Purification of Androgen Receptors in Human Sebocytes and Hair

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Human sebaceous glands (SG) and hair follicles (HF) are target structures in the skin for androgen action. They contain steroid enzymes, capable of transforming weak androgens into the target-tissue-active androgens testosterone (T) and dihydrotestosterone (DHT), which bind to the androgen receptor (AR) to regulate cellular transcription. The AR from HF and SG from human scalp tissue has been purified >86,000 times by phenyl-sepharose, DEAE-sephacel, gel filtration chromatography, and ultrafiltration. Sucrose density gradient analysis and non-denaturing gradient polyacrylamide gel electrophoresis (PAGE) and sodium dodecyl sulfate (SDS)-PAGE revealed two molecular species of AR, an active form called monomer, capable of binding DHT with great specificity (4S, m = 62,000 Da, Kd = 0.6 nM, Bmax 8260 fmol/μg protein), and the other, an inactive form of the monomer called tetramer (10.8S, m = 252,000 Da, Kd = 2.9 nM). The two species are interconvertible, and after purification each appeared as a single band on SDS-PAGE. The conversion of the monomer to the tetramer AR form is influenced by reduced and oxidized glutathione, and possibly by an endogenous disulfide converting factor (DCF). Furthermore, biochemical events in the androgenic signal transduction sequence were shown to be stimulated by androgens via the AR. These include the total nuclear AR content, chromatin binding of AR complexes, and stimulation of RNA polymerase II, thus influencing gene expression, which is important in understanding regulation of HF growth and SG proliferation. J Invest Dermatol 98:92S–96S, 1992

Human sebaceous gland (SG) and human hair follicle (HF) are androgen-sensitive tissues, and several dermatologic conditions, including androgenetic alopecia (AGA), acne vulgaris, hirsutism, and even lichen sclerosis et atrophicus, have been attributed to the action of androgens.

The biochemical mechanisms in which androgens and steroids act in the HF, SG, and skin in general are not fully known. It is known that active metabolism of androgens and steroids takes place in the skin and a number of important enzymes, such as 3β-hydroxysteroid dehydrogenase (3β-HSD), 17β-HSD, 5α-reductase (5α-R), and cytochrome P-450 aromatase enzyme (AT enzyme), have been biochemically and immunohistochemically located in the HF and SG of human skin [1–5].

A microdissection method for isolating human SG and HF without enzyme digestion has been described [2], and studies have shown that these skin appendages are able to transform dehydroepiandrosterone (DHEA) to 4-androstenedione (AD), then to T 5α-dihydrotestosterone (DHT). The sequence of reactions produces metabolites with increasing androgenic potency at each step, and the end product, DHT, is the target-tissue-active androgen. In recent biochemical and immunohistochemical studies [4,5], findings that AD and T can be transformed to estrone and estradiol in outer root sheath of HF and SG suggested that the AT enzyme may be important for regulating the local production of androgens, perhaps influencing HF cycles and SG function.

My studies in the last few years have focused on the androgen receptor (AR), with purification and characterization of the AR in isolated human HF and SG from scalp tissue. This review will describe purification of the AR and findings that the AR has two molecular species, an active form of the AR, called monomer, and a larger, multisubunit complex of AR called tetramer, each with different biochemical binding characteristics, suggesting that the AR is the same protein, but takes different forms in mediating androgen action.

Studying the biochemical events mediating androgen action will enable us to understand the way androgens regulate hair growth and SG proliferation in men and women, and the way they may also relate to other hormone-mediated disorders or diseases where androgens influence gene expression.

MATERIALS AND METHODS

Chemicals Radioactive and nonradioactive [17α-methyl-3H]-methyltripenolone, R-1881, (86 Ci/mmole) were purchased from Dupont New England Nuclear (Boston, MA). Other nonradioac-
tive steroids, DHT, T, AD, and DHEA, were purchased from Sigma Chemical Co. (St. Louis, MO). Both radiolabeled and unlabeled steroids were purified by thin-layer chromatography prior to use.

Isolation of HF and SG from Human Scalp Skin plugs (4 mm) removed from the recipient and donor sites of men and women were collected during hair transplant surgery for AGA. Tissues were dissected under a stereo microscope to isolate SG and HF as described previously [2]. The isolated HF and SG were immediately placed in HBSS, pH 7.4, and kept at 4°C.

AR Assays Preparation of cytosol and nuclear fractions from HF and SG, and assays for AR binding activity by the dextran-coated charcoal method and steroid exchange assays, were as previously described [6]. After correction for nonspecific binding, the data assessing AR binding were plotted according to Scatchard [7] to obtain the binding characteristics, Kd, and Bmax.

