Protein Chemistry and Structure:

Ligand-induced Conformational Alterations of the Androgen Receptor Analyzed by Limited Trypsinization: STUDIES ON THE MECHANISM OF ANTIANDROGEN ACTION

Cor W. Kuil, Cor A. Berrevoets and Eppo Mulder

J. Biol. Chem. 1995, 270:27569-27576.
doi: 10.1074/jbc.270.46.27569

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 270, No. 46, Issue of November 17, pp. 27569–27576, 1995

Printed in U.S.A.

Ligand-induced Conformational Alterations of the Androgen Receptor Analyzed by Limited Trypsinization

STUDIES ON THE MECHANISM OF ANTIANDROGEN ACTION*

(Received for publication, April 17, 1995, and in revised form, June 23, 1995)

Cor W. Kuil‡, Cor A. Berrevoets, and Eppo Mulder

From the Department of Endocrinology and Reproduction, Erasmus University Rotterdam, P. O. Box 1738, 3000 DR Rotterdam, The Netherlands

Limited proteolysis of *in vitro* produced human androgen receptor was used to probe the different conformations of the receptor after binding of androgens and several antiandrogens. The results provide evidence for five different conformations of the receptor, as detected by the formation of proteolysis resisting fragments: 1) an initial conformation of the unoccupied receptor not resisting proteolytic attack; and receptor conformations characterized by 2) a 35-kDa proteolysis resisting fragment spanning the ligand binding domain and part of the hinge region, obtained with most antagonists, and in an initial step after agonist binding; 3) a 29-kDa proteolysis resisting fragment spanning the ligand binding domain, obtained in the presence of agonists after an activation process; 4 and 5) 30- and 25-kDa fragments, derived from 2 and 3, but missing part of the C terminus, obtained with RU486 (RU486 has antiandrogenic properties, besides its effects as an antiprogestagen/antiglucocorticoid). Concomitantly with the change from 2 to 3 (and of 4 to 5 for RU486), dissociation of the 8 S complex of receptor with associated proteins occurred. With a mutant receptor (LNCaP cell mutation in C-terminal region), some antagonists activated transcription analogous to agonists, and induced the activated receptor conformation 3. A mutant lacking the C-terminal 12 amino acids bound RU486 but not androgens, and formed with RU486 conformation 5. These data imply that, after the initial rapid binding of ligand, androgens induce a conformational change of the receptor, a process that also involves release of associated proteins. RU486 induces an inappropriate conformation of the C-terminal end, similar as found for its effect on the progesterone receptor. In contrast, the other antiandrogens act at a different step in the mechanism of action: they do not induce an abnormal conformation, but act earlier and prevent a conformation change by stabilizing a complex with associated proteins.

The biological effects of androgens and other steroid hormones are mediated through intracellular receptors, belonging to the steroid and thyroid hormone receptor superfamily (1). Upon activation by the hormone, steroid receptors interact with specific DNA sequences, located in the flanking regions of target genes, resulting in modulation of the expression of these genes (2–4). Steroid hormone receptor antagonists inhibit the biological effects of agonists, and are frequently used in the treatment of hormone-based dysfunctions in human. Furthermore, the synthetic antagonists are important tools to define the molecular mechanism of transactivation by steroid hormones (5, 6).

Agonists and antagonists may change the spatial structure of the receptor in distinct ways, as was first indicated by gel retardation experiments: antagonist- and agonist-receptor-DNA complexes showed slightly different mobilities (7–10). Recently, limited proteolysis of progesterone, estrogen, and glucocorticoid receptors pinpointed the distinction in conformation between hormone- and antihormone-bound receptor at the very C-terminal end of the receptor. Hormone treatment induced a pronounced conformational change in receptor structure, resulting in a prominent proteolysis resisting fragment. Several antagonists, including those which inhibit binding of the receptor to a hormone responsive element *in vitro*, induced an equally dramatic but distinct change in the receptor conformation; the proteolysis resisting fragments induced by binding of the different antagonists to their receptors were smaller than that induced by hormone binding (11–13). In addition, the monoclonal antibody C-262, raised against the last 14 amino acids of the progesterone receptor, could discriminate agonist- and antagonist-bound progesterone receptors. The antibody bound to the full-length receptor only in the presence of antagonist, whereas progesterone prevented the recognition of the receptor by C-262 (14, 15). Deletion of 42 amino acids abolished the binding of progesterone, but had no effect on binding of the antagonist RU486. Functional characterization showed that the mutant receptor induced transcription upon addition of RU486 (14). These results led to the hypothesis that the activity of antagonists is based on the induction of a non-functional conformation at the C terminus of steroid hormone receptors (4–6, 11).

Androgen binding to the androgen receptor (AR)[1] changed the receptor structure in such a way that the entire ligand binding domain resisted proteolytic degradation (10, 16, 17). However, for the antiandrogen-bound receptor, the outcome of preliminary studies on resistance against proteolytic degradation varied in different investigations. An unaltered conformation of the ligand binding domain, similar as seen in the absence of ligand, was reported by Kallio *et al*. (10). A structural difference between agonist- and antagonist-receptor complexes in the area around the hinge region of the receptor was deduced from studies in our laboratory. In the presence of an antiandrogen, a part of the hinge region in addition to the ligand

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Dept. of Endocrinology and Reproduction, Erasmus University Rotterdam, P.O. Box 1738, 3000 DR Rotterdam, The Netherlands. Tel.: 3110-4087338; Fax: 3110-4366832.

[1] The abbreviations used are: AR, wild-type human androgen receptor; ARL, LNCaP mutant androgen receptor; HSP(s), heat-shock protein(s); LUC, luciferase; MMTV, mouse mammary tumor virus.

binding domain resisted proteolysis (16). Finally, Zeng *et al.* (17) observed no difference in the size of the proteolysis resisting fragments in the presence of either the agonist dihydrotestosterone or the antagonists cyproterone acetate and hydroxyflutamide. Despite these differences in results, none of these three studies provided evidence for the involvement of the C terminus of the androgen receptor in the mechanism of antiandrogen action. Therefore, the differences in structural alteration of the homologous ligand binding domains of steroid hormone receptors by antiandrogens and by other steroid receptor antagonists may reflect a difference in the mechanisms of antagonist action.

In this paper we extend the studies on androgen receptor conformation by inclusion of conditions that affect the transformation process of receptors. In addition, RU486 was included. Due to its broad steroid specificity, this compound also binds to the androgen receptor, although with a much lower affinity than for the progesterone and glucocorticoid receptor (18, 19). Furthermore, we used the mutant receptor ARL (LNCaP mutant)(20) to assess a correlation between the ligand-induced conformational alteration of the receptor and the ability to activate transcription in transfection studies. The results indicate a similarity in conformational alterations for the different steroid hormone receptors, but a distinction in mechanism of action between most antiandrogens and antagonists for other steroid hormone receptors.

