Localization and hormonal stimulation of phosphorylation sites in the LNCaP-cell androgen receptor

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Phosphorylation of the androgen receptor in human prostate tumour cells (LNCaP) is increased by addition of androgens to intact cells. Double-label studies, using [^{35}S]methionine incorporation into receptor protein, and [^{32}P]P_i to label metabolically receptor phosphorylation sites, have enabled us to determine the phosphate content, relative to receptor protein, of both non-transformed and transformed androgen receptors generated in intact LNCaP cells. No net change in the phosphorylation of the intact 110 kDa steroid-binding component of the androgen-receptor complex was found upon transformation to the tight nuclear binding form in the intact cell. Partial proteolysis of androgen receptor protein metabolically labelled with [^{32}P]P_i and photolabelled with [^{3}H]R1881 (methyltrienolone) revealed that phosphorylation occurs mainly in the N-terminal *trans*-activation domain, whereas no phosphorylation was detected in the steroid- and DNA-binding domains. The location of most (> 90%) of the hormonally regulated phosphorylation sites in the N-terminal *trans*-activation domain suggests a role of phosphorylation of the androgen receptor in transcription regulation.

INTRODUCTION

Steroid-hormone receptors are *trans*-acting gene-regulating proteins, involved in the accomplishment of steroid-hormone-induced cellular responses. Upon binding of hormone, the receptor–hormone complex undergoes a conformational change called transformation, which is thought to precede binding of the complex to hormone-responsive elements in the target-cell genome [1].

A putative role of receptor phosphorylation in steroid-hormone action has long been recognized. On the basis of effects of ATP on hormone binding and transformation of steroid receptors, it has been suggested that these two processes are influenced by phosphorylation/dephosphorylation events ([2], and references therein). Ample evidence has now been provided that the progesterone, glucocorticoid, oestrogen, androgen and vitamin D_3 receptors exist as phosphoproteins even in the absence of ligand [3–7]. Additional phosphorylation has been observed upon hormone binding [2]. It has been shown that basal phosphorylation is indispensable for the acquisition of ligand-binding activity of the oestradiol receptor [5]. Indirect evidence has been provided for a similar role of phosphorylation in ligand binding of several other steroid receptors [2]. Various functional roles for hormone-induced receptor phosphorylation have been proposed, e.g. dissociation of associated proteins (such as hsp 90), interaction with other transcription factors and specific binding to hormone-responsive elements [2]. All steroid receptors thus far described have multiple phosphorylation sites [8–14].

We have studied androgen-receptor heterogeneity and synthesis in the human LNCaP cell line (Lymph-Node Carcinoma of the Prostate cells) [7,15]. In these cells, the androgen receptor is a heterogeneous protein which is synthesized as a single 110 kDa protein, which becomes rapidly phosphorylated to a 112 kDa protein [15]. Metabolic labelling experiments using [^{32}P]P_i indicated that the androgen receptor is a phosphoprotein in hormone-depleted cells [7]. Immunoprecipitation of androgen receptors from total cell lysates showed an almost 2-fold increase in receptor phosphorylation within 30 min after hormone administration as compared with control cells [16].

It has also been shown that the amount of androgen-receptor protein in nuclear extracts of LNCaP cells reaches a near-optimum within 30 min after addition of hormone, whereas the cytosolic fraction is not completely depleted [15,16]. The fact that transformation to the tight nuclear binding form and hormone-induced hyperphosphorylation both reach an optimum within 30 min after addition of hormone to LNCaP cells suggests that there might be a link between both processes.

In the present study we have used monoclonal antibodies against the androgen receptor to purify cytosolic non-transformed and nuclear transformed complexes from ^{32}P-labelled and ^{35}S-labelled LNCaP cells exposed to androgens at 37 °C. Data obtained indicated that there is a similar degree of phosphorylation of the androgen receptor, before and after transformation to the tight nuclear binding form in the intact cell.

Using limited proteolysis it was established that most of the phosphorylation sites are localized in a *trans*-activation domain in the N-terminal part of the androgen-receptor protein.

MATERIALS AND METHODS

Materials

[^{35}S]Methionine (sp. radioactivity > 800 Ci/mmol) and [^{32}P]P_i (carrier free) was obtained from Amersham (Little Chalfont, Bucks., U.K.). Culture media were obtained from Seromed (Berlin, Germany). Fetal-calf serum was obtained from Serolab (U.K.). The synthetic androgen 17β-hydroxy-17α-[^{3}H]-methyl-4,9,11-oestratrien-3-one ([^{3}H]R1881; sp. radioactivity ~ 87 Ci/mmol) and unlabelled R1881 were purchased from NEN–Dupont (Dreieich, Germany).

