Forskolin-Induced Dephosphorylation of the Androgen Receptor Impairs Ligand Binding

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ABSTRACT: When androgen receptor containing cells are cultured in the presence of the PKA stimulator forskolin, a rapid dephosphorylation of the androgen receptor occurs resulting in a decrease in the amount of 112 kDa androgen receptor isoform and an increase in 110 kDa androgen receptor isoform on SDS−PAGE. To establish which amino acid residues in the androgen receptor were phosphorylated in control and forskolin-treated cells, trypsin-digested androgen receptors were subjected to RP-HPLC analysis and subsequently to Edman degradation. It was observed that serine residues 506, 641, and 653 were potentially phosphorylated in control cells, while after forskolin treatment strong evidence was obtained that phosphorylation of serines 641 and 653 was significantly reduced. When the dephosphorylated androgen receptor was analyzed for its transcription activation capacity, it was observed that androgen-induced transcriptional regulation of two endogenous genes (PSA and β1-subunit of Na,K-ATPase), in cells cultured in the presence of forskolin, was inhibited as compared to the control situation. The observation that the dephosphorylated androgen receptor was transcriptionally less active was further strengthened by the finding that the dephosphorylated androgen receptor was markedly impaired in ligand binding (B_{max} was found to be reduced by approximately 40%). The current investigations show for the first time a clear function for the rapid phosphorylation which occurs directly after synthesis of the androgen receptor, namely, effective ligand binding.

Transcription factors are often phosphorylated as a mechanism to regulate their functional activities. For example, phosphorylation of the IκB regulatory subunit triggers nuclear import of NF-κB; phosphorylation of threonine 231 and/or serine 249 of c-Jun inhibits DNA binding, and phosphorylation in the C-terminal region of c-Fos is required for transrepression (see review *1*). Steroid receptors are also phosphorylated, and in addition to the already mentioned functions for phosphorylation, regulation of ligand binding and receptor dimerization can also be affected by phosphorylation.

For the estrogen receptor, protein kinase A (PKA) and protein kinase C (PKC) induced phosphorylation is important for ligand-independent transcriptional activation (*2*−*5*). Furthermore, for the human estrogen receptor, it was observed that src-kinase-induced phosphorylation of tyrosine 537 was involved in ligand binding, receptor dimerization, and DNA binding (*6*−*9*).

Ligand-induced glucocorticoid receptor phosphorylation occurs in a cell cycle dependent manner. In the S-phase, when receptor activity is high, phosphorylation is also at its peak (*10*, *11*). These experiments are an indication that glucocorticoid resistance during the cell cycle may be regulated by receptor phosphorylation (*12*, *13*). Mutation analysis of the mouse glucocorticoid receptor revealed that transcriptional activation by mutated glucocorticoid receptors of a minimal GRE_2TATA driven CAT reporter was reduced by 75% (*14*).

For the human progesterone receptor B, cell cycle dependent phosphorylation is also suggested (*15*). Furthermore, the chicken progesterone receptor can ligand-independently be activated by 8-bromo-cAMP, okadaic acid, vanadate, EGF, and dopamine (*16*−*19*). Mutation analysis revealed that in the chicken progesterone receptor serine residue 530 affects ligand binding (*20*, *21*).

Phosphorylation of the androgen receptor was first reported by van Laar et al. (*22*, *23*). A 1.8-fold increase in phosphorylation of the receptor was observed after 30 min of incubation of LNCaP[1] cells in the presence of 10 nM of the synthetic androgen R1881. Phosphorylation of the androgen receptor also displays itself in the formation of different androgen receptor isoforms. The androgen receptor is synthesized as a nonphosphorylated protein and migrates as a 110 kDa protein during SDS−PAGE. Within 15 min after synthesis, this nonphosphorylated androgen receptor becomes phosphorylated and runs as a 110−112 kDa doublet

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[1] Abbreviations: SDS-PAGE, sodium dodecyl sulfate−polyacryl amide gel electrophoresis; PSA, prostate-specific antigen; LNCaP, lymph node carcinoma derived from a human prostate; ATCC, American type culture collection; PBS, phosphate-buffered saline; TFA, trifluoroacetic acid; SD, standard deviation, RP-HPLC, reverse-phase high-performance liquid chromatography.

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during SDS−PAGE (*24*). Upon addition of androgens to the culture medium, a third (114 kDa) androgen receptor isoform appears (*25*). The formation of this third isoform will usually take more than 12 h (*26*). Amino acid residues which are known to be involved in androgen receptor phosphorylation are the serine residues located at positions 80, 93, and 641[2] (*25*, *27*). Using mutation analysis, it was established that the introduction of other amino acids in these positions affected the phosphorylation of the receptor molecule (*25*−*27*). However, when the function of these mutated receptor molecules was evaluated, only in the case of substitution of serine 641 by alanine, a 30% reduction in the transcription capacity of the androgen receptor was observed (*27*).

