Anti-Androgens and the Mutated Androgen Receptor of LNCaP Cells: Differential Effects on Binding Affinity, Heat-Shock Protein Interaction, and Transcription Activation

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ABSTRACT: Previous studies from this laboratory have described that LNCaP prostate tumor cells contain an androgen receptor (AR) with a point mutation in the steroid-binding domain (codon 868, Thr to Ala). This defect leads to a change in specificity of the AR. Estrogens, progestagens, and some anti-androgens (e.g., cyproterone acetate, hydroxyflutamide, nilutamide) stimulate LNCaP cell growth rate through the AR. The present studies indicate that not all anti-androgens showed agonistic effects with the mutated receptor. The growth rate of LNCaP cells did not increase with the anti-androgen ICI 176 334, nor could this compound increase transcription activation of the reporter gene construct via the mutant receptor in a cotransfection system [HeLa cell cotransfection system with an androgen-regulated reporter gene construct (pG29G-tk-CAT) and the mutant receptor as trans-vector]. Interaction of the AR of LNCaP cells with heat-shock proteins was studied by isolation of the receptor with a specific monoclonal antibody and characterization of associated proteins. Hsp90, hsp70, and hsp56 were found to coprecipitate with the AR. Incubation of the cells at 37 °C with androgen (R1881, 10 nM) or the anti-androgen hydroxyflutamide, prior to receptor isolation, resulted in dissociation of the AR–heat-shock protein complex. This dissociation is paralleled by the transformation to a tight nuclear-binding form of the AR. In contrast, ICI 176 334 could not induce a release of heat-shock proteins and did not increase nuclear binding, but inhibited the transformation process induced by R1881. From these results, we propose a mechanism of action of anti-androgens in LNCaP cells in which these compounds affect different steps in the processes of receptor transformation and transcription activation. In LNCaP cells, ICI 176 334 shows decreased affinity for the AR and affects steps before DNA binding occurs. In contrast, other anti-androgens including hydroxyflutamide show increased affinity for the mutant AR, transform the receptor to the DNA-binding state, and permit interaction of the receptor with the transcription machinery.

Effects of steroids in target cells are mediated by their respective receptors. After binding of the hormone, these ligand-dependent transcription factors are transformed to a DNA-binding form with high affinity for hormone responsive elements (HREs)[1] of target genes. Subsequently, the transcription of these genes is modulated by binding of the transformed steroid–receptor complex and interaction with other transcription factors (Beato, 1989). All steroid hormone receptors appear to be composed of several functional domains, including a large C-terminal ligand-binding domain and a central basic region responsible for DNA binding. In addition, domains involved in the transactivating function of the receptor have been identified in both the N- and the C-terminal part of progesterone, glucocorticoid, and estrogen receptors (Carson-Jurica et al., 1990). The primary structure of the androgen receptor has been determined, but the exact location of domains involved in transcription activation has not yet been described (Chang et al., 1988; Trapman et al., 1988; Lubahn et al., 1988; Faber et al., 1989).

In the absence of hormones, steroid receptors are thought to exist in a non-DNA-binding (nontransformed)[2] state, associated with several other proteins. The 90-kDa heat-shock protein (hsp90) was shown to be associated with the androgen, progestagen, glucocorticoid, and estrogen receptors (Joab et al., 1984; Sullivan et al., 1985). Another component of the receptor complex is a protein of 56–59 kDa. The antibody EC1, developed by Nakao et al. (1985), reacts specifically with a 59-kDa protein present in rabbit progesterone–, glucocorticoid–, androgen–, and estrogen–receptor complexes. Recently, it was shown that this protein also is a heat-shock protein (Sanchez, 1990). In addition to the 90- and 56–59-kDa proteins, the 70-kDa heat-shock protein (hsp70) has been found in the nontransformed progesterone– and glucocorticoid–receptor complexes (Kost et al., 1989; Smith et al., 1990; Sanchez et al., 1990). Thus far, association of hsp70 with other steroid receptors has not been shown.

The large multiprotein–receptor complex is considered to dissociate upon hormone binding, thereby revealing the DNA-binding domain of the receptor. The receptor then dimerizes, binds to the responsive element of the regulated gene, and interacts with other participants in the transcription machinery (Carson-Jurica et al., 1990).

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[1] Abbreviations: AR, androgen receptor; LNCaP, lymph node carcinoma of the prostate; GRE, glucocorticoid responsive element; CAT, chloramphenicol acetyltransferase; hsp90, 90-kDa heat-shock protein; hsp70, 70-kDa heat-shock protein, hsp56, 56-kDa heat-shock protein; TAF, transcription activation function; HRE, hormone responsive element; LH, luteinizing hormone; DTT, dithiothreitol; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; RBA, relative binding affinity.

[2] It should be noted that the term "transformation" will be used to describe the process whereby the steroid-bound receptor is converted from a non-DNA-binding state to a tight nuclear-binding form.

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The precise mechanisms of the effects of steroid receptor antagonists at the receptor level are not known. Several mechanisms have been proposed, ranging from induction of an abnormal conformation (Moudgil et al., 1989), impaired translocation of the receptor to the nucleus (Lindemyer et al., 1990; Segnitz & Gehring, 1990), impaired dissociation of the heteromeric receptor complex (Moudgil & Hurd, 1987; Segnitz & Gehring, 1990), impaired receptor dimerization, and binding of the receptor to DNA (Berry et al., 1990; Fawell et al., 1990; Klein-Hitpass et al., 1991) to impaired interaction of the DNA-bound receptor with transcription factors (Guiochon-Mantel et al., 1988; Berry et al., 1990; Sabbah et al., 1991; Klein-Hitpass et al., 1991). Anti-androgens act by inhibition of the binding of androgens to the receptor, but their precise molecular mechanisms of action are at present not known. With respect to their physiological effect, anti-androgens can be divided into two groups. The nonpure (steroidal) anti-androgens (e.g., cyproterone acetate, megestrol acetate, medroxyprogesterone acetate, chlormadione acetate) block androgen action, but in addition have progestational and glucocorticoid activities. The pure (nonsteroidal) anti-androgens (e.g., flutamide, nilutamide) block the action of androgens and have a stimulating effect on the hypothalamus–pituitary–gonadal axis and consequently lead to increased LH and testosterone levels (Mowszowicz et al., 1974; Neumann & Töpert, 1986; Raynaud & Ojassoo, 1986). ICI 176 334 is a pure but peripherally-selective anti-androgen in rats and dogs (Furr et al., 1987; Chandolia et al., 1991), but in a clinical study in men it caused a small but significant elevation of serum LH and testosterone, suggesting that it does affect androgen receptors at the hypothalamic level in men (Mahler & Denis, 1990).