Sucrose Gradient Sedimentation Linear 5–30% (w/v) sucrose gradients of 5 ml were prepared as previously described [6]. Stokes radii and sedimentation coefficients of standard proteins were used to determine molecular weights and fractional ratios of the AR [8].

Purification of AR Hydrophobic chromatography: the cytosol (164,000 x g) fraction from HF and SG were subjected to hydrophobic chromatography on phenyl-Sepharose (PS) (Pharmacia, Piscataway, NJ) for initial purification of AR. Columns (1.5 x 10 cm) containing 5 ml of PS were equilibrated with 0.1 M NaCl, 10 mM Tris-HCl, pH 7.4. A 5-ml aliquot of cytosol, containing 12–25 mg of protein, was applied to each column. The columns were washed with equilibrating buffer to remove unretainable materials, until absorbance at 280 nm returned to baseline. Each fraction was assayed for AR activity and fractions containing AR were combined for further purification.

Ion-Exchange Chromatography The combined fractions from PS columns containing AR were applied to 1.5 x 10 cm columns containing 10 ml of DEAE-Sephascl (Pharmacia), equilibrated with 10 mM Tris. After the absorbance at 280 nm of washings with buffer containing 0.25 M NaCl returned to baseline, the columns were eluted sequentially with buffers of increasing ionic strength, and 5–8 ml fractions were collected. Each fraction was assessed for AR activity.

Gel Filtration and Ultrafiltration Sephacyrl S-150 and 100 columns (2.5 x 50 cm) were equilibrated with Tris buffer containing 0.3 M NaCl. Samples of 3 ml were applied, and columns were eluted under hydrostatic pressure of 15 cm, and 3 ml fractions collected. Fractions were assessed for AR activity. Marker proteins were located by measuring optical density at 280 nm. The column was calibrated with blue dextran, ferritin, bovine globulin, aldolase, bovine serum albumin, ovalbumin, chymotrypsinogen A, and ribonuclease A. The Stokes radius was estimated by graphical analysis as previously described [8]. Column fractions with AR activity were placed in ultrafiltration cartridges with 30 K filtration membranes (Amikon, Danvers, MA). Filtered samples were assessed for AR specific activity, and samples used for PAGE analysis.

Chromatofocusing A modification of the chromatofocusing technique as previously described [9] was used to determine the isoelectric point of the AR.

Non-Denaturing PAGE Non-denaturing PAGE was performed after the AR samples were allowed to bind to the 3H-ligand by incubation at 2°C for 1 h. Pre-cast 4–30% gradient gels (Pharmacia) were purchased and samples applied for electrophoresis. Gels were cut in 2-mm sections and extracted with ethanol/dichloromethane (2:1, v/v) for radioassay. Parallel gels were silver stained to visualize protein bands.

SDS-PAGE SDS-PAGE was performed according to O’Farrell [10] using the discontinuous buffer system of Laemmli [11] in 12%, 15%, or 20% PAGE slab gels. After electrophoresis, the gels were either silver stained (Bio-Rad) or stained with Coomassie bright blue.

Interconversion of AR Monomer to Tetramer Purified AR monomer (8400 fmol/µg protein) was incubated with various concentrations of reduced and oxidized glutathione (5–12 mM), according to Gilbert [12]. The incubation mixture was degassed and tubes sealed during incubations. After incubation for 20 min at 2°C, 3H-ligand (12 nM) was added to each tube for assay of AR activity. Aliquots were placed on 4–30% gradient non-denaturing PAGE to assess formation of the tetramer AR.

Conversion of Tetramer to Monomer AR Purified tetramer was shaken with buffered 5 mM DTT or 5 mM mercaptoethanol under the same incubation conditions as above. Control samples were kept at 2°C without DTT and mercaptoethanol. 3H-Ligand was then introduced for the AR assay. Aliquots were applied to non-denaturing PAGE to evaluate monomer AR formation.

Assay for RNA Polymerase II After incubation with ligand-AR complexes, nuclear solutions (20–50 µg of DNA) were incubated at 27°C for 15 min in the presence of adenosine, guanosine, cytosine, and 3H-uridine triphosphates, in the absence or presence of 10% DTT. Incorporation of [3H]UTP was terminated by adding 10% trichloroacetic acid. Polymerase II activity was calculated by subtracting radioactivity incorporated in the presence of a-amanitin from that incorporated in the absence of the inhibitor, as previously described [13].

