Phosphotryptic Peptide Analysis of the Human Androgen Receptor: Detection of a Hormone-Induced Phosphopeptide†

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ABSTRACT: Phosphorylation of the androgen receptor (AR) in human prostate tumor cells (LNCaP) is increased by androgens. The AR is expressed as two isoforms with apparent molecular masses of 110 and 112 kDa. Metabolic labeling experiments with [^{32}P]orthophosphate revealed that only the 112 kDa isoform is radioactively labeled. Phosphoamino acid analysis revealed only phosphorylation on serine residues. Phosphotryptic peptide analysis of human AR protein by two-dimensional peptide mapping and by reverse-phase HPLC showed phosphorylation at multiple sites. Comparison of phosphopeptide maps of AR protein from cells incubated in the absence or presence of the synthetic androgen R1881 indicated that the ligand-stimulated phosphorylation is probably due to induction of phosphorylation at a new site rather than increased phosphorylation at an existing site. This result suggests that hormone-dependent AR phosphorylation might play a role in the signal transduction pathway of androgens.

Steroid hormone receptors are transacting gene-regulatory 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 receptor to hormone-responsive elements in the target cell genome (Bagchi et al., 1992; Jensen, 1991).

Ample evidence has been provided that the progesterone, glucocorticoid, estrogen, androgen, and 1,25-dihydroxyvitamin D_3 receptors are phosphoproteins (Moudgil, 1990; Orti et al., 1992; Kuiper & Brinkmann, 1994). Additional phosphorylation has been observed upon hormone binding. All steroid receptors investigated thus far have multiple phosphorylation sites (Moudgil, 1990; Orti et al., 1992). Steroid hormone receptor phosphorylation has been directly implicated in activation of hormone binding capacity, nuclear−cytoplasmic shuttling of steroid receptors, modulation of binding to hormone response elements, and regulation of receptor transactivation function (Takimoto & Horwitz, 1993; Kuiper & Brinkmann, 1994).

Previously we have studied androgen receptor (AR)[1] synthesis and heterogeneity in the human LNCaP cell line (Van Laar et al., 1990; Kuiper et al., 1991). In these cells, the AR is synthesized as a protein with an apparent molecular mass of 110 kDa, which becomes rapidly phosphorylated, resulting in an upshift to 112 kDa on SDS−PAGE (Kuiper et al., 1991). In addition, metabolic labeling experiments using [^{32}P]P_i indicated that the AR is a phosphoprotein in hormone-depleted LNCaP cells, and also in COS cells after transfection with an AR expression vector (Van Laar et al., 1990; Kemppainen et al., 1992). Upon addition of hormone to LNCaP cells, a rapid 2-fold increase in AR phosphorylation degree was observed (Van Laar et al., 1991; Kuiper et al., 1993). Partial proteolysis of AR protein labeled with [^{32}P]P_i revealed that phosphorylation occurs mainly in the N-terminal transactivation domain (Kuiper et al., 1993). Also progesterone, glucocorticoid, and estrogen receptors are predominantly phosphorylated in the N-terminal transcription activation domain, suggesting that phosphorylation might play a role in regulation of receptor transactivation function (Orti et al., 1992; Kuiper & Brinkmann, 1994).

The N-terminal region of the AR, found to be predominantly phosphorylated, contains 12 potential phosphorylation sites for Ser/Thr-Pro-directed kinases, casein kinases, and double-stranded DNA-dependent kinase, on the basis of published consensus sequences (Kennelly & Krebs, 1991; Finnie et al., 1993; Kuiper et al., 1993). These kinases have been shown to phosphorylate various transcription factors and are supposed to be involved in the regulation of transcription factor activity (Hunter & Karin, 1992). No data are available on the number of AR phosphorylation sites, their regulation by hormone, and the kinases involved. In the present study, we have used monoclonal antibodies against the AR to purify receptor protein from total cell lysates of LNCaP cells, after metabolic labeling with [^{32}P]-P_i. The purified AR protein was digested with trypsin, to estimate the number of phosphorylation sites and the effect of hormone incubation on the various sites. The resulting phosphopeptides were resolved by 2D phosphopeptide mapping and by reverse-phase HPLC. It is described herein that the AR contains multiple phosphorylation sites and that a new phosphopeptide is detectable upon hormone treatment, indicating the induction of phosphorylation at a site which

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[1] Abbreviations: TFA, trifluoroacetic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; DTT, dithiothreitol; HPLC, high-performance liquid chromatography; AR, androgen receptor; LNCaP, lymph node carcinoma of the prostate; MEM, minimum essential medium; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone; TLCK, *N*-tosyl-L-lysine chloromethyl ketone; R1881, 17β-hydroxy-17α-methyl-4,9,11-estratrien-3-one; DOC, sodium deoxycholate; PMSF, phenylmethanesulfonyl fluoride; BSA, bovine serum albumin; TCA, trichloroacetic acid.