Phosphorylation of the androgen receptor can also be influenced by the PKA pathway. Blok et al. (*26*) showed androgen receptor dephosphorylation in transfected COS-1 cells by the PKA activator forskolin. Nazareth and Weigel (*29*) reported on ligand-independent activation of the human androgen receptor by forskolin. Ligand-independent transcriptional activation of the androgen receptor may be relevant in view of the inescapable development of androgen-independent prostate cancer during androgen-ablation therapy. Because of the possible implications for prostate cancer therapy, PKA effects on phosphorylation and transcriptional capacity of the androgen receptor were further investigated in the human prostate cancer cell line LNCaP.

MATERIALS AND METHODS

Cells. LNCaP cells (derived from a metastatic lesion of a human prostate by Horoszewicz et al., *30*) were obtained from ATCC and were used between passages 28 and 34. The cells were cultured in RPMI-1640 supplemented with 7.5% fetal calf serum (FCS, Gibco-BRL/Life technologies, Breda, The Netherlands) in the presence of antibiotics. For the experiments, cells were passaged into RPMI-1640 supplemented with 5% stripped serum [dextran-coated charcoal-treated fetal calf serum (dcc-FCS)], were allowed to attach to the plastic, and were cultured for the indicated times with or without hormones. Forskolin (Sigma, St. Louis, MO) was added to a final concentration of 20 μM. R1881 (NEN, Boston, MA) was added in the indicated amounts.

Phosphorylation of the AR. After culture of LNCaP cells in RPMI-1640 + 7.5% FCS, the cells were washed twice at room temperature with saline. Subsequently, the cells were cultured in RPMI-1640 medium without phosphate (Sigma), supplemented with 5% dccFCS which had been dialyzed against saline. During the last 16 h ^{32}P was added (final concentration 300 μCi/mL; Amersham International plc, Buckinghamshire, England) in combination with or without forskolin. After incubation, cells were washed twice at room temperature with PBS, and lysed in ice-cold immunoprecipitation buffer A [40 mM Tris-HCl (pH 7.4), 5 mM EDTA, 10% glycerol, 10 mM sodium phosphate, 10 mM sodium molybdate, 50 mM sodium fluoride, 0.5 mM sodium orthovanadate, 10 mM dithiothreitol, 0.6 mM phenylmethanesulfonyl fluoride, 0.5 mM bacitracin] supplemented with 1% Triton X-100, 0.5% deoxycholic acid, and 0.08% sodium dodecyl sulfate (SDS) at 4 °C. After 5 min, the lysate was centrifuged at 100000*g* for 30 min at 4 °C. The supernatant was then incubated with a specific antibody (F39.4.1) raised against amino acid residues 301−320 of the human androgen receptor (*31*). This antibody had been linked to goat anti-mouse agarose (Sigma). After 2 h of incubation at 4 °C, the lysates were washed 3 times in immunoprecipitation buffer A supplemented with 1% Triton X-100, 0.5% deoxycholic acid and 0.08% SDS, 3 times in buffer A supplemented with 0.2% Triton X-100 and 0.4 M NaCl, and 3 times in buffer A without additions. During these washes, the total amount of radioactivity was reduced by approximately a 100 000-fold. The immunoprecipitated androgen receptor was separated on SDS−PAGE and blotted to a nitrocellulose filter either for X-ray film exposure or for immunodetection, or was dissected from the gel for trypsin digestion and subsequent RP-HPLC analysis.

Western Blotting of the Androgen Receptor. The androgen receptor containing immunoblot was incubated for 1 h in the presence of a polyclonal antiserum (SP061) raised against the same amino acid residues as the monoclonal antibody (F39.4.1). After extensive washing [4 × 15 min with PBS−Tween (0.05%)], the blot was incubated with a goat anti-rabbit alkaline phosphatase conjugated antibody (1 h, at room temperature). Again the blot was washed (4 × 15 min with PBS−Tween). Fresh alkaline phosphatase substrate was added in a 0.2 M Tris-HCl (pH 9.1), 10 mM $MgCl_2$ buffer, and allowed to develop in the dark for several minutes. Subsequently the blot was washed in H_2O and dried to air.

