# Hormone-Induced Dissociation of the Androgen Receptor–Heat-Shock Protein Complex: Use of a New Monoclonal Antibody To Distinguish Transformed from Nontransformed Receptors

Jos Veldscholte,*,‡ Cor A. Berrevoets,‡ Netty D. Zegers,§ Theodorus H. van der Kwast,⊥ J. Anton Grootegoed,‡ and Eppo Mulder‡

*Department of Endocrinology & Reproduction and Department of Pathology, Erasmus University of Rotterdam, P.O. Box 1738, 3000 DR Rotterdam, The Netherlands, and Department of Immunology, MBL-TNO, P.O. Box 45, 2280 AA Rijswijk, The Netherlands*

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ABSTRACT: The hormone-induced transformation process of the androgen receptor in the androgen-responsive human prostatic carcinoma cell line LNCaP was studied. Immunoprecipitation of the nontransformed cytosolic receptor (8S on sucrose gradients) with a specific monoclonal antibody (F39.4.1) resulted in coprecipitation of three heat-shock proteins (hsp90, hsp70, and hsp56). Upon incubation of the cells with the synthetic androgen R1881, the sedimentation value of the receptor complex decreased to an intermediate form of 6S, and an almost complete loss of coprecipitating heat-shock proteins was observed. After a 2-h incubation, the receptor was recovered in considerable part from the nuclear fraction (extraction with high salt; 4.6S form). By use of the bifunctional cross-linker dimethyl pimelimidate, dissociation of the 8S complex, but not of the 6S complex, was blocked. A newly developed monoclonal antibody (F52.24.4), directed against the C-terminal part of the DNA-binding domain of the androgen receptor, specifically recognized both the 4.6S and the 6S forms of the receptor but did not react with the nontransformed 8S form. It is concluded that the unoccupied androgen receptor is associated with several heat-shock proteins and that transformation of the receptor to the tight nuclear-binding form is a multistep process that involves the dissociation of heat-shock proteins from the receptor.

Steroid hormone receptors act as ligand-dependent transcription factors in the process of steroid-induced effects on target cells. Upon steroid binding, the receptor is converted from a non-DNA-binding state to a tight nuclear-binding form. It is believed that binding of the receptor to DNA takes place in specialized regions, called the hormone-responsive elements, mostly present in front of the regulated genes. This ligand-induced, specific interaction between the receptor and a target gene, results in interaction of other transcription factors with the gene and ultimately in modulation of transcription (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 involved in DNA binding. For the progesterone, glucocorticoid, and estrogen receptors, domains involved in transcription activation have been identified in both the N- and the C-terminal part of the receptor (Carson-Jurica et al., 1990). The primary structure of the androgen receptor was determined some years ago (Chang et al., 1988; Lubahn et al., 1988; Trapman et al., 1988; Faber et al., 1989) but only recently, transcription activation functions have been ascribed to the N-terminal domain and were suggested for the steroid-binding region (Jenster et al., 1991; Simental et al., 1991).

The process of steroid hormone receptor transformation[1] to a tight nuclear-binding form has been studied extensively (Grody et al., 1982; Joab et al., 1984; Sullivan et al., 1985; Bailly et al., 1986; Mendel et al., 1986; Tai et al., 1986; Arányi et al., 1988; Denis et al., 1988; Howard & Distelhorst, 1988; Kost et al., 1989) [for recent reviews, see Pratt (1987) and Pratt et al. (1989)]. Most investigations have focused on in vitro transformation of receptors and have shown that, after cell rupture, the untransformed (non-DNA-binding) receptor is associated with several other proteins. It is now generally accepted that a 90-kDa heat-shock protein (hsp90)[2] is associated with androgen, progesterone, glucocorticoid, and estrogen receptors (Joab et al., 1984; Sullivan et al., 1985). Another component of the receptor complex is a protein of 54–60 kDa, with small variations in size for different species. It was shown that this protein is also a heat-shock protein and was therefore called hsp56 (Sanchez, 1990). 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 (Tai et al., 1986). Recently, Yem et al. (1992) identified a 60-kDa protein of which the N-terminal sequence was identical to that of hsp56 and showed immunosuppressant-binding properties. Also recently, the cDNA of a similar protein (p59) from rabbit liver was cloned (Lebeau et al., 1992). The sequence in the N-terminal part showed a considerable homology to peptidyl-prolyl isomerase. It was speculated that these 56–60-kDa immunosuppressant-binding proteins play a role in intracellular trafficking of heterooligomeric forms

* To whom correspondence should be addressed.
‡ Department of Endocrinology & Reproduction, Erasmus University Rotterdam.
§ Department of Immunology, MBL-TNO.
⊥ Department of Pathology, Erasmus University Rotterdam.

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

[2] Abbreviations: hAR, human androgen receptor; hGR, human glucocorticoid receptor; hPR, human progesterone receptor; cPR, chicken progesterone receptor; hER, human estrogen receptor; LNCaP, lymph node carcinoma of the prostate; hsp90, 90-kDa heat-shock protein; hsp70, 70-kDa heat-shock protein; hsp56, 56-kDa heat-shock protein; DMP, dimethyl pimelimidate; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; MAb, monoclonal antibody; ELISA, enzyme-linked immunosorbent assay.

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of steroid hormone receptors (Lebeau et al., 1992). A third member of the group of heat-shock proteins associated with steroid receptors is a 70-kDa heat-shock protein, hsp70, shown to be present in nontransformed progesterone, glucocorticoid, and androgen receptor complexes (Kost et al., 1989; Smith et al., 1990a; Sanchez et al., 1990a; Veldscholte et al., 1992).