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is not phosphorylated in AR from cells incubated in the absence of hormone.

MATERIALS AND METHODS

Materials. [^{35}S]Methionine (>1000 Ci/mmol) and [^{32}P]-orthophosphate (carrier free) were obtained from Amersham (Little Chalfont, Bucks, U.K.). Culture media were obtained from Gibco (Life Technologies, Breda, The Netherlands). The synthetic androgen 17β-hydroxy-17α-methyl-4,9,11-estratrien-3-one (R1881) was purchased from NEN−Dupont ('s Hertogenbosch, The Netherlands). The monoclonal antibody against the AR (F39.4.1, epitope amino acid residues 301−320) and the polyclonal antiserum sp061 have been described previously (Van Laar et al., 1989, 1990). Trypsin (TPCK-treated) was from Sigma (St. Louis, MO), and bovine α-chymotrypsin was from Merck (Darmstadt, Germany). Sequencing-grade endoproteinase Glu-C (V8 protease) and TLCK (trypsin inhibitor) were obtained from Boehringer Mannheim (Germany). Thin-layer cellulose plates and all solvents for 2D phosphopeptide mapping were obtained from Merck. HPLC reagents were from Merck, and from J. T. Baker Chemical Co. (Phillipsburg, NY). All other chemicals and reagents were from commercial sources and high purity.

Cell Culture. The LNCaP cell line was cultured as described previously (Van Laar et al., 1991). LnCaP cells between the 66th and 74th passage in vitro were used for the present studies.

Metabolic Labeling. For phosphorylation studies, LNCaP cells were incubated for 12−16 h at 37 °C in phosphate-free MEM (Sigma) with 0.25 mCi/mL [^{32}P]orthophosphate, in the presence or absence of 10 nM R1881. Each 175 cm^2 flask contained about 7×10^7 cells in 10 mL of medium, and was kept in a humidified atmosphere of 5% CO_2 in air. For labeling with [^{35}S]methionine, 7×10^7 LNCaP cells were incubated for 60 min at 37 °C with 20 μCi/mL [^{35}S]-methionine in 5 mL of methionine-free RPMI 1640 medium. Incubations were stopped by removal of the medium, immediately followed by a wash with PBS at 20 °C.

Preparation of Cellular Lysates. Cells were lysed at 6−8 °C with 1.5 mL per flask of lysis buffer [40 mM Tris-HCl (pH 7.4), 5 mM EDTA, 10 mM sodium phosphate, 10 mM DTT, 10% v/v glycerol, 10 mM sodium molybdate, 50 mM NaF, 0.5 mM sodium orthovanadate, 1% Triton X-100, 0.5% w/v DOC, 0.08% SDS, 0.6 mM PMSF, and 0.5 mM bacitracin]. The lysate was cleared by centrifugation (30 min, 100000g) at 6 °C.

Immunoprecipitation and Gel Electrophoresis. Cell lysates were immunoprecipitated with anti-AR F39.4.1 monoclonal antibody linked to agarose beads (Van Laar et al., 1991). Typically, 1.5 mL portions of lysate were incubated with 25 μL (packed gel) of F39−agarose (~1 μL of F39 ascites) for 2 h at 4 °C with gentle shaking. As a control, lysates were immunoprecipitated with anti-PSA (prostate-specific antigen) monoclonal antibody linked to agarose beads. The agarose beads were then washed extensively with detergent and salt-containing buffers, as described previously (Kuiper et al., 1991). Proteins were extracted with sample buffer [40 mM Tris-HCl (pH 6.8), 5% (v/v) glycerol, 2% (w/v) SDS, and 10 mM DTT], boiled for 3 min, and centrifuged. The supernatants were separated on 7% SDS−PAGE gels in a mini Protean II cell (Bio-Rad) and subsequently blotted to nitrocellulose as described previously (Kuiper et al., 1991). The nitrocellulose membrane was dried for 30 min or longer, and incubations with polyclonal AR antiserum sp061 and secondary alkaline phosphatase-conjugated antibodies were done as described (Kuiper et al., 1991). The blot was air-dried and autoradiographed using Amersham Hyperfilm MP.