In the present study, we have used the LNCaP cell line, derived from a human lymph node carcinoma of the prostate, for investigations on the mechanism of action of anti-androgens. The LNCaP cell line is the only available human cell line that shows both hormone dependency and continuous growth in vitro (Horoszewicz, 1983). Although the cells do not contain steroid receptors other than the androgen receptor, growth can also be stimulated by progesterone, estradiol, and the anti-androgens cyproterone acetate and nilutamide (Schuurmans et al., 1988, 1990; Wilding et al., 1989). The LNCaP cell line contains an androgen receptor with a mutation in the ligand-binding domain: amino acid 868, Thr replaced by Ala (Veldscholte et al., 1990a). In transfected cells, the mutant receptor was found to enhance transcription from an androgen-regulated reporter gene construct (GRE-tk-CAT), not only in the presence of androgens and different other steroids but also in the presence of some anti-androgens (Velscholte et al., 1990a). In the present study, it is shown that not all anti-androgens have similar, stimulatory effects through the mutant receptor of LNCaP tumor cells. The anti-androgens used in this study have different effects on dissociation of the receptor–heat-shock protein complex, on tight nuclear binding of the receptor, and on transactivation of an androgen receptor regulated gene.

Experimental Procedures

Materials. [^{3}H]R1881 (87 Ci/mmol), unlabeled R1881 (methyltrienolone), and R5020 (promegestone) were purchased from NEN (Boston, MA); triamcinolone acetonide and butyryl-CoA were from Sigma (St. Louis, MO). Nilutamide ("Anandron", RU 23908) was a gift from Roussel Uclaf (Paris, France), cyproterone acetate from Schering (Berlin, FRG), hydroxyflutamide from Schering, USA (Bloomfield, NJ), and ICI 176 334 (trademark "Casodex") from ICI Pharmaceuticals (Macclesfield, Cheshire, U.K.). ICI 176 334 was freshly dissolved before each experiment. All other steroids were purchased from Steraloids (Wilton, NH). [^{14}C]Chloramphenicol (50–60 mCi/mmol) was obtained from Amersham (U.K.).

The glucocorticoid/progestagen/androgen responsive CAT construct pG29GtkCAT (Schüle et al., 1988) was generously provided by Dr. R. Renkawitz. The mouse monoclonals AC88, N27, and KN382/EC1 were generously provided by Dr. D. O. Toft, Dr. W. J. Welch, and Dr. L. E. Faber, respectively.

Cell Culture. LNCaP prostate tumor cells, obtained from Dr. Horoszewicz, were cultured in RPMI 1640 as described previously (Veldscholte et al., 1990b). COS-1 cells and HeLa cells were cultured in Eagle's minimal essential medium (GIBCO, Breda, The Netherlands) supplemented with 5% heat-inactivated fetal calf serum (Sera Lab, Uden, The Netherlands), antibiotics, and nonessential amino acids (GIBCO) (medium A). Before transfection, cells were cultured in medium A with 5% (v/v) dextran–charcoal-treated serum (medium B).

Growth Studies. LNCaP cells (passage 20) were plated in 24-multi-well dishes (Falcon, Oxnard, CA) at a density of 2 $\times$ 10^4 cells/cm^2, in RPMI 1640 medium supplemented with 5% (v/v) dextran-charcoal-treated serum (medium C). After 2 days, medium was changed, and cells were kept on experimental medium (medium C with R1881, hydroxyflutamide, and ICI 176 334, at the indicated concentrations) with one medium change after 3 days. At day 6, cells were washed twice with phosphate-buffered saline, pH 7.5 (buffer I), and dissolved in 1 M NaOH for determination of DNA content (Hinegardner, 1976).

Incubation of LNCaP Cells and Subcellular Fractionation. LNCaP cells at confluency were kept on medium C for 2–4 days and washed twice with buffer I. Half of the number of flasks (175 cm^2) were put on ice, ice-cold serum-free RPMI 1640 medium with either R1881, hydroxyflutamide, or ICI 176 334 at the indicated concentrations was added, and the cells were incubated for 30 min at 4 °C. The cells in the other half of the flasks were incubated at 37 °C for 30 min with the same experimental media. Subsequently, the cells were washed with ice-cold buffer I and scraped in ice-cold buffer II [10 mM sodium phosphate, 1.5 mM EDTA, 12 mM 1α-thioglycerol, 10 mM DTT, 10 mM sodium molybdate, 0.6 mM PMSF, 0.25 mM leupeptin, 0.5 mM bacitracin, and 10% (v/v) glycerol, pH 7.4]. The cells were then homogenized with a glass/Teflon homogenizer and centrifuged at 800*g* for 5 min. The supernatant was then centrifuged for 30 min, 105000g, at 2 °C. The supernatant (cytosol) was used for immunopurification of the receptor complexes and Western blot analysis. The crude nuclear (800*g*) pellet was resuspended in buffer II with 0.2% (v/v) Triton X-100. After 5 min, the nuclei were pelleted and washed with buffer II. Nuclear extracts were made by incubating the nuclei with an extraction buffer [0.5 mL/flask; 40 mM Tris-HCl, 1.5 mM EDTA, 10 mM DTT, 0.6 mM PMSF, 0.25 mM leupeptin, 0.5 mM bacitracin, 0.5 M NaCl, and 10% (v/v) glycerol, pH 8.5] for 1 h at 4 °C. After centrifugation for 30 min, 105000*g* at 2 °C, the supernatant was used for receptor immunoprecipitation and Western blot analysis. Experiments were performed in triplicate.

