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ANDROGEN RECEPTOR HETEROGENEITY AND PHOSPHORYLATION IN HUMAN LNCaP CELLS

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Androgen receptor heterogeneity and phosphorylation were studied in the human LNCaP cell line. Fluorography after photoafffinity labeling as well as immunoblotting with a specific polyclonal antibody revealed that the human androgen receptor migrated as a closely spaced 110 kD doublet on SDS-polyacrylamide gels. A time-dependent change in the ratio between the two isoforms was not observed after R1881 treatment of intact cells. In nuclear extracts of LNCaP cells that were incubated with [^{32}P]orthophosphate in the presence of 10 nM R1881, a 110 kD phosphorylated protein was demonstrated after immunopurification using a monoclonal antibody against the human androgen receptor. Only a very small amount of this phosphoprotein was detected in the nuclear fraction from cells not treated with R1881. These results indicate that the human androgen receptor in LNCaP cells can be phosphorylated. © 1990 Academic Press, Inc.

Steroid hormone receptors are intracellular proteins which are involved in transcriptional regulation of specific genes in target tissues. The first step in steroid hormone action is ligand binding. This results in transformation of the steroid-receptor complex from a loosely nuclear binding form to a form which is tightly bound in the cell nucleus (1,2). The molecular mechanism by which the receptor molecule changes upon ligand binding into a DNA-binding protein which regulates gene transcription is not completely clear.
Phosphorylation of the steroid receptor molecule might play an important role in the mechanism of action of steroid hormones. Ample evidence has been provided that steroid hormone receptors can exist in intact cells as phosphoproteins (3-6). It has been postulated that an initial phosphorylation step is necessary for the acquisition of the hormone binding capacity (7-9). A second, hormone dependent, phosphorylation step might be involved in the transformation process and in the regulation of gene transcription (4,10,11). Steroid receptor phosphorylation may result in receptor heterogeneity (4,12). An increased incorporation of [^{32}P]orthophosphate and an upshift in apparent

Abbreviations: LNCaP, Lymph Node Carcinoma of the Prostate; SDS-PAGE, sodium dodecyl sulphate poly acrylamide gel electrophoresis.

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molecular mass on SDS-gels have been observed after hormone treatment of target cells for the progesterone, glucocorticoid, oestradiol and vitamin D receptors (4,11-16).

It is not known whether phosphorylation of the androgen receptor may also occur and might be involved in androgen action. With the recent development of specific polyclonal (17) and monoclonal antibodies against a region within the N-terminal domain of the human androgen receptor, tools were provided to investigate phosphorylation of this receptor molecule. In the present study it is shown that the androgen receptor in human LNCaP cells is a heterogeneous protein that migrates as a doublet on SDS-PAGE. In addition, the results described herein provide evidence that the androgen receptor in these human cells is phosphorylated.

MATERIALS AND METHODS

Materials

17β-hydroxy-17α-[^{3}H]methyl-4,9,11-estratrien-3-one ([^{3}H]R1881) (87 Ci/mmol) and radioinert R1881 were purchased from NEN-Dupont (Dreieich, F.R.G.). Dihydrotestosterone was obtained from Steraloids (Wilton, NH, USA).

Cell culture

The LNCaP cell line was cultured as described previously (18). Two to four days before use the cells were kept on medium containing 5% heat-inactivated, charcoal stripped fetal calf serum.

Preparation of nuclear salt extracts

The preparation of nuclear salt extracts has been described previously (17). 50 mM sodium fluoride and 10 mM sodium molybdate were added as phosphatase inhibitors to all buffers.

In situ photolabeling, preparation of cell lysates and gel slicing have been described elsewhere (18).

SDS-PAGE

SDS-PAGE was carried out according to Laemmli (19) using 6% linear polyacrylamide gels. High molecular weight markers (29,000-205,000 D, Sigma,St. Louis, MO, USA) were used as references for molecular weight estimation.