Transformation of the non-DNA-binding receptor complex (8–9 S on sucrose density gradients), either by warming in the presence of hormone or by high salt treatment (0.4–0.5 M), leads to a decrease in size (4–5 S) and induces the ability of this smaller receptor form to bind to nuclei and DNA or other polyanions [reviewed by Pratt (1987) and Pratt et al. (1989)]. It has been shown for the glucocorticoid and progesterone receptors that hsp90 and hsp56 dissociate from the complex during this process (Mendel et al., 1986; Denis et al., 1988; Kost et al., 1989; Sanchez et al., 1990a; Smith et al., 1990a). The in vitro studies suggest that the receptor complex dissociates to a 4–5S form, thereby revealing the DNA-binding domain, resulting in binding of the receptor to the hormone-responsive element. Additional arguments in favor of this unmasking hypothesis are that nonliganded glucocorticoid and progesterone receptors also have a high affinity for DNA if they are free of associating proteins (Bailly et al., 1986; Willmann & Beato, 1986) and that nonliganded thyroid receptors do not bind hsp90 and readily associate with DNA (Dalman et al., 1990). Furthermore, it was found that glucocorticoid receptor mutants which were constitutively active, when transfected into COS cells, were recovered in the 4S form, whereas the steroid-inducible forms were recovered as 9S complexes (Pratt et al., 1988). In vivo studies indicated that hormone-induced dissociation of hsp90 from the glucocorticoid and progesterone receptors indeed does occur (Howard & Distelhorst, 1988; Smith et al., 1990a). In the latter study, however, it was shown that in vivo treatment with hormone does not result in complete dissociation of the receptor complexes. The association of hsp70 with the progesterone receptor seems not to be lost, even after hormone injection in vivo (Smith et al., 1990a). Hsp70 is not involved in stabilization of the receptor complex to DNA (Onate et al., 1992). It has been shown that hsp70 functions as a protein chaperone and assists in unfolding and renaturation of proteins (Palleros et al., 1991; Smith et al., 1992), but as for the other heat-shock proteins, its function in steroid receptor transformation is not understood.

In the present study, the composition of the androgen receptor protein complex was investigated during the process of hormone-induced receptor transformation in vivo, in intact cells. The composition of the protein complex was probed with antibodies recognizing the heat-shock protein hsp90, hsp70, and hsp56, respectively, and with a newly developed antibody that specifically reacts with the DNA-binding domain of the androgen receptor. We found that, prior to tight nuclear binding, the receptor complex undergoes large rearrangements resulting in sequential loss of the different heat-shock proteins, leading to disclosure of the antigenic epitope in the DNA-binding domain of the androgen receptor.

## MATERIALS AND METHODS

*Materials.* [$^3$H]R1881 (87 Ci/mmol), unlabeled R1881 (methyltrienolone), [$^3$H]R5020 (72.4 Ci/mmol), and unlabeled R5020 (promegestone) were purchased from NEN (Boston, MA). [$^3$H]Oestradiol (94 Ci/mmol) and [$^3$H]dexamethasone (94 Ci/mmol) were obtained from Amersham (Cardiff, U.K.). Dimethyl pimelimidate (DMP) was obtained from Sigma (St. Louis, MO). AMPPD alkaline phosphatase substrate, Sapphire Chemiluminescence Amplifier, I-Block reagent, and Nitro-Block reagent were obtained from Trophix, Inc. (Bedford, MA). All other reagents were of analytical grade. Mouse monoclonal antibody F39.4.1 (Sanbio, Uden, The Netherlands) was prepared against the N-terminal domain of the androgen receptor (Zegers et al., 1991). The mouse monoclonals AC88 (recognizing hsp90), N27 (recognizing hsp70), and KN382/EC1 (recognizing hsp56) were generously provided by Dr. D. O. Toft (Mayo Clinic, Rochester, MN), Dr. W. J. Welch (School of Medicine, San Francisco, CA), and Dr. L. E. Faber (Medical College of Ohio, Toledo, OH), respectively.

*Buffer Solutions.* Buffer I, phosphate-buffered saline, pH 7.5; buffer II, 10 mM sodium phosphate, 1.5 mM EDTA, 12 mM $\alpha$-thioglycerol, 10 mM $Na_2MoO_4$, 0.6 mM PMSF, 0.25 mM leupeptin, 0.5 mM bacitracin, and 10% (v/v) glycerol, pH 7.4; buffer III, buffer II supplemented with 0.2% (v/v) Triton X-100, but without leupeptin; buffer IV, 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; buffer V, 10 mM sodium phosphate, 1.5 mM EDTA, 12 mM $\alpha$-thioglycerol, and 10% (v/v) glycerol, pH 7.4; buffer VI, buffer I, containing 0.1% (v/v) Tween 20; buffer VII, 0.05 M $Na_2CO_3$, 1 mM $MgCl_2$, pH 9.5.

*Cell Culture.* LNCaP prostate tumor cells (Horoszewicz et al., 1983), obtained from Dr. Horoszewicz, were cultured in RPMI 1640 as described previously (Veldscholte et al., 1990). Culture of cell lines NHIK, MCF-7, and T47D was described previously (Veldscholte et al., 1990; Berns et al., 1984; Van Laar et al., 1989).

*Development of Monoclonal Antibody F52.24.4.* MAb F52.24.4 was developed essentially as described for MAb F39.4.1., which recognizes amino acids 301–320 (Zegers et al., 1991). Briefly, synthetic peptides homologous to amino acid sequence 593–612 (Thr-Ile-Asp-Lys-Phe-Arg-Arg-Lys-Asn-Cys-Pro-Ser-Cys-Arg-Leu-Arg-Lys-Cys-Tyr-Glu) in the DNA-binding region of the human androgen receptor were synthesized on RapidAmide resin beads and coupled to keyhole limpet hemocyanin for immunization of mice. Sera were tested in a direct ELISA for anti-peptide response, and in an immunoprecipitation assay for androgen receptor specificity. From specific serum antibody producing mice, spleen cells were fused with SP2/0 cells. Antibody-producing clones were first identified in a primary selection in anti-peptide ELISA, and then MAbs were selected for the ability to immunoprecipitate androgen receptors prepared from LNCaP cell nuclear extract. Balb/c mice were injected intraperitoneally with 0.5 mL of pristane (2,6,10,14-tetramethylpentadecane, 96%, Egachemie, Steinheim, F.R.G.). Seven days later, the mice were injected with $10^6$ monoclonal hybridoma cells in 0.25 mL of buffer I. Ascitic fluid was collected under anesthesia.