Immunoaffinity Purification of the Receptor Complexes and Western Blot Analysis. The monoclonal antibody F39.4.1 directed against amino acids 301–320 in the N-terminal domain of the androgen receptor (Zegers et al., 1991) was chemically cross-linked directly to protein A–Sepharose by the method of Schneider et al. (1982). Ascitic fluid (400 μL) was

used to prepare 1 mL of affinity matrix. In each experiment, 15 μL of matrix was used for immunoprecipitation of the receptor from cytosol (1.4 mg of cytosolic protein) or nuclear extract (0.6 mg of nuclear protein). The immunoprecipitation was performed at 4 °C by incubating the affinity resin with the cytosols or extracts for 2 h under rotation. The resin was then washed 3 times with buffer I (buffer I with 10 mM sodium molybdate after binding of cytosolic receptors). Before the last washing step, the resin was transferred to a new vial. Thereafter, the pellet was boiled for 2–3 min in SDS–sample buffer, and SDS–PAGE was carried out according to Laemmli (1970) using 7% polyacrylamide gels on a Mini Protean II system (Bio-Rad). After electrophoresis, the slab gel was subjected to Western blotting essentially as described previously (Van Laar et al., 1989). The Mini Protean II system was used for the transfer of the protein onto nitrocellulose (Schleicher & Schuell) for 1 h at 100 V.

The monoclonal antibodies F39.4.1, specific for the androgen receptor, AC88, specific for hps90 (Riehl et al., 1985), N27, specific for hsp70 (Vass et al., 1988), and KN382/EC1, specific for hsp56-59 (Nakao et al., 1985), were used as primary antibodies for protein detection. F39.4.1, AC88, and KN382/EC1 were used at a concentration of 10 μg/mL. N27 was used at a dilution of 1:1000. Alkaline phosphate conjugated goat anti-mouse IgG (Sigma) was used as secondary antibody to detect the proteins on the blot.

Construction of the Expression Vectors and Transfections. Construction of expression vectors (pAR0 for wild type, pARL for LNCaP mutant androgen receptor) was described previously (Veldscholte et al., 1990a). Transfection of COS-1 and HeLa cells was done by the calcium phosphate precipitation method (Chen & Okayama, 1987). For binding studies, 12 dishes (75 cm^2, Nunclon) each with 1.2×10^6 COS cells were transfected with either 10 μg of pAR0 or 10 μg of pARL and 10 μg of pTZ (Pharmacia) carrier plasmid per dish. For transcription regulation studies, 5×10^5 HeLa cells/dish (30 cm^2, Nunclon) were transfected with either 1 μg of pAR0 or 1 μg of pARL (the optimal amount for this assay, unpublished results) and 1 μg of pG29GtkCAT reporter gene (Schüle et al., 1988). Carrier DNA (pTZ) was added to a total of 10 μg per dish. After 1 day, cells were washed, and experimental media (medium B with hormones at the indicated concentrations) were added. Two days after the transfection, cells were harvested for the CAT assay.

Hormone-Binding Assay. COS cells transfected with either pAR0 or pARL were collected by scraping in buffer and homogenized, and a cytosol fraction was prepared as described previously (Veldscholte et al., 1990b). The cytosol was incubated overnight at 4 °C with 5 nM [^{3}H]R1881 in the presence of unlabeled steroids ranging from 0 to 1000-fold the concentration of the label. Separation of bound and unbound steroid was achieved by protamine sulfate precipitation (Veldscholte et al., 1990b). Relative binding affinity (RBA, expressed in percent) represents the ratio of the amount of nonlabeled R1881 and competing compound which are needed for 50% inhibition of the binding of tritiated R1881.

CAT Assays. The CAT (chloramphenicol acetyltransferase) assay was essentially performed as described by Seed and Sheen (1988), using the method of xylene extraction of butyrylated chloramphenicol. The CAT activity per dish was calculated; background CAT activity (vehicle only; 0.2% ethanol) was set at 0%. For each steroid (or combination of steroids) tested, the amount of CAT activity after subtraction of background activity was expressed as a percentage of the highest level of CAT activity that was found for cells incubated with R1881. Background activity was less then 5% of the highest levels of CAT activity (at 10^{-9}–10^{-8} M R1881). Experiments were performed in triplicate.

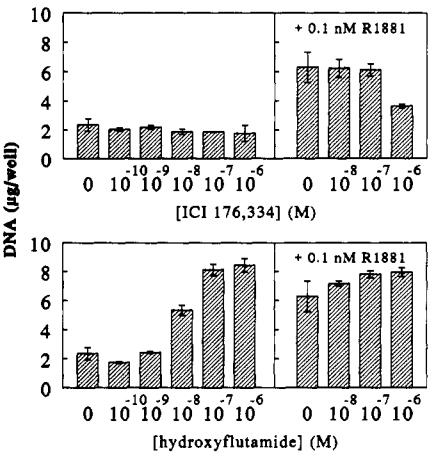

FIGURE 1: Effects of ICI 176 334 and hydroxyflutamide on growth of LNCaP cells. Various concentrations of ICI 176 334 (upper panels) and hydroxyflutamide (lower panels) were added alone (left panels) or in combination with 0.1 nM R1881 (right panels) with one medium change after 3 days, as described under Experimental Procedures. DNA content was determined after 6-days culture. Means and standard deviations of four measurements are shown.

RESULTS

LNCaP Growth Studies. The synthetic androgen R1881 increases the growth rate of LNCaP cells in charcoal-stripped medium at concentrations of 10^{-11} M and higher, with maximal stimulation at 10^{-10} M (Schuurmans et al., 1988). In the present experiments, 10^{-10} M R1881 gave a 2.7-fold increase in DNA content versus control cultures (Figure 1 compare the first bar in the left panels with the first bar in the right panels). ICI 176 334 did not have any effect on the growth rate from 10^{-10} up to 10^{-6} M (Figure 1). However, ICI 176 334 partly inhibited the effect of 10^{-10} M R1881 on the cell growth at 10^{-6} M (upper right panel of Figure 1).