Generation of Phosphopeptides and Two-Dimensional Separation. Immunopurified receptors were separated on SDS−PAGE gels as described. The gels were washed with water and dried during 12 h. Dried gels were autoradiographed, and the autoradiogram was used as a guide to excise AR protein. Dried gel slices containing ^{32}P-labeled AR (equivalent to one flask; see Metabolic Labeling) were rehydrated in 0.75 mL of 50 mM NH_4HCO_3 (pH 8.0) and incubated for 16 h at 37 °C with 50 μg of TPCK-treated trypsin. Three more additions of 25 μg of trypsin each were made with 6−12 h intervals. The fractions were combined and lyophilized. For two-dimensional separation, tryptic phosphopeptides were separated first on thin-layer cellulose plates, by electrophoresis at 500 V for 45 min at room temperature in 1% w/v ammonium carbonate buffer (pH 9.1) in a chamber as described by Gracy et al. (1977). After drying, chromatography in the second dimension was done for 6−8 h at room temperature in butanol/isobutyric acid/pyridine/glacial acetic acid/water (2:65:5:3:29) (Scheidtmann et al., 1982). Plates were dried and subjected to autoradiography for 3−5 weeks at −70 °C.

HPLC Analysis. Immunopurified AR proteins from five to seven flasks of LNCaP cells (total 10−15 mCi of [^{32}P]-orthophosphate) were separated on SDS−PAGE gels and treated with trypsin as described. After lyophilization, the phosphopeptides were taken up in 30 μL of 0.1% TFA. Peptides were separated by reverse-phase HPLC using a 2 × 150 mm Waters Delta Pak C18 column (Waters Chromatography Division, Millipore Corp., Milford, MA). The HPLC equipment consisted of a Waters 625LC system with a 600E system controller and a Waters 486 UV detector. The sample (25 μL) was applied on the column in 0.1% TFA, and a linear gradient of 0−60% acetonitrile in 0.1% TFA was generated in 240 min. The flow rate was 0.18 mL/min, and fractions were collected every 1.5 min with an ISCO Retriever II fraction collector. Radioactivity of the fractions was determined by Cerenkov counting in a Packard 2500 TR counter (approximately 30% efficiency).

Phosphoamino Acid Analysis. Immunopurified AR protein was eluted from the F39 mAb−agarose beads with 0.75 mL of buffer [50 mM NH_4HCO_3 (pH 8.0)/0.1% SDS/10 mM DTT] for 3 min at 90 °C. After addition of 100 μg of BSA as carrier protein, precipitation occurred with 20% w/v TCA on ice. The precipitate was dissolved in 100 μL of 6 N ultrapure HCl (Pierce Chemical Co., Rockford, IL) and hydrolyzed for 1 h at 110 °C. After repeated lyophilization steps, the sample was resuspended in 10 μL of bidest containing 2 mg/mL phosphoamino acid standards each (Sigma) and spotted on a cellulose thin-layer plate. Two-dimensional electrophoretic separation of the partial hydrolysis products was done at pH 1.9 in formic acid/acetic acid/bidest (50:156:1794), and at pH 3.5 in acetic acid/pyridine/bidest (100:10:1890), for 90 min at 500 V in each dimension (van der Geer et al., 1993). Plates were autoradiographed for 1−2 weeks at −70 °C.

Stoichiometry of Phosphorylation. These experiments were performed as described for pp60$^{v\text{-}src}$ and phospholipase C-γ (Meisenhelder et al., 1989; Sefton et al., 1982; Sefton, 1991). In brief, (2−3) × 10^7 LNCaP cells were incubated for 24 h in 11 mL of a mixture of methionine-free and phosphate-free MEM/phosphate-free MEM (80:20), supplemented with $NaH_2PO_4 \cdot H_2O$ to a final concentration of 0.95 mM. To this medium was added either approximately 100 μCi of [^{35}S]methionine or 4 mCi of [^{32}P]orthophosphate. The specific activity of the phosphate in the medium was calculated from the measured amount of ^{32}P in the medium at the end of the incubation and the known amount of phosphate. Cells were lysed at 6−8 °C, and the AR protein was immunoprecipitated as described and separated on a 7% SDS−PAGE gel. Following autoradiography, either the ^{35}S- or the ^{32}P-labeled AR bands were cut out from the dried gel and solubilized with Soluene (Packard) at 60 °C for 4 h before being counted in scintillation fluid. Total cellular protein was measured in a sample of the lysate by the Bradford dye method, using BSA as standard (Rubin & Warren, 1977). The radioactivity (^{35}S) of the cellular protein was measured in a fraction of the lysate, by TCA precipitation of protein onto a glass fiber filter which was counted in parallel with the ^{35}S-labeled gel bands.

