unit/ml). The fluorograph shown is representative of three experiments.

present in the incubation (9, 10), it was of considerable interest to determine whether DHT-BA was also capable of high efficiency covalent labeling. The amount of protein specifically radiolabeled in cytosol by [^{3}H]DHT and [^{3}H]DHT-BA, respectively, was estimated by quantitating the radioactivity in the major peak from gel filtration chromatography. [^{3}H]DHT binding in that peak averaged 29 ± 10 fmol/mg cytosolic protein (n = 3), in good agreement with reported values for cytosolic binding assays for the androgen receptor (15). In contrast, [^{3}H]DHT-BA binding in this same peak averaged 850 ± 90 fmol/mg protein (n = 12). As noted above, this radiolabeled protein was indistinguishable from the classically defined androgen receptor on the basis of hydrodynamic properties, isoelectric point, or hydrophobicity. Similar results were obtained using the noncovalent and covalent ligands to radiolabel proteins in intact monolayers of cells (Table 1). In six experiments using [^{3}H]DHT as the radioligand an average of 51 ± 11 fmol specific binding/mg cellular protein was found. Published values for normal genital fibroblasts in an identical assay average 33 ± 3 fmol/mg protein (23). In contrast, using [^{3}H]DHT-BA as the radioligand, the amount of specific binding averaged 500 ± 29 fmol/mg cellular protein in experiments using a single saturating concentration of radioactive affinity ligand. When the covalently radiolabeled products from experiments using intact cells were analyzed by SDS-PAGE only the single radiolabeled protein of 58,000 mol wt was seen (Fig. 7).

Discussion

The data reported here show that the human genital skin fibroblast cytosolic protein covalently radiolabeled by the affinity ligand DHT-BA is indistinguishable under nondenaturing conditions from the noncovalently labeled human androgen receptor on the basis of a variety of physicochemical properties. These properties include the Stokes radius and sedimentation coefficient of the covalently and noncovalently radiolabeled proteins as well as their isoelectric points and hydrophobicity.

Hydrodynamic studies reveal the protein labeled by either ligand to have an apparent Stokes radius of 4.4 nm and a sedimentation coefficient of 9S. The mol wt of the protein calculated from these data is 170,000. A wide variety of apparent mol wt of nondenatured putative androgen receptors from different species and tissues have been reported. Under nondenaturing conditions of ionic strength similar to those used in our experiments these mol wt range from 25,000–167,000 (reviewed in Ref. 24). Interspecies differences in the receptor or proteins associated with it in broken cell preparations as well as tissue-specific protease activity differences have been thought to underlie these diverse observations (24).

The affinity-labeled samples analyzed by denaturing techniques show a single radiolabeled protein of 58,000 mol wt. Under denaturing conditions in experiments using putative affinity ligands a variety of mol wt have also been reported. The rat prostate androgen receptor analyzed under such conditions has been reported to have mol wt of 46,000 (6) and 50,000 (7) as well as 86,000 (12). In bovine seminal vesicle the apparent mol wt of the androgen receptor under denaturing conditions is 60,000 (3). The affinity-labeled receptor from the Dunning transplantable prostatic tumor has been found to have a mol wt of 118,000 (25), although in identical experiments later reported from the same laboratory the majority of saturable covalent binding appeared to be to proteins with mol wt of 25,000 and 30,000 (24). A human autoantibody reactive with the androgen receptor has been reported to recognize a protein of 118K in cytosol from this same tumor (26). Consistent with these more recent protein chemical (24) and immunochemical (26) studies, data from the molecular cloning of partial length cDNAs for the human androgen receptor suggested a mol wt of considerably greater than 58,000 (27–29). However, at least *in vitro*, mRNA transcribed from full-length human androgen receptor cDNAs can be translated beginning at a number of start sites, yielding receptor-related molecules of 94K, 76K, 70K, and 46K (30). In addition, translation products of 55K, 32K, and 30K are produced and are presumed to be the result of proteolysis *in vitro* (30). The heterogeneity of mol wt results reported in protein studies may be accounted for by the multiplicity of translational start sites as well as lability of the protein product. The latter explanation seems most likely for our observation of the 58K species labeled by DHT-BA.

In the present experiments it was not possible to demonstrate any effect of protease inhibitors on receptor size under denaturing conditions, although detectable receptor quantity was increased in the presence of such inhibitors. Furthermore, the receptor species observed

TABLE 1. Quantitation of androgen receptors in cytosol and intact cells using covalent and noncovalent ligands

	Androgen receptor content (fmol/mg protein)	
	[^{3}H]DHT	[^{3}H]DHT-BA
Cytosolic assay		
Present study	29 ± 10 (3)	850 ± 90 (12)
Literature[a]	31 ± 3 (8)	Not reported
Whole cell assay		
Present Study	51 ± 11 (6)	500 ± 29 (3)
Literature[b]	33 ± 3 (8)	Not reported

Receptor content values are expressed as the mean ± SEM for the number of experiments shown in parentheses.