EXPERIMENTAL PROCEDURES

Materials—[^{3}H]R1881 (87 Ci/mmol) and unlabeled R1881 (methyltrienolone) were purchased from DuPont NEN. [^{3}H]RU486 (38.4 Ci/mmol), unlabeled RU486 (RU 38486, mifepristone), and nilutamide (RU 23908) were gifts from Roussel Uclaf (Romainville, France); cyproterone acetate was from Schering AG (Berlin, Germany); hydroxyflutamide from Schering (Bloomfield, IL); and ICI 176.334 ("Casodex") from ICI Pharmaceuticals (Macclesfield, United Kingdom). L-[^{35}S]Methionine (>1000 mCi/mM) was obtained from Amersham (Buckinghamshire, UK); RNA transcription kit and pBluescript KS− from Stratagene; rabbit reticulocyte lysate and recombinant RNasin from Promega; trypsin (type III), goat anti-mouse agarose, and goat anti-rabbit agarose from Sigma; and BM chemiluminescence Western blotting kit from Boehringer Mannheim (Mannheim, Germany). The mouse monoclonal antibody AC88 (recognizing HSP90) was generously provided by Dr. D. O. Toft (Mayo Clinic, Rochester, MN).

Plasmid Construction—The expression vectors pSVAR (encoding a human AR of 910 amino acids) (21) and pSVARL (LNCaP mutation; codon 868, Thr to Ala of an AR of 910 amino acids) (20) were used for subcloning in pBluescript to obtain pBSAR and pBSARL, respectively (16). The plasmid pBSAR 1–898, encoding a receptor with a C-terminal deletion of 12 amino acids, was constructed from pSVAR 1–898 (22). The mouse mammary tumor virus-luciferase (MMTV-LUC) reporter plasmid was kindly provided by Organon (Oss, The Netherlands).

Cell Culture and Transfection—HeLa cells were cultured and transfected, with some modifications, as described previously (23). For transcription regulation studies, 1.5×10^5 HeLa cells/well (10 cm^2) were transfected with either 0.5 μg of pSVAR or 0.5 μg of pSVARL, and 0.5 μg of MMTV-LUC, per well. Carrier DNA (pTZ) was added to a total of 5 μg/well. After 1 day, a glycerol shock (15% (w/v) glycerol in serum-free minimal essential medium, 1.5 min) was performed, whereafter the cells were washed and experimental media were added. Two days after transfection, cells were harvested for the luciferase assay.

Luciferase Assay—For the luciferase (LUC) assay, 200 μl of lysis buffer (25 mM Tris phosphate (pH 7.8), 15% glycerol, 1% Triton X-100, 8 mM $MgCl_2$, 1 mM dithiothreitol) was added to the phosphate-buffered saline-washed cells. After an incubation for 10 min, supernatants were collected, 100 μl of luciferine solution (1 mM luciferine, 1 mM ATP in lysis buffer) added, and luciferase activity was measured with a LUMAC Biocounter M2500. In the absence of hormone, LUC activity was less then 10% of the highest levels of LUC activity (at 10^{-9}-10^{-8} nM R1881). Experiments were performed in triplicate.

In Vitro Transcription and Translation—Both *in vitro* transcription and translation, in the presence of unlabeled or [^{35}S]methionine, were performed according to the manufacturer's instruction. Sense mRNA produced from pBSAR, pBSARL, and pBSAR 1–898 was used for translation.

Limited Trypsinization Assay—Two μl of labeled translation mixture was incubated at 25 °C with 3 μl of ligand solution diluted in water for the indicated time. Limited trypsinization was performed by addition of 5 μl of trypsin solution (dissolved in water) either at 25 °C (15 min; 40 μg/ml trypsin) or at 0 °C (1 h; 100 μg/ml trypsin). After incubation, 20 μl of 1.5 × SDS sample buffer was added. Samples were electrophoresed (24) and autoradiography was performed overnight. In some experiments, immunoadsorbed receptor protein was used. Of the resuspended resin with adsorbed receptor protein, 80 μl was incubated with 10 μl of ligand solution for 1 h at 25 °C, whereafter 10 μl of trypsin solution (50 μg/ml) was added to the incubation mixture. After this incubation 25 μl of 5 × SDS sample buffer was added, samples were boiled, the resin removed by centrifugation, and 25 μl was loaded on the gel.

Immunoprecipitation of Receptor Fragments and Intact Receptor—Soybean trypsin inhibitor was added to the digestion mixture to a final concentration of 200 μg/ml. Goat anti-rabbit or goat anti-mouse agarose (100 μl, diluted 1:4 in phosphate-buffered saline) was incubated for 2 h at 4 °C with 1 μl of polyclonal rabbit antiserum SP066 (epitope amino acids 892–910) (25) or mouse monoclonal antiserum F52 (epitope amino acids 593–612) (26). Following this incubation, the resin was washed with phosphate-buffered saline and added to the limited proteolytic digest of the receptor. After incubation for 2 h at 4 °C, the resin was washed with phosphate-buffered saline, and 25 μl of sample buffer was added. Electrophoresis was performed as described above (24).

Immunoadsorbed intact receptor was prepared with polyclonal rabbit antiserum SP197 (epitope amino acids 1–20)(27) from a non-digested translation mixture. After incubation for 2 h at 4 °C, the resin was washed with 0.5 M NaCl in buffer A (40 mM Tris/HCl (pH 7.4), 10 mM EDTA, 10% (w/v) glycerol), and resuspended in 1 ml of buffer A, containing 1 mg/ml bovine serum albumin, for proteolytic digestion experiments.

Western Immunoblot Analysis—Imunoaffinity purification and Western immunoblot analysis of androgen receptor complexes from LNCaP cells was performed as described previously (23). In short, the monoclonal antibody F39.4.1, specific for the androgen receptor (25), was chemically cross-linked to protein A-Sepharose and incubated with cytosol for 2 h at 4 °C under rotation. Subsequently, the resin was washed in buffer A with or without 0.5 M NaCl, whereafter electrophoresis and Western blotting was performed. The monoclonal antibodies AC88, specific for HSP90 (28), and F39.4.1 were used as primary antibodies in a chemiluminescence protein detection method, performed as described by the manufacturer (Boehringer Mannheim).

Sucrose Density Gradients—Sucrose density gradients (5–20% (w/v) sucrose) were prepared in buffer B (40 mM Tris/HCl (pH 7.4), 10% (w/v) glycerol, 10 mM dithiothreitol, 10 mM Na_2MoO_4, 50 mM NaCl). In experiments with labeled ligands, reticulocyte lysate containing unlabeled translation products (25 μl) was incubated with either 10 nM [^{3}H]R1881 or 100 nM [^{3}H]RU486 to label the receptor. For determination of nonspecific binding, 100-fold unlabeled steroid was added. Labeling occurred either for 2 h at 4 °C or 1 h at 25 °C, before addition of 25 μl of buffer B. Thereafter, free steroid was removed by incubation with 25 μl of dextran-coated charcoal suspension (0.1% (w/v) dextran, 1% (w/v) charcoal in 40 mM Tris/HCl (pH 7.4)) and centrifugation. Samples of 65 μl were loaded onto the gradients and run for 2 h at 60,000 rpm in a VTi 65 rotor (Beckman) at 4 °C. Fractions were collected from the bottom and assayed for radioactivity. Hemoglobin (4.6 S), alkaline phosphatase (6.2 S), and ^{14}C-labeled aldolase (7.9 S) were used as markers.

When no labeled ligand (antiandrogens) was available, [^{35}S]methionine was used to label translation products, and incubation occurred in the presence of unlabeled ligands. After sucrose density gradient centrifugation, fractions were denatured and electrophoresed as described under limited trypsinization assay. Autoradiograms were scanned, and density was calculated from recorded data.