RESULTS

Androgen Receptor Protein Heterogeneity and Phosphorylation. Pulse-labeling studies with [^{35}S]methionine showed that the AR is synthesized as a 110 kDa protein which is rapidly converted to a 112 kDa protein. Alkaline phosphatase treatment of cytosols either from LNCaP cells or from COS cells transiently expressing AR protein caused an elimination of the 112 kDa isoform with a concomitant increase of the 110 kDa isoform (Kuiper et al., 1991; G. Jenster et al., submitted for publication). These experiments indicated that the 112 kDa AR isoform is phosphorylated, but did not provide information on the phosphorylation status of the 110 kDa AR isoform. Therefore, LNCaP cells were metabolically labeled with [^{35}S]methionine for 1 h, and the AR protein was immunoextracted and separated on SDS−PAGE gels. The autoradiogram as well as the immunoblot showed an AR doublet of 110−112 kDa molecular mass, indicating that de novo synthesized as well as preexisting AR proteins exist as two isoforms in LNCaP cells (Figure 1, lanes 2 and 4). In a similar experiment, LNCaP cells were metabolically labeled with [^{32}P]orthophosphate for 16 h. The half-life of the AR protein in LNCaP cells is between 2 and 3 h, allowing the turnover of essentially all AR molecules during 16 h incubation (Van Laar et al., 1991). The autoradiogram (Figure 1, lane 6) showed only one radioactively labeled band, comigrating with the 112 kDa AR isoform. Also after incubation of cells with 10 nM of the synthetic androgen R1881, only the 112 kDa band was radioactively labeled (Figure 1, lane 7). From this experiment, we concluded that the 112 kDa AR isoform represents phosphorylated AR and that the 110 kDa AR isoform represents nonphosphorylated AR protein. From the immunoblots (Figure 1, lanes 1 and 2), it is not possible to calculate the ratio between both AR isoforms.

In an attempt to determine the extent to which AR protein is phosphorylated in LNCaP cells, we labeled cells for 24 h with either [^{35}S]methionine or [^{32}P]orthophosphate, and isolated AR protein by immunoprecipitation and SDS−PAGE. The number of moles of phosphate incorporated per mole of AR protein was calculated, with the assumptions that (1) after 24 h the specific activity of the phosphoamino acids in AR protein is the same as that of the phosphate in the labeling medium and that (2) the specific activity of the methionine in AR protein is the same as that in bulk cellular protein (see Materials and Methods). It was calculated that the AR protein contains 1.0 mol of phosphate per mole of protein (n = 3) when the LNCaP cells were not incubated with R1881 (see Table 1). Since only an aggregate value was measured, we cannot calculate exactly which fraction of the total population of AR molecules is phosphorylated. Since also after R1881 incubation of LNCaP cells the AR protein is detected as a 110−112 kDa doublet on immunoblots (Kuiper et al., 1991), we conclude that a significant fraction of AR molecules remains nonphosphorylated (110 kDa isoform) in the presence of hormone.

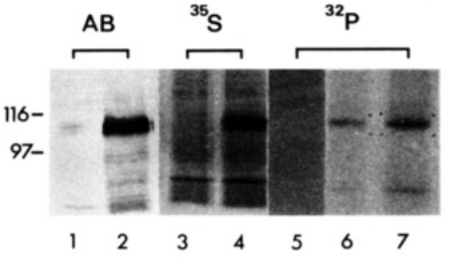

FIGURE 1: AR protein heterogeneity and phosphorylation. LNCaP cells were incubated for 16 h with [^{32}P]orthophosphate (lanes 5, 6, and 7), in the presence (lane 7) or absence (lanes 5 and 6) of 10 nM R1881. Another set of cells was incubated with [^{35}S]methionine for 1 h (lanes 1−4). Receptors were immunoprecipitated from total cell lysates with the F39 monoclonal antibody (lanes 2, 4, 6, and 7) or nonspecific mouse IgG (lanes 1, 3, and 5). After SDS−PAGE on a 7% gel, the proteins were transferred to a nitrocellulose membrane and incubated with polyclonal AR antiserum sp061 and secondary alkaline phosphatase-conjugated antibodies (AB), as described (lanes 1 and 2). The blots prepared from the LNCaP cells incubated with [^{35}S]methionine (lanes 3 and 4) or [^{32}P]-orthophosphate (lanes 5−7) were autoradiographed. Molecular mass markers (kDa) are indicated on the left.

