Androgen Receptor Phosphorylation

REGULATION AND IDENTIFICATION OF THE PHOSPHORYLATION SITES*

Received for publication, April 29, 2002, and in revised form, May 14, 2002
Published, JBC Papers in Press, May 15, 2002, DOI 10.1074/jbc.M204131200

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Activation of signal transduction kinase cascades has been shown to alter androgen receptor (AR) activity. Although it has been suggested that changes in AR phosphorylation might be directly responsible, the basal and regulated phosphorylations of the AR have not been fully determined. We have identified the major sites of AR phosphorylation on ARs expressed in COS-1 cells using a combination of peptide mapping, Edman degradation, and mass spectrometry. We describe the identification of seven AR phosphorylation sites, show that the phosphopeptides seen with exogenously expressed ARs are highly similar to those seen with endogenous ARs in LNCaP cells and show that specific agonists differentially regulate the phosphorylation state of endogenous ARs in LNCaP prostate cancer cells. Treatment of LNCaP cells with the synthetic androgen, R1881, elevates phosphorylation of serines 16, 81, 256, 308, 424, and 650. Ser-94 appears constitutively phosphorylated. Forskolin, epidermal growth factor, and phorbol 12-myristate 13-acetate increase the phosphorylation of Ser-650. The kinetics of phosphorylation of most sites in response to hormone or forskolin is temporally delayed, reaching a maximum at 2 h post-stimulation. The exception is Ser-81, which continues to display increasing phosphorylation at 6 h. These data provide a basis for analyzing mechanisms of crosstalk between growth factor signaling and androgen in prostate development, physiology, and cancer.

The steroid hormone receptors are ligand-activated transcription factors. In addition to regulation by steroids, they are also regulated by post-translational modifications generated by signal transduction pathways. Thus, they function not only as transcription factors but also as nodes that integrate multiple signal transduction pathways. Therefore, they function not only as transcription factors but also as nodes that integrate multiple signal transduction pathways. An example is the androgen receptor (AR) which is regulated directly or indirectly by phosphorylation of multiple residues; the basal phosphorylation sites, including serines 81, 102, and 162, are rapidly induced in the presence of steroid (1). However, phosphorylation of serines 102, 294, and 345 in response to hormone is temporally delayed, reaching a maximum at 2 h (2). Phosphorylation of AR on Ser-676 in the hinge region has recently been identified (3); the analogous site in the chicken PR is also phosphorylated. When this site is mutated to alanine, sub saturating levels of hormone show severalfold less transcriptional activity compared with wild type (4). At least seven phosphorylation sites have been identified on the glucocorticoid receptor, and the relative level of phosphorylation of these sites appears to be cell cycle-regulated (5–7). Recent evidence indicates that the phosphorylation status of the glucocorticoid receptor plays a prominent role in receptor protein turnover (8). Growth factors are known to stimulate the ligand-independent activity of the estrogen receptor through the activation of the mitogen-activated protein kinase (MAPK) cascade and the direct phosphorylation of estrogen receptor by MAPK at Ser-118 (9). Hormone binding also regulates the phosphorylation of Ser-118; however, this phosphorylation is not mediated by MAPK (10). The estrogen receptor is phosphorylated by pp90rsk on Ser-167; phosphorylation of Ser-167 aids in regulating the transcriptional activity of AF-1 (11).

A substantial body of literature suggests that the androgen receptor (AR) is regulated directly or indirectly by phosphorylation. Kulig et al. (12) investigated the stimulatory effects of growth factors on AR-mediated transcription (12). In this study, DU145 cells, a prostate cancer cell line that expresses neither AR nor prostate-specific antigen (PSA), were co-transfected with an expression vector encoding the AR and chlorampheicol acetyltransferase reporter constructs driven by either a synthetic androgen response element or by the PSA promoter. Insulin-like growth factor 1, EGF, and keratinocyte growth factor were able to stimulate reporter gene expression. Similar studies by Nazareth and Weigel (13) demonstrate that a protein kinase A (PKA) activator could activate the androgen receptor in the absence of androgen (13). This activation can be blocked by the AR antagonists casodex and flutamide, indicating that the activation effect was dependent on the AR. Furthermore, Sadar (14) finds that treatment of LNCaP cells with PKA activators resulted in a dose- and time-dependent increase in PSA mRNA levels (14). Moreover, the AR antagonist bicalutamide blocked the PKA-dependent increase in PSA mRNA, suggesting that these transcription changes required the AR even though they were driven by PKA. Studies by Janne and co-workers (15, 16) using androgen response element and mouse mammary tumor virus-driven CAT reporter constructs showed EGF, insulin-like growth factor 1, and PKA