Receptor specificity of subclone F52.24.4 was tested with a double immunoprecipitation assay of [$^3$H]R1881-labeled hAR, [$^3$H]oestradiol-labeled hER, [$^3$H]R5020-labeled hPR, and [$^3$H]dexamethasone-labeled hGR preparations from nuclear extracts obtained from LNCaP, MCF-7, T47D and NHIK cells, respectively (Zegers et al., 1991). F39.4.1 and F52.24.4 ascites (0.5 $\mu$L) were incubated at 4 °C for 2 h with goat anti-mouse agarose in buffer I, and after extensive washing of the resin with buffer I, nuclear extracts containing comparable amounts of labeled receptors ($1.2 \times 10^4$ dpm) were added. After incubation for 2 h at 4 °C, the resin was washed and the amount of precipitated receptor was estimated by scintillation counting. The amount of labeled receptors present in the nuclear extracts was measured in a protamine sulfate assay as described by Veldscholte et al. (1990).

*Receptor Transformation.* LNCaP cells (passage 65–72) at confluency were kept on RPMI 1640 medium with 5% dextran–charcoal-stripped fetal calf serum for 2–8 days and washed twice with buffer I; then serum-free RPMI 1640 medium with 10 nM [$^3$H]R1881 was added. For sucrose gradient experiments, the control cells (containing nontransformed receptors) were incubated with 10 nM [$^3$H]R1881 on ice for 2 h to label the receptors. For all transformation studies, the cells were transferred to a water bath of 37 °C for 1–3 min and then transferred to the incubator (37 °C) in the case of longer incubation times. Receptor transformation was stopped by putting the flasks on ice. In the indicated experiments, labeling of the receptors with tritiated R1881 was stopped by adding a 100-fold excess of unlabeled R1881. The cells were washed with ice-cold buffer I and scraped in ice-cold buffer II. 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 at 105000*g*, at 2 °C. The high-speed supernatant (cytosol) was used for cross-linking studies, sucrose density gradient analysis, and Western immunoblot analysis. The crude nuclear pellet (800*g* pellet) was resuspended in buffer III. After 5 min the nuclei were pelleted and washed with buffer III without Triton X-100. Nuclei were extracted by incubation in buffer V for 1 h at 4 °C. After centrifugation for 30 min at 150000*g*, at 2 °C, the supernatant was used for sucrose gradient analysis, receptor immunoprecipitation, and Western immunoblot analysis. The amount of labeled receptors present in the nuclear extracts was measured in a protamine sulfate assay as described by Veldscholte et al. (1990).

*Cross-Linking of the Receptor Complexes.* Protein–protein cross-linking was performed in cytosol made in buffer II supplemented with 10 mM DTT, by the method of Arányi et al. (1988). In brief, the pH was adjusted to pH 9.0 with $^1/_{10}$ volume of a 2.2 M triethanolamine buffer. Then $^1/_5$ volume of a 0.1 M dimethyl pimelimidate (DMP), freshly dissolved in a 0.2 M triethanolamine buffer was added. Cross-linking was performed for 30 min at 10 °C. The reaction was stopped by adding $^1/_{100}$ volume of a 5 M hydroxylamide solution.

*Interaction of Monoclonal Antibodies F39.4.1 and F52.24.4 with Receptor.* Cytosols were prepared from LNCaP cells grown on RPMI 1640 with 5% dextran–charcoal-treated serum. The cells were harvested by trypsinization, and the reaction was stopped with an excess of trypsin inhibitor. After two washings with buffer I, the cells were homogenized in ice-cold buffer II with 15 strokes of a glass–glass homogenizer and spun at 105000*g* for 30 min, at 2 °C. The supernatant was incubated with 10 nM [$^3$H]R1881 for 2 h, and unbound label was removed by dextran–charcoal adsorption (Mulder et al., 1978). Then, 100 μL of cytosol was incubated for 2 h with 2 μL of ascitic fluid either with monoclonal antibodies F39.4.1 or F52.24.4 or with a nonspecific antibody, in the presence or absence of 0.5 M NaCl. Interaction of the antibodies with the receptor complex was assayed by sucrose density gradient centrifugation.

*Sucrose Density Gradient Centrifugation.* The samples were treated with dextran-coated charcoal to remove the unbound label and then were applied on sucrose gradients (10–30% sucrose) prepared in buffer V. In the cross-linking experiments, and in the experiments where interaction of the antibodies with the salt-dissociated receptor was investigated, 0.5 M NaCl was included in the gradient. The gradients were run for 20 h at 250000*g*, at 2 °C. $^{14}$C-Labeled bovine serum albumin (4.6 S) and alkaline phosphatase (6.2 S) were used as internal sedimentation markers. Fractions of the gradients were collected from the bottom and assayed for radioactivity and alkaline phosphatase activity.

Table I: Percentage of Steroid Hormone Receptors Precipitated by Two Monoclonal Antibodies[a]

| MAb | labeled receptor precip (%) | | | |
|---|---|---|---|---|
| | hAR | hGR | hER | hPR |
| F52.24.4 | 38 | 53 | 6.5 | 3.6 |
| F39.4.1 | 39 | 0 | 2.2 | 0.1 |

[a] Nuclear extracts of LNCaP, NHIK, MCF7, and T47D cells were incubated for 2 h at 4 °C with goat anti-mouse agarose. The amount of precipitated radioactivity was expressed as the percentage of the total amount (12 000 dpm) added.

*Immunoaffinity Purification of the Receptor and Western Immunoblot Analysis.* The MAb F39 [against amino acids 301–320; Zegers et al. (1991)] and the MAb F52 (against amino acids 593–612) were 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. This affinity matrix was used for immunoprecipitation of the receptor. In each experiment, either 15 (F39) or 25 μL (F52) of matrix was used for immunoprecipitation of the receptor from either cytosol (1.2 mg of cytosolic protein) or nuclear extract (3.6 mg of nuclear protein). The immunoprecipitation and Western immunoblot analysis was performed as described previously (Veldscholte et al., 1992). In most experiments, bound antibodies were detected by chemiluminescence as described below.

*Chemiluminescence Detection of Proteins.* Chemiluminescence detection was performed essentially as described by the manufacturer (Trophics, Bedford, MA). After transfer of proteins to the nitrocellulose membrane, the membrane was dried for 30 min or longer, then washed for 5 min in buffer VI, and subsequently incubated in 0.2% (w/v) I-Block reagent in buffer VI. The membrane was then washed in buffer VI for 5 min, and incubations with primary and secondary alkaline phosphatase conjugated antibodies were performed as described previously (Veldscholte et al., 1992), except that buffer VI (0.1% Tween) instead of buffer I with 0.05% Tween was used. The membrane was then washed 2 × 5 min in buffer VII, incubated for 5 min in Nitro-Block reagent (0.5 mg/mL in buffer VII), washed 2 × 5 min in buffer VII, and subsequently incubated for 2 h in a AMPPD alkaline phosphatase substrate solution (0.24 mM AMPPD and 1 mg/mL Sapphire amplifier in buffer VII) for formation of the chemiluminescence product. The immunoblots were wrapped in catering foil and placed in contact with X-ray film (Hyperfilm MP, Amersham, Cardiff, U.K.) for 1–45 min, depending on the intensity of chemiluminescence. Films were developed according to standard procedures.