RP-HPLC Analysis. RP-HPLC analysis was performed essentially as described by Kuiper et al. (*32*). Gel slices containing phosphorylated androgen receptor were incubated for 16 h in a 50 mM NH_4HCO_3 (pH 8.0) buffer containing 50 μg of TPCK-treated trypsin (Sigma) at 37 °C. During the next 24 h, three more additions of 25 μg of trypsin each were made. The gel slices were taken from the buffer, and the trypsin-digested androgen receptor fragments (phospho-peptides) were freeze-dried. The phospho-peptides were dissolved in 0.1% TFA. HPLC analyses started by applying 25 μL of this solution to a 2 × 150 mm Waters delta pack C18 column (Waters Chromatography Division, Millipore Corp., Milford, MA). A linear gradient of 0−70% 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. Radioactivity in the collected fractions was determined by Cerenkov counting in a Packard 2500 TR counter.

Edman Degradation of Peptide Fractions 7, 33/34, and 56/57. To obtain enough radioactively labeled peptide for Edman degradation, the androgen receptor was transiently expressed in COS-1 cells and subjected to the same procedures for labeling and purification as in LNCaP cells (*26*). Androgen receptor peptides (RP-HPLC fractions 7, 34, and 54) were freeze-dried and redissolved in 30 μL of 50% acetonitrile. Subsequently the fractions were spotted on an arylamine−sequelon disk. The spots were allowed to dry for 5 min at 50 °C. The peptides were covalently linked to the disk by incubation in 100 μL of 0.1 M 2-morpholinoethanesulfonic acid monohydrate (pH 5.0) + 1 mg of 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide.

[2] Throughout the text, the amino acid numbering is based on a 910 amino acid-androgen receptor, containing 21 glutamine and 16 glycine residues (*28*).

After 30 min of incubation at room temperature, the disks were extensively washed with water and then extracted 5 times with TFA to remove unbound peptide and 3 times with methanol before Edman degradation. Edman degradation was performed essentially as described by Sullivan and Wong (*33*).

RNA Isolation and Hybridization. Total RNA was isolated as described by Blok et al. (*34*). In short, cells were lysed in a solution containing 3 M lithium chloride and 6 M urea (*35*). DNA was sheared using an Ultra-Turrax homogenizer. RNA was precipitated by ultracentrifugation. Total RNA (20 μg/lane) was electrophoresed on a denaturing gel for 100 min at 100 V (*36*). RNAs were blotted onto a Hybond N membrane (Amersham). PSA (*34*) and the β1-subunit of Na,K-ATPase (*37*) were detected as androgen-regulated genes using differential display PCR. Probes were isolated using cDNA amplification by PCR. Hybridization was allowed to continue at 42 °C for 48 h in a buffer containing 45% formamide, 5 × SSC, 0.5% SDS, 10% Denhardt's solution, 10 mM phosphate buffer, 15% dextran sulfate, and 100 mg/L herring sperm DNA (*38*, *39*). The blots were washed for 2 × 15 min at 1 × SSC/0.25% SDS and exposed to phosphorimager analysis or X-ray film.

Scatchard Analysis. LNCaP cells were cultured to 50% confluency in 6 well plates, in RPMI-1640 + 7.5% FCS. Subsequently, the cells were cultured for 24 h in RPMI-1640 + 5% dccFCS before forskolin was added. Culture in the presence or absence of forskolin proceeded for another 24 h before the incubations with ^{3}H-R1881 in the presence or absence of 200-fold cold R1881 were started. ^{3}H-R1881 (1.25 nM, 0.63 nM, 0.45 nM, 0.32 nM, 0.16 nM, 0.08 nM) was added to the culture for 2 h before extensive washing with PBS at 4 °C. Cells were lysed in 0.5 M NaOH for 30 min at 56 °C. Protein concentration was measured using Bradford reagents, and 50 μL samples were counted using liquid scintillation. Scatchard plots were constructed and K_d and B_{max} values calculated.

RESULTS

To influence phosphorylation of the androgen receptor in LNCaP cells, these cells were cultured for 4 h in the presence of forskolin. In nontreated cells (control), the upper 112 kDa androgen receptor isoform is phosphorylated, while the lower 110 kDa isoform represents the nonphosphorylated androgen receptor (*26*). Upon treatment of the cells with forskolin, the intensity of the 112 kDa band was reduced while the intensity of the lower 110 kDa band increased. This change in distribution of the androgen receptor over its different isoforms coincided with an overall decrease in androgen receptor phosphorylation by approximately 50% as measured by phosphorimage analysis of incorporated ^{32}P-orthophosphate. The total amount of androgen receptor protein observed by immunoanalysis remained unchanged (Figure 1).