* This work was supported by a gift from CaP CURE, a grant from the Mellon Prostate Cancer Research Institute at the University of Virginia, and United States Public Health Service Grants CA39076, CA76465, CA40042 (to M. J. W.), and GM37537 (to D. F. H.). The costs of publication of this article were defrayed in part by the payment of page charges. This paper is available online at http://www.jbc.org

‡ Supported by NCI, National Institutes of Health Grants T32 CA09109 and T32 DK07320 and the American Foundation for Urologic Disease/Scott Fund.

¶ Supported by NCI, National Institutes of Health Grants T32 CA09169 and T32 DK07320 and the American Foundation for Urogologic Disease/Scott Fund.

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1 The abbreviations used are: PKC, protein kinase C; FSK, forskolin; MS, mass spectrometry; MS/MS, tandem MS; IMAC, immobilized metal affinity chromatography; HPLC, high performance liquid chromatography; Bis, bisindolylmaleimide.
agonist stimulation of the AR and sensitization of the AR to low levels of androgen.

Taken together, these studies strongly suggest that kinase cascades regulate AR function in part by activating the AR in the absence of ligand or sensitizing the AR to reduced levels of ligand. The ability of signaling cascades to influence AR function may play a significant role in the development and progression of prostate cancer where an increase in signal transduction activity has been associated with the acquisition of androgen-independent disease. However, despite this association, there has not previously been a systematic and direct analysis of the in vivo phosphorylation sites on the AR.

Previous studies infer candidate phosphorylation sites on the AR by in vitro phosphorylation reactions and/or by identifying kinase consensus sites and then mutating them. Sites so identified include serines 81, 94, 213, 515, 650, and 791 (18–22). However, these determinations, although a useful first step, are not definitive because in vitro kinase reactions are often not selective, and mutagenesis can alter the phosphorylations on sites distinct from the ones mutagenized. Recently, Ser-308 was directly identified as a phosphorylation site in baculovirus-expressed AR using mass spectrometry (23). This is the only site identified in living cells either by mass spectrometry or by in vivo metabolic labeling. Because unequivocally identifying the in vivo sites of AR phosphorylation is fundamental to understanding the interactions of the AR and cell signaling, we undertook an extensive study of AR phosphorylation to explore regulated changes in AR phosphorylation as a possible mechanism for activation/sensitization of AR-dependent gene expression by cell surface receptors and their downstream signaling effectors.

Here we describe the identification of seven AR phosphorylation sites by a combination of mass spectrometry and metabolic labeling with 32P in COS-1 cells expressing exogenous AR. We also show that these phosphopeptides are identical or similar to those seen on endogenous AR in LNCaP cells. Our studies show that specific agonists differentially regulate the phosphorylation state of endogenous LNCaP ARs. Treatment of LNCaP prostate cancer cells with either the synthetic androgen, R1881, or FSK revealed differences in the relative phosphorylation state of endogenous LNCaP ARs. Treatment of LNCaP prostate cancer cells with either the synthetic androgen, R1881, or FSK revealed differences in the relative phosphorylation state of endogenous LNCaP ARs. Treatment of LNCaP prostate cancer cells with either the synthetic androgen, R1881, or FSK revealed differences in the relative phosphorylation state of endogenous LNCaP ARs.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Transfections—**LNCaP cells (a gift from Dr. L. W. K. Chung, Emory University) were grown in T-media (Invitrogen) supplemented with 5% fetal calf serum (Invitrogen). CV-1 and COS-1 were grown in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal calf serum. All cultures were maintained in a humidified chamber at 37 °C with 5% CO2.

Transfections were performed using LipofectAMINE (Invitrogen) according to the manufacturer's instructions. For transfections of wild type AR and AR phosphorylation site mutants into COS-1 cells, 5.5 μg of DNA/100-mm dish or 12.4 μg of DNA/150-mm dish was used. For transfections into CV-1 cells for luciferase assays, a total of 0.85 μg of DNA/6 wells was used; 0.375 μg of pGL3-PSA, 0.025 μg of tkRL, and 0.45 μg total of AR phosphorylation mutant plus empty vector (the amount of AR phosphorylation mutant was manipulated to equalize expression; the range of mutant AR DNA concentrations used was 0.15–0.45 μg).