Fluorography

After SDS-PAGE, the slabgel was fixed in 50% methanol/10% acetic acid for 45 min and soaked in Amplify (Amersham, Buckinghamshire, U.K.) for 20 min, dried under vacuum, and exposed to Hyperfilm-MP (Amersham) for 10 weeks at -80°C.

Western blotting and autoradiography

Western blot analysis, using Sp061 antiserum in a 1:1000 dilution, has been described previously (17). If receptor preparations were labeled with [^{32}P]orthophosphate, the filter was air-dried after colour development and exposed to hyperfilm-MP (Amersham) with two intensifying screens for 8 days at -80°C.

Metabolic labeling with [^{32}P]orthophosphate

For phosphorylation studies LNCaP cells were preincubated for 1 hour at 37°C with a solution of 118.3 mM NaCl, 4.75 mM KCl, 25 mM $NaHCO_3$, 1.2 mM $NaSO_4$, 2.5 mM $CaCl_2$, 0.2% glucose, essential and non-essential amino acids (GIBCO, Grand

Island, NY, USA) and glutamine (GIBCO) in appropriate dilutions at pH 7.3 and subsequently incubated for 4 h with 0.2 mCi/ml [^{32}P]orthophosphate (carrier-free, Amersham). 30 min before the end of the incubation, R1881 (10 nM; final concentration) or vehicle were added. After the incubation period nuclear salt extracts were prepared and androgen receptors were isolated by immunopurification as described below.

Preparation of monoclonal antibodies

A synthetic peptide, corresponding to the amino acids 301-320 of the human androgen receptor (20) was coupled to keyhole limpet hemocyanin and used for immunization. The same peptide has been successfully used for the generation of polyclonal antibodies (17). A Balb/c mouse (12-weeks old) was immunized subcutaneously with the peptide conjugate; 3 times at 5 weeks intervals with 25 µg of the peptide conjugate in Specol (21). Fusion of approximately 10^8 spleen cells with 2.10^7 mouse myeloma cells (SP2/0) was achieved with 40% polyethylene glycol 4000 (Merck) and 5% dimethyl sulfoxide. Fused cells were cultured in RPMI-1640 medium in the presence of azaserine (1 µg/ml) and hypoxanthine (0.1 mM). After 10 days hybridoma's were cultured in RPMI-1640 medium containing hypoxanthine (0.1 mM). Hybridoma supernatants were screened for reactivity with the peptide in an ELISA. Some positive cultures were cloned by limiting dilution and then plated out at one cell per well density.

Immunoprecipitation

200 µl hybridoma supernatant, containing monoclonal mouse anti-androgen receptor antibodies, was mixed with 50 µl anti-mouse IgG-agarose (packed gel) (Sigma) and 150 µl phosphate-buffered saline and incubated for 2 h at 4°C under constant rotation. Following centrifugation (10 sec, 2000 x g) the supernatant was removed and the pellet was washed three times with TEDGF buffer (40 mM Tris, 1 mM EDTA, 10% glycerol, 10 mM dithiothreitol, 50 mM sodium fluoride, 10 mM sodium molybdate, pH 7.4). A portion of 400 µl of the nuclear extract from [^{32}P]orthophosphate labeled LNCaP cells was added together with 2 ml TEDGF buffer, containing 1% Triton X-100, 0.5% desoxycholate and 0.08% SDS. The mixture was incubated for 2 h at 4°C under constant rotation and washed 3 times with the incubation buffer, 3 times with TEDGF buffer containing 0.4 M NaCl and 0.2% Triton X-100, and 3 times with TEDGF buffer without further additions. The pellet was mixed with 100 µl sample buffer (40 mM Tris, pH 6.8, 5% glycerol, 2% SDS, 10 mM DTT), boiled for 2 min and centrifuged (2000 x g, 5 min). The supernatants were subjected to SDS-PAGE.