To follow more precisely which changes were taking place in androgen receptor molecules produced in the presence of forskolin, the androgen receptor preparations from control and forskolin-treated cells were subjected to RP-HPLC analysis. Before separation, the immunoprecipitated receptor was digested using trypsin. It was observed that in control

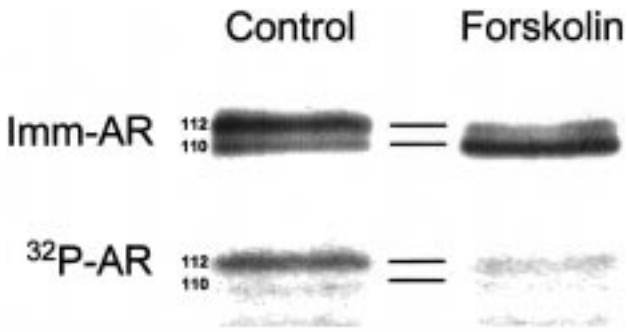

FIGURE 1: Forskolin-induced dephosphorylation of the androgen receptor. LNCaP cells were cultured for 16 h in the presence of radioactive orthophosphate and during the last 4 h also in the presence (Forskolin) or absence (Control) of forskolin. Cells were harvested, and the androgen receptor was immunopurified before separation on SDS−PAGE. Western blotting was performed as outlined under Materials and Methods. Imm-AR = immunostained androgen receptor; ^{32}P-AR = autoradiogram of the immunostained androgen receptor. 110 and 112 indicate the 110 and 112 kDa androgen receptor isoforms, respectively.

cells three major phosphorylated peptides were present: fractions 7, 33/34, and 56/57, respectively (Figure 2A). After incubation in the presence of forskolin, however, the radioactivity in fractions 33/34 and 56/57 was substantially reduced (Figure 2B).

Edman degradation of phosphopeptides isolated after RP-HPLC analysis of trypsin digested androgen receptor from transiently transfected COS-1 cells (fractions 7, 33, and 54) was performed to determine which amino acid residues could potentially be dephosphorylated by forskolin in the androgen receptor. Edman degradation of fraction 7 resulted in release of the majority of radioactivity in cycle 5 (Figure 3A), Edman degradation of fraction 34 (LNCaP fractions 33/34) resulted in loss of almost all radioactivity in cycle 4 (Figure 3B), and Edman degradation of fraction 54 (LNCaP fractions 56/57) resulted in the loss of virtually all radioactivity in cycle 12 (Figure 3C).

From previous studies, it is known that the phosphorylated amino acid residues in the androgen receptor are serines (*32*). Furthermore, it is possible to predict which peptides are generated after trypsin digestion. Taking this information into account, all potential phosphopeptides (produced after trypsin digestion) were analyzed for serine residues on positions 5, 4, and 12, respectively. Five peptides were found with a serine on position 4; 4 peptides with a serine on position 5; and 3 peptides with a serine on position 12 (Table 1). As indicated in Table 1, only three of these serines were found to be part of a putative phosphorylation consensus site: serine 506 (position 5) can potentially be phosphorylated by a mitogen-activated protein kinase (MAPK) and a proline-directed kinase and serine 641 (position 4) by a proline-directed kinase, and serine 653 (position 12) is part of a casein kinase II site.

To investigate whether the forskolin-induced dephosphorylation of the androgen receptor results in a change in functional activity, endogenous androgen-regulated gene expression was investigated. Androgen regulation of PSA mRNA expression has been extensively documented in the literature (*40*−*42*); however, androgen regulation of the β1-subunit of Na,K-ATPase is a novel finding (*37*). The expression level of mRNA from the β1-subunit of Na,K-