The monoclonal antibody against the androgen receptor (F39.4.1, epitope amino acid residues 301–320) has been de-

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scribed previously [16–18]. The polyclonal antisera against the androgen receptor sp061 (epitope amino acid residues 301–320), sp066 (epitope amino acid residues 899–917) and sp197 (epitope amino acid residues 1–20) were prepared by previously published procedures [19]. The polyclonal antiserum (sp066) recognizes the 110–112 kDa androgen receptor on Western immunoblots, but is unable to interact with the native androgen receptor in solution complexed with radioactive ligand [19]. The polyclonal antisera sp197 (designed as described in [20]) and sp061 both contain high-titre antibodies against the androgen receptor, as was shown by immunoprecipitation and Western blotting [18].

All other chemicals and reagents were purchased from either Sigma Chemical Co. (St. Louis, MO, U.S.A.) or Merck (Darmstadt, Germany).

Cell culture

The LNCaP cell line was cultured as described previously [16].

Metabolic labelling with [^{35}S]methionine and/or [^{32}P]P_i

For phosphorylation studies, LNCaP cells were incubated for 4 h at 37 °C in a phosphate-free Krebs–Ringer buffer at pH 7.3 (118 mM NaCl, 4.75 mM KCl, 25 mM $NaHCO_3$, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$) containing 0.2% (w/v) glucose and amino acids (minus methionine) according to the formulation of Eagle's Minimum Essential Medium, and 0.15 mCi/ml [^{32}P]P_i and 10 μCi/ml [^{35}S]methionine when appropriate.

Incubations were stopped by removal of the medium, immediately followed by a wash with PBS at 20 °C. Subsequently, the cells were lysed in buffer A [40 mM Tris/HCl, pH 7.4, 1 mM EDTA, 10% (v/v) glycerol, 10 mM dithiothreitol, 10 mM Na_2MoO_4, 50 mM NaF], supplemented with 1% (v/v) Triton X-100, 0.5% (w/v) sodium deoxycholate, 0.08% (w/v) SDS, 0.6 mM phenylmethanesulphonyl fluoride and 0.5 mM bacitracin at 4 °C. The lysate was centrifuged (30 min, 100000 *g*) and androgen receptor was immunoprecipitated from the supernatant.

Preparation of cytosol and nuclear extract

Approx. 6×10^7 cells were collected in 5 ml of buffer A supplemented with 0.6 mM phenylmethanesulphonyl fluoride, 0.5 mM bacitracin and 0.2 mg/ml leupeptin. Cells were homogenized, the homogenate was centrifuged for 10 min at 800 *g*, and the cytosol was prepared by centrifugation of the supernatant at 105000 *g* for 30 min at 4 °C. The nuclear pellet was washed with buffer A containing 0.2% Triton X-100, and then with buffer A without additions. Subsequently, the nuclear pellet was extracted with buffer A (pH 8.5) containing 0.4 M NaCl for 1 h at 4 °C. The nuclear extract was centrifuged at 105000 *g* for 30 min.

Immunoprecipitation, electrophoresis, blotting and autoradiography

These were done by previously published procedures [15,16].

Double labelling of LNCaP-cell androgen receptor with [^{3}H]R1881 and ^{32}P and cleavage with α-chymotrypsin

LNCaP cells were incubated with [^{32}P]P_i as described for 4 h. Then [^{3}H]R1881 was added to a final concentration of 10 nM and the incubation was continued for 30 min at 37 °C. After washing with ice-cold PBS, the cells were irradiated for 2 min at the surface of an u.v. trans-illuminator (wavelength 300 nm, Chromato-Vue-transilluminator; UV Products Inc., San Gabriel, CA, U.S.A.). A cell lysate was prepared, and 1 ml portions of it (corresponding to approx. 3×10^7 cells) were precipitated with the F39.4.1 monoclonal antibody against the androgen receptor, bound to goat anti-(mouse IgG)–agarose [15,16]. The pellets were transferred to a clean Eppendorf tube, and 100 μl of TEG buffer (40 mM Tris/HCl, pH 7.5, 1 mM EDTA and 10% glycerol) was added. Digestions were carried out with 0.5–1.0 μg of α-chymotrypsin (Merck; trace amounts of trypsin activity present) for 30 min at 4 °C, with constant mixing. At the end of the digestion, 25 μl of 5-fold-concentrated SDS/PAGE sample buffer was added, and the tubes were heated at 95 °C for 3 min. Samples were then centrifuged at 10000 *g* for 2 min and subjected to SDS/PAGE (11%-acrylamide gel). Lanes were cut into 2 mm slices, and the slices were incubated overnight in 1 ml of 0.1% SDS/TEG buffer. The radioactivity of 750 μl samples was determined with a Packard Tri-Carb 2500 TR liquid-scintillation counter, with a double-label setting for ^{3}H and ^{32}P.

Measurement of receptor-bound phosphate of transformed and non-transformed complexes after whole-cell incubation with R1881

Metabolic labelling of LNCaP cells with [^{32}P]P_i and [^{35}S]methionine was performed as described herein. Between 5 and 30 min before the end of the labelling period, R1881 was added to a final concentration of 10 nM and incubation was continued at 37 °C. Subsequently the cells were homogenized, and cytosolic fractions and nuclear extracts were made. After immunoprecipitation with the F39.4.1 monoclonal antibody, SDS/PAGE and blotting to nitrocellulose, the blots were incubated with the polyclonal antibody sp061 [16]. After colour development, each lane was cut into 2 mm slices and the slices were dissolved in 10 ml of Filtercount cocktail (Packard). Radioactivity was determined with a Packard Tri-Carb 2500 TR liquid-scintillation counter, with a double-label setting for ^{32}P and ^{35}S.