## RESULTS

*Generation of the Monoclonal F52.* The procedure to obtain antibodies recognizing the epitope in the DNA-binding domain of the androgen receptor (amino acids 593–612) resulted in a clone producing antibodies of the IgG1 isotype. In an immunoprecipitation assay, ascitic fluid of clone F52.24.4 (in short, F52) was incubated with nuclear extracts containing equal amounts of either androgen receptor, glucocorticoid receptor, estrogen receptor, or progesterone receptor, labeled with the respective, receptor-specific, tritiated ligands. The antibody–receptor complexes were precipitated with goat anti-mouse agarose, and the amount of precipitated receptor was estimated and expressed as a percentage of the total amount of receptors in the reaction mixture (Table I). The percentages

of receptors precipitated with MAb F52 were compared with the percentages obtained with MAb F39.4.1 (in short, F39), which recognizes amino acids 301–320. MAb F52 precipitated about 40% of the added labeled androgen receptor from LNCaP cell nuclear extracts (Table I). Such a relatively low percentage was also found for MAb F39, directed against the N-terminal domain of the receptor. This might be caused by dissociation of the ligand from the receptor during the precipitation procedure. Equivalent results have also been described for a set of polyclonal antisera against the androgen receptor (Van Laar et al., 1989). In addition to binding of the antibody to the androgen receptor, F52 could also precipitate considerable amounts of other steroid receptors (Table I). This cross-reactivity of the MAb is probably due to the high level of homology between the different receptors in this region. Of the 20 amino acid residues in the peptide used for the immunization of the mice, 14 are conserved in the human progesterone receptor and in the human glucocorticoid receptor and 16 are conserved in the human estrogen receptor (Misrahi et al, 1987; Hollenberg et al., 1985; Green et al., 1986). In contrast, the antibody F39, developed against an amino acid sequence in the N-terminal region (Zegers et al., 1991), is highly specific, as shown by the low amounts of glucocorticoid, estrogen, and progesterone receptors precipitated with this latter antibody (Table I). In our studies with LNCaP cells, the low receptor specificity of MAb F52 does not influence the interpretation of the results, because these cells only contain androgen receptors and no other receptors of the same family.

*Transformation of the Androgen Receptor in Intact Cells Resulting in a Decreased Size of the Receptor Complex and Changed Protein Interactions.* To describe the transformation of the AR in terms of changes in the configuration of the heterogeneous receptor protein complex, the receptor was analyzed on sucrose density gradients after various time periods of hormone-induced transformation. The nontransformed androgen receptor in LNCaP cells was recovered as one peak, sedimenting approximately as an 8S complex (Figure 1, panel A). After incubation of the cells with the tritiated synthetic androgen R1881 for 5 min at 37 °C, in addition to the 8S receptor, a second receptor form with a lower sedimentation value appeared (Figure 1, panel B). Incubation of the cells with tritiated R1881 for 30 min at 37 °C led to a decrease in sedimentation value of the receptor to approximately 6S (Figure 1, panel C). Concurrently, the total amount of labeled receptor in the cytosol fraction was decreased and an increasing amount of receptor was found in the nuclear fraction. During a period up to 2 h, the amount of labeled receptor recovered from the nuclear fraction steadily increased (Figure 6; discussed below).

The association of proteins with the receptor was further investigated with a bifunctional cross-linker dimethyl pimelimidate (DMP). This cross-linker covalently links lysine residues at a spatial distance of approximately 9 Å from each other, provided that these residues are accessible for the reagent. Cross-linking for 30 min at 10 °C with DMP was found to be optimal for stabilization of the 8S form of the receptor complex and prevented it from dissociating in the presence of 0.5 M NaCl (Figure 1, panel D). The small amount of receptor present in the 4.6S region at 0-min incubation indicates either that not all receptor molecules are initially present in the 8S form or that the cross-linking efficiency is below 100%. The 6S receptor complex which is formed on incubation with hormone (see Figure 1, panels B and C) was not prevented from dissociating in high salt after

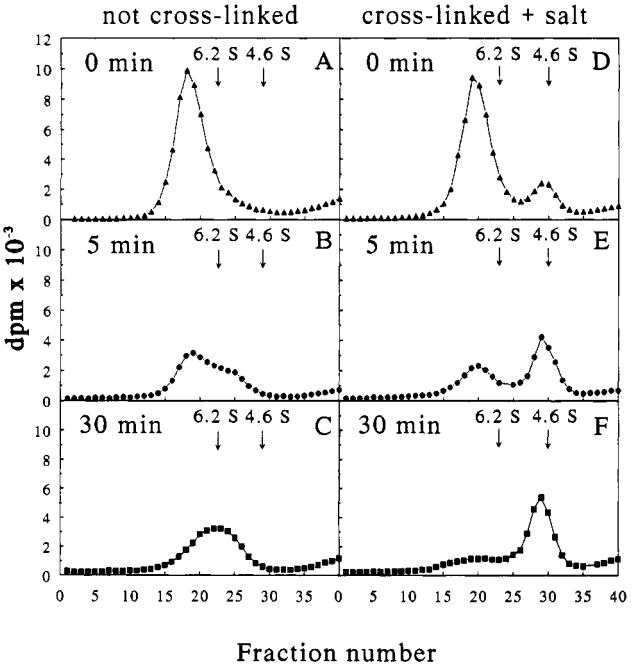


FIGURE 1: Sucrose density gradient profiles of androgen receptor from LNCaP cells. Cells were incubated for 2 h at 0 °C in the presence of 10 nM [$^3$H]R1881 and subsequently incubated for 0 (A and D), 5 (B and E), or 30 min (C and F) at 37 °C. Cytosol was prepared, and half of it was run on a 10–30% sucrose gradient without additional salt, as described under Materials and Methods (A–C). The other half was treated with the cross-linker DMP and run on a 10–30% sucrose gradient containing 0.5 M NaCl (D–F). Alkaline phosphatase (6.2 S) and bovine serum albumin (4.6 S) were used as internal sedimentation markers.

reaction with the cross-linking reagent DMP, and a smaller, approximately 4.6S form of the receptor was obtained (Figure 1, panels E and F). We conclude that the cross-linker does not couple the proteins contained in the 6S receptor complex.