Hormone Binding Assay—The hormone binding assays were performed with reticulocyte lysate containing unlabeled translation products. Lysate (10 μl) was incubated overnight at 4 °C with increasing concentrations of [^{3}H]RU486 (0.3–100 nM). In parallel incubations, 100-fold unlabeled RU486 was included to assess nonspecific binding. Separation of bound and free steroid was done with protamine precipitation as described previously (20). Scatchard plot analysis was performed to determine binding constants.

RESULTS

Transcription Activation—To compare the antiandrogenic properties of several antiandrogens, transcription activation was studied in a transient transfection system with either the wild-type AR or a mutant androgen receptor (ARL). The AR and ARL expression vectors were transfected into HeLa cells, together with the androgen receptor-sensitive reporter plasmid MMTV-LUC. The non-metabolizable, synthetic androgen R1881 induced LUC activity in a dose-dependent manner (Fig. 1). Cyproterone acetate and RU486 showed a limited partial agonistic effect with AR at concentrations up to 100 nM, whereas partial agonistic activity was not observed for hydroxyflutamide and ICI 176.334. In contrast, in cells with the mutant receptor ARL, cyproterone acetate and hydroxyflutamide induced LUC activity to the same levels as that observed with R1881. A partial agonistic activity of RU486 on ARL was found, whereas ICI 176.334 did not activate ARL. HeLa cells contain a limited amount of glucocorticoid receptor and RU486 is able to bind to these receptors. However, effects of RU486 on transcription activation were not due to this receptor system; in the absence of AR or ARL expression vectors, RU486 failed to induce LUC activity from the transfected MMTV-LUC reporter plasmid (not shown). Thus, with respect to its antiandrogenic properties, RU486 acted as an antagonist in the transient transfection system with AR and ARL, and did not show a higher agonistic activity for ARL than for AR.

Protease Resistance of Androgen- and Antiandrogen-Receptor Complexes—For study of the ligand-induced conformational changes of the AR, *in vitro* produced AR was incubated in the presence of ligand for different periods of time at 25 °C, whereafter limited trypsinization was performed at 0 °C and proteolysis resisting fragments were analyzed (Fig. 2*A*). When both the androgens R1881 (10 nM), testosterone (100 nM), or dihydrotestosterone (10 nM) and the trypsin were added simultaneously (*i.e.* no preincubation of receptor and steroid at 25 °C), a 35-kDa proteolysis resisting fragment of the AR was formed (result shown for R1881: Fig. 2*A*, *lane 4*). In the absence of ligand the receptor is completely degraded (Fig. 2*A*, *lane 2*). Addition of hormone therefore immediately induces a change in the conformation of the AR in such a way that the receptor was no longer completely degraded. With increasing incubation times at 25 °C, a shift in abundance of the 35-kDa proteolysis resisting fragment to a 29-kDa proteolysis resisting fragment of the AR occurred (Fig. 2*A*, *lanes 4–7*). This shift in size of the proteolysis resisting fragment indicates a second conformational change of the AR induced by androgens. The antiandrogens hydroxyflutamide (10 μM), cyproterone acetate (1 μM), ICI 176.334 (10 μM), and RU 23908 (1 μM) also immediately induced resistance of a 35-kDa fragment of AR against trypsinization, but no conformational alteration was observed within an incubation period of 60 min at 25 °C (result shown for hydroxyflutamide: Fig. 2*A*, *lanes 8–11*). In contrast to these antiandrogens, the presence of RU486 (1 μM) resulted in formation of 35- and 30-kDa proteolysis resisting fragments of the AR, and in a time-dependent shift toward shorter 29- and 25-kDa fragments (Fig. 2*A*, *lanes 12–15*). Next, antisera were used to study the differences in composition of the proteolysis resisting fragments of the AR, incubated for 60 min at 25 °C before trypsinization. The C terminus of the ligand binding domain of the RU486-bound AR did not resist trypsinization, as the 25-kDa fragment could not be immunoprecipitated with the antiserum SP066 (epitope at C terminus). In contrast, both the 35- and 29-kDa fragments could be precipitated with this antiserum (Fig. 2*B*, *lanes 16–21*). Because both these last two fragments contain the C terminus of the ligand binding domain, the differences in size are due to differences in extension into the hinge region, a region located at approximately 250 amino acids from the C terminus. The antiserum F52 recognizes an epitope in the DNA binding domain, adjacent to the hinge region. None of the proteolysis resisting fragments could

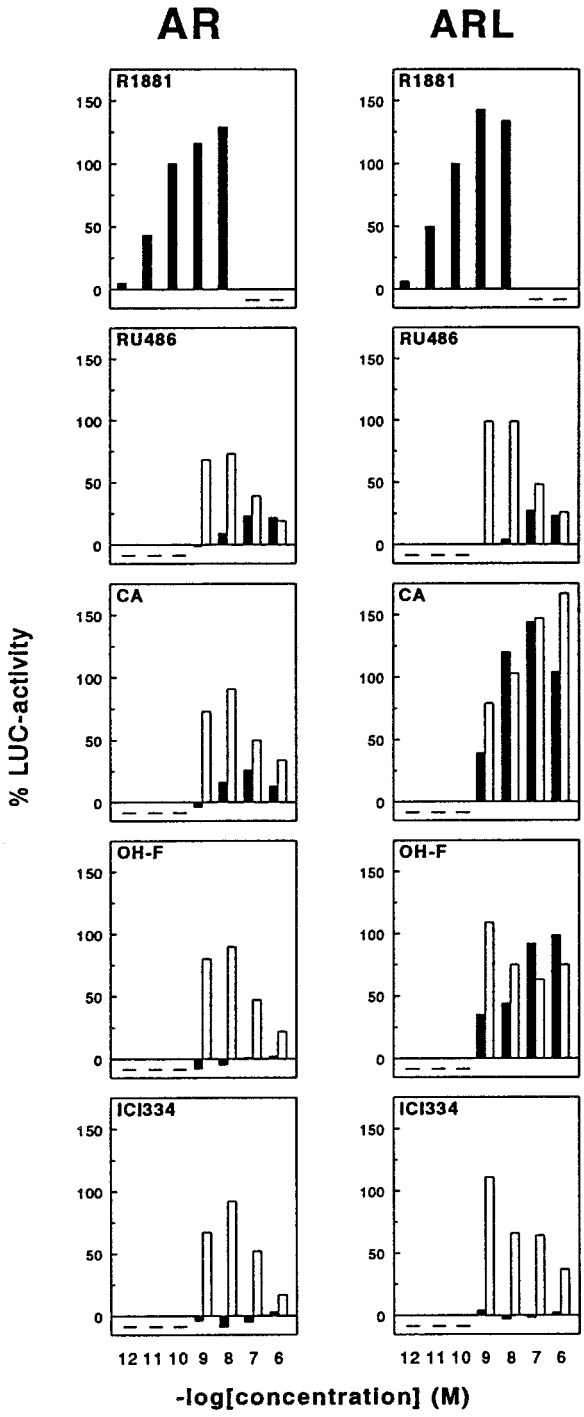

FIG. 1. **Transcriptional activity of the AR and LNCaP androgen receptor (ARL) in the presence of androgen, antiandrogens, and RU486.** LUC activity was determined in HeLa cells co-transfected with pSVAR or pSVARL and MMTV-LUC. After transfection, cells were incubated with R1881, hydroxyflutamide (*OH-F*), cyproterone acetate (*CA*), ICI 176.334 (*ICI334*) or RU486. LUC activity is indicated as percentage of the activity induced by 0.1 nM R1881 (=100%). LUC activities, induced by the indicated ligands are represented by *closed bars*. *Open bars* represent competition of 0.1 nM R1881 with the other ligands. The mean values of at least two experiments are shown. A LUC activity of <5% of that induced by 0.1 nM R1881 was observed with 10 nM R1881 in the absence of pSVAR or pSVARL, or in the absence of hormone.