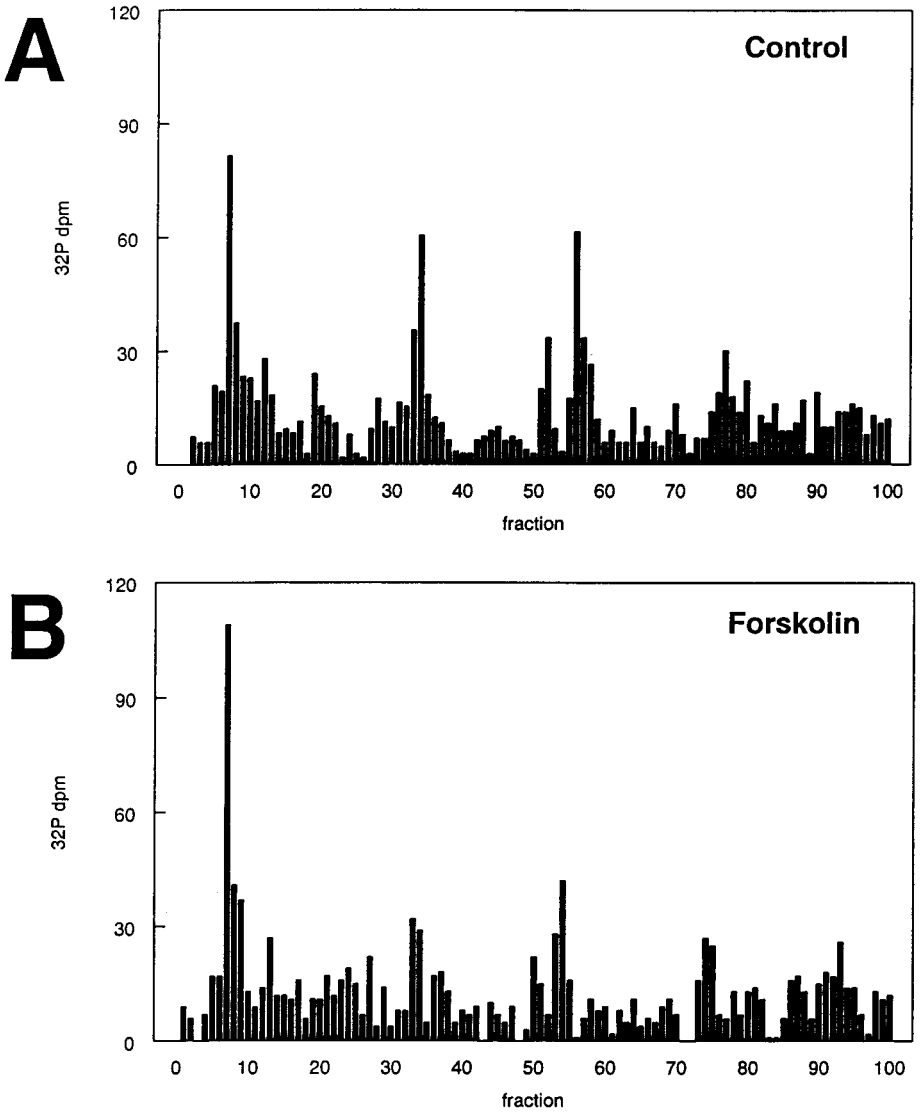

FIGURE 2: HPLC analysis of trypsin-digested androgen receptor preparations. LNCaP cells were cultured for 16 h in the presence of radioactive orthophosphate and during the last 4 h also in the presence (Forskolin, B) or absence (Control, A) of forskolin. Cells were harvested, and the androgen receptor was immunopurified before separation on SDS−PAGE. Western blotting was performed as outlined under Materials and Methods. The androgen receptor protein band was dissected from the gel and digested using excess trypsin. The tryptic fragments were separated by RP-HPLC on a C18 column as indicated under Materials and Methods. Radioactivity in the collected fractions was determined by Cerenkov counting and depicted on the *y*-axis.

ATPase is normally decreased upon culture of the cells in the presence of androgens. When in addition to androgens the LNCaP cells were also cultured in the presence of forskolin, down-regulation of the mRNA expression became less pronounced (Figure 4). Similar observations were done for androgen-regulated expression of PSA mRNA: PSA mRNA expression is normally highly up-regulated by androgens, but addition of forskolin to the culture medium resulted in reduced up-regulation (Figure 4). It should be noted that PSA mRNA expression is reduced in the presence of forskolin alone. This can be explained because the high androgen receptor concentration, which is present in LNCaP cells, may stimulate PSA transcription marginally in the absence of ligand. This stimulation will also be inhibited by forskolin.

The differences between the mRNA expression patterns in the presence or absence of forskolin are reproducible, and β-actin mRNA expression does not change in these experiments (Figure 4). Therefore, it was concluded that forskolin suppresses androgen receptor mediated modulation of gene expression.

To verify K_d and B_{max} of the forskolin-treated androgen receptor, Scatchard analysis was performed on LNCaP cells which were cultured for 24 h in the presence or absence of forskolin. It was observed that the K_d values of control and forskolin-treated cells were not significantly different (0.252 $\pm$ 0.33 nM versus 0.204 $\pm$ 0.18 nM, mean $\pm$ SD). The B_{max} of forskolin-treated cells, however, was found to be 43% reduced as compared to the B_{max} of control cells (369 $\pm$ 51 fmol/mg of protein versus 641 $\pm$ 87 fmol/mg of protein, mean $\pm$ SD) (Figure 5). This result was unexpected because immunoanalysis of the amount of forskolin-treated androgen receptor did not reveal such a difference (Figure 1).