These results of the gradient centrifugation studies indicate that the transformation of the receptor to the tight nuclear-binding form is a multistep process with regard to changes in size and conformation of the proteins. First the receptor complex changes in sedimentation value from 8 S to approximately 6 S, and then it gains high affinity for the nucleus and is no longer recovered in the cytosol fraction.

*Exposure of a Specific Epitope on the Surface of the Receptor Complex during the Transformation Process.* To demonstrate that rearrangements of proteins on the surface of the receptor complex have occurred during the transformation process, we used the monoclonal antibody described above (MAb F52) that is directed against an epitope in the DNA-binding region of the receptor. This antibody caused a shift of the 4.6S androgen receptor to higher sedimentation values (Figure 2A) but did not provoke a shift of the 8S complex on sucrose gradients (Figure 2B). In contrast, MAb F39, recognizing an epitope in the N-terminal domain of the receptor, caused shifts of both the 4.6S and 8S forms of the receptor to complexes with higher sedimentation values (Figure 2). These results show that the epitope for MAb F52 is exposed in the 4.6S receptor but not in the nontransformed 8S receptor complex. Mab F39 shifted the receptor complex over a greater distance than did F52 (Figure 2A). Because the sedimentation behavior of proteins is affected by the shape of the proteins, it can be envisaged that the conformation of the protein complexes is different when an antibody is bound either to the central DNA-binding domain or to the more distal N-terminal domain of the receptor.

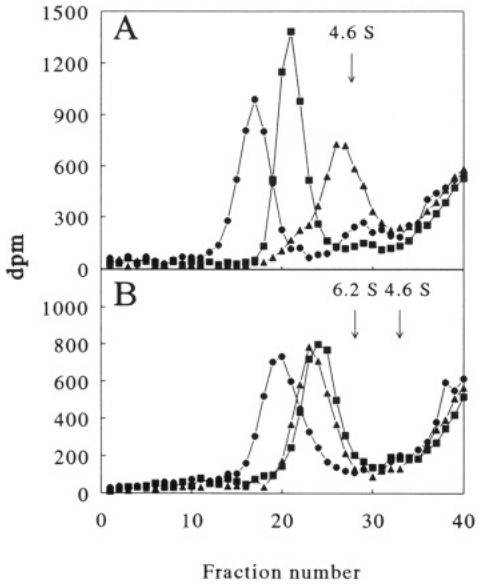


FIGURE 2: Sucrose density gradient profiles of androgen receptor from LNCaP cells. Cytosol was labeled with [$^3$H]R1881 for 2 h, and excess label was removed as described under Materials and Methods. The cytosol (100 $\mu$L) was then incubated for 2 h either with 2 $\mu$L of ascitic fluid of the androgen receptor antibody F39 (●) or F52 (■) or with 2 $\mu$L of ascitic fluid of a nonspecific antibody (▲). Incubation of the cytosol with antibodies and running of the 10–30% sucrose gradients was performed either in the presence (A) or in the absence (B) of 0.5 M NaCl. Bovine serum albumin and alkaline phosphatase were used as internal 4.6S and 6.2S sedimentation markers, respectively.

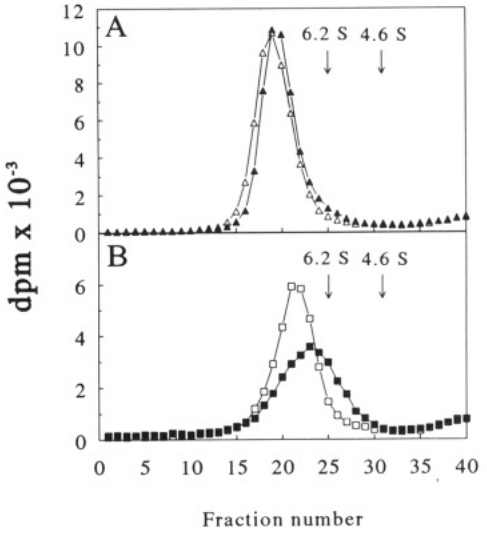


FIGURE 3: Sucrose density gradient profiles of androgen receptor from LNCaP cells. Cells were incubated in the presence of 10 nM [$^3$H]R1881, either for 2 h at 4 °C (untransformed receptor) or for 30 min at 37 °C (transformed receptor). Cytosols were prepared and cleared from unbound steroid by dextran-coated charcoal and then incubated for 2 h at 4 °C in the presence or absence of 2.5 $\mu$L of ascites F52. The samples were run on 10–30% sucrose gradients without additional NaCl, as described under Materials and Methods. Alkaline phosphatase (6.2 S) and bovine serum albumin (4.6 S) were used as internal sedimentation markers: (A) untransformed receptor, incubated either with (△) or without (▲) antibody; (B) transformed receptor, incubated either with (□) or without (■) antibody.

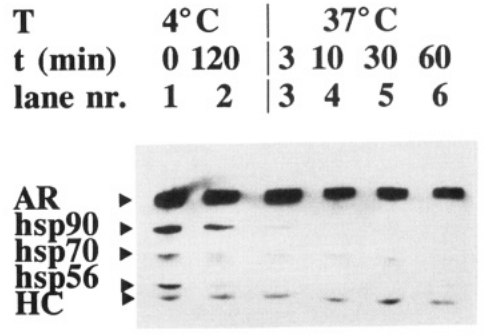


FIGURE 4: Immune purification with monoclonal F39 of androgen receptor complexes from cytosols of LNCaP cells incubated for various time periods with R1881. Receptor complexes were purified using F39–protein A–Sepharose and subjected to electrophoresis. Chemiluminescence exposures of Western immunoblots were prepared as described under Materials and Methods. MAbs F39, AC88, N27, and EC1 were used to identify the AR, hsp90, hsp70, and hsp56, respectively. HC, antibody heavy chain. Cells incubated without hormone, with 10 nM R1881 for 2 h at 4 °C, and with 10 nM R1881 at 37 °C for 3, 10, 30, and 60 min are represented by lanes 1–6, respectively.