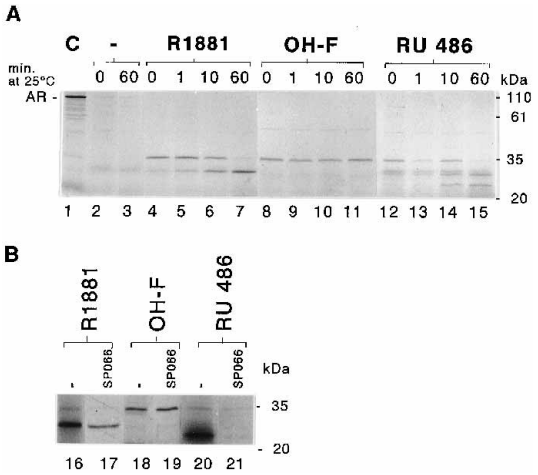

FIG. 2. **Limited trypsinization of androgen- and antiandrogen-bound AR.** *A, in vitro* produced [^{35}S]AR was incubated for the indicated time (min) at 25 °C in the presence of vehicle (−), R1881 (10 nM), hydroxyflutamide (*OH-F*, 10 μM) or RU486 (1 μM) before limited tryptic (100 μg/ml) digestion for 1 h at 0 °C. The trypsin-treated samples were denatured and electrophoresed on a 12.5% acrylamide gel. Autoradiography was performed overnight. *B, in vitro* produced [^{35}S]AR was incubated for 1 h at 25 °C in the presence of R1881 (*lanes 16* and *17*), hydroxyflutamide (*lanes 18* and *19*), or RU486 (*lanes 20* and *21*) and subjected to limited trypsinization (20 μg trypsin/ml, 15 min at 25 °C); thereafter samples were either left on ice (−; *lanes 16*, *18*, and *20*) or immunoprecipitated with the polyclonal antiserum SP066 (SP066; *lanes 17*, *19*, and *21*).

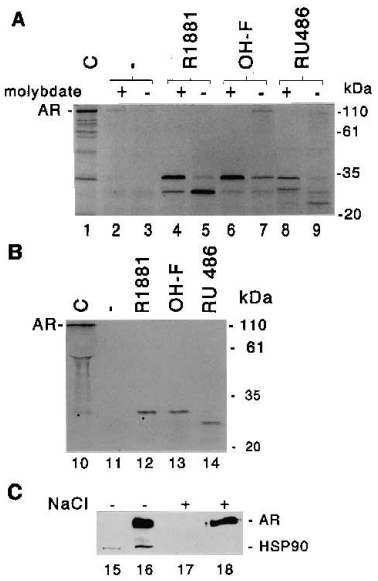

FIG. 3. **Effect of molybdate and removal of associated proteins on limited trypsinization of AR.** *A, in vitro* produced [^{35}S]AR was incubated (1 h at 25 °C) in the presence of vehicle (−), R1881 (10 nM), hydroxyflutamide (*OH-F*; 10 μM), or RU486 (1 μM), with (+) or without (−) molybdate (20 mM). Before limited trypsinization (20 μg trypsin/ml, 15 min at 25 °C), molybdate was added to 20 mM in all samples. *B*, [^{35}S]AR was immunoprecipitated with the antiserum SP197 and salt-washed (0.5 M NaCl), before incubation with ligands as above. Limited digestion was performed with trypsin (5 μg/ml, 15 min at 25 °C), and samples were analyzed as described in the legend of Fig. 2. *C*, HSP90 interaction with the AR. AR in cytosol obtained from LNCaP cells was immunoprecipitated with the antibody F39.4.1, washed without (−; *lane 16*) or with (+; *lane 18*) NaCl (0.5 M), and after electrophoresis visualized on a Western blot. *Lanes 15* and *17* show nonspecific binding of HSP90 to the affinity resin without antibody F39.4.1. AR and HSP90 were stained with the specific antibodies F39.4.1 and AC88, respectively.

be immunoprecipitated with the antiserum F52 (not shown), indicating the absence of the DNA binding domain in all fragments.

In conclusion, these results show differences in interaction with the receptor between antiandrogens cyproterone acetate, hydroxyflutamide, ICI 176.334, and RU 23908 and the antiandrogen RU486. Furthermore, they emphasize that receptors complexed with antiandrogens (except RU486) do not contain a trypsin-sensitive site available for the cleavage of the C-terminal 30–40 amino acids.

Effect of Molybdate and Immunoadsorption on Proteolysis Resisting Conformation—Transformation by hormones of steroid hormone receptor complexes into an activated form is thought to be accompanied by the release of several associated proteins, mainly belonging to the family of heat shock proteins (HSPs; reviewed in Refs. 29 and 30). A 90-kDa heat shock protein (HSP90) is the major component in this complex and has been shown unambiquously to be part of unactivated steroid receptor complexes, both *in vivo* and *in vitro*. The HSPs are predominantly bound to the ligand binding domain of steroid receptors. Release of the HSPs may be involved in the time-dependent conformational changes in the ligand binding domain that are detected with the limited trypsinization procedure in androgen- and RU486-bound receptors. Molybdate is known to stabilize steroid receptor-HSP interactions (29). It was observed, that the time-dependent conformational change of AR induced by androgens and by RU486 was also delayed by the addition of molybdate (Fig. 3*A*). With antiandrogens, addition of molybdate increased the amount of the 35-kDa proteolysis resisting fragment (Fig. 3*A*, *lanes 6* and *7*). In the absence of ligand (Fig. 3*A*, *lanes 2* and *3*), addition of molybdate had no effect; protected fragments were still not detectable. These results indicate that molybdate stabilized the first proteolysis resisting conformation of the receptor that is initially formed after binding of the ligand, and suggest a role for associated (heat-shock) proteins in the process of time-dependent conformation change.

The effect of removal of associated proteins on the protease resistance of ligand-bound AR was examined with an immunoadsorbed receptor. *In vitro* produced receptor was adsorbed to an agarose matrix with an immobilized antibody that recognizes the N terminus of the AR. Subsequently, the resin was washed with salt, which was reported to remove most of the associated proteins from glucocorticoid and progesterone receptors (31–33). The direct demonstration of the release of the major heat shock protein HSP90 from the *in vitro* produced AR was not feasible, due to the low amount of AR in the reticulocyte lysate and the relatively high nonspecific binding of HSP90 to the affinity resin. In an experiment with a larger amount of unlabeled androgen receptors, obtained from LNCaP cells, it was shown that the interaction of HSP90 with an immunopurified AR is disturbed upon a salt wash, whereas ligand binding remained (dissociation of HSP90 shown in Fig. 3, *lane 15–18*). Strikingly, when the *in vitro* produced ^{35}S-labeled AR on the affinity matrix was liganded with an antiandrogen, trypsinization now resulted in the formation of a 29-kDa fragment (Fig. 3, *lane 13*), a fragment with the same size as formed with agonists (Fig. 3, *lane 12*). Trypsinization of unliganded, and of androgen- or RU486-bound, immunoadsorbed AR showed results similar as seen with the non-immunoabsorbed receptor (compare Fig. 3, *lanes 11*, *12*, and *14* with Fig. 2*A*, *lanes 7*, *11*, and *15*). These results suggest that, after removal of associated proteins, antiandrogens, except RU486, are able to stabilize the same protease resisting conformation as agonists.