Labelling of LNCaP-cell androgen receptor with ^{32}P and partial proteolytic cleavage with α-chymotrypsin

LNCaP cells were incubated with [^{32}P]P_i as described herein. After labelling for 4 h, R1881 was added to a final concentration of 10 nM and the incubation was continued for 30 min at 37 °C. A total cell lysate was prepared and the androgen receptor was immunoprecipitated from 1 ml portions of the cell lysate (corresponding to about 3×10^7 cells) with the F39.4.1 monoclonal antibody against the androgen receptor bound to goat anti-(mouse IgG)–agarose [15,16]. Digestions were carried out with α-chymotrypsin (Merck) in the range 12.5–150 ng in 100 μl of TEG buffer (pH 7.4), for 30 min at 4 °C with constant mixing. At the end of digestion, SDS/PAGE sample buffer was added, and the tubes were heated at 95 °C for 3 min. Samples were then centrifuged at 10000 *g* for 2 min and subjected to SDS/PAGE (11%-acrylamide gel) and blotted on to nitrocellulose as described previously [15]. The filter was air-dried and exposed to Hyperfilm-MP (Amersham) with intensifying screens for 16–72 h at −80 °C. Thereafter blots were incubated with receptor-specific polyclonal antisera as described previously [15].

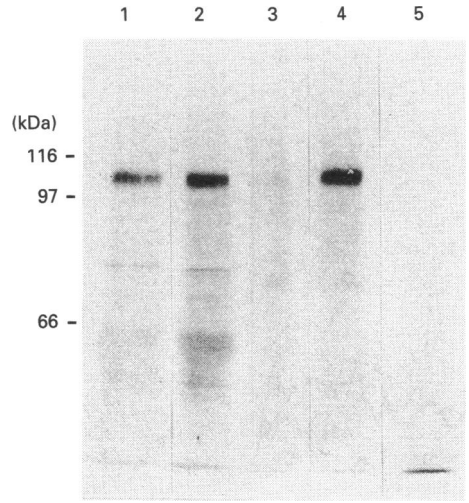

Figure 1 Hormone-dependent androgen-receptor phosphorylation and tight nuclear binding

LNCaP cells cultured for 4 h with [^{32}P]P_i were incubated in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of 10 nM R1881. R1881 was added 30 min before the end of the incubation with [^{32}P]P_i. Receptors were immunoprecipitated from cytosol (lanes 1, 2 and 5) and nuclear extracts (lanes 3 and 4) with the F39.4.1 monoclonal antibody (lanes 1–4) or non-specific mouse IgG (lane 5). After SDS/PAGE on a 7% gel, the proteins were transferred to nitrocellulose. The blot was exposed to X-ray film for 18 h at −80 °C before development. Molecular-mass markers (kDa) are indicated on the left.

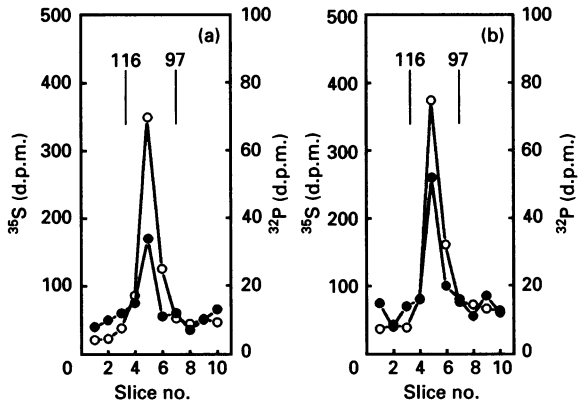

Figure 2 Hormone-dependent androgen-receptor phosphorylation

The Figure shows the radioactivity in the nitrocellulose slices which contained the immunopurified receptor protein from cytosolic extracts of LNCaP cells after SDS/PAGE and immunoblotting. The cells were incubated for 4 h at 37 °C with [^{32}P]P_i and [^{35}S]methionine, (**a**) without or (**b**) with 10 nM R1881 for the last 30 min. The migration and mass (kDa) of molecular-mass markers are indicated at the top. ○, ^{35}S; ●, ^{32}P.

RESULTS

Receptor phosphorylation and tight nuclear binding

In the absence of hormone, androgen receptors are only loosely bound to chromatin, and are easily extracted in the cytosolic fraction. Upon addition of hormone, androgen receptors are transformed to a form which is tightly bound in the cell nucleus, and can only be recovered after extraction of nuclei with high salt (0.4 M NaCl). From the fact that transformation of the androgen receptor to the tight nuclear binding form and hormone-induced hyperphosphorylation both reach an optimum within 30 min after addition of hormone to LNCaP cells, it could be suggested that there is a link between both processes.