Next we examined whether the epitope for F52 (in the DNA-binding region of the receptor) was also exposed in the intermediate 6S receptor complex. Therefore, LNCaP cells were first incubated with tritiated R1881, and cytosol fractions obtained from these cells were probed for interaction with MAb F52. When analyzed on sucrose density gradients, in the absence of antibodies, a receptor peak of approximately 8 S was found when cells were incubated in the cold and a 6S receptor peak was shown after the cells were warmed for 30 min at 37 °C (Figure 3). The 8S receptor complex did not interact with MAb F52 and remained in the same position (Figure 3A). The 6S receptor complex was shifted toward higher sedimentation values in the presence of MAb F52 (Figure 3B). These results show that the 6S, but not the larger 8S, receptor complex exposes the epitope for the MAb F52.

*Loss of Receptor-Associated Heat-Shock Proteins during the Transformation Process.* To investigate whether the hormone-induced transition of the 8S to the 6S form is the result of dissociation of associated proteins from the larger complex, these receptor complexes were immunopurified and screened for coprecipitating proteins that are known to be present in other steroid hormone receptor complexes. We used buffers containing 10 mM molybdate, a condition known to stabilize the 8S complex during isolation.

When the androgen receptor was immunoprecipitated with MAb F39 from the cytosol obtained from LNCaP cells incubated at 4 °C, the heat-shock proteins hsp90, hsp70, and hsp56 were coprecipitated. Incubation of the cells with R1881 at 37 °C led to a fast decrease in the amount of coprecipitating hsp90 and hsp56 (Figure 4, lanes 1 and 3–6). Coprecipitated hsp56 was already absent after 3 min of incubation, and most hsp90 had dissociated within 10 min. The amount of coprecipitated hsp70 also decreased, but this was a somewhat slower process. The observed loss of coprecipitated proteins is also observed after incubation of the cell at 4 °C, although at a much lower rate (Figure 4; compare lanes 1 and 2).

Coinciding with a decrease in the amount of receptor in the cytosol fraction, an increase in the amount of immunoprecipitable nuclear receptor was found (Figure 5). The Western immunoblot only shows immunodetectable androgen receptor. We did not detect coimmunoprecipitated heat-shock proteins after extraction of nuclei with 0.5 M NaCl. This high-salt condition increases the dissociation rate of heteromeric complexes but is required to release the receptors that are tightly bound in the nucleus to DNA. At lower ionic strength, most AR remained in the nuclear pellet. Not only the amount of immunodetectable receptors in the nuclear fraction increased on incubation of the cells at 37 °C, but in addition,

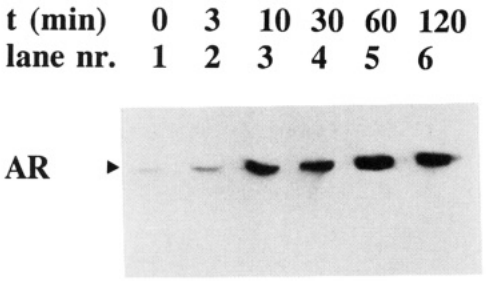


FIGURE 5: Androgen receptor isolated from LNCaP cell nuclear extracts of cells incubated for various time periods with R1881. Receptor molecules were purified using F39–protein A–Sepharose and subjected to electrophoresis. Chemiluminescence exposures of Western immunoblots were prepared as described under Materials and Methods. Cells incubated at 37 °C for 0, 3, 10, 30, 60, and 120 min are represented by lanes 1–6, respectively.

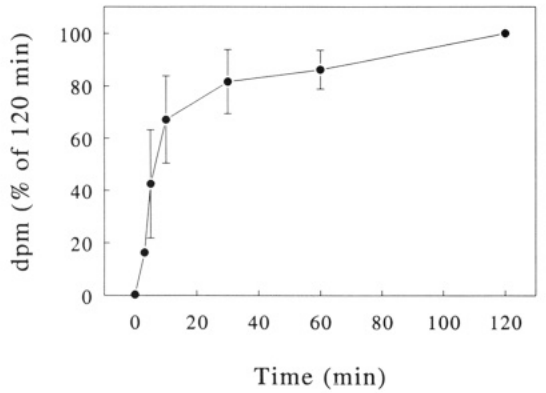


FIGURE 6: Labeling of androgen receptor extracted from nuclei of LNCaP cells incubated for various time periods with [$^3$H]R1881 at 37 °C. At the end of the labeling period, 1 $\mu$M of unlabeled R1881 was added to stop the specific labeling. The amount of label is expressed as the percentage of the amount found after 120 min of incubation. Preparation of nuclear extracts and the protamine sulfate assay for measurement of the amount of labeled receptor are described under Materials and Methods.

the amount of tightly nuclear bound receptor labeled with [$^3$H]R1881 increased after long incubation times with the radioactive androgen (Figure 6). This indicates that the receptor becomes tightly bound to the nucleus after it has bound ligand. After 2 h of incubation, the amount of androgen receptor present in both cytosol and nuclear extract was estimated by protamine sulfate precipitation. The results showed that about 40% of the total amount of labeled receptor was at that time present in the nuclear extract.

*Lack of Coimmunopurification of Hsp90 and Hsp56 with the 6S Receptor Complex.* The antibody F52 forms a complex with the intermediate-sized 6S receptor protein complex, but not with the large 8S receptor complex (sucrose gradients studies shown above; see Figure 2). Antibody F52 can therefore be used to isolate the 6S complex from mixtures of both 6S and 8S receptor complexes. LNCaP cells were kept at 4 °C either with or without androgen, and subsequently, receptor complexes were immunoprecipitated with MAb F52. Then, in addition to the receptor band, a hsp70 band and a faint band of hsp90 were visible on the Western immunoblot (Figure 7, lanes 1 and 2). Hsp90 was absent when the last wash step of the resin was extended for several hours, which is indicative of a low binding affinity of hsp90 in the complex precipitated with F52 antibody (not shown). Hsp56 was not visible on the blots.