RESULTS

LNCaP cells were photolabeled with the synthetic androgen [^{3}H]R1881 in the presence or absence of a 100-fold molar excess of unlabeled dihydrotestosterone. After photolabeling cell lysates were prepared and analyzed by SDS-PAGE. Gel slicing revealed that two proteins of 110 kD and 43 kD were labeled covalently with [^{3}H]R1881 (Fig. 1A and ref.18). The radiolabeled 110 kD protein represents the human androgen receptor (18). Fluorography after SDS-PAGE of lysates prepared from photolabeled LNCaP cells revealed that the androgen receptor migrated as a closely spaced doublet around 110 kD (Fig. 1B). Further evidence for heterogeneity of the androgen receptor was obtained after Western blotting of a similar receptor preparation (Fig. 1C). Most of the androgen receptor protein was present in the higher molecular weight form according to the difference in intensity between the two bands. (Figs. 1B and 1C).

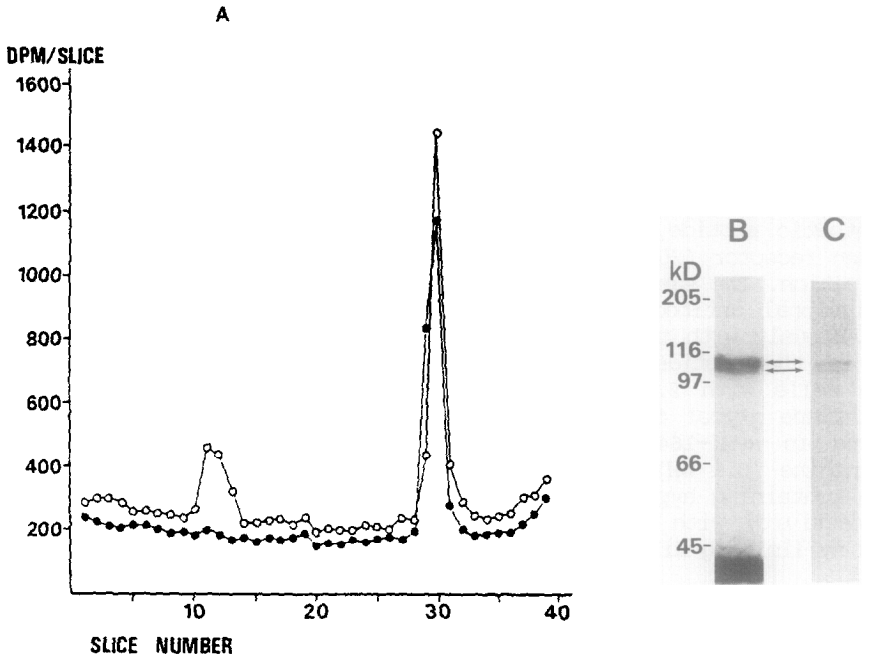

Figure 1
SDS-PAGE of the androgen receptor from LNCaP cell lysates.
A: The cells were incubated for 1 h with 10 nM [^{3}H]R1881 in the presence (●) or absence (○) of a 100-fold molar excess of dihydrotestosterone and were photolabeled. Cell lysates were analyzed on SDS-PAGE. Gel slices were counted for radioactivity.
B: Fluorography of androgen receptor in lysates, prepared after incubation of the cells with 10 nM [^{3}H]R1881 for 1 h and subsequent photolabeling in situ.
C: Immunoblot of the androgen receptor in cell lysates prepared after incubation of the cells with 10 nM R1881 for 1 h.