Table 1 Phosphorylation of androgen receptors in cytosolic (C) and nuclear (N) extracts

The experiment was performed as described in the legend to Figure 1. After colour development, 110 kDa receptor bands were excised and subjected to liquid-scintillation counting (see Figure 2). The amounts of each radioisotope (^{32}P and ^{35}S) associated with the androgen receptor were calculated after subtraction of the background under the peak. The ^{32}P/^{35}S ratios of two independent experiments are presented (* not determined)

	^{32}P/^{35}S ratio			
	Expt. 1		Expt. 2	
Incubation time with R1881 (min)	C	N	C	N
0 (no hormone)	0.11	–	0.06	–
5	0.12	–	*	–
15	0.14	0.15	0.07	0.07
30	0.19	0.19	0.11	0.11

In order to address this question, receptors were isolated by immunoprecipitation from both cytosol and nuclear salt extracts of ^{32}P-labelled LNCaP cells that were incubated at 37 °C for 30 min, in either the presence or the absence of hormone (R1881). Androgen receptor was subjected to SDS/PAGE, and blotted on to nitrocellulose. In Figure 1, an autoradiogram of such an experiment is shown. In the absence of hormone, almost all of the phosphorylated receptor is recovered in the cytosolic fraction (compare lanes 1 and 3 with lanes 2 and 4) and is therefore regarded as non-transformed. Upon addition of hormone, hyperphosphorylated androgen receptor is present in the nuclear extract (lane 4). However, not all androgen receptor becomes transformed to the tight nuclear binding form, because in the cytosolic fraction (lane 2) also hyperphosphorylated receptor is present.

The extent of receptor phosphorylation was determined in double-label experiments, using [^{35}S]methionine to measure receptor protein and [^{32}P]P_i to measure receptor phosphate. This approach enables the determination of the phosphate content, relative to the amount of receptor protein, of both the non-transformed androgen receptor and the androgen-receptor molecules that are transformed to the tight nuclear binding form in intact LNCaP cells exposed to R1881 at 37 °C. In Figures 2(a) and 2(b) the profiles of ^{35}S and ^{32}P radioactivity associated with the 110 kDa androgen-receptor protein are shown. In the presence as well as in the absence of hormone an equal amount of receptor protein was immunoprecipitated (^{35}S label). Incubation of the cells with hormone, however, increased the amount of radioactive phosphate associated with the receptor (Figure 2b). Data from two independent experiments in which cells were incubated for various time periods with R1881 are summarized in Table 1. The androgen receptor is already phosphorylated in the absence of hormone, and upon addition of hormone a 1.8-fold stimulation of phosphorylation for the cytosolic extract was observed. However, the ratio of ^{32}P to ^{35}S radioactivity specifically associated with the 110 kDa androgen-receptor protein did not change upon transformation to the tight nuclear binding form.

Localization of phosphorylation sites with regard to the functional domains of the androgen receptor

The androgen receptor from LNCaP cells after photolabelling *in situ* with [^{3}H]R1881 is a protein of 110 kDa [18,21]. Upon limited

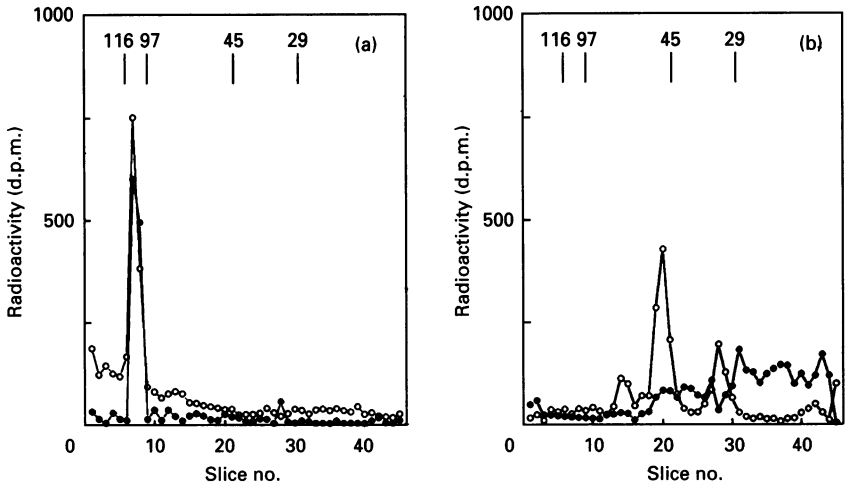

Figure 3 Chymotrypsin cleavage of ^{32}P- and ^{3}H-R1881-photolabelled androgen receptor

Androgen receptor from LNCaP cells was labelled with [^{32}P]P_i and ^{3}H-R1881. Portions of total cell lysate were immunoadsorbed to anti-(mouse IgG)–agarose with anti-androgen-receptor antibody. After the multiple-wash procedure, the immunoadsorbed receptor was incubated for 30 min at 4 °C, in the absence (**a**) or the presence (**b**) of 1.0 μg of α-chymotrypsin. The digestion products were resolved by SDS/PAGE, the gel was sliced, and the radioactivity in the slices was counted for ^{32}P (●) and ^{3}H (○). The migration of molecular-mass markers (kDa) is indicated at the top.