The amount of androgen receptor present on the blots increased when the cells were incubated with R1881 at 37 °C (Figure 7, lanes 3–5), indicating that more F52-precipitable

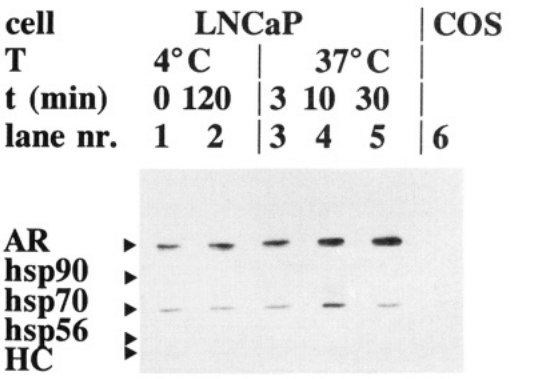


FIGURE 7: Immune precipitation with monoclonal F52 of androgen receptor complexes from cytosols of LNCaP cells incubated for various time periods with R1881. Receptor complexes were purified using F52–protein A–Sepharose and subjected to electrophoresis. Chemiluminescence exposures of Western immunoblots were prepared as described under Materials and Methods. MAbs F39, AC88, N27, and EC1 were used to identify AR, hsp90, hsp70, and hsp56, respectively. HC, antibody heavy chain. Cells incubated without hormone, with 10 nM R1881 for 2 h at 4 °C, and with 10 nM R1881 at 37 °C for 5, 10, and 30 min are represented by lanes 1–5, respectively. The sample in lane 6 is an immunoprecipitate from COS-1 cells (control).

receptor complexes are formed upon prolonged exposure of the cells to the receptor ligand. Hsp70 was present in all precipitates, but the amount of this heat-shock protein recovered varied considerably between different experiments. When the immunoprecipitation with MAb F52 was performed on a cytosol from a control cell line without androgen receptors (COS-1 cells), a small amount of hsp70 was detected on the blot (Figure 7, lane 6). This result shows that hsp70 also binds nonspecifically to MAb F52. With the antibody F39, which precipitates both large (8 S) and intermediate (6 S) receptor complexes, we observed a lower association of the receptor complex with hsp70 after prolonged incubation with androgen than found with F52 (compare Figures 4 and 7). This difference is an extra indication that hsp70 binds nonspecifically to the F52 resin. These results suggest that the 6S complex contains mainly androgen receptor, probably in the form of a dimer (the monomer sediments at 4.6 S). However, we cannot exclude the presence of small amounts of hsp70 or other proteins that are not detected by the antibodies used in this study.

## DISCUSSION

In the absence of hormones, steroid receptors are present in the cells as heteromeric complexes with several different proteins and have a low affinity for DNA. Ligand-induced release of regulatory proteins is thought to be an important step required for activation of the DNA-binding function of the receptor. In this process, called transformation, the heteromeric complex dissociates, thereby unmasking the DNA-binding domain of the receptor (Carson-Jurica et al., 1990; Pratt, 1990). In the present study, we analyzed the composition of the androgen receptor complex during ligand-induced receptor transformation in vivo, in intact LNCaP prostate tumor cells. In this process, an untransformed 8S form from the cytoplasmic cell fraction is converted to a 4.6S nuclear form. In addition, intermediate size complexes (6 S) were observed in the cytosolic cell fraction. We did not address the question of the localization of the different receptor forms in the intact cell. Receptors isolated from cytosolic fractions may actually have been located in the intact cell as a nuclear "docking place" (Pratt, 1990). Recent evidence from im-

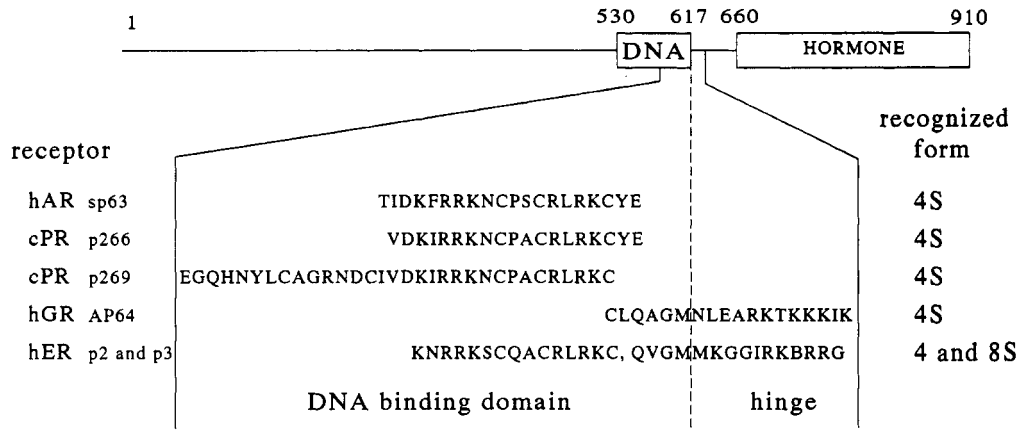


FIGURE 8: Schematic presentation of the peptides used for raising antibodies against steroid receptors. The peptide sp63 was used in the development of MAb F52. The peptides p266 and p269 are derived from the chicken glucocorticoid and progesterone receptor sequences in the homologues' region (Smith et al., 1988; Wilson et al., 1988). The antiserum AP64 (Urda et al., 1989) contains antibodies raised against the hGR sequence $Cys_{500}$–$ys_{517}$, overlapping only the three amino acid residues that are homologues to the three carboxy-terminal amino acid residues of sp63. The peptides p2 and p3 are derived from human estrogen receptor sequences $Lys_{213}$–$Cys_{245}$, totally overlapping the homologues' sequence of peptide sp63, and $Glu_{247}$–$Gly_{261}$, homologues to part of AP64, respectively. DNA, DNA-binding domain; hormone, hormone-binding domain; hinge, hinge region. The numbers indicate the amino acid residue numbers at the domain boundaries of the androgen receptor (Trapman et al., 1988; Faber et al., 1989).

munohistochemical studies (Jenster et al., 1991) suggests that in fact most androgen receptor molecules are located in the nucleus or are associated with perinuclear structures.