Whether the relative amounts of the isoforms change during hormone treatment was investigated by analyzing the receptor pattern at different times following the administration of 10 nM R1881 to LNCaP cells. Fig. 2 shows a

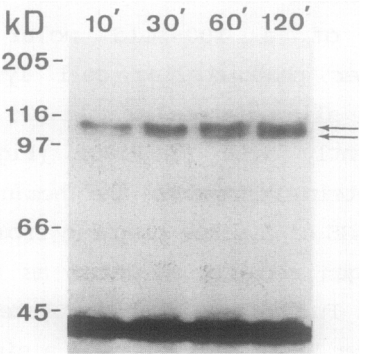

Figure 2
Fluorography after SDS-PAGE of androgen receptor in lysates prepared from photolabeled LNCaP cells. The cells were incubated for 10-120 min with 10 nM [^{3}H]R1881 before photolabeling.

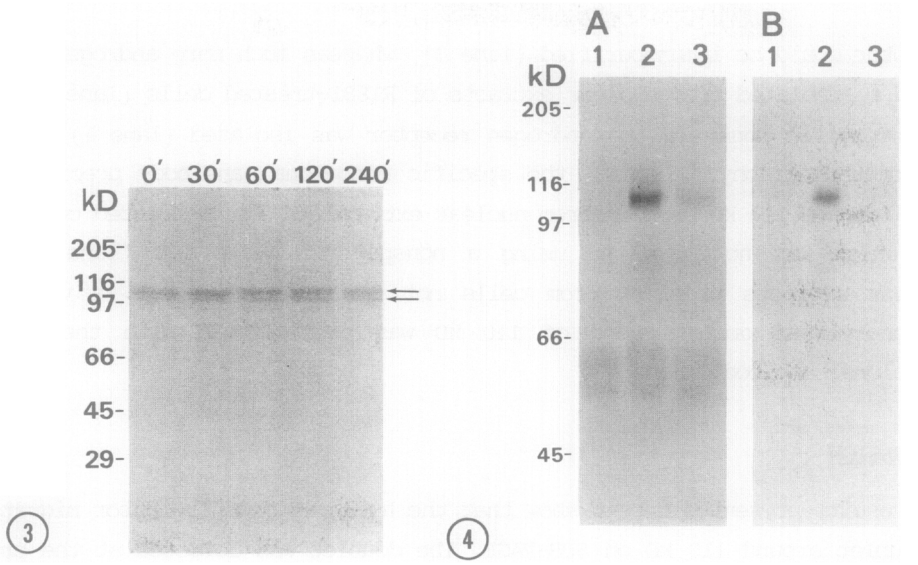

Figure 3
Western blot of androgen receptor in LNCaP cell lysates after exposure of the cells for 0-240 min to 10 nM R1881.

Figure 4
Western blot (4A) and autoradiogram (4B) of androgen receptor immunopurified from nuclear extracts of R1881-treated (lanes 1 and 2) or untreated (lane 3) LNCaP cells. The cells were incubated with 0.2 mCi/ml [^{32}P]orthophosphate for 4 h either in the presence or absence of R1881. Androgen receptors were immunopurified using a specific monoclonal antibody (lanes 2 and 3) or nonspecific mouse IgG (lane 1) and analyzed after SDS-PAGE and Western blotting. Equal amounts of protein from nuclear extracts were used for immunopurification in all cases. The immunoblot was autoradiographed.

fluorogram of lysates of LNCaP cells that were photolabeled with [^{3}H]R1881 at 10-120 min after hormone administration. A pronounced time-dependent change in the ratio of the receptor isoforms in the presence of R1881 could not be detected. The relatively low intensity of photolabeling at 10 min after [^{3}H]R1881 administration might be due to incomplete labeling of the androgen receptor. Based on additional experiments, labeling of the androgen receptor was optimal 30 min after administration of 10 nM [^{3}H]R1881 to LNCaP cells. Western blot analysis of lysates, prepared from LNCaP cells after incubation with 10 nM R1881 for different time periods up to 240 min, also revealed no time dependent changes of the ratio between the androgen receptor isoforms (Fig. 3).