Table 2 Ratio of ^{32}P and ^{3}H-R1881 of the intact receptor and the generated chymotryptic fragments

The experiment was performed as described in the legend to Figure 3. The amounts of ^{3}H and ^{32}P radioactivity (d.p.m.) associated with each fragment was calculated from the data in Figure 3 after subtraction of background obtained.

Androgen receptor and fragments	^{32}P (d.p.m.)	^{3}H (d.p.m.)	Ratio $^{32}P/^{3}H$
Intact	1067	875	1.2
~ 70 kDa fragment	18	123	0.14
~ 45 kDa fragment	85	695	0.12
~ 33 kDa fragment	15	230	0.06

proteolysis, a tryptic fragment of ~ 30 kDa containing the steroid-binding domain and a ~ 45 kDa chymotryptic fragment containing the DNA- and steroid-binding domain are generated [22]. We have now used photoaffinity labelling with [^{3}H]R1881 and metabolic labelling with [^{32}P]P_i of the LNCaP-cell androgen receptor to detect possible phosphorylation sites within the DNA- and/or ligand-binding domain. Double-labelled receptors were purified with the F39.4.1 monoclonal antibody against the androgen receptor, before proteolysis with α-chymotrypsin. Fragments were subsequently analysed by SDS/PAGE, followed by liquid-scintillation counting of gel slices. Figure 3(a) shows the result for the intact androgen receptor, and Figure 3(b) shows the result after partial digestion with α-chymotrypsin. Three ^{3}H-labelled fragments were identified: a ~ 33 kDa fragment containing the ligand-binding domain, a ~ 45 kDa fragment encompassing the DNA- and ligand-binding domain, and a fragment of ~ 70 kDa which, in addition to the ~ 45 kDa fragment, also contains part of the N-terminal domain of the receptor (see also Figure 5). All three fragments reacted on immunoblots with the sp066 antiserum, which is directed to the C-terminus of the androgen-receptor protein (epitope amino acid 899–917), indicating that the fragments contained at least the whole ligand-binding domain (result not shown). The degrees of phosphorylation of the various fragments generated by α-chymotrypsin and the intact receptor were determined and expressed as the $^{32}P/^{3}H$ ratio (Table 2). The $^{32}P/^{3}H$ ratio for the intact receptor protein was more than 10 times that for the various fragments generated containing the DNA- and steroid-binding domains.

It can be concluded that 90 % or more of the phosphorylation sites are outside the DNA- and ligand-binding domain. In Figure 3(b) a large amount of ^{32}P is associated with fragments of < 29 kDa, probably proteolytic fragments of the N-terminal domain of the androgen receptor.

To analyse in more detail the proposed N-terminal domain phosphorylation of the LNCaP-cell androgen receptor, immunoprecipitated receptor was incubated with limited amounts (12.5–100 ng) of α-chymotrypsin. The partial digests of ^{32}P-labelled androgen receptor were analysed by SDS/PAGE, immunoblotting and autoradiography. The results of these experiments are shown in Figure 4. In this Figure, lane 10, containing the intact androgen receptor, displayed the presence of a single phosphorylated species of 110 kDa. Lanes 1–3 reflect the receptor preparations incubated with decreasing amounts of α-chymotrypsin. Strongly phosphorylated bands of 110 kDa, 85–90 kDa, ~ 50 kDa, ~ 40 kDa, ~ 25 kDa and a weakly phosphorylated band of ~ 15 kDa were observed.

In lanes 4–6 the corresponding immunoblot is shown after staining with the sp061 antiserum (epitope amino acid residues 301–320). All the phosphorylated bands from the autoradiogram (lanes 1–3) react with this antibody, except for the ~ 25 kDa band. Particularly interesting is the ~ 50 kDa fragment, which reacts with the sp061 antibody and is strongly phosphorylated, showing that indeed the N-terminal domain is heavily phosphorylated. This ~ 50 kDa fragment also reacts on blots with the sp197 antiserum, which is directed against an epitope at the N-terminus (amino acid residues 1–20), showing that this fragment encompasses the whole N-terminal domain of the receptor (see lanes 8 and 9). The ~ 50 kDa fragment does not react with the sp066 antiserum (epitope amino acid residues 899–917; result not shown). On the immunoblot an additional fragment of ~ 70 kDa is not phosphorylated (cf. lanes 3 and 6).