Protein/DNA Assays Protein content of samples was measured by the method of Lowry [14]. Protein content of dilute samples was also assessed spectrophotometrically [15]. DNA was assayed by the method of Burton [16].

RESULTS

After cytosols from SG or HF were placed on phenyl-Sepharose columns, AR activity was eluted with 10 mM Tris-HCl, pH 7.4. The columns retained the AR, as no androgen-binding activity was recovered in the eluate fractions containing unretained materials. On further purification by DEAE-sephacel (Fig 1B), AR activity was recovered obtaining two peaks of AR activity. The fractions from peak 1 were combined and placed on a Sephadex G-150 column and the fractions from peak 2 were combined and placed on a Sephadex G-100 column. Fractions eluted by gel filtration were assayed for AR activity. The results obtained from 30 SG and HF preparations (30 patients with AGA) were nearly identical. The overall purification (Table I) achieved was > 86,000 times. Binding characteristics of the two AR forms was assessed by Scatchard plot analysis. Results showed that peak 1 versus peak 2 were different; however, peaks 1 and 2 show the same values whether from HF or SG, indicating that the same AR protein is in HF and SG. The binding characteristics for peak 2, monomer AR, Kd 0.64 ± 0.09 nM, with a Bmax 8260 fmol/µg p, after purification of > 86000. Peak 1, the tetramer form of the AR, had different binding characteristics of Kd 3.0 ± 0.32 nM, with Bmax of 2400 fmol/µg p, and dissociates from the monomer form with use of sulfhydryl reducing reagents, such as DTT and mercaptoethanol (Table II).

Samples from peak 1 and 2 DEAE columns were placed on non-denaturing polyacrylamide gel electrophoresis (PAGE) revealing 252 kDa and 62 kDa bands, respectively (Fig 2). As well, separate aliquots from DEAE peaks 1 and 2 were treated with 0.01 M Tris, pH 8.0, containing 1 mM EDTA, 1% sodium dodecyl sulfate (SDS), and 5% mercaptoethanol, then subjected to 12% SDS-PAGE. Only a single 62-kDa band was found. The disappearance of the 252-kDa band from non-denaturing PAGE suggests that this larger AR molecular species could have dissociated into the monomer subunit form. Because the 252-kDa form is nearly four times larger than the smaller 62-kDa form, the names tetramer and monomer were given to describe these bands. Further experiments showing the addition of reduced and oxidized sulfhydryl-reducing reagents suggested that intramolecular disulfide bonds may be important in this tetramer AR form (Table II).
Figure 1. (A) Phenyl-Sepharose chromatography. A 25-mg cytosol sample from HF/SG was placed on the column (see Materials and Methods) containing 5 ml packed gel volume of phenyl-Sepharose. Elution is with buffers as indicated, and fractions are collected for determining AR activity. Samples 10–14 were pooled for placing on DEAE-Sepacel. (B) Sample is equilibrated and eluted with buffers as indicated. The NaCl concentration of eluting buffer increased, as indicated. Five-millimeter fractions were collected and assayed for AR activity.

Other characteristics of the AR such as its isoelectric point were found by chromatofocusing. Fractions were labeled with 16 nM [3H-R1881] and applied to gel rods, ampholine pH 3–10. The isoelectric point of the AR proteins was 5.0 for both forms from HF and SG, indicating the same monomer protein for both AR forms.

The Stokes radius of the purified AR was determined. The AR monomer and tetramer forms were applied to a gel filtration column as indicated in Materials and Methods, and eluted with buffer. Two peaks of binding activity were eluted from the column, corresponding to Stokes radii of 34 A for the monomer and 52 A for the tetramer AR. An aliquot of both peak fractions was subsequently analyzed on 5–30% sucrose gradients in Tris buffer system, again finding two peaks of bound radioactivity with sedimentation coefficients of 4.0 S and 10.8 S, for the monomer and tetramer AR, respectively. This value can be combined with the estimated Stokes radii of 34 and 52 A, respectively, to calculate a molecular weight for the two AR forms of 62 kDa and 252 kDa, using the formula of Siegel and Monty [17], which is identical to the results found using non-denaturing PAGE.