Limited Trypsinization of Mutated Androgen Receptors—As described in the section on transcription activation, hydroxyflutamide and cyproterone acetate could activate the mutant receptor ARL, whereas ICI 176.334 and RU486 were antago-

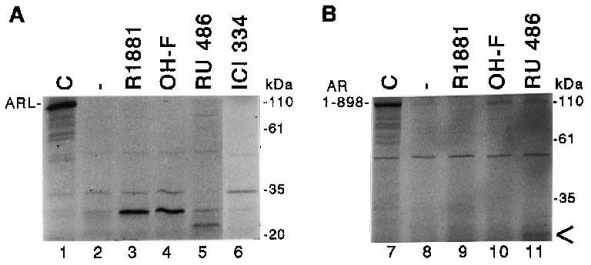

FIG. 4. **Limited trypsinization of mutated androgen receptors.** Both *in vitro* produced ARL (*A*) and AR 1–898 (*B*) were incubated (1 h at 25 °C) in the presence of vehicle (−), R1881 (10 nM), hydroxyflutamide (*OH-F*, 10 μM) or RU486 (1 μM), before limited trypsinization (20 μg/ml, 15 min at 25 °C). *Lanes 1* and *6* are controls (*C*) without trypsin added. In *lane 11* the position of the 25-kDa fragment is indicated (<). The band below 61 kDa is nonspecific.

nists also for ARL. Binding of hydroxyflutamide and cyproterone acetate to ARL induced an increase of the resistance of a 29-kDa fragment to trypsinization, indicating that both antiandrogens could induce a proteolysis resisting conformation of the receptor normally only seen with androgen receptor agonists (Fig. 4, result shown for R1881 and hydroxyflutamide: *lanes 3* and *4*). For ICI 176.334 only a minor increase in intensity of the 35-kDa fragment is seen, and no formation of a 29-kDa fragment (Fig. 4, *lane 6*). RU486 protected a fragment of 25 kDa to trypsinization, similar as seen for the wild-type receptor (Fig. 4, *lane 5*). So, it appears that the mutation in ARL did not affect the conformational changes of ARL induced by these last two antagonists.

Deletion of the last 12 amino acid residues of the ligand binding domain of the AR completely abolished [^{3}H]R1881 binding (22). In studies with the wild-type androgen receptor described above, we observed protection by RU486 of a fragment of the receptor lacking the C-terminal amino acids. To study the effect of a deletion of 12 amino acid residues at the C terminus in a limited proteolytic digestion assay, AR 1–898 was produced *in vitro* and incubated with different ligands. Limited proteolytic digestion resulted for both agonists and antagonists, except RU486, in a complete digestion of AR 1–898 into fragments not detectable after electrophoresis (Fig. 4, *lanes 9* and *10*), similar as observed in the absence of ligand (Fig. 4, *lane 8*). The complete digestion of AR 1–898 indicates the necessity of ligand binding for resistance against trypsinization. RU486 protected a 25-kDa fragment of AR 1–898 against proteolytic degradation, indicating that the C-terminal amino acids are not required for RU486 binding (Fig. 4, *lane 11*). The efficiency of protection of the 25-kDa fragment, however, is lower in comparison with wild-type AR. The binding affinity of [^{3}H]RU486 was determined by Scatchard plot analysis (results not shown). The observed lower binding affinity of RU486 for AR 1–898 (K_d 5.4 nM) as compared to AR (K_d 1.6 nM) may explain the lower efficiency of protection of AR 1–898 by RU486. The study with the mutant AR 1–898 provides additional evidence for a different mechanism of action of the antagonist RU486.

Sucrose Density Gradient Centrifugation—The experiments with molybdate-stabilized and salt-treated receptor complexes provided indirect evidence for a process, in which a change in proteolysis resisting conformation occurred simultaneously with a change in interaction with other proteins. Additional indications for such a process were obtained from the estimation of sedimentation values of ligand-receptor complexes on sucrose density gradients. *In vitro* produced AR was complexed with [^{3}H]R1881 or [^{3}H]RU486, or labeled with [^{35}S]methionine and complexed with unlabeled ligands. Incubation was performed at 0 °C or at 25 °C, to mimic the incubation conditions as used for trypsinization of the receptor. AR liganded at 0 °C, with either the androgen [^{3}H]R1881 or the antiandrogen [^{3}H]RU486, sedimented as an 8 S complex (Fig. 5, *A* and *B*). Incubation at 25 °C resulted in formation of a 5 S complex with R1881 and a 4 S complex with RU486. Therefore, both ligands, which induced a second conformational change of the AR at 25 °C as detected by limited trypsinization, also transformed an initially present 8 S receptor complex into a smaller complex on warming. Incubation of ^{35}S-AR complexed with non-radioactive R1881 showed similar 8 S and 4 S receptor forms as with [^{3}H]R1881-labeled receptor (compare Fig. 5, *B* and *C*). In contrast, only 8 S complexes were present after an incubation at 25 °C of the AR in the absence of ligand or in the presence of the antagonists cyproterone acetate, hydroxyflutamide, ICI 176.334, and RU 23908 (Fig. 5, *D* and *E*).

The antagonists hydroxyflutamide and cyproterone acetate could activate transcription with the mutant ARL and protected a 29-kDa fragment of ARL against trypsinization, indicative for the release of associated proteins. The effect of binding of these ligands to ARL on the sedimentation value of the receptor complex was also studied. Indeed, both ligands partly shifted the initial 8 S complex to a 5 S complex after an incubation at 25 °C (Fig. 5*F*, result shown for hydroxyflutamide). A shift in sedimentation value of ARL from 8 S to 5 S in the presence of agonists, or to 4 S in the presence of RU486, was seen on warming, equivalent to the results obtained with the wild-type AR. Unliganded and ICI 176.334-bound ARL sedimented as an 8 S complex also at 25 °C, a result similar as found for the wild-type AR (results not shown).

In summary, the sucrose gradient centrifugation studies show that an 8 S receptor complex was present under conditions when a 35-kDa fragment could be demonstrated by limited trypsinization. A 5 S complex correlated with a proteolysis resisting fragment of 29 kDa, and was characteristic for the receptor form able to induce transcription. Binding of RU486 to the AR resulted in formation of a complex different from the agonist-receptor complex in both proteolysis resisting conformation and sedimentation characteristics (4 S) (results are summarized in Fig. 6).