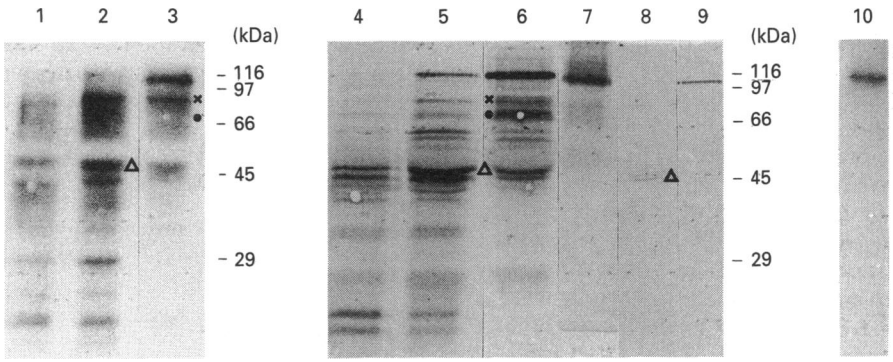

Figure 4 Proteolytic digestion of phosphorylated androgen receptors with α-chymotrypsin

Androgen receptor was labelled with [^{32}P]P_i, immunopurified from total cell lysates and digested with α-chymotrypsin. The digestion products were resolved by SDS/PAGE and the proteins were transferred to nitrocellulose. Lanes 1–3 show the autoradiogram of equal amounts of receptor preparations digested for 30 min at 4 °C with 100 ng (lane 1), 50 ng (lane 2) and 12.5 ng (lane 3) of α-chymotrypsin. Lanes 4–6 show the corresponding blot after staining with the sp061 antiserum. Lane 10 shows the autoradiogram of intact androgen receptor (no protease) and lane 7 the corresponding immunoblot. Lanes 8 and 9 show the immunoblot after staining with the sp197 antiserum, for the undigested androgen receptor (lane 9) and the receptor preparation digested with α-chymotrypsin (lane 8). Molecular-mass markers (kDa) are indicated. The symbols used indicate the fragments depicted in Figure 5.

This is most likely identical with the ~ 70 kDa fragment shown in Figure 3(b), which is essentially non-phosphorylated. In addition, a strongly phosphorylated fragment of 85–90 kDa was observed (cf. lanes 3 and 6). This fragment reacts on immunoblots with the sp061 antibody (lane 6) and also with the sp066 antiserum directed against an epitope at the C-terminus (amino acid residues 899–917; result not shown).

From these results, it is very likely that the main phosphorylation sites of the LNCaP-cell androgen receptor are located in the N-terminal domain, probably in the region of amino acid residues 1–300 (Figure 5).

Partial proteolysis of phosphorylated androgen receptor from control cells (not incubated in the presence of hormone) revealed a similar phosphopeptide pattern to that shown in Figure 4 for receptor in the presence of hormone. The only difference was the less strong labelling intensity of the intact receptor and the proteolytic fragments.

DISCUSSION

In the present study we have compared the degree of phosphorylation, in hormone-treated cells, of cytosolic receptors and 0.4 M NaCl-extracted receptors from nuclei. It was concluded that there is no net change in degree of phosphorylation between non-transformed receptor and receptor transformed to the tight nuclear binding form. This is analogous to results obtained for the glucocorticoid receptor in mouse L cells [23] and WEHI-7 cells [24].

Since our methods only measure the total amount of phosphate associated with the receptor, it is still possible that these phosphates are rearranged upon transformation. For the progesterone receptor in T47D cells, it has been shown that there are at least two different phosphopeptides in the transformed nuclear receptor, compared with the cytosolic non-transformed receptor [25]. Studies on phosphorylation of progesterone, glucocorticoid, 1,25-dihydroxyvitamin D_3 and oestrogen receptors in various systems provided evidence for hormone-dependent extra phosphorylation [6,12,14,26–29].

Also in the present study, using double labelling with [^{32}P]P_i and [^{35}S]methionine, we show that there is an almost 2-fold increase in degree of receptor phosphorylation on addition of hormone. This accounts for the cytosolic as well as for the nuclear-extractable androgen receptor. This hormone-induced extra phosphorylation is a rapid process, reaching an optimum within 30 min. The half-life of the androgen receptor in LNCaP cells is 2–3 h, in the presence as well as in the absence of ligand [16,30]. Only after longer incubations of LNCaP cells with androgens (24 h or longer) could a significant increase of androgen receptor protein be detected [31]. A possible stabilizing effect of androgens on receptor protein, mimicking receptor hyperphosphorylation, as recently shown for the androgen receptor in COS cells [32], can therefore be excluded.