In contrast, hydroxyflutamide induced cell growth at concentrations ranging from 10^{-8} to 10^{-6} M. In cells submaximally stimulated with R1881, this anti-androgen further increased the growth rate (not shown).

Binding of Heat-Shock Proteins to Androgen Receptors. To investigate the effects of androgens and anti-androgens on the interaction of the androgen receptor with heat-shock proteins, the LNCaP cells were incubated either at 4 °C (control) or at the physiological temperature of 37 °C with androgens or anti-androgens. The receptor complexes were isolated from the cytosol using an antibody specific for the human androgen receptor and subjected to electrophoresis. Subsequently, Western blots were incubated with antibodies specific for the androgen receptor and the heat-shock proteins hsp90, hsp70, and hsp56-59, respectively.

When only vehicle (ethanol) was added to the cells, incubation at 37 °C did not induce changes in the interactions of the AR with the three different heat-shock proteins (Figure 2, compare lanes 1 and 2). Incubation of the cells with the androgen receptor agonist R1881 at 37 °C resulted in a loss of hsp90 and hsp56 from the receptor complex and in a decrease in the amount of hsp70 bound (Figure 2, compare lanes 3 and 4). Incubation of the cells with hydroxyflutamide at 37 °C, both in the absence and in the presence of R1881, resulted in dissociation of the receptor complex (Figure 2, compare lane 7 with lane 8, and lane 11 with lane 12). In contrast, ICI 176 334 did not affect receptor complex disso-

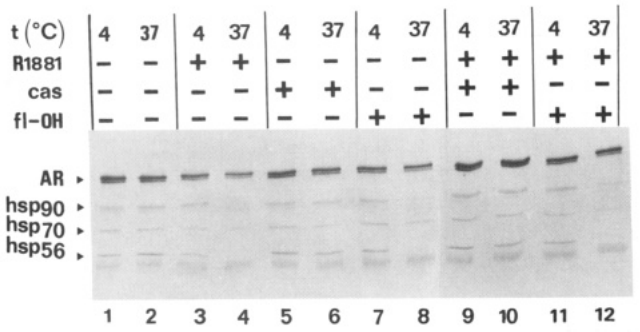

FIGURE 2: Heat-shock protein interaction with the androgen receptor isolated from LNCaP cell cytosol. The cells were incubated for 30 min at 4 °C (lanes 1, 3, 5, 7, 9, and 11) or at 37 °C (lanes 2, 4, 6, 8, 10, and 12). The androgen receptor was immunopurified from the cytosol with the monoclonal antibody F39.4.1 and after SDS electrophoresis visualized on a Western blot with the same antibody. Equal amounts of cytosolic protein (1.4 mg) were used for the immunopurification procedure. Hsp90, hsp70, and hsp56 were stained with the specific antibodies AC88, N27, and KN382/EC1, respectively. In all lanes, staining of IgG is visible. AR, androgen receptor; cas, ICI 176 334; fl-OH, hydroxyflutamide. Compounds tested: vehicle only (lanes 1 and 2); 10^{-8} M R1881 (lanes 3 and 4); 5×10^{-5} M ICI 176 334 (lanes 5 and 6); 5×10^{-5} M hydroxyflutamide (lanes 7 and 8); 10^{-8} M R1881 + 5×10^{-5} M ICI 176 334 (lanes 9 and 10); 10^{-8} M R1881 + 5×10^{-5} M hydroxyflutamide (lanes 11 and 12).

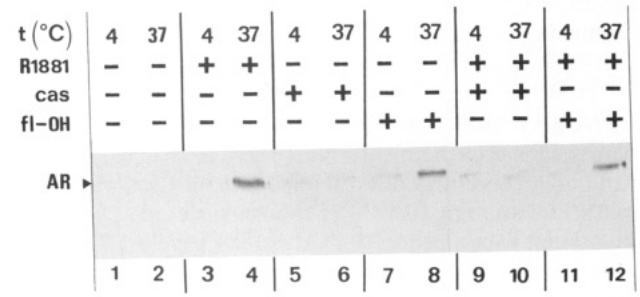

FIGURE 3: Retention of the androgen receptor in the nucleus of LNCaP cells. The cells were incubated for 30 min at 4 °C (lanes 1, 3, 5, 7, 9 and 11) or at 37 °C (lanes 2, 4, 6, 8, 10, and 12). The androgen receptor was immunopurified from nuclear extracts with the monoclonal antibody F39.4.1 and after SDS electrophoresis visualized on a Western blot with the same antibody. AR, androgen receptor; cas, ICI 176 334; fl-OH, hydroxyflutamide. Equal amounts of nuclear protein (0.6 mg) were used for the immunopurification procedure. Compounds tested: vehicle only (lanes 1 and 2); 10^{-8} M R1881 (lanes 3 and 4); 5×10^{-5} M ICI 176 334 (lanes 5 and 6); 5×10^{-5} M hydroxyflutamide (lanes 7 and 8); 10^{-8} M R1881 + 5×10^{-5} M ICI 176 334 (lanes 9 and 10); 10^{-8} M R1881 + 5×10^{-5} M hydroxyflutamide (lanes 11 and 12).

ciation (Figure 2, compare lanes 5 and 6) and antagonized the effect of the androgen R1881 (Figure 2, compare lanes 9 and 10).