Experiments showing the interconversion of the monomer and tetramer are shown in Table II. When monomer was incubated

### Table II. Interconversion of AR Monomer and Tetramer

<table>
<thead>
<tr>
<th></th>
<th>Tetramer (%)</th>
<th>Monomer (%)</th>
<th>Bmax (fm/μg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monomer (control)</td>
<td>0</td>
<td>100</td>
<td>2800</td>
</tr>
<tr>
<td>Monomer + (GSSG/GSH)</td>
<td>23</td>
<td>77</td>
<td>1678</td>
</tr>
<tr>
<td>Tetramer (control)</td>
<td>91</td>
<td>9</td>
<td>569</td>
</tr>
<tr>
<td>Tetramer + DTT</td>
<td>22</td>
<td>78</td>
<td>1767</td>
</tr>
<tr>
<td>Tetramer + MeSH</td>
<td>19</td>
<td>81</td>
<td>2029</td>
</tr>
</tbody>
</table>

* Monomer AR was incubated with 5 mM reduced GSH and 10 mM GSSG as described in Materials and Methods. The tetramer was incubated with 5 mM DTT or 5 mM mercaptoethanol at 2°C for 60 min. Control samples were kept at the same temperature. After the incubation, samples were assayed for [3H-ligand-AR binding. Aliquots were applied to nondenaturing PAGE for separation of AR forms. Gel lanes were cut into 2 mm sections, extracted, and radioassayed. Results are expressed as percent distribution of monomer and tetramer.

### Table I. Purification of AR-Monomer/Tetramer from Human HF and SG

<table>
<thead>
<tr>
<th>Tissue Sample</th>
<th>Total Protein (μg)</th>
<th>Specific Activity (fmol/μg P)</th>
<th>Purification Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytosol</td>
<td>25,200</td>
<td>0.096</td>
<td>1.0</td>
</tr>
<tr>
<td>phenyl-Sepharose</td>
<td>1,190</td>
<td>6.720</td>
<td>70.0</td>
</tr>
<tr>
<td>DEAE-Sephacel</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peak 1 (tetramer)</td>
<td>15</td>
<td>675</td>
<td>7,030</td>
</tr>
<tr>
<td>Peak 2 (monomer)</td>
<td>12</td>
<td>3,920</td>
<td>31,500</td>
</tr>
<tr>
<td>Sephadex G-150*/G-100** Gel filtration</td>
<td>**Monomer-AR</td>
<td>8</td>
<td>8,260</td>
</tr>
</tbody>
</table>

* The 164,000 × g supernatant from microdissected HF and SG was applied to columns as stated in Materials and Methods. Samples were equilibrated on the column, then eluted with the indicated buffers (see Fig 1A, B).
Figure 3. (A) Uptake of ligand-AR complex by nuclei. $^3$H-ligand-AR complex was incubated with fresh nuclei (800 x g pellet of cell homogenates) obtained from SG. Incubations were at 37°C for times indicated. Results are expressed as fmoles $^3$H-ligand-AR complex/μg nuclear DNA. (B) AR binding to DNA-cellulose. Various amounts of $^3$H-ligand-monomer or tetramer AR were incubated with DNA-cellulose. The monomer AR binds in a dose-dependent fashion; however, the tetramer AR had minimal binding to DNA. Nonspecific binding was subtracted from both curves.

with 5 mM reduced GSH and 10 mM oxidized GTT, the formation of tetramer was approximately 23%. Formation of the monomer from tetramer in the presence of DTT or mercaptoethanol was evidenced by a band corresponding to the monomer on non-denaturing PAGE, and also by the increased Bmax as the monomer form has (nearly four times greater than the tetramer form). This interconversion was apparently through intramolecular disulfide bonds. Figure 3A, B describes experiments where AR monomer and tetramer were incubated with fresh nuclei (800 x g pellet of cell homogenates) obtained from SG and HF. Nuclear integrity was verified by trypan blue exclusion and nuclear enzyme marker, 5' nucleotidase. $^3$H-R1881/DHT-AR complex was incubated at 37°C for various times. Results are expressed as fmoles $^3$H-ligand-AR complex/μg nuclear DNA. It is apparent that the monomer AR form is preferentially taken up by the nuclei, perhaps by translocation, in comparison to the tetramer AR. Analysis of the tetramer AR found in the nuclei revealed that dissociation occurred to the monomer AR form. Parallel to this, monomer AR preferentially binds to DNA-cellulose (Fig 3B), where minimal binding of $^3$H-ligand tetramer AR to DNA occurred.

Experiments showing the effects androgens have on the androgen signal transduction sequence were done, specifically looking at stimulation of RNA polymerase II activity. Aliquots of DHT-monomer AR were pre-incubated with nuclei from SG. $^3$H-UTP incorporation was measured. In parallel tubes the amount of $^3$H-ligand-AR to DNA was assessed, and results shown in Fig 4. Results show that increasing levels of AR complex incubated with SG nuclei have an effect in stimulating RNA polymerase II activity, as measured by UTP incorporation. As well, an increase in total nuclear AR content, AR-DNA binding, and stimulation of RNA polymerase II were shown, thereby indicating the effects androgens have on influencing gene expression.