Table 1: Stoichiometry[d] of AR Phosphorylation in LNCaP Cells

	flask				
	1	2	3	4	$X \pm$ SD[e]
^{32}P (cpm)	1180	1058	894		
^{32}P SA[a] (cpm/pmol)	660	659	614		
P in AR (pmol)	1.78	1.61	1.46		1.62 ± 0.16 (n = 3)
^{35}S (dpm)	323	481	666	431	
^{35}S SA[b] (dpm/μg)	2790	3022	2982	3216	
AR[c] (pmol)	1.18	1.62	2.28	1.37	1.61 ± 0.47 (n = 4)

[a] Specific activity per picomole of phosphate. [b] Specific activity per microgram of protein. [c] For the conversion of micrograms of AR protein to picomoles of AR protein, it was assumed that 10 pmol of AR protein equals 0.98 μg of AR protein. [d] Stoichiometry = (P in AR)/(AR) = (1.62 pmol)/(1.61 pmol) = 1.0 mol of P/mol of AR protein. [e] Mean ± standard deviation.

Phosphoamino Acid Analysis of AR Protein. In order to obtain some information on the type of kinases which phosphorylate human AR protein, we analyzed the phosphoamino acids of AR protein isolated from LNCaP cells incubated in the presence or absence of R1881. Conditions of acid hydrolysis (1 h) were optimal for the preservation of phosphoamino acids, especially phosphotyrosine (Duclos et al., 1991). After two-dimensional electrophoresis of the acid hydrolysate of immunopurified AR protein from cells incubated with 10 nM R1881, only phosphorylation on serine

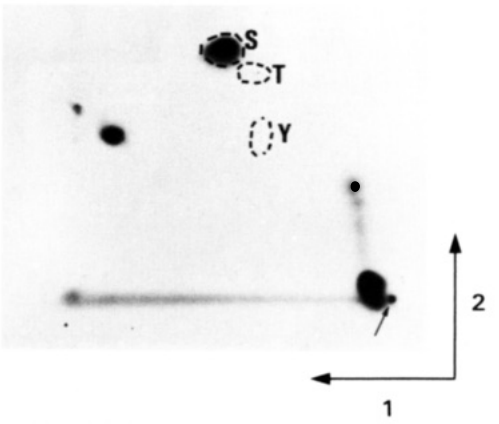

FIGURE 2: Phosphoamino acid analysis of AR protein. LNCaP cells were incubated for 16 h with [^{32}P]orthophosphate, and subsequently AR protein was immunopurified as described under Materials and Methods. The ^{32}P-labeled AR protein was subjected to partial acid hydrolysis (6 N HCl, 1 h at 110 °C). Partial hydrolysis products were mixed with unlabeled phosphoamino acid standards and resolved on thin-layer cellulose plates by electrophoresis at pH 1.9 (first dimension, 1) prior to electrophoresis at pH 3.5 (second dimension, 2). Unlabeled phosphoamino acids were visualized by ninhydrin staining and ^{32}P-labeled phosphoamino acids by autoradiography. The position of the phosphoserine (S), phosphothreonine (T), and phosphotyrosine (Y) standards is indicated. The origin is indicated by a small arrow.