A new monoclonal antibody (F52) against the DNA-binding domain of the androgen receptor was generated. This antibody was used to show that androgen receptor complex intermediates are formed in the transformation process and that the epitope for this antibody in the DNA-binding region becomes exposed during this process. It was demonstrated that the epitope for this antibody is exposed not only in the 4.6S receptor but also in the 6S intermediate form of the receptor. Other antibodies against synthetic peptides have been described which also specifically or preferably recognize monomeric receptor forms (Wilson et al., 1988; Smith et al., 1988; Urda et al., 1989). In Figure 8, two peptide sequences derived from the progesterone receptor are shown, which overlap the homologous sequence in the androgen receptor to which the F52 antibody was raised [p266, p269; Smith et al. (1988) and Wilson et al. (1988)]. The antiserum AP64 (Urda et al., 1989) contains antibodies raised against the human glucocorticoid receptor sequence $Cys_{500}$–$Lys_{517}$, overlapping the carboxy-terminal end of the DNA-binding domain and the amino terminus of the hinge region (Figure 8). Antisera developed against a region of the human estradiol receptor homologous to the region in the DNA-binding domain of the other steroid receptors, however, did bind the nontransformed estradiol receptor on sucrose gradients [Traish et al. (1989); sequence p2 and p3 in Figure 8]. This might be due to differences in tertiary structure of the regions flanking the peptide sequence in the less homologous estradiol receptor. However, specific recognition of the transformed receptor only was also observed for antibodies raised against the chicken progesterone receptor sequence $Leu_{523}$–$Pro_{536}$ (Weigel et al., 1989). This sequence is located in the hinge region, indicating that antigenic sites outside the DNA binding-domain also become exposed after transformation of the receptor.

Using the bifunctional cross-linker dimethyl pimelimidate for cross-linking of the androgen receptor complex, we have shown formation of a covalently linked complex sedimenting at 8S that did not dissociate on sucrose gradient in the presence of salt. In studies with glucocorticoid receptors it has been shown that, in the untransformed complex, the cross-linker dimethyl suberimidate could cross-link the receptor to two hsp90 molecules and one 50-kDa unknown protein (Rexin et al., 1988). The 6S, intermediate size, androgen receptor complex was not stabilized by DMP, indicating a change in structure of the complex which prevents receptor cross-linking to other proteins. This suggests that the receptor can be covalently linked only to one of the fast-dissociating proteins (e.g., hsp90 or hsp56) that are absent in the 6S form of the receptor complex. Alternatively, in the intermediate form of the receptor complex, the distances between the reactive amino acid residues (lysines) have changed in a way that makes coupling of the receptor with the other proteins impossible. In cross-linking reactions with bis(imidates), the distance between the reacting residues is very important for optimal coupling of the proteins (Arányi et al., 1988).

In the present study, two different monoclonal antibodies against the androgen receptor were used for the characterization of heat-shock proteins interacting with the receptor. The antibody F39, recognizing an epitope in the N-terminal region, precipitates all different forms of the receptor and was used to study initial steps in heat-shock protein release. Before incubation of the LNCaP cells with hormone, the heat-shock proteins hsp90, hsp70, and hsp56 were precipitated together with the receptor by antibody F39. Other proteins might also be present in the complexes, as has been described for the progesterone receptor (Smith et al., 1990a), but the repertoire of antibodies against heat-shock proteins used in this study does not permit their detection. The dissociation rate of hsp70 from the androgen receptor complex, during incubation of the cells with hormone, is lower than that observed for hsp90 and hsp56. However, after 1 h of incubation of the cells with hormone, the associated hsp70 level was reduced considerably. Investigations of Sanchez et al. (1990b) suggested that hsp56 exists in cytosol in a higher order complex containing hsp70 and hsp90. Furthermore, p59 (the rabbit homologue of hsp56) is bound to hsp90 and not to the hormone-binding subunit of steroid receptor complexes (Renoir et al., 1990a, Lebeau et al., 1992). This implies that hsp56 dissociates from the receptor complex either together with or in advance of hsp90. For the progesterone and glucocorticoid receptor, a similar steroid-induced dissociation process of the multiprotein receptor complex was found. As in our study of the androgen receptor, also for the progesterone and glucocorticoid receptor, hsp70 remained partly bound to the receptor. In contrast to

our observation, however, in the latter studies, hsp70 also remained bound to the receptor complex in the presence of high concentrations of salt (Kost et al., 1989; Sanchez et al., 1990a; Smith et al. 1990a; Smith et al., 1990b). ATP is probably required for the in vitro dissociation of hsp70 from the progesterone receptor. (Smith et al., 1992).

Immunoprecipitation of the intermediate 6S form of the androgen receptor with the newly developed F52 antibody resulted in coprecipitation of hsp70, but not of hsp56. Only limited amounts of hsp90 were coprecipitated, and after extended washing of the precipitate, this heat-shock protein was no longer present. This indicates that the carboxy-terminal part of the DNA-binding domain is exposed to the F52 antibody, after removal of hsp56 and most of hsp90. In contrast, hsp70 was coprecipitated and could not be removed by washing of the antibody–receptor complex. However, as described under Results, the experiments indicate that a considerable part of the hsp70 is nonspecifically bound. This nonspecifically bound (not receptor associated) hsp70 may have prevented the detection of a small amount of specifically bound (receptor associated) hsp70.

In summary, it appears that the first step of androgen receptor transformation of the 8S androgen receptor complex results in loss of association of hsp90 and hsp56, leaving a smaller 6S receptor complex. This intermediate receptor complex, in contrast to the 8S complex, cannot be stabilized by cross-linking with DMP, indicative of the changes in association of the receptor with the associating proteins. Furthermore, F52, a monoclonal antibody raised against part of the DNA-binding domain of the androgen receptor (F52), binds to the 6S as well as to the 4.6S form, but not to the 8S receptor form, on sucrose gradients. This demonstrates that the C-terminal part of the DNA-binding domain is exposed in the 6S, hormonally transformed receptor. It cannot be excluded that the 6S receptor form detected on sucrose gradients consists of heteromeric complexes with hsp70, or other as yet undefined proteins. The possibility that receptor-bound protein factors might play a role in transcription activation should not be excluded (Lewin, 1990). Alternatively, the 6S complex may predominantly consist of homodimeric receptor complexes. This is not unlikely, since formation of homodimers preceding receptor binding to DNA has been shown for human and chick progesterone receptors (Demarzo et al., 1991; Rodrigues et al., 1990). Further analysis of the 6S intermediate forms of the androgen receptor will be the next step in the study of the hormone-induced receptor transformation process in intact cells.

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