Nuclear Retention of the Androgen Receptor. The presence of receptors in nuclear extracts is indicative for the transformation process of the steroid–receptor complex to a tight nuclear-binding form (Beato, 1989). To investigate the effects of androgens and anti-androgens on the binding of the androgen receptor in the nucleus, LNCaP cells were incubated either at 4 °C or at 37 °C with androgens or anti-androgens. Androgen receptors were isolated from nuclear extracts of these cells and subjected to electrophoresis and Western blotting and staining with a specific antibody for the androgen receptor. A small amount of receptor was found in the nuclear extracts after incubation of the cells at 4 °C in the absence of hormones and in the presence of R1881, ICI 176 334, or hydroxyflutamide, respectively (Figure 3, lanes 1, 3, 5, and 7). The amount of tight nuclear-bound receptor increased when the cells were incubated at 37 °C only in the presence of R1881 (lane 4) and hydroxyflutamide (lane 8), but not in

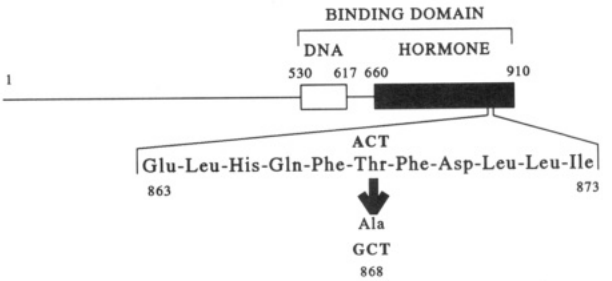

FIGURE 4: Androgen receptor of LNCaP cells. In codon 868, A is replaced by G, which results in the substitution of an alanine for a threonine residue. The numbers indicate the amino acid residue numbers at the domain boundaries [from Veldscholte et al. (1990a)].

the presence of ICI 176 334 (lane 6) or in the absence of hormones (lane 2). Furthermore, ICI 176 334 inhibits tight nuclear binding of the receptor induced by R1881 (Figure 3, lanes 9 and 10).

Binding Affinities. To compare the binding affinities of different compounds under identical conditions, expression vectors containing either the wild-type sequence (normal androgen receptor; pAR0) or the mutant sequence (LNCaP cell androgen receptor; pARL; see Figure 4) were transiently expressed in COS cells. Competition experiments performed on the cytosols of these cells showed that the two receptors had similar relative binding affinities (RBA's) for androgenic compounds (dihydrotestosterone and R1881) but showed striking differences for some nonandrogenic compounds (increased RBA of progestagens and estradiol for the mutant receptor; Veldscholte et al., 1990a). For a series of anti-androgens, a slight increased RBA was observed for the mutant receptor: cyproterone acetate, 2.6 vs 1.4; nilutamide, 0.4 vs 0.1; hydroxyflutamide, 2.4 vs 0.4. The RBA for ICI 176 334 is negatively influenced by the mutation (RBA 0.1 vs 0.3, mutant vs wild-type AR).

CAT Induction. Cotransfection of the expression vector for either the wild-type or the mutant androgen receptor with an androgen-regulated reporter gene was performed to study differences in effect on transcription of the anti-androgens. It has been shown that the glucocorticoid responsive element (GRE) can also act as androgen responsive element (Beato, 1989). Therefore, the GRE-driven vector pG29GtkCAT (Schüle et al., 1988) was used as reporter gene in the cotransfection experiments. The androgen R1881 stimulated CAT activity, when added to cells containing either the wild-type or the mutant androgen receptor (Figure 5). When ICI 176 334 was tested in this cotransfection system, this compound did not stimulate CAT activity in cells containing either the normal or the mutant receptor. However, hydroxyflutamide stimulated CAT activity of cells expressing the mutant receptor. The ability of the two anti-androgens to antagonize the CAT induction by R1881 was also tested (Figure 6). In the presence of the wild-type receptor, both hydroxyflutamide and ICI 176 334 antagonized the effect of R1881. The antagonistic effect of hydroxyflutamide was observed at concentrations of 1000-fold or higher the concentration of R1881, and ICI 176 334 mediated antagonism was observed at 10 000-fold or higher the concentration of R1881. When the mutant receptor was expressed, hydroxyflutamide had only limited effects on R1881-mediated CAT induction, but ICI 176 334 showed an antagonistic effect, as in the case of the wild-type receptor.

Discussion

The androgen receptor, like other members of the steroid hormone receptor family, is thought to be present in its un-

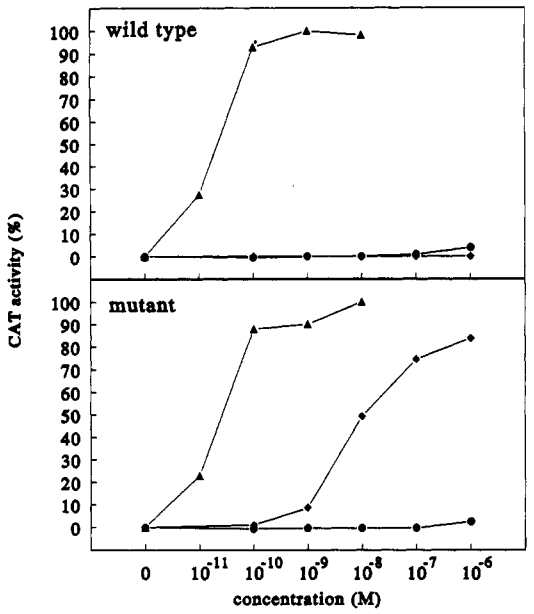

FIGURE 5: Induction of CAT activity in transfected HeLa cells. The cells were cotransfected with the expression vector either encoding the wild-type androgen receptor (left panels) or coding for the LNCaP mutant receptor (right panels) and a GRE-tk-CAT construct. CAT activity was determined after incubation of the cells with R1881 (▲), hydroxyflutamide (♦), or ICI 176 334 (●) as described under Experimental Procedures.