Since isoforms of other steroid hormone receptors appear to be due to differences in their phosphorylation states, it was investigated whether the androgen receptor is also a phosphoprotein. To this end LNCaP cells were incubated with [^{32}P]orthophosphate in the presence or absence of R1881. Androgen receptors were isolated from nuclear extracts with a specific monoclonal antibody and subsequently analyzed by SDS-PAGE, Western blotting and autoradiography (Fig. 4). Fig. 4A shows that from nuclear extracts of cells incubated in the absence of R1881, only a very small amount of androgen

receptor could be immunopurified (lane 3), whereas much more androgen receptor could be isolated from nuclear extracts of R1881-treated cells (lane 2). Using a nonspecific mouse IgG no androgen receptor was isolated (lane 1). As shown by autoradiography (Fig. 4B) the specific monoclonal antibody precipitated a [^{32}P]labeled 110 kD protein from nuclear extracts of R1881-treated cells (lane 2), which was not isolated using a nonspecific mouse IgG (lane 1). From nuclear extracts prepared from cells not treated with R1881, a very faint phosphorylated protein band of 110 kD was precipitated with the specific monoclonal antibody (lane 3).

DISCUSSION

The results presented herein show that the human androgen receptor migrates as a doublet around 110 kD on SDS-PAGE. The doublet was detected at the protein level (using Western blot analysis) and at the steroid binding level (using fluorography), which indicates that both isoforms bind ligand. In addition strong evidence is provided that the human androgen receptor is a phosphoprotein. First, the phosphorylated protein was precipitated specifically using a monoclonal antibody raised against the human androgen receptor. Second, the immunopurified protein co-migrated with the 110 kD androgen receptor on SDS-PAGE. Third, the presence of the phosphorylated protein in nuclear extracts of LNCaP cells was hormone dependent. Isoforms of other steroid hormone receptors reflect differences in their phosphorylation states. The heterogeneous properties of the androgen receptor found in the present investigation might also be due to differentially phosphorylated androgen receptor forms. This variability may reflect differences in the extent as well as in the sites of phosphorylation (22,23).

A time dependent upshift in apparent molecular weight, as shown for the progesterone, estradiol and vitamin D receptors after ligand binding (12,15,16) was not observed for the androgen receptor upon R1881 treatment of LNCaP cells. This might indicate that phosphorylation of the androgen receptor in LNCaP cells does not change markedly after ligand binding. Possibly, ligand-induced phosphorylation of steroid hormone receptors is cell specific, as may be suggested on basis of results on the glucocorticoid receptor (11,14,24).

Within 30 min after R1881 administration to the LNCaP cells the androgen receptor was transformed to the tight nuclear binding form and could be recovered completely from nuclear extracts. Prolonged exposure to R1881 did not result in the extraction of more androgen receptor (not shown). In the absence of ligand estradiol and progesterone receptors are loosely bound to the nucleus, while untransformed glucocorticoid receptor is present both in

the nucleus and in the cytoplasm (25-28). These receptor proteins are recovered from the cytosol after cell fractionation in the absence of ligand. The subcellular localization of the androgen receptor in the absence of hormone is still not known. In the present investigation a small amount of androgen receptor was extracted from nuclear salt extracts of cells that were not treated with R1881, as shown using Western blot analysis. Presently it is not clear whether this small amount of androgen receptor reflects a more tightly bound nuclear receptor population or represents a residual fraction associated to the nucleus due to incomplete washing of the nuclei.

The present experiments with [^{32}P]orthophosphate indicate that the transformed androgen receptor recovered from nuclear extracts of R1881-treated LNCaP cells is a phosphoprotein. It remains to be investigated whether the untransformed androgen receptor is also phosphorylated. The function of androgen receptor phosphorylation remains to be elucidated, but might be essential for ligand binding (7-9) or transcriptional regulation (4,10,11).