To clarify this discrepancy, LNCaP cells were metabolically labeled using [^{35}S]methionine in the presence or absence of forskolin. It was observed that after 0, 4, or 24 h of culture in the presence of forskolin, total [^{35}S]methionine incorporation did not change significantly (measured relative radio-

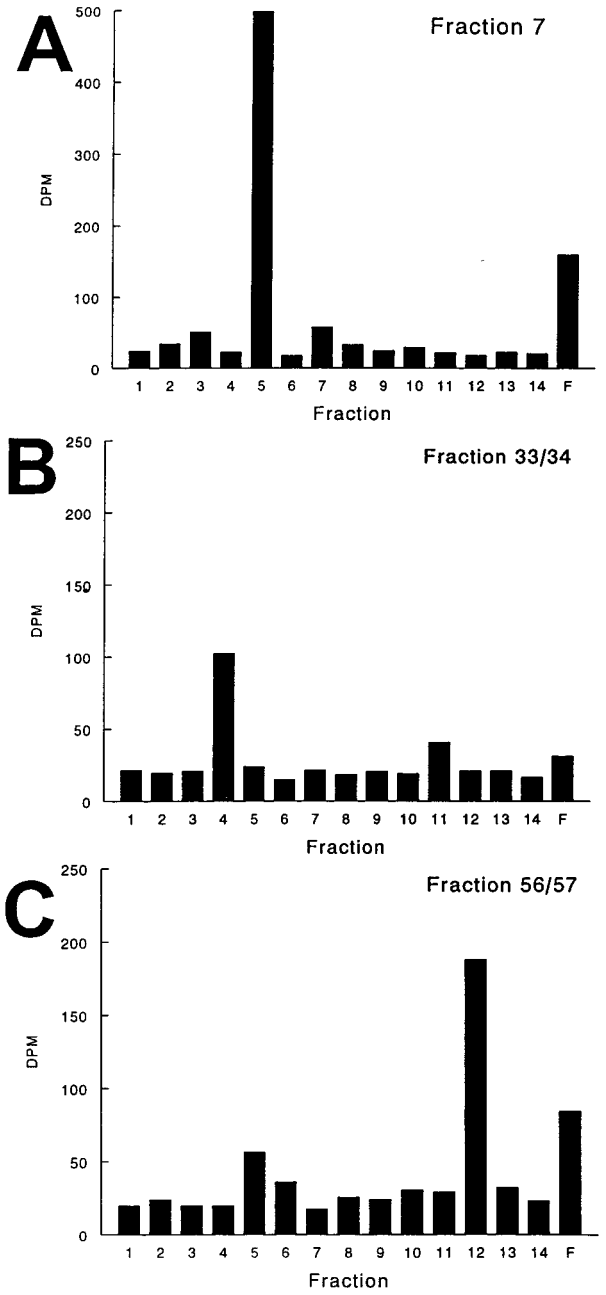

FIGURE 3: Edman degradation analysis of radioactively labeled peptides corresponding to fractions 7 (A), 33/34 (B), and 56/57 (C). Edman degradation was performed as outlined under Materials and Methods. Edman degradation cycle number is indicated on the *x*-axis. F = counts which are still present on the arylamine−sequelon disks after 14 cycles. Radioactivity in the collected fractions was determined after the addition of liquid scintillation fluid and depicted on the *y*-axis.

activity values were 89% of control at 4 h and 117% of control at 24 h; Figure 6). The decrease of the 112 kDa androgen receptor isoform and the increase of the 110 kDa androgen receptor isoform, indicative for dephosphorylation, however, did occur. These results indicate that under the current culture conditions, the dephosphorylated androgen receptor is partly impaired in ligand binding.

DISCUSSION

Reversible phosphorylation is an important regulatory mechanism which allows the cell to respond adequately to

Table 1: Potential Sites for Androgen Receptor Phosphorylation[a]

239	A-V-S-V-**S**-M-G-L-G-V-E-A-L-X_9-R
502	V-*P*-Y-*P*-***S***-*P*-T-C-V-K
584	Y-L-C-A-**S**-R
875	S-H-M-V-**S**-V-D-F-P-E-M-M-A-X_8-R
180	D-I-L-**S**-E-A-S-T-M-Q-L-L-Q-X_{14}-R
601	N-C-P-**S**-C-R
650	L-T-V-***S***-*H*-*I*-*E*-G-Y-E-C-Q-P-X_{38}-R
839	N-P-T-**S**-C-S-R
853	L-L-D-**S**-V-Q-P-I-A-R
316	G-L-E-G-E-S-L-G-C-S-G-**S**-A-X_{17}-R
347	S-G-A-L-D-E-A-A-A-Y-Q-**S**-R
630	L-Q-E-E-G-E-A-S-S-T-T-***S***-*P*-X_6-K

[a] All potential phosphopeptides (produced by trypsin digestion of the androgen receptor) were analyzed for serines on positions 5, 4, and 12. Five peptides were found with a serine on position 4; four peptides with a serine on position 5; and three peptides with a serine on position 12. The potential phosphorylated serine residues are indicated in boldface type. The phosphorylation consensus recognition motifs are indicated in italic. The peptides which were potentially recovered in RP-HPLC fractions 7, 33/34, and 56/57 are underlined.