Figure 5 presents a model for the location of phosphorylation sites with respect to the functional domains of the androgen receptor. It is possible that, when the receptor is cleaved by a protease, certain fragments become more sensitive to dephosphorylation, thereby giving the false impression that a certain fragment is not phosphorylated. However, digestions were carried out with immunoadsorbed receptor, which had been

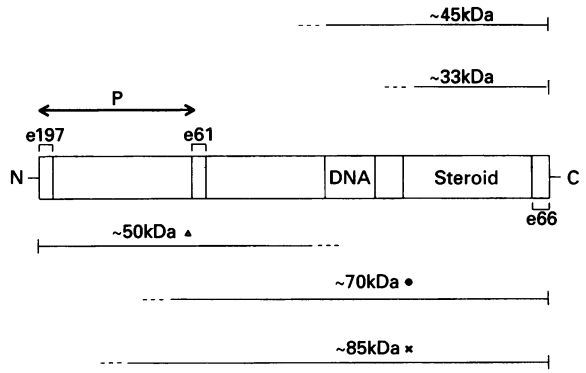

Figure 5 Model of phosphorylated region and functional domains of the human androgen receptor

Positions of the functional domains of the 110 kDa human androgen receptor (917 amino acid residues) are as described previously [17]. The epitopes (e197, e61 and e66) for the anti-peptide antisera used are indicated. The continuous lines represent the various chymotryptic fragments generated in this study. The positions of these fragments are based on previous work [22] and on probing with the indicated anti-peptide antisera. Where boundaries are not exactly known, lines are dashed. Fragments indicated by symbols correspond to fragments indicated by the same symbols in Figure 4. The phosphorylated region (see the Discussion section) is indicated by an arrow and the letter 'P'.

thoroughly washed free of cellular constituents, and we regard this possibility as highly unlikely. The chymotryptic fragments of ~45 kDa (Figure 3) and ~50 kDa (Figure 4) react with polyclonal antibodies directed against the C- and N-terminus respectively. The possibility of missing phosphorylated sites in both termini can therefore be excluded. The predominant localization of phosphorylation sites in the N-terminal domain of the androgen receptor is consistent with reports on progesterone and glucocorticoid receptors [8–12]. Recently seven phosphorylated sites were identified in the N-terminus (between amino acids 120 and 320) of the mouse glucocorticoid receptor [13]. Also, after transfection of progesterone [33] and glucocorticoid [12] receptors into COS or CV-1 cells, it was shown that all phosphorylated sites were located in the N-terminal domain.

It is noteworthy that the region shown to be heavily phosphorylated (amino acid residues 1–300) has also been shown to be essential for transcriptional activation [17]. A deletion mutant lacking amino acids 51–211 (pAR7) showed a strongly decreased transcriptional activation capacity, which was only 5% of that of the wild-type androgen receptor. The wild-type androgen receptor expressed in COS-1 cells migrated as a 110–112 kDa doublet when analysed by SDS/PAGE. This doublet represents androgen-receptor isoforms produced by phosphorylation [15,17,30]. The deletion mutant pAR7, however, migrated as a single band with an apparent molecular mass of ~84 kDa after expression in COS cells [17]. It is tempting to speculate that in the deleted region one or more serine or threonine residues become phosphorylated in the wild-type androgen receptor, giving rise to the described doublet.

A possible mechanism by which phosphorylation might modulate transcription is by altering the transcriptional activation potential of a transcription factor. Evidence for this type of regulation has been provided by studies in which a correlation was found between the phosphorylation state of certain transcription factors and their ability to activate transcription, despite phosphorylation having no effect on the ability of these factors to bind to DNA [34]. Such a model could also be applied to members of the steroid-receptor superfamily. No definitive evidence has been provided so far that phosphorylation of steroid receptors directly influences DNA binding capacity [2]. Also, in the present study we have shown that there is no net change in the degree of receptor phosphorylation upon transformation in the intact cell.

The most definitive indication for a role of phosphorylation in regulating members of the receptor superfamily of transcription factors has been provided for the progesterone receptor and the v-*erb* A protein [35,36]. It was shown by site-directed mutagenesis that phosphorylation of a particular serine residue in the N-terminal part of v-*erb* A regulates biological activity, without affecting nuclear localization or DNA-binding capacity [35]. The progesterone receptor in the chicken oviduct is a phosphoprotein, and treatment with progesterone *in vivo* stimulates phosphorylation of the receptor [14]. In CV-1 cells transfected with the chicken progesterone receptor, it was shown that treatment of cells with kinase activators or phosphatase inhibitors mimicked progesterone-dependent receptor-mediated transcription in the absence of progesterone [36]. This suggested that phosphorylation of the progesterone receptor or other proteins in the transcription complex modulate progesterone-receptor-mediated transcription.

The N-terminal receptor region defined here to be predominantly phosphorylated in the LNCaP-cell androgen receptor contains a total of 12 potential phosphorylation sites for Ser-Pro-directed kinase, casein kinases and double-stranded-DNA-dependent kinase, on the basis of published consensus sequences [37]. Identification of the actual sites of phosphorylation should allow definitive determination of the role of phosphorylation in the mode of action of the androgen receptor.

This work was supported by The Netherlands Organization for Scientific Research (NWO) through GB-MW (Medical Sciences), and by a grant from The Netherlands Cancer Society.