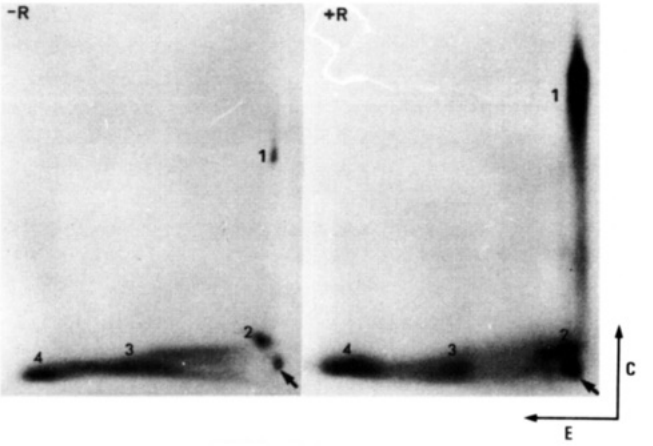

FIGURE 3: Two-dimensional phosphopeptide maps of trypsin-digested AR protein. AR protein was labeled in LNCaP cells with [^{32}P]orthophosphate during an incubation for 12 h, in the presence or absence of 10 nM R1881, and subsequently immunoprecipitated from total cell lysates. The proteins were resolved on a 7% SDS−PAGE gel (see Figure 1, lanes 6 and 7), and phosphoproteins were detected by autoradiography of the dried gel. The labeled AR protein was excised from the gel and digested with trypsin. Two-dimensional maps of the phosphopeptides were prepared on thin-layer cellulose plates through electrophoresis (E, anode on the left) and chromatography (C), as described under Materials and Methods. Shown are the autoradiograms (3 weeks exposure at −70 °C) of AR protein from cells incubated in the presence of 10 nM R1881 (+R) or without R1881 (−R). The origins are indicated by arrows.

residues was observed (see Figure 2). During one-dimensional electrophoresis, there was, apart from ^{32}P activity comigrating with the phosphoserine standard, also ^{32}P activity wholly or partially overlapping with the phosphotyrosine and phosphothreonine standards (result not shown). However, during 2D electrophoresis, this ^{32}P activity separated from the phosphotyrosine and phosphothreonine standards (see Figure 2), and probably results from nucleoside monophosphates, arising from RNA degradation during acid hydrolysis (Duclos et al., 1991). Also when AR protein was isolated from cells incubated without R1881, or after elution of intact AR protein from SDS−PAGE gels, only phosphorylation on serine residues was detected (not shown).

Peptide Analysis of the ^{32}P-Labeled Androgen Receptor. In previous studies, it was shown that the phosphorylation degree of the AR protein in LNCaP cells increases 2-fold upon incubation of cells with R1881 and that predominantly the N-terminal domain is phosphorylated (Van Laar et al., 1991; Kuiper et al., 1993). In order to obtain information on the number of AR phosphorylation sites and the effect of hormone treatment on individual sites, two-dimensional phosphopeptide maps were prepared. Immunopurified and SDS−PAGE gel-purified ^{32}P-labeled AR was digested with TPCK-treated trypsin (see Materials and Methods), and the phosphopeptides were separated on thin-layer cellulose plates by electrophoresis in the first dimension and by chromatography in the second dimension. In initial studies, various buffers for electrophoresis and chromatography were used, and the maps obtained were analyzed. When electrophoresis was performed at pH 3.5 or pH 1.9, all the phosphopeptides remained at or near the application site (results not shown). A possible explanation for this lack of mobility could be the fact that the N-terminal domain of the human AR is very hydrophilic and contains a large number of acidic amino acid residues, especially between amino acid residues 100 and 325 (Faber et al., 1989). When the pH of the electrophoresis buffer was raised to 9.1, most phosphopeptides moved toward the anode, indicating the relative enrichment of acidic amino acid residues in these peptides (see Figure 3). During chromatography in "isobutyric acid buffer", designed to resolve hydrophilic phosphopeptides (Scheidtmann et al., 1982), only one phosphopeptide moved in the second dimension, showing that most AR phosphopeptides are extremely hydrophilic (see Figure 3). In total, four phosphopeptides were identified, although it should be emphasized that the resolution of the hydrophilic peptides which do not move in the second dimension during chromatography is not optimal. In Figure 3, a comparison is shown between tryptic phosphopeptide maps of ^{32}P-labeled AR protein isolated from cells incubated either in the presence or in the absence of 10 nM R1881 (see also Figure 1, lanes 6 and 7). The location and number of phosphopeptides were the same in each case. A significant increase in the phosphorylation degree of the peptides which are moving in the second dimension during chromatography, however, was observed after hormone treatment of the cells. No significant changes in the phosphorylation degree of the other peptides were detected. The hormone-induced phosphopeptide spot is very broad, possibly due to phosphorylation of the same peptide at different positions, which affects its hydrophobicity to different degrees due to the context of the phosphorylation site (Boyle et al., 1991).