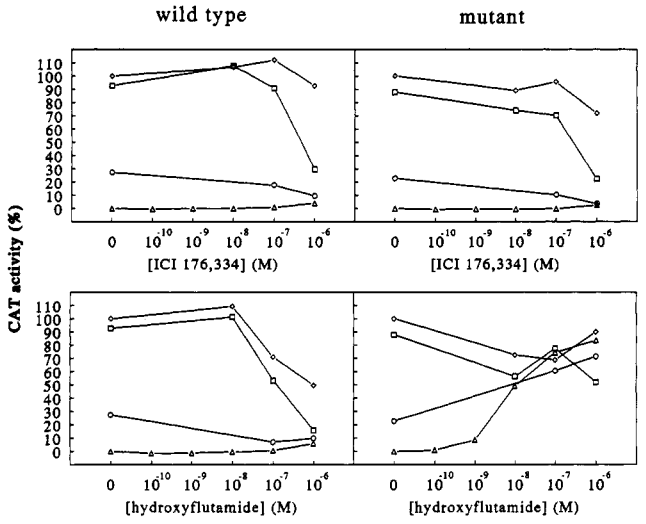

FIGURE 6: Effects of R1881, ICI 176 334, and hydroxyflutamide on the induction of CAT activity in transfected HeLa cells. The cells were cotransfected with the expression vector either encoding the wild-type androgen receptor or encoding the LNCaP mutant receptor, and a GRE-tk-CAT construct. CAT activity was determined after incubation of the cells with ICI 176 334 or hydroxyflutamide alone (△) or in combination with R1881 at a concentration of 0.01 (○), 0.1 (□), or 1 nM (◇) as described under Experimental Procedures.

transformed state as a heteromolecular complex, containing several proteins, including heat-shock proteins. Heat-shock proteins are predominantly cytoplasmic, while most steroid receptors (with the exception of the glucocorticoid receptor) are primarily nuclear (Carson-Jurica et al., 1990). In one model explaining the action of steroid hormones, the ligand induces a dissociation of the heteromeric complex (step 1), thereby revealing the DNA-binding domain which can interact with the hormone responsive element (Pratt et al., 1989; Renoir et al., 1990). Receptor dimerization (step 2) has been shown to play a role in receptor binding to the glucocorticoid and estradiol responsive elements (Chalepakis et al., 1990; Fawell et al., 1990). For progesterone receptors, the ability to form stable dimers in the absence of DNA was found to correlate with the release of 90-kDa heat-shock protein (DeMarzo et al., 1991). Interaction of the receptor with transcription factors (step 3) is the final step leading to transcription regulation of target genes. Anti-hormones may exert their effect at one or more steps in this scheme, and as a consequence differ in their mechanism with respect to inhibition of steroid-induced transcription.

In the present study, we have used the androgen-sensitive LNCaP prostate tumor cell line to study the mechanism of action of some anti-androgens. The androgen receptor in the LNCaP cells contains a mutation in the steroid-binding domain (Thr to Ala at position 868; Veldscholte et al., 1990a). Two structurally related, nonsteroidal anti-androgens (called "pure" anti-androgens because their mechanism of action is thought to interfere only in androgen action) showed opposite effects on growth of the tumor cells. While ICI 176 334 inhibited LNCaP tumor growth, hydroxyflutamide behaved as an agonist and stimulated LNCaP cell proliferation. In previous studies, the agonistic properties of some anti-androgens on growth of LNCaP cells have been described (Wilding et al., 1989; Schuurmans et al., 1990). Also the secretion of prostatic acid phosphatase by LNCaP cells is increased not only by androgens but also by estradiol and the anti-androgens cyproterone acetate and nilutamide (Schuurmans et al., 1990). Cyproterone acetate gives a down-regulation of the androgen receptor mRNA, indicative of an agonistic effect (Quarmby et al., 1990). To prove that the deviant effects of anti-androgens on LNCaP cells are solely due to altered ligand-binding characteristics and to an altered transcription activation mechanism of the mutant androgen receptor, HeLa cells were transfected with a reporter gene and androgen receptor expressing plasmid constructs differing only with respect to the bases coding for the mutated amino acid residue. Similar results were obtained as in the growth studies: the anti-androgen ICI 176 334 retained inhibitory characteristics for both the mutant and wild-type androgen receptor, whereas hydroxyflutamide behaved as an inhibitor of normal androgen receptor function but as a stimulator of the mutant receptor. For the estrogen and glucocorticoid receptors, partial agonistic properties of some antagonists have been shown. It was theorized that, depending on cell type and promoter context, the N-terminally located TAF-1 (transcription activation function of the receptor) was activated by those compounds (Meyer et al., 1990; Klein-Hitpass et al., 1991). We did not observe any agonistic action of anti-androgens for the wild-type receptor transfected into HeLa cells together with a GRE-tk-CAT-containing reporter gene construct.

To compare the binding characteristics of anti-androgens for wild-type and mutant receptors, these receptors were overexpressed in COS cells, and competition analysis was performed. The binding affinity of the anti-androgens for the AR was only a few percent of the affinity of androgens. For ICI 176 334, the binding affinity was decreased for the mutant receptor as compared with the wild-type receptor, whereas the affinity of hydroxyflutamide was increased for the mutant receptor. The lower affinity of ICI 176 334 for the mutant receptor might be related to a faster dissociation rate. A strict relationship between the dissociation rate of anti-androgens and antagonistic activity, however, has not been found (Wakeling et al., 1981). It is therefore unlikely that the differences in affinity alone could explain agonistic or antagonistic properties of the anti-androgens for the receptor in LNCaP cells.

Our in vitro studies with LNCaP cell cytosol showed that the androgen receptor in its transcriptionally inactive state (i.e., the steroid–receptor complex before transformation to the DNA-binding state) is present as a heteromolecular complex with different heat-shock proteins. Isolation of the androgen receptor with a monoclonal antibody against the receptor resulted in coprecipitation of hsp90, hsp70, and hsp56. Association of the androgen receptor with hsp90 and hsp56 was shown before (Joab et al., 1984; Sullivan et al., 1985; Nakao et al., 1985; Tai et al., 1986). The association with hsp70 was shown for both the progesterone receptor and the glucocorticoid receptor (Kost et al., 1989; Sanchez et al., 1990) but, thus far, not for the androgen receptor. Incubation of intact LNCaP cells with the synthetic androgen R1881 at 37 °C resulted in dissociation of heat-shock proteins from the receptor complex. In line with the agonistic properties of hydroxyflutamide in LNCaP cells, this compound also induced release of heat-shock proteins at 37 °C. ICI 176 334 exerted a stabilizing effect on the heteromeric androgen–receptor complex. Furthermore, its antagonistic properties in LNCaP cells were displayed by the inhibitory effects on androgen-induced heat-shock protein release. Similarly, stabilizing effects on a multiprotein heteromeric complex by glucocorticoid and progesterone receptor antagonists have been proposed as a mechanism of antagonism (Lefebvre, 1988; Segnitz & Gehring, 1990; Distelhorst & Howard, 1990; Renoir et al., 1990). Stabilization of the complex is thought to prevent the receptor from dimerizing and binding to regulatory sequences of responsive genes. We therefore also measured the effect of ICI 176 334 and hydroxyflutamide on binding of the androgen receptor to the nucleus in LNCaP cells. The loss of association of the receptor with heat-shock proteins is accompanied by an increase in the amount of tight nuclear-bound receptor, an indication that a transformed, DNA-binding form of the receptor is obtained. R1881 and hydroxyflutamide, both agonistic in LNCaP cells, increase the amount of tight nuclear-bound receptor in these cells, whereas the antagonist ICI 176 334 does not stimulate tight nuclear binding of the receptor but rather inhibits the effect mediated by R1881. Our data indicate that in LNCaP cells ICI 176 334 acts as an antagonist by inhibiting both dissociation of the heteromeric complex of the AR with heat-shock proteins and the subsequent high-affinity binding of the receptor to the nucleus.