DISCUSSION

The AR has been the most difficult of all receptors in the steroid classification to isolate and purify [8,9]. Even though the AR has been cloned and sequenced [18], it is of great importance to obtain the AR protein from the specific tissue source, so that specific studies can be directly correlated with biochemical processes taking place in that tissue, or species, i.e., to show AR-mediated effects, such as the androgen signal transduction sequence, to better understand the mechanisms in signaling gene expression.

At the present time, the AR in human skin has not been purified, or fully characterized, possibly because of the proteases in whole skin tissue. The HF and SG are highly specialized structures in skin and may contain considerably fewer proteolytic enzymes. In comparison, human prostate, which is another source of AR, is found to contain a high content of hydrolytic and proteolytic enzymes to permit purification and characterization of AR.

Previous reported molecular weights for the monomeric steroid binding subunit of the AR have ranged from 25–167 kDa [8]. The molecular weight varied among different species and target tissues that were studied [8,9]. A common monomer form of the AR was determined in various animal target tissues by Johnson et al [8], ranging from 40 to 110 kDa. Abnormal AR complexes were detected by McEwan et al [19], where the AR was partially purified and the characteristics of the AR studied from human skin fibroblasts of patients with androgen insensitivity syndrome. Their studies revealed a 60–65-kDa protein with a Stokes radius of 32 A, similar to present findings in this report for human HF and SG.

The studies in this report describe purification and isolation of the AR in two forms, a monomer and a tetramer form. The amounts of these two AR forms were found to be different in active, anagen HF versus in regressed, receded, balding HF [20]. Differences in AR quality and quantity have become more apparent in recent studies where inactive forms of steroid receptors have been implicated with disease processes [15,19].

Results from the purification show that the monomer AR form has a molecular weight 62,000 by SDS-PAGE (silver staining and Coomassie blue), with a Stokes radius of 34 A, a sedimentation coefficient of 4.0S, and with a calculated molecular weight of 61,700, close to the experimental value obtained by SDS gel analysis. The binding characteristics between the two forms were also different, with the monomer form having greater specificity of binding and greater Bmax. Perhaps AR activity is regulated in the form of the tetramer complex, which may be the less active state of the receptor, where minimal hormone binding occurs due to the hormone binding site being occluded from oxidation of disulfide bonds in the tetramer state. Investigators have found that sulphydryl

Figure 4. RNA Polymerase II activity and nuclear acceptor site occupancy. Aliquots of DHT-monomer AR were incubated with nuclei of cells from SG. $^3$H-UTP incorporation was measured to assess RNA polymerase II activity as by previous methods [13]. Results indicate that increasing AR incubated with SG nuclei has an effect in stimulating RNA polymerase II activity.
groups in the other steroid receptors, such as the estrogen receptor, as involved with the binding of estradiol [21], indicating the importance of maintaining steroid receptors in a sulphydryl-reduced state for optimum hormone binding to occur [22].

As well, the AR seems to be sensitive to oxidized and reduced sulphydryl agents, such as DTT, mercaptoethanol, GSH, and GSSG. The binding characteristics were different for the monomer versus tetramer form of AR, in the presence of sulphydryl-reducing agents such as DTT, and GSH, showing greater Bmax and more specific binding of the hormone, DHT, or R1881, as reflected in the Kd. Previous studies have indicated that the sulphydryl-reducing environment is important in steroid receptor activation. Various endogenous enzyme systems, such as the thioredoxin-reductase enzyme system and protein disulfide isomerases, have been mentioned as important in maintaining the sulphydryl environment [23]. As well, the finding of a specific endogenous disulfide factor (DCF) [24] may be important in mediating androgen action in SG and HF of skin. Grippo et al. [23] demonstrated that an endogenous thioredoxin system is involved in glucocorticoid receptor stabilization, by keeping the receptor in the reduced state necessary for specific hormone binding.

The studies performed with the purified monomer AR show that hormone-AR complex localizes in the nucleus, binds to DNA, and stimulates RNA polymerase II. All of these are necessary events in the androgen signal transduction pathway, indicating that androgens affect cellular regulation in HF and SG.

In summary, purification of the monomeric steroid-binding form of the AR is described. The method described is suitable for obtaining purified AR to be used in specific biochemical studies, i.e., the androgen signal transduction sequence, which is important to better understand the way androgens influence gene expression in HF and SG.

REFERENCES