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REFERENCES

1. Evans, R.M. (1988). Science 240, 889-895.
2. Beato, M. (1989). Cell 56, 335-344.
3. Housley, P.R., and Pratt, W.B. (1983). J. Biol. Chem. 258, 4630-4635
4. Sheridan, P.L., Krett, N.L., Gordon, J.A. and Horwitz, K.B. (1988) Mol. Endocrinol. 2, 1329-1342.
5. Dougherty, J.J., Puri, R.K and Toft, D.O. (1984). J. Biol. Chem. 259, 8004-8009.
6. Migliaccio, A., Rotondi, A. and Auricchio, F. (1986). The EMBO J. 5, 2867-2872.
7. Housley, P.R., Dahmer, M.K. and Pratt, W.B. (1982). J. Biol. Chem. 257, 8615-8618.
8. Migliaccio, A., Di Domenico, M., Green, S., de Falco, A., Kajtaniak, E.L., Blasi, F., Chambon, P., and Auricchio, F. (1989). Mol. Endocrinol. 3, 1061-1069.
9. Golsteyn, E.J., Graham, J.S., Goren, H.J. and Lefebvre, Y.A. (1989). The Prostate 14, 91-101.
10. Sigler, P.B. (1988). Nature 333, 210-212.
11. Hoeck, W., Rusconi, S. and Groner, B. (1989). J. Biol. Chem. 264, 14396-14402.
12. Sullivan, W.P., Smith, D.F., Beito, T.G., Kroo, C.J. and Toft, D.O. (1988). J. of Cell. Biochem. 36, 103-119.
13. Logeat, F., Le Cunff, Pamphile, R. and Milgrom, E. (1985). Biochem. Biophys. Res. Commun. 131, 421-427.
14. Orti, E., Mendel, D.B., Smith, L.I. and Munck, A. (1989). J. Biol. Chem. 264, 9728-9731
15. Golding, T.S. and Korach, K.S. (1988). Proc. Natl. Acad. Sci 85, 69-73.
16. Pike, J.W. and Sleator, N.M. (1985). Biochem. Biophys. Res. Commun. 131, 378-385.

17. van Laar, J.H., Voorhorst-Ogink, M.M., Zegers, N.D., Boersma, W.J.A., Claassen, E., van der Korput, J.A.G.M., Ruizeveld de Winter, J.A., van der Kwast, Th.H., Mulder, E., Trapman, J. and Brinkmann, A.O. (1989). Mol. Cell. Endocrinol. 67, 29-38.
18. van Laar, J.H., Bolt-de Vries, J.J., Voorhorst-Ogink, M.M. and Brinkmann, A.O. (1989). Mol. Cell. Endocrinol. 63, 39-44.
19. Laemmli, U.K. (1970). Nature 227, 680-685.
20. Faber, P.W., Kuiper, G.G.J.M., van Rooij, H.C.J., van der Korput, J.A.G.M., Brinkmann, A.O. and Trapman, J. (1989). Mol. Cell. Endocrinol. 61, 257-262.
21. Bokhout, B.A., van Gaalen, C. and van der Heyden, J. (1981). Vet. Immunol. 2, 491-498.
22. Wegener, A.D. and Jones, L.R. (1984). J. Biol. Chem. 259, 1834-1841.
23. DePaoli-Roach, A.A., Ahmed, Z., Camic, M., Lawrence, Jr. J.C. and Roach, P.J. (1983). J. Biol. Chem. 258, 10702-10709.
24. Tienrungroj, W., Sanchez, E.R., Housley, P.R., Harrison, R.W. and Pratt, W.B. (1987) J. Biol. Chem. 262, 17342-17349.
25. Perrot-Applanat, M., Logeat, F., Groyer-Picard, M.T. and Milgrom, E. (1985). Endocrinology 116, 1473-1484.
26. King, W.J. and Greene, G.L. (1984). Nature 307, 745-747.
27. Welshons, W.V., Lieberman, M.E. and Gorski, J. (1984). Nature 307, 747-749.
28. Wikström, A., Bakke, O., Okret, S., Brönegaord, M. and Gustafsson, J. (1987). Endocrinology 120, 1232-1242.