The progestagen and estrogen antagonists have been tentatively divided into two classes depending on their level of action (Klein-Hitpass et al., 1991; Green, 1990). The so-called "type I" or "pure" antagonists interfere with the binding of the receptor to DNA (Berry et al., 1990; Fawell et al., 1990: Klein-Hitpass et al., 1991). Impaired receptor dimerization and subsequent binding to DNA in vitro were shown for ICI 164 384, an estrogen receptor antagonist (Fawell et al., 1990), although recently a stimulatory effect on receptor–DNA binding was also observed for this compound (Sabbah et al., 1991; Pham et al., 1991). The other class of anti-hormones (type II; including, e.g., the progesterone/glucocorticoid receptor antagonist RU486) does induce DNA binding of the receptor but blocks the transcription activation function TAF-2, a region located in the C-terminal steroid-binding domain of the receptor (Meyer et al., 1990). According to this scheme, the anti-androgen ICI 176 334 would be classified for LNCaP cells as a type I antagonist, interfering with the transformation of the androgen–receptor complex to the DNA-binding state (the receptor form that interacts with the hormone responsive element). It is tempting to speculate that in LNCaP cells, due to the mutation, antagonist do not impair functioning of a TAF-2-like transcription activation function in the androgen receptor.

In conclusion, we propose from our results a mechanism of action of anti-androgens in LNCaP cells in which these compounds affect different steps in the processes of receptor transformation and transcription activation. In LNCaP cells, ICI 176 334 shows decreased affinity for the AR and affects steps before DNA binding occurs. In contrast, other anti-androgens including hydroxyflutamide show increased affinity for the mutant AR, transform the receptor to the DNA-binding state, and permit interaction of the AR with the transcription machinery.

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Registry No. ICI 176334, 90357-06-5; hydroxyflutamide, 52806-53-8.

References

Beato, M. (1989) *Cell 56*, 335–344.

Berry, M., Metzger, D., & Chambon, P. (1990) *EMBO J. 9*, 2811–2818.

Carson-Jurica, M. A., Schrader, W. T., & O'Malley, B. (1990) *Endocr. Rev. 11*, 201–220.

Chalepakis, G., Schauer, M., Cao, X., & Beato, M. (1990) *DNA Cell Biol. 9*, 355–368.

Chandolia, R. K., Weinbauer, G. F., Behre, H. M., & Nieschlag, E. (1991) *J. Steroid Biochem. Mol. Biol. 38*, 367–375.

Chang, C., Kokontis, J., & Liao, S. (1988) *Science 240*, 324–326.

Chen, C., & Okayama, H. (1987) *Mol. Cell. Biol. 7*, 2745–2752.

DeMarzo, A. M., Beck, C. A., Oñate, S. A., & Edwards, D. P. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 72–76.

Distelhorst, C. W., & Howard, K. J. (1990) *J. Steroid Biochem. 36*, 25–31.

Faber, P. W., Kuiper, G. G. J. M., Van Rooij, H. C. J., Van der Korput, J. A. G. M., Brinkmann, A. O., & Trapman, J. (1989) *Mol. Cell. Endocrinol. 61*, 257–262.

Fawell, S. E., White, R., Hoare, S., Sydenham, M., Page, M., & Parker, M. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 6883–6887.

Furr, B. J. A., Valcaccia, B., Curry, B., Woodburn, J. R., Chesterton, G., & Tucker, H. (1987) *J. Endocrinol. 113*, R7–R9.

Green, S. (1990) *J. Steroid Biochem. Mol. Biol. 37*, 747–751.

Guiochon-Mantel, A., Loosfelt, H., Ragot, T., Bailly, A., Atger, M., Misrahi, M., Perricaudet, M., & Milgrom, E. (1988) *Nature 336*, 695–698.

Hinegardner, R. T. (1976) *Anal. Biochem. 72*, 248–254.

Horoszewicz, J. S., Leong, S. S., Kawinski, E., Karr, J. P., Rosenthal, H., Ming Chu, T., Mirand, E. A., & Murphy, G. P. (1983) *Cancer Res. 43*, 1809–1818.

Joab, I., Radanyi, C., Renoir, M., Buchou, T., Catelli, M.-G., Binard, N., Mester, J., & Baulieu, E.-E. (1984) *Nature 308*, 850–853.

Klein-Hitpass, L., Cato, A. C. B., Henderson, D., & Ryffel, G. U. (1991) *Nucleic Acids Res. 19*, 1227–1234.

Kost, S. L., Smith, D., Sullivan, W., Welch, W. J., & Toft, D. O. (1989) *Mol. Cell. Biol. 9*, 3829–3838.

Laemmli, U. K. (1970) *Nature 227*, 680–685.