DISCUSSION

Proteolytic analysis has proven to be a powerful method to analyze agonist- and antagonist-induced conformational changes of progesterone (11, 12), estrogen (13), glucocorticoid (11), androgen (10, 16, 17), and retinoic acid receptors (34). In the present study, a detailed analysis of protease resisting fragments permitted the identification of two conformational changes after the binding of androgens. Immediately upon binding of an agonist to the wild-type AR, the ligand binding domain (amino acids 662–910) and part of the hinge region (amino acids 612–662) resisted trypsinization (*form 2* in Fig. 6), indicative for a more compact structure of the liganded protein than in the absence of ligand. After this rapid initial structural alteration, a second conformational change occurred as detected with limited trypsinization, concomitantly with a shift of a receptor complex sedimenting at 8 S to a complex sedimenting at 5 S (*form 3* in Fig. 6). In analogy to other steroid hormone receptors (reviewed in Ref. 28), the high molecular mass form of the AR presumably represents a complex of several proteins, including the 90-kDa heat-shock protein (23, 35, 36). The second conformational change likely results in dissociation of this complex and precedes the formation of a transcriptionally active complex. The antiandrogens cyproterone acetate and hydroxyflutamide, which are agonists for the mutant ARL, induced in this mutant the two conformational changes, and released associated proteins. Previous studies with ARL already indicated that for the formation of the tran-

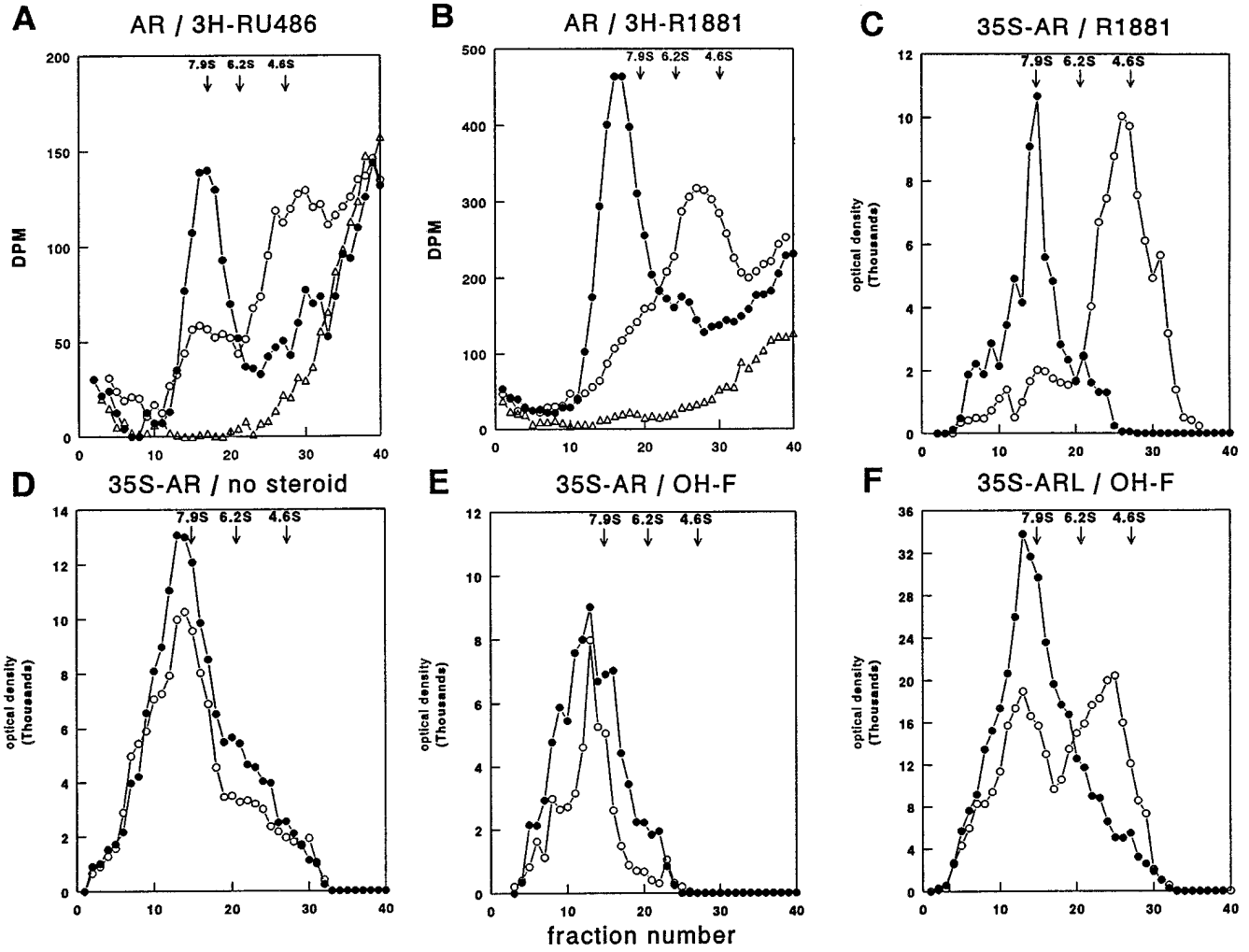

FIG. 5. **Sucrose gradient centrifugation of androgen receptors.** Sucrose density gradient (5–20%) centrifugation of radioactive labeled receptor, incubated for 2 h at 0 °C (●) or 1 h at 25 °C (○). *A* and *B*, labeling of AR occurred with [^{3}H]steroid. Excess of unbound [^{3}H]steroid was removed by dextran-coated charcoal; nonspecific binding was determined by incubation in the presence of 100-fold excess of unlabeled steroid for 2 h at 0 °C ( ). *C-F*, ^{35}S-labeled receptor was incubated in the absence or presence of ligand as above, prior to sucrose density gradient centrifugation. *Panel F*, results for mutant receptor ARL are shown. Fractions were subjected to electrophoresis, followed by autoradiography. Autoradiograms were scanned to determine optical density (arbitrary unit). Aldolase (7.9 S), alkaline phosphatase (6.2 S), and hemoglobin (4.6 S) were used as standards. *OH-F*, hydroxyflutamide.

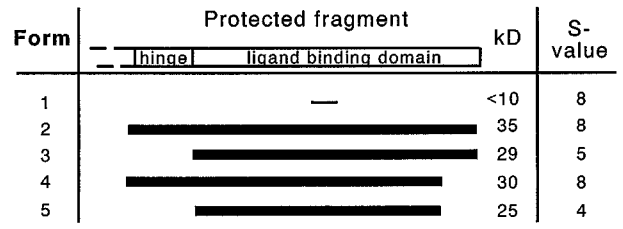

FIG. 6. **Different forms of the androgen receptor.** Five different forms of the androgen receptor can be detected with limited trypsinization and sucrose density gradient centrifugation. *Form 1* represents the unoccupied receptor. *Form 2* is obtained in the presence of either one of the various antagonists, except RU486, and also after the initial binding of agonists at 4 °C. Upon incubation of the androgen receptor with the agonist at 25 °C, *Form 3* is found. *Forms 4* and *5*, derived of *Forms 2* and *3*, are found in the presence of RU486. Part of the domain structure of the androgen receptor is shown schematically on top of the figure.

scriptionally active form the release of HSPs is essential (26). It can be assumed that the single amino acid change in the ARL made it possible for cyproterone acetate and hydroxyflutamide to induce both the second conformational change and HSP dissociation, which results in agonistic activity.