Although the two-dimensional thin-layer separation method was sensitive to quantitative changes in the phosphorylation degree of AR phosphopeptides, the resolution of most of the hydrophilic peptides was not optimal. Therefore, separation of tryptic phosphopeptides by reverse-phase HPLC was performed. The peptides were generated by tryptic digestion of AR protein as described (Materials and Methods) and applied to a C18 column in 0.1% trifluoroacetic acid. A total of 15 separate ^{32}P-labeled peaks above background (20 ± 2 cpm) were detected (see Figure 4). This was clearly

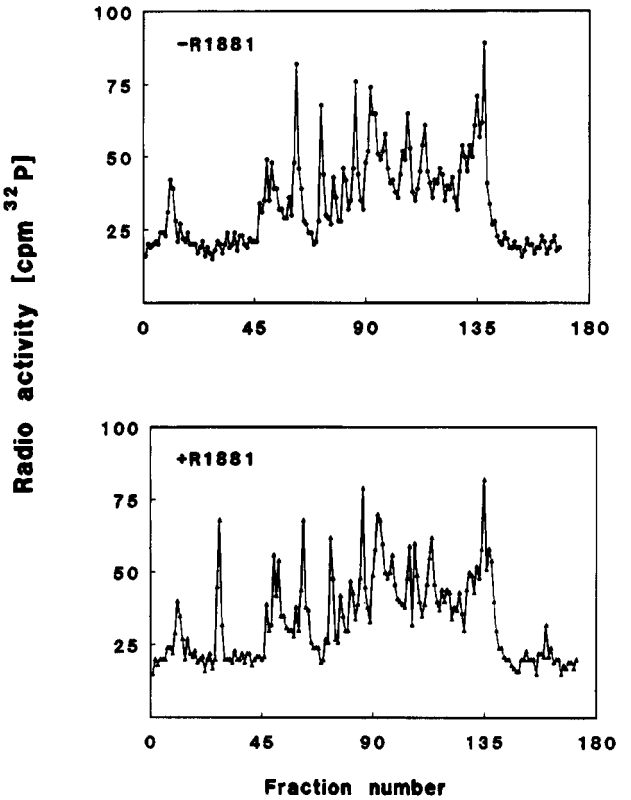

FIGURE 4: HPLC elution profiles of tryptic phosphopeptides of AR protein from LNCaP cells. AR protein was labeled in LNCaP cells with [^{32}P]orthophosphate during incubation for 12 h in the presence (▲; lower graph) or absence (●; upper graph) of 10 nM R1881, and immunoprecipitated from total cell lysates. Following electrophoresis, the labeled AR protein was excised and digested with trypsin. The peptides were acidified and applied to a C18 column. The column was developed with a linear 0−60% acetonitrile gradient in 0.1% TFA, in 240 min. The flow rate was 0.18 mL/min, and fractions were collected every 1.5 min. Fractions were analyzed by Cerenkov counting.

more than the number of phosphopeptides detected by thin-layer separation (see Figure 3). Incubation of LNCaP cells with R1881 induced phosphorylation of a site in a peptide eluting in fractions 27−28 (retention time 40 min). Only minimal differences in the phosphorylation degree and elution positions of the other peptides were observed (Figure 4), indicating that the hormone-stimulated AR protein hyperphosphorylation is to a large extent due to phosphorylation of a new site.

DISCUSSION

Steroid hormone receptors, including the AR, are heterogeneous proteins that exist as multiple isoforms, migrating as doublets or triplets on SDS−PAGE gels. Upon ligand binding, the isoforms of progesterone, androgen, estradiol, and vitamin D receptors undergo an increase in apparant molecular mass, concomitantly with additional phosphorylation (Jurutka et al., 1993; Brown & DeLuca, 1990; Le Goff et al., 1994; Washburn et al., 1991; Beck et al., 1992; G. Jenster, personal communication). The reason for these mobility shifts is not clear, since the actual increase in molecular mass of the protein by phosphorylation is very small. A discrepancy exists between the theoretical calculated molecular mass of the AR protein (98 kDa) and the apparent molecular mass on SDS−PAGE gels (110−112 kDa). In the present study, it was shown that the nonphosphorylated AR protein has an apparant molecular mass of 110 kDa, indicating that this discrepancy is not due to phosphorylation. In another study, this discrepancy has been ascribed to some unknown features of the region between amino acid residues 51 and 211 of the human AR protein (Jenster et al., 1991). For the mouse glucocorticoid receptor, a similar discrepancy in molecular mass has been ascribed to the region between amino acid residues 108−324 (Hutchison et al., 1993). The two regions have in common a relatively high content of acidic amino acid residues, which might influence in both cases the association of SDS and thereby the mobility during SDS−PAGE. An alternative posttranslational modification, such as glycosylation, causing anomalous behavior on SDS−PAGE gels, cannot be excluded.