[a] Reference 15.

[b] Reference 23.

under a variety of nondenaturing conditions were found to have an apparent mol wt of 58,000 when analyzed by denaturing electrophoresis. Thus, we could not definitively demonstrate that proteolytic degradation of a larger mol wt form of the receptor results in generation of the stable 58K species. Such negative results, of course, cannot be interpreted to exclude a role for proteolytic action in the generation of the 58K species.

The affinity-labeled protein was indistinguishable from the authentic androgen receptor on the basis of isoelectric point. Chromatofocusing of either the covalently or noncovalently radiolabeled proteins revealed two isoforms with pI values of 5.4 and 4.5. The predominant isoform (pI 5.4) constituted greater than 75% of the total affinity-labeled receptor; the ratio of isoforms was more variable when the noncovalent ligand was used to radiolabel the receptor. A number of isoelectric points for androgen receptors from various species and target tissues have been reported from experiments using noncovalent ligands for conventional isoelectric focusing as well as chromatofocusing. The values found include pI 5.8 in rat prostate (31), 5.8 in rat epididymis (32), 6.0 in human hyperplastic prostate (33), 4.9 and 5.7 in mouse kidney (34), 6.6 in steer seminal vesicle (3), and 6.8 in human hyperplastic prostate (35).

The hydrophobicity of the androgen receptor has not been previously examined. The proteins radiolabeled with either the covalent or noncovalent ligand demonstrated a high degree of hydrophobicity. The radiolabled receptor preparations required water elution from the phenyl-Sepharose column, while the majority of proteins eluted in higher ionic strength buffers. This high degree of hydrophobicity may indicate an ability of the receptor to associate with hydrophobic surfaces, such as lipid membranes, or to form multimeric structures.

While no qualitative differences in the proteins radiolabeled by DHT and DHT-BA were noted, a remarkable quantitative difference was found. The amount of high affinity saturable cytosolic protein bound by the affinity ligand is 10- to 30-fold higher than estimates of receptor concentration obtained using the noncovalently bound ligand in a variety of assay systems. The affinity-labeled protein eluted from gel filtration columns could not be dissociated from the affinity ligand by a variety of denaturing conditions (SDS, guanidine, high pH), indicating that all of the observed radioligand binding is covalent. Denaturing SDS-PAGE of the gel filtration column eluate shows that only the single saturable 58K protein is labeled. The radiolabeled DHT used for the synthesis of the affinity ligand is of the same specific activity as that used for the noncovalent receptor binding studies. Although the specific activity of the synthesized affinity ligand was not redetermined, a 10- to 30-fold increase in specific activity during the synthetic reaction would be necessary to account for the increased amount of binding observed with the affinity ligand. Such an alteration seems unlikely. One plausible explanation for the large discrepancy in receptor content measured by the two ligands is dissociation of the noncovalent ligand during various assay and chromatographic procedures. Alternatively, proteolytic breakdown of the unoccupied receptor might occur during the prolonged incubations required to reach saturation with a noncovalent ligand. Such an analysis would imply that quantitation of the androgen receptor using noncovalent ligand binding assays may tremendously underestimate the actual amount of receptor present. Alternatively, it seems more likely that the affinity ligand labels a subclass of receptors that do not bind the physiological noncovalent ligand. Such subclasses of receptors might be incompletely processed receptors or recycling receptors that have been altered during transmission of the hormonal signal. This formulation has been favored by Nickel *et al.* (36), who, we have found in preliminary collaborative experiments (not shown), have characterized the identical protein by two-dimensional gel electrophoresis. Whatever physicochemical differences exist among such subclasses of receptors would have to be sufficiently subtle to escape detection by the analytical methods used in the present experiments. Finally, although unlikely, the affinity ligand might also label a protein totally unrelated to the androgen receptor. Again, however, such an unrelated molecule would have to be physicochemically indistinguishable from the noncovalently labeled androgen receptor under the diverse nondenaturing conditions used in our experiments.

The results of the present work support previous observations that DHT-BA is an efficient affinity ligand for the human androgen receptor or a fragment thereof. The ligand binds with high affinity to a single saturable protein of 58,000 mol wt that is present in cells containing androgen receptors assayable by conventional methods and is absent from receptor-negative cells. Specificity of binding of the affinity ligand was previously demonstrated both by its ability to displace DHT from the classically defined androgen receptor as well as by the ability of the noncovalent ligand DHT to inhibit the rapid covalent binding of the affinity ligand. The present experiments show that under a variety of nondenaturing conditions the protein covalently radiolabeled by [^{3}H]DHT-BA is indistinguishable from the authentic human androgen receptor noncovalently labeled with [^{3}H]DHT. This affinity ligand may serve as a useful tool for the characterization of various human syndromes thought to be the result of mutations in the androgen receptor protein and will facilitate direct purification of the covalently radiolabeled species by techniques that have not been applicable to noncovalently labeled receptors.

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