Two nonexclusive models have previously been postulated to explain the mechanism of action of steroid receptor antagonists. In the first model, two types of antagonists are proposed (37–39). Both types of compounds compete with agonists for binding to the receptor, but they differ in their effect on subsequent steps in the mechanism of receptor activation. The so called "pure antagonists" have no partial agonistic activity, probably due to decreased dimerization (38, 40), decreased binding of the receptor complex to DNA (41–42), or increased turnover (43). The other type of antagonists ("non-pure antagonists" with partial agonistic activity) provokes the transformation of the receptor to the DNA binding form but fails to promote its transcriptional activity, probably through the induction of an inappropriate conformation of the C-terminally located transcription activation region (AF-2) (44, 45). The cell and gene specific partial agonistic effects of some estrogen and progesterone receptor antagonists have been explained by the action of the ligand-independent transcription activation region (AF-1) (38, 41, 46). This division in two types of antagonists has been challenged. For the "pure antiestrogen" ICI 164.384 (40), DNA binding was observed in some studies (9, 47). For the antiprogestagen ZK 98299 the failure of the receptor to interact with DNA (38) was subscribed to the lower

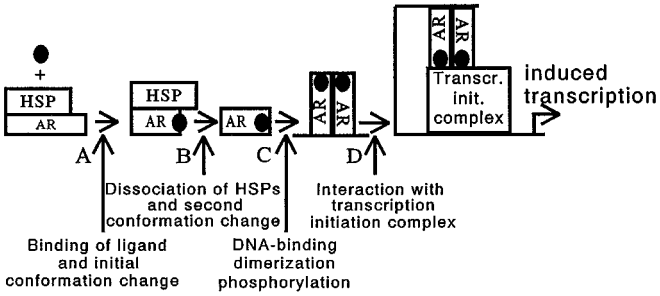

FIG. 7. **Model of the sequential steps in androgen receptor activation.** Binding of an agonist to the inactive AR, complexed with HSP, induces an initial conformation change (*A*). Upon HSP-dissociation and a second conformational change (*B*), the receptor dimerizes, binds to DNA and phosphorylation occurs (*C*). The DNA-bound receptor interacts with the transcription initiation complex to regulate transcription (*D*). One, or more of the steps *B*, *C*, and *D* may be blocked after binding of an antagonist.

binding affinity of the ligand for the receptor (48) and could be overcome by increasing the ligand concentration. However, ZK 98299 could prevent the agonistic activity of the ("non-pure") antiprogestagen RU486 after stimulation of the cAMP signaling pathways (49). A not yet well understood difference in phosphorylation of the receptor could possibly play a role (48, 50).

In a second model explaining the mechanism of antagonists, described recently by O'Malley and collaborators (4, 5, 11), the importance of a conformational change of the ligand binding domain is emphasized. The model argues that agonists and antagonists recognize distinct regions of the ligand binding domain. Antagonists induce an incomplete conformational change that results in dimerization and DNA binding, but leave the C terminus of the ligand binding domain in a form still available for protease (11) and antibody recognition (14, 15). As a result, a surface repressor function is not removed and the receptor is not able to induce transcription.

We would propose to incorporate both models described above in one scheme, and add an additional step that might be inhibited by antagonists, to explain the results of our studies with the antiandrogens cyproterone acetate, hydroxyflutamide, ICI 176.334, and RU 23908 (Fig. 7). The key point of this model is the step between a ligand-occupied complex of the receptor and associated proteins, and a receptor that has a changed conformation and has released these proteins. This step (*step B* in Fig. 7) is partly or completely blocked by the antiandrogens cyproterone acetate, hydroxyflutamide, ICI 176.334, and RU 23908. This step in the process of receptor activation has been previously proposed as a step that is impaired by antagonists (51–53), but recent observations of DNA-bound receptors occupied with antagonists do not favor this step as critical for the action of most antiprogestogens and antiestrogens (6). We like to stress that more than one step may be involved in the mechanism of antagonist action, and that a strict division of antagonists in distinct categories might not be appropriate. The pure antagonists of the estrogen and progesterone receptor induce a partial conformational change, and might block the process of receptor activation at step C (DNA-binding, dimerization, and/or phosphorylation by nuclear kinases) in Fig. 7. However, from our studies on antiandrogens, we have no new arguments to support an absolute distinction between antagonists blocking either step C or step D (interaction with the transcription initiation complex) in Fig. 7. RU486, when bound to the androgen receptor, induced an inappropriate conformational change of the receptor, but also showed partial agonistic properties apart from its antagonistic action. This might indicate inhibition of step D in Fig. 7, and emphasized that antiandrogenic activity may be exerted by different mechanisms.

Several data support the inhibition of step B (Fig. 7) in the above described model for the action of the androgen receptor antagonists cyproterone acetate, hydroxyflutamide, ICI 176.334, and RU 23908. Binding of these antiandrogens first induced a rapid initial change within the AR, similar as seen in the presence of agonists and indicative for a comparable initial binding site on the receptor. However, in the presence of the above mentioned antiandrogens no indication for the induction of a second conformational change or release of associated proteins *in vitro* could be detected. Furthermore, no support was found for the involvement of the C terminus of the AR in the mechanism of action of the four antiandrogens mentioned above. Despite the presence of several protease degradation sites, the C terminus was always present in the proteolysis resisting fragment, obtained after limited proteolysis of these antiandrogen-receptor complexes. In addition, the mutant AR 1–898, with a C-terminal deletion of 12 amino acids, does not bind androgens (22) and could not induce a protease resisting conformation in the presence of androgens and the four antiandrogens. This suggests that the C terminus is involved in binding of both androgens and the four antiandrogens, and consequently that these antiandrogens must interfere with another step in the process of receptor activation.

In conclusion, our data indicate that the antiandrogens cyproterone acetate, hydroxyflutamide, ICI 176.334, and RU 23908 act at a different step in the mechanism of action of steroid hormones than several antiprogestagens and antiestrogens. These antiandrogens do not induce or stabilize an inappropriate conformation of the C-terminal end of the receptor, but act earlier and prevent a conformation change presumably by stabilizing a complex with different associated proteins. RU486 has a broad steroid specificity, and also binds to the androgen receptor. This latter compound showed that antiandrogenic activity might also be accomplished in a different way, by induction of a non-functional conformation of the ligand binding domain, similar as found for its effect on the progesterone receptor.

Acknowledgments—We thank Dr. J. A. Grootegoed for helpful discussions and critical reading of the manuscript. We gratefully acknowledge the gifts of radioactive RU486 from Roussel Uclaf and the mouse monoclonal antibody AC88 from Dr. D. O. Toft.