Lefebvre, P., Danze, P.-M., Sabloniere, B., Richard, C., Formstecher, P., & Dautrevaux, M. (1988) *Biochemistry 27*, 9186–9194.
Lindemeyer, R. G., Robertson, N. M., & Litwack, G. (1990) *Cancer Res. 50*, 7985–7991.
Lubahn, D. B., Joseph, D. R., Sar, M., Tan, J., Higgs, H. N., Larson, R. E., French, F. S., & Wilson, E. M. (1988) *Mol. Endocrinol. 2*, 1265–1275.
Mahler, C., & Denis, L. (1990) *J. Steroid Biochem. Mol. Biol. 37*, 921–924.
Meyer, M. E., Pornon, A., Ji, J. W., Bocquel, M. T., Chambon, P., & Gronemeyer, H. (1990) *EMBO J. 9*, 3923–3932.
Moudgil, V. K., & Hurd, C. (1987) *Biochemistry 26*, 4993–5001.
Moudgil, V. K., Anter, M. J., & Hurd, C. (1989) *J. Biol. Chem. 264*, 2203–2211.
Mowszowicz, I., Bieber, D. E., Chung, K. W., Bullock, L. P., & Bardin, C. W. (1974) *Endocrinology 95*, 1589–1599.
Nakao, K., Myers, J. E., & Faber, L. E. (1985) *Can. J. Biochem. Cell Biol. 63*, 33–40.
Neumann, F., & Töpert, M. (1986) *J. Steroid Biochem. 25*, 885–895.
Pham, T. A., Elliston, J. F., Nawaz, Z., Mcdonnell, D. P., Tsai, M. J., & Omalley, B. W. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 3125–3129.
Pratt, W. B., Sanchez, E. R., Bresnick, E. H., Meshinchi, S., Scherrer, L. C., Dalman, F. C., & Welsh, M. J. (1989) *Cancer Res. 49*, 2222s–2229s.
Quarmby, V. E., Yarbrough, W. G., Lubahn, D. B., French, F. S., & Wilson, E. M. (1990) *Mol. Endocrinol. 4*, 22–28.
Raynaud, J.-P., & Ojasoo, T. (1986) *J. Steroid Biochem. 25*, 811–833.
Renoir, J. M., Radanyi, C., Jung-Testas, I., Faber, L. E., & Baulieu, E.-E. (1990) *J. Biol. Chem. 265*, 14402–14406.
Riehl, R. M., Sullivan, W. P., Vroman, B. T., Bauer, V. J., Pearson, G. R., & Toft, D. O. (1985) *Biochemistry 24*, 6586–6591.
Sabbah, M., Gouilleux, F., Sola, B., Redeuilh, G., & Baulieu, E.-E. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 390–394.
Sanchez, E. R. (1990) *J. Biol. Chem. 265*, 22067–22070.
Sanchez, E. R., Faber, L. E., Henzel, W. J., & Pratt, W. B. (1990) *Biochemistry 29*, 5145–5152.
Schneider, C., Newman, D., Sutherland, R., Asser, U., & Greaves, M. F. (1982) *J. Biol. Chem. 257*, 10766–10769.
Schüle, R., Muller, M., Kaltschmidt, C., & Renkawitz, R. (1988) *Science 242*, 1418–1420.
Schuurmans, A. L. G., Bolt, J., Voorhorst, M., Blankenstein, R. A., & Mulder, E. (1988) *Int. J. Cancer 42*, 917–922.
Schuurmans, A. L. G., Bolt, J., Veldscholte, J., & Mulder, E. (1990) *J. Steroid Biochem. Mol. Biol. 37*, 849–853.
Seed, B., & Sheen, J.-Y. (1988) *Gene 67*, 271–277.
Segnitz, B., & Gehring, U. (1990) *J. Biol. Chem. 265*, 2789–2796.
Smith, D. F., Faber, L. E., & Toft, D. O. (1990) *J. Biol. Chem. 265*, 3996–4003.
Sullivan, W. P., Vroman, B. T., Bauer, V. J., Puri, R. K., Riehl, R. M., Pearson, G. R., & Toft, D. O. (1985) *Biochemistry 24*, 4214–4222.
Tai, P. K., Maeda, Y., Nakao, K., Wakim, N. G., Duhring, J. L., & Faber, L. E. (1986) *Biochemistry 25*, 5269–5275.
Trapman, J., Klaassen, P., Kuiper, G. G. J. M., Van der Korput, J. A. G. M., Faber, P. W., Van Rooij, H. C. J., Geurts van Kessel, A., Voorhorst, M. M., Mulder, E., & Brinkmann, A. O. (1988) *Biochem. Biophys. Res. Commun. 153*, 241–248.
Van Laar, J. H., Voorhorst-Ogink, M. M., Zegers, N. D., Boersma, W. J. A., Claassen, E., van der Korput, J. A. G. M., Ruizeveld de Winter, J. A., van der Kwast, Th. H., Mulder, E., Trapman, J., & Brinkmann, A. O. (1989) *Mol. Cell. Endocrinol. 67*, 29–38.
Vass, K., Welch, W. J., & Nowak, T. S., Jr. (1988) *Acta Neuropathol. 77*, 128–135.
Veldscholte, J., Ris-Stalpers, C., Kuiper, G. G. J. M., Jenster, G., Berrevoets, C., Claassen, E., VanRooij, H. C. J., Trapman, J., Brinkmann, A. O., & Mulder, E. (1990a) *Biochem. Biophys. Res. Commun. 173*, 534–540.
Veldscholte, J., Voorhorst-Ogink, M. M., Bolt-de Vries, J., VanRooij, H. C. J., Trapman, J., & Mulder, E. (1990b) *Biochim. Biophys. Acta 1052*, 187–194.
Wakeling, A. E., Furr, B. J. A., Glen, A. T., & Hughes, L. R. (1981) *J. Steroid Biochem. 15*, 355–359.
Wilding, G., Chen, M., & Gelmann, E. P. (1989) *Prostate (N.Y.) 14*, 103–115.
Zegers, N. D., Claassen, E., Neelen, C., Mulder, E., Van Laar, J. H., Voorhorst, M. M., Berrevoets, C. A., Brinkmann, A. O., Van der Kwast, Th. H., Ruizeveld de Winter, J. A., Trapman, J., & Boersma, W. J. A. (1991) *Biochim. Biophys. Acta 1073*, 23–32.