REFERENCES

1 Beato, M. (1989) Cell **56**, 335–344
2 Moudgil, V. K. (1990) Biochim. Biophys. Acta **1055**, 243–258
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 Migliaccio, A., Rotondi, A. and Auricchio, F. (1986) EMBO J. **5**, 2867–2872
6 Pike, J. W. and Sleator, N. M. (1985) Biochem. Biophys. Res. Commun. **131**, 378–385
7 Van Laar, J. H., Bolt-de Vries, J., Zegers, N. D., Trapman, J. and Brinkmann, A. O. (1990) Biochem. Biophys. Res. Commun. **166**, 193–200
8 Sheridan, P. L., Evans, R. M. and Horwitz, K. B. (1989) J. Biol. Chem. **264**, 6520–6528
9 Sullivan, W. P., Madden, B. J., McCormick, D. J. and Toft, D. O. (1988) J. Biol. Chem. **263**, 14717–14723
10 Dalman, F. C., Sanchez, E. R., Lin, A. L. Y., Perini, F. and Pratt, W. B. (1988) J. Biol. Chem. **263**, 12259–12267
11 Smith, L. I., Mendel, D. B., Bodwell, J. E. and Munck, A. (1989) Biochemistry **28**, 4490–4498
12 Hoeck, W. and Groner, B. (1990) J. Biol. Chem. **265**, 5403–5408
13 Bodwell, J. E., Orti, E., Coull, J. M., Pappin, D. J. C., Smith, L. I. and Swift, F. (1991) J. Biol. Chem. **266**, 7549–7555
14 Denner, L. A., Schrader, W. T., O'Malley, B. W. and Weigel, N. L. (1990) J. Biol. Chem. **265**, 16548–16555
15 Kuiper, G. G. J. M., de Ruiter, P. E., Grootegoed, J. A. and Brinkmann, A. O. (1991) Mol. Cell. Endocrinol. **80**, 65–73
16 Van Laar, J. H., Berrevoets, C. A., Trapman, J., Zegers, N. D. and Brinkmann, A. O. (1991) J. Biol. Chem. **266**, 3734–3738
17 Jenster, G., van der Korput, H. A. G. M., van Vroonhoven, C., van der Kwast, T. H., Trapman, J. and Brinkmann, A. O. (1991) Mol. Endocrinol. **5**, 1396–1404
18 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
19 Zegers, N. D., Claassen, E., Neelen, C., Mulder, E., van Laar, J. H., 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
20 Husmann, D. A., Wilson, C. M., McPhaul, M. J., Tilley, W. D. and Wilson, J. D. (1990) Endocrinology (Baltimore) **126**, 2359–2368
21 Brinkmann, A. O., Kuiper, G. G. J. M., Bolt-de Vries, J. and Mulder, E. (1988) J. Steroid Biochem. **30**, 257–261
22 De Boer, W., Bolt, J., Kuiper, G. G. J. M., Brinkmann, A. O. and Mulder, E. (1987) J. Steroid Biochem. **28**, 9–19
23 Tienrungroj, W., Sanchez, E. R., Housley, P. R., Harrison, R. W. and Pratt, W. B. (1987) J. Biol. Chem. **262**, 17342–17349
24 Mendel, D. B., Bodwell, J. E. and Munck, A. (1987) J. Biol. Chem. **262**, 5644–5648
25 Sheridan, P. L., Evans, R. M. and Horwitz, K. B. (1989) J. Biol. Chem. **264**, 6520–6528
26 Jones, B. J., Jurutka, P. W., Haussler, C. A., Haussler, M. R. and Whitfield, G. K. (1991) Mol. Endocrinol. **5**, 1137–1145
27 Orti, E., Mendel, D. B., Smith, L. I. and Munck, A. (1989) J. Biol. Chem. **264**, 9728–9731
28 Brown, T. A. and DeLuca, H. F. (1990) J. Biol. Chem. **265**, 10025–10029
29 Denton, R. R., Koszewski, N. J. and Notides, A. C. (1992) J. Biol. Chem. **267**, 7263–7268
30 Kuiper, G. G. J. M., de Ruiter, P. E. and Brinkmann, A. O. (1992) J. Steroid Biochem. Mol. Biol. **41**, 697–700
31 Krongrad, A., Wilson, C. M., Wilson, J. D., Allman, D. R. and McPhaul, M. J. (1991) Mol. Cell. Endocrinol. **76**, 79–88
32 Kemppainen, J. A., Lane, M. V., Sar, M. and Wilson, E. M. (1992) J. Biol. Chem. **267**, 968–974

33 Chauchereau, A., Loosfelt, H. and Milgrom, E. (1991) J. Biol. Chem. **266**, 18280–18286
34 Jackson, S. P., MacDonald, J. J., Lees-Miller, S. and Tjian, R. (1990) Cell **63**, 155–165
35 Glineur, C., Zenke, M., Beug, H. and Ghysdael, J. (1990) Genes Dev. **4**, 1663–1676
36 Denner, L. A., Weigel, N. L., Maxwell, B. L., Schrader, W. T. and O'Malley, B. W. (1990) Science **250**, 1740–1743
37 Pearson, R. B. and Kemp, B. E. (1991) Methods Enzymol. **200**, 62–81

Received 31 July 1992/19 October 1992; accepted 27 October 1992