REFERENCES

1. Evans, R. M. (1988) *Science* **240,** 889–895
2. Beato, M. (1989) *Cell* **56,** 335–344
3. Gronemeyer, H. (1991) *Annu. Rev. Gen.* **25,** 89–123
4. Tsai, M.-J., and O'Malley, B. W. (1994) *Annu. Rev. Biochem.* **63,** 451–486
5. Baniahmed, A., and Tsai, M. J. (1993) *J. Cell. Biochem.* **51,** 151–156
6. Agarwal, M. K. (1994) *Int. J. Biochem.* **26,** 341–350
7. El-Ashry, D., Oñate, S. A., Nordeen, S. K., and Edwards, D. P. (1989) *Mol. Endocrinol.* **3,** 1545–1558
8. Lees, J. A., Fawell, S. E., White, R., and Parker, M. G. (1990) *Mol. Cell. Biol.* **10,** 5529–5531
9. Sabbah, M., Gouilleux, F., Sola, B., Redeuilh, G., and Baulieu, E.-E. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 390–394
10. Kallio, P. J., Jänne, O. A., and Palvimo, J. J. (1994) *Endocrinology* **134,** 998-1001
11. Allan, G. F., Leng, X., Tsai, S. Y., Weigel, N. L., Edwards, D. P., Tsai, M.-J., and O'Malley, B. W. (1992a) *J. Biol. Chem.* **267,** 19513–19520
12. Allan, G. F., Tsai, S. Y., Tsai, M.-J., and O'Malley, B. W. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 11750–11754
13. Beekman, J. M., Allan, G. F., Tsai, S. Y., Tsai, M.-J., and O'Malley, B. W. (1993) *Mol. Endocrin.* **7,** 1266–1274
14. Vegeto, E., Allan, G. F., Schräder, W. T., Tsai, M.-J., McDonnell, D. P., and O'Malley, B. W. (1992) *Cell* **69,** 703–713
15. Weigel, N. L., Beck, C. A., Estes, P. A., Prendergast, P., Altmann, M., Christensen, K., and Edwards, D. P. (1992) *Mol. Endocrinol.* **6,** 1585–1597
16. Kuil, C. W., and Mulder, E. (1994) *Mol. Cell. Endocrinol.* **102,** R1-R5
17. Zeng, Z., Allan, G. F., Thaller, C., Cooney, A. J., Tsai, S. Y., O'Malley, B. W., and Tsai, M.-J. (1994) *Endocrinology* **135,** 248–252
18. Baulieu, E. E. (1989) *Science* **248,** 1351–1357
19. Philibert, D., Costerousse, G., Gallaird-Moguilewski, M., Nedelec, L., Nique, F., Tournemine, C., and Teutsch, G. (1991) *Front. Horm. Res.* **19,** 1–17
20. Veldscholte, J., Ris-Stalpers, C., Kuiper, G. G. J. M., Jenster, G., Berrevoets, C., Claassen, E., Van Rooij, H. C. J., Trapman, J., Brinkmann, A. O., and

Mulder, E. (1990) *Biochem. Biophys. Res. Commun.* **173,** 534–540
21. Brinkmann, A. O., Faber, P. W., Van Rooij, H. C. J., Kuiper, G. G. J. M., Ris, C., Klaassen, P., Van der Korput, J. A. G. M., Voorhorst, M. M., Van Laar, J. H., Mulder, E., and Trapman, J. (1989) *J. Steroid Biochem.* **34,** 307–310
22. Jenster, G., Van der Korput, H. A. G. M., Van Vroonhoven, C., Van der Kwast, Th. H., Trapman, J., and Brinkmann, A. O. (1991) *Mol. Endocrinol.* **5,** 1396–1404
23. Veldscholte, J., Berrevoets, C. A., Brinkmann, A. O., Grootegoed, J. A., and Mulder, E. (1992) *Biochemistry* **31,** 2393–2399
24. Laemmli, U. K. (1970) *Nature* **227,** 680–685
25. Zegers, N. D., Claassen, E., Neelen, C., Mulder, E., Van Laar, J., Voorhorst, M., Berrevoets, C. A., Brinkmann, A. O., Van der Kwast, Th. H., Ruizeveld de Winter, J. A., Trapman, J., and Boersma, W. J. A. (1991) *Biochim. Biophys. Acta* **1073,** 23–32
26. Veldscholte, J., Berrevoets, C. A., Zegers, N. D., Van der Kwast, T. H., Grootegoed, J. A., and Mulder, E. (1992) *Biochemistry* **32,** 7422–7430
27. Kuiper, G. G. J. M., de Ruiter, P. E., Trapman, J., Boersma, W. J. A., Grootegoed, J. A., and Brinkmann, A. O. (1993) *Biochem. J.* **291,** 95–101
28. Riehl, R. M., Sullivan, W. P., Vroman, B. T., Bauer, V. J., Pearson, G. R., and Toft, D. O. (1985) *Biochemistry* **24,** 6586–6591
29. Pratt, W. B., Sanchez, E. R., Bresnick, E. H., Meshinchi, S., Scherrer, L. C., Dalman, F. C., and Welsh, M. J. (1989) *Cancer Res.* **29,** 2222s-2229s
30. Smith, D. F., and Toft, D. O. (1993) *Mol. Endocrinol.* **7,** 4–11
31. Smith, D. F., Schowalter, D. B., Kost, S. L., and Toft, D. O. (1990) *Mol. Endocrinol.* **4,** 1704–1711
32. Smith, D. F. (1993) *Mol. Endocrinol.* **7,** 1418–1429
33. Pratt, W. B. (1993) *J. Biol. Chem.* **268,** 21455–21458
34. Keidel, S., LeMotte, P., and Apfel, C. (1994) *Mol. Cell. Biol.* **14,** 287–298
35. Marivoet, S., Van Dijck, P., Verhoeven, G., and Heyns, W. (1992) *Mol. Cell. Endocr.* **88,** 165–174
36. Nemeto, T., Ohara-Nemeto, Y., and Ota, M. (1992) *J. Steroid Biochem.* **42,** 803–812
37. Reese, J. C., and Katzenellenbogen, B. S. (1991) *Nucleic Acids Res.* **19,** 6595–6602
38. Klein-Hitpass, L., Tsai, S. Y., Weigel, N. L., Allan, G. F., Riley, D., Rodriquez, R., Schräder, W. T., Tsai, M.-J., and O'Malley, B. W. (1990) *Cell* **60,** 247–257
39. Gronemeyer, H., Benhamou, B., Berry, M., Bocquel, M. T., Gofflo, D., Garcia, T., Lerouge, T., Metzger, D., Meyer, M. E., Tora, L., Vergezac, A., and Chambon, P. (1992) *J. Steroid Biochem. Molec. Biol.* **41,** 217–221
40. Fawell, S. E., White, R., Hoare, S., Sydenham, M., Page, M., and Parker, M. G. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 6883–6887
41. Berry, M., Metzger, D., and Chambon, P. (1990) *EMBO J.* **9,** 2811–2818
42. Bocquel, M. T., Ji, J., Ylikoni, T., Benhamou, B., Vergezac, A., Chambon, P., and Gronemeyer, H. (1993) *J. Steroid Biochem. Mol. Biol.* **45,** 205–215
43. Gibson, M. K., Nemmers, L. A., Beckman, W. C., Jr., Davis, V. L., Curtis, S. W., and Korach, K. S. (1991) *Endocrinology* **129,** 2000–2010
44. Meyer, M.-E., Pornon, A., Ji, J., Bocquel, M.-T., Chambon, P., and Gronemeyer, H. (1990) *EMBO J.* **9,** 3923–3932
45. Danielian, P. S., White, R., Lees, J. A., and Parker, M. G. (1992) *EMBO J.* **11,** 1025–1033
46. Green, S. (1990) *J. Steroid Biochem. Mol. Biol.* **37,** 747–751
47. Pham, T. A., Elliston, J. F., Nawaz, Z., McDonnell, D. P., Tsai, M.-J. and O'Malley, B. W. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 3125–3129
48. Delarbre, K., Guiochon-Mantel, A., and Milgrom, E. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 4421–4425
49. Beck, C. A., Estes, P. A., Bona, B. J., Muro-Cacho, C. A., Nordeen, S. K., and Edwards, D. P. (1993) *Enocrinology* **133,** 728–740
50. Takimoto, G. S., Tasset, D. M., Eppert, E. C., and Horwitz, K. B. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 3050–3054
51. Moudgil, V. K., and Hurd, C. (1987) *Biochemistry* **26,** 4993–5001
52. Segnitz, B., and Gehring, U. (1990) *J. Biol Chem.* **265,** 2789–2796
53. Renoir, J.-M., Radanyi, C., Jung-Testas, I., Faber, L. E., and Baulieu, E.-E. (1990) *J. Biol. Chem.* **265,** 14402–14406