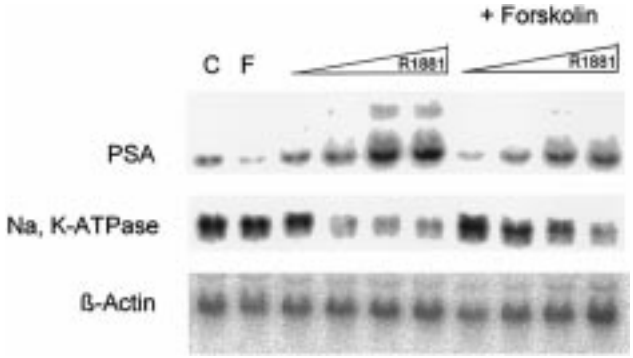

FIGURE 4: Forskolin effect on androgen-regulated gene expression in LNCaP cells. LNCaP cells were treated for 24 h prior to total RNA isolation as indicated at the top of the figure. C = nontreated control cells; F = cells which have only been cultured in the presence of forskolin; R1881 concentrations are 0.01, 0.1, 1, and 10 nM; [forskolin] = 20 μM. Total RNA (20 μg/lane) was loaded on the gel and after separation blotted onto nitrocellulose. Hybridization to PSA, β1-subunit, and actin cDNA probes was performed as outlined under Materials and Methods.

extracellular stimuli. Androgens are such extracellular stimuli resulting in hyperphosphorylation of the androgen receptor. Using different androgen receptor mutants, Jenster et al. could show that androgen-induced hyperphosphorylation of the androgen receptor occurred during or after transcription activation, suggesting a possible role for hyperphosphorylation in androgen receptor transcriptional activation (*25*). Dephosphorylation of the androgen receptor can be caused by stimulation of the protein kinase A pathway

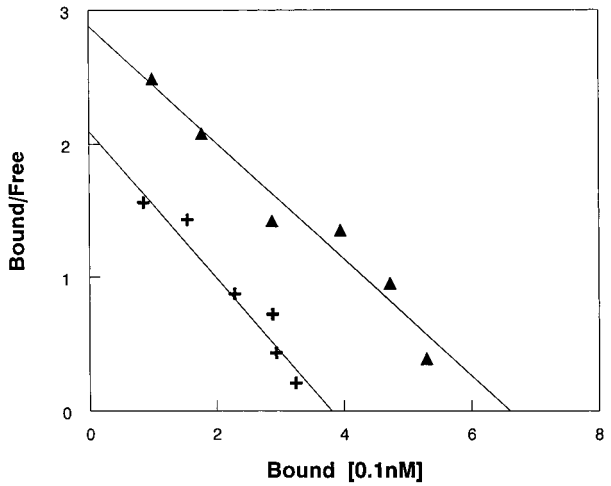

FIGURE 5: Binding characteristics of the androgen receptor in the presence and absence of forskolin. LNCaP cells were cultured for 24 h in the presence (+) or absence (▲) of forskolin (20 μM) before ^{3}H-R1881 binding was measured. Growing cells were exposed to 1.25, 0.63, 0.45, 0.32, 0.16, and 0.08 nM ^{3}H-R1881 in the presence or absence of a 200-fold molar excess of unlabeled R1881 for 1 h at 37 °C. On the *x*-axis, bound ligand concentration is depicted; on the *y*-axis, bound ligand concentration is divided by free ligand concentration. This experiment was repeated 3 times; this figure is a representative experiment.

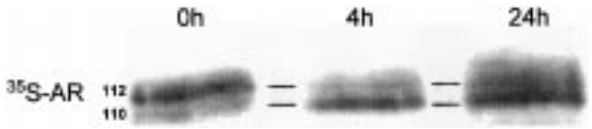

FIGURE 6: Forskolin effect on androgen receptor expression. LNCaP cells were cultured in medium containing [^{35}S]methionine (24 h), in the presence of forskolin for 0, 4, or 24 h. The androgen receptor was immunoprecipitated and Western blotted (^{35}S-AR). 110 and 112 indicate the 110 and 112 kDa androgen receptor isoforms, respectively.

(PKA). When COS-1 cells, transfected with wild-type androgen receptor, were treated with dbcAMP or forskolin (both stimulators of PKA), a clear dephosphorylation of the receptor was observed (*26*). Androgen receptor dephosphorylation is a rapid process; within 10 min the first signs are visible, and after 30 min the process is completed. The swift nature of this process suggests that androgen receptor dephosphorylation is an active process which involves activation of phosphatases rather than inhibition of kinases. Indeed, the activity of some phosphatases is known to be regulated by stimulation of PKA. For example, the nuclear protein phosphatase-1 (PP-1N) is activated by PKA-induced phosphorylation of NIPP-1 (nuclear inhibitor of protein phosphatase-1) (*43*−*45*). Dephosphorylation of the androgen receptor may involve this phosphatase.