In the present study, it was calculated that the AR protein contains 1 mol of phosphate/mol of protein. The stoichiometry measurements may be an underestimation, if the specific activity of the phosphoamino acids in the AR protein is not equal to that of the phosphate in the labeling medium or if cellular phosphatases are active during cell lysis. It was attempted to minimize these factors by labeling the cells for 24 h and by adding phosphatase inhibitors to the lysis buffer. Phosphorylation after cell lysis was blocked by the addition of EDTA and nonradioactive phosphate to the lysis buffer. Given the rapid turnover of phosphate groups on proteins, this renders the labeling of the phosphoryl moieties in phosphoproteins to equilibrium much easier than the labeling of the polypeptide backbone. The average half-life of cellular proteins (40 h) is long relative to the length of time that many kinds of cells can tolerate radiolytic damage (Weber, 1972; Sefton, 1991). On the other hand, the specific activity ([^{35}S]methionine labeling) of a protein with a short half-life would be greater than that of bulk cellular protein if the labeling period was too short. The AR half-life is between 2 and 3 h (van Laar et al., 1991; Syms et al., 1985), so that after a labeling period of 24 h all AR protein molecules have been renewed.

In the present investigation, it is shown that the AR protein is exclusively phosphorylated on serine residues. Nuclear, but not cytosolic, AR protein from rat ventral prostate was shown previously to react with anti-phosphotyrosine antibodies, indicating that these receptors or associated protein(s) are possibly phosphorylated on tyrosine residues (Golsteyn et al., 1989; 1990). However, it should be noted that no intact 110 kDa AR protein was identified in these studies. Phosphoamino acid analysis has revealed that the glucocorticoid, progesterone, and 1,25-dihydroxyvitamin D_3 receptors are phosphorylated on serine residues (Dalman et al., 1988; Sheridan et al., 1988; Brown & DeLuca, 1991). Results on phosphoamino acid analysis of estrogen receptors are not conclusive. While Auricchio (1989) showed that estrogen receptors are only phosphorylated on tyrosine residues, other investigators reported phosphorylation only on serine residues (Washburn et al., 1991; Denton et al., 1992; LeGoff et al., 1994; Lahooti et al., 1994). Separation of tryptic phosphopeptides from human AR protein revealed that the AR is phosphorylated at multiple sites. When a comparison was made between cells incubated with R1881 and cells incubated without R1881, it was found that one additional phosphopeptide appeared after hormone treatment. No further significant changes (quantitatively and/or qualitatively) were detected in the two separation systems which were used.

It is difficult to give an accurate estimation on the number of AR protein phosphorylation sites. The N-terminal region shown to be heavily phosphorylated in the AR protein contains 33 serine residues and 12 consensus phosphorylation sites for casein kinases, Ser-Pro-directed kinases, and DNA-dependent kinase. Partial trypsin digestion products can be generated at adjacent arginine and/or lysine residues and also at arginine/lysine−aspartic acid/glutamic acid bonds (Boyle et al., 1991). Between amino acid residues 1 and 300 of the AR protein, six of these combinations known to cause partial digests are present, so that it is possible that a single phosphorylation site gives rise to several different phosphopeptides. Nevertheless, from the present results, it can be inferred that the number of AR phosphorylation sites is in the same order as those reported for the glucocorticoid and progesterone receptors. In the mouse glucocorticoid receptor, seven phosphorylation sites were identified, and in the chicken progesterone receptor, four sites were found (Poletti & Weigel, 1993; Bodwell et al., 1991; Poletti et al., 1993). Digestion of AR protein with α-chymotrypsin produced seven phosphopeptides after HPLC (result not shown). Digestions with V8 protease were not conclusive. The appearance of new phosphopeptides after hormone treatment was also shown by phosphopeptide mapping for the human and chicken progesterone receptor, the calf uterus estrogen receptor, and the rat glucocorticoid receptor (Denner et al., 1990; Sheridan et al., 1989; Nakao et al., 1992; Denton et al., 1992; DeFranco et al., 1991). In contrast, for the human estrogen receptor and the avian progesterone receptor, it was found that hormone-induced phosphorylation was not due to phosphorylation at a new site, but rather increased phosphorylation of existing sites (Sullivan et al., 1988; LeGoff et al., 1994).

Efforts will now be focused on the identification of the hormone-stimulated AR phosphorylation site(s), in order to be able to identify their significance in nuclear cytoplasmic AR shuttling, DNA binding of the AR protein, and transcriptional activation by the AR. Since the amount of AR protein which can be purified from LNCaP cells is rather limited, this will involve the use of an AR protein overexpression system in yeast cells or insect cells.

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