Dephosphorylation of a protein upon stimulation of PKA is not new. For example, ribosomal protein S6 and the retinoblastoma gene product (Rb) are dephosphorylated as a result of stimulation of the protein kinase A pathway (*46*, *47*). The phosphorylation status is very important for the function of Rb: the dephosphorylated Rb prevents cell proliferation by binding transcription factors involved in cell cycle regulation (E2F). Upon phosphorylation, retinoblastoma releases these transcription factors and cell proliferation can proceed. Dephosphorylation, which occurs under normal circumstances in the beginning of G1, was also found to be caused by PKA-inducible protein phosphatases as described before (*47*).

In this paper forskolin-induced dephosphorylation of the androgen receptor in LNCaP cells is described. However, the amount of ^{32}P-labeled androgen receptor which can be recovered from LNCaP cells is not sufficient for Edman degradation. Therefore, COS-1 cells were transiently transfected with a human androgen receptor expression plasmid. After trypsin digestion and RP-HPLC separation, radioactivity was recovered in fractions 7, 34, and 54. These three fractions seem identical to the fractions isolated after RP-HPLC analysis of ^{32}P-labeled androgen receptor from LNCaP cells. Using RP-HPLC analysis and Edman degradation of the trypsin-digested androgen receptor, strong evidence is presented that serine residues 506, 641, and 653 were phosphorylated. Forskolin treatment of the LNCaP cells resulted in dephosphorylation of serine residues 641 and 653. It is interesting to note that Zhou et al. (*27*) showed that substituting serine 641 into alanine reduced the overall phosphorylation of the androgen receptor. Their finding that this mutant receptor was also reduced in transcription activation capacity by 30% corresponds with the current results. Serine 641 is located in the hinge region of the androgen receptor and part of a proline-directed kinase site. For the chicken progesterone receptor, serine 530 is located in the same region, and also part of a proline-directed kinase site. When the serine 530 residue of the chicken progesterone receptor was mutated into alanine, the transcriptional capacity of the mutated receptor was found to be reduced. The reason for this reduction in transcriptional activity was that ligand binding became partly impaired (*20*). These data are also in correspondence with the here presented data.

Nazareth et al. (*29*) have shown that forskolin induces ligand-independent activation of the androgen receptor. Blok et al. (*26*) showed that forskolin induces dephosphorylation of the androgen receptor. Combining these two sets of data suggests that the dephosphorylated nonliganded androgen receptor may be transcriptionally active (ligand-independent activation). The study of ligand-independent activation of the androgen receptor is important because the treatment of invasive prostate cancer is hampered by the unescapable development of ligand-independent tumor growth. It is hypothesized that a subset of hormone-independent tumors can function because of ligand-independent transcriptional activation of the androgen receptor. The LNCaP cell line represents a prostate cancer cell model which is well characterized for its androgen-responsiveness (*48*−*50*). Using these cells in the current investigations, it was observed that the androgen receptor was dephosphorylated upon culture in the presence of stimulators of the PKA pathway. Androgen-induced transcriptional regulation of endogenous genes, in the presence of forskolin, however, was found to be inhibited as compared to the control situation. These observations were further strengthened by the finding that the dephosphorylated androgen receptor was partly impaired in ligand binding (B_{max} was found to be reduced by approximately 40%). These data are not in agreement with the finding of ligand-independent transcriptional activation of the dephosphorylated androgen receptor by Nazareth et al. (*29*). The nature of the discrepancy between the data observed by Nazareth et al. (*29*) and the currently presented data is not entirely clear, but it is likely that cellular

differences (CV1 versus LNCaP) and differences in reporter genes (nonrecombinant adenoviral-mediated DNA transfer versus endogenous genes) are at the basis of it.

Since the affinity (K_d) of the androgen receptor for its ligand has not significantly changed (indicating that R1881 binding to the receptor is normal), it is possible that the decrease in B_{max} value either is caused by a reduced stability of ligand binding (in this case the ligand is easily released from the receptor resulting in reduced B_{max} values during Scatchard analysis) or results from nonphosphorylated androgen receptors being unable to bind ligand (in this case only the phosphorylated androgen receptor is measured during Scatchard analysis). In both explanations, the amount of androgen receptor measured on Western blot would remain normal. The nature of the partial ligand binding deficiency caused by androgen receptor dephosphorylation will be the subject of further investigations.

In summary, forskolin-induced androgen receptor dephosphorylation in the prostate cancer cell line LNCaP causes reduced ligand binding which adversely affects the transcriptional capacity of the androgen receptor. To our knowledge, this is the first report on a clear function for the rapid phosphorylation which occurs directly after synthesis of the androgen receptor.

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