**Plasmid Construction and Mutagenesis—**The human AR was NH3-terminally FLAG-tagged using the Expand High Fidelity PCR system (Roche Molecular Biochemicals) from pCMV-hAR (provided by Dr. C. Gao of the University of Illinois). FLAG-AR was cloned into pcDNA3 (Invitrogen), and the final construct was completely sequenced to ensure no mutations were introduced. pGL3-PSA was constructed by inserting the 5.8-kb HindIII fragment from the PSA promoter cloned by Dr. A. Lundwall into pGL3 basic vector (Promega, Madison WI) (24, 25).

**Transfection—**COS-1 cells were transfected as above and allowed to recover for 1 day. Transfected cells were then stably transfected as with LNCaP cells. Cultures were then labeled in labeling media containing 3 μCi of carrier-free 32P/ml for 6 h. Cells were lysed in radioimmune precipitation buffer (1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 7.5, 2 mM EDTA, 150 mM NaCl, 0.01% sodium deoxycholate, 50 mM NaF) plus the following protease and phosphatase inhibitors: 1 μg/ml leupeptin, 1 μg/ml pepstatin, 2 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, 20 μM orthovanadate, 2 μM sodium orthovanadate, and 0.4 μM microcystin. Metabolically labeled AR was immunoprecipitated with 15 μg of anti-human AR raised against a peptide corresponding to the first 21 amino acids of the AR (Upstate Biotechnology, Lake Placid, NY) (100-mm dish; this is sufficient antibody to clear the AR (data not shown). Immunoprecipitates were washed 5 times with lysis buffer. Precipitates were resuspended in SDS-PAGE sample buffer, resolved by SDS-PAGE, and transferred to nitrocellulose.

Tryptic phosphopeptide mapping was performed essentially as described (26). N-Tosyl-l-phenylalanine chloromethyl ketone-treated trypsin from Worthington Biochemical Corp. (Freehold, NJ) and endoproteinase Glu-C from Calbiochem (San Diego CA) were used. For the trypsin and Glu-C double digest, the trypsin digestion was done to completion, and the peptides were washed and dried. Peptides were then reloaded in 50 μl of 50 mM ammonium bicarbonate and digested with 2 μg of Glu-C for 1.5 h at 37°C. An effort was made to load equal counts on each TLC plate; ~10,000 cpm/TLC plate was loaded.

**Phosphoamino Acid Analysis and Western Blotting—**Phosphoamino acid analysis was done as described (26), except electrophoresis was performed at pH 2.5–27. Plates were subject to autoradiography after staining with ninhydrin.

For quantitative Western blotting, 250 μg total LNCaP cell lysate was separated on a 7% SDS-PAGE and transferred to nitrocellulose. The primary anti-AR antibody was 441 (Santa Cruz) raised to the amino acid 464–473 of the human AR. This antibody is specific for the anti-AR antibody. We used an anti-AR antibody specific for the human AR and found that the anti-AR antibody cross-reacted with the mouse AR. The secondary antibody was horseradish peroxidase-conjugated goat anti-mouse IgG (PerkinElmer Life Sciences) at 1 μg/ml. Edman Degradation—For Edman degradation, phosphopeptides were extracted from TLC plates with pH 1.9 buffer. The sample was then covalently coupled to a Sequelon® amyl amine membrane (Milligen) through carboxyl groups in a reaction with a carbodiimide. After coupling, the membrane was washed 4 times with 1 ml of 27% acetic acid, 20% trifluoroacetic acid in water and twice with 50% methanol. The sample was then subjected to repetitive Edman degradation in an Applied Biosystems Procise protein sequencer. The amino acids released from the peptide were extracted from the Sequelon® membrane with 100% trifluoroacetic acid and collected in a fraction collector without the normal conversion from ATZ amino acid to PTH amino acid. The tubes were counted by Cerenkov counting to determine the cycle of 32P release. Dr. John Shannon in the Biomolecular Research Facility at the University of Virginia performed the Edman degradation.

**Purification of FLAG-AR—**Fifteen 150-mm dishes of COS-1 cells were transfected with FLAG-AR. Cells were stimulated with 5 nM R1881 for 6 h. All subsequent operations were performed at 4°C. Cells were washed 1× with ice-cold calcium- and magnesium-free phosphate-buffered saline and lysed in Triton lysis buffer (0.01% Triton, 50 mM Tris, pH 7.5, 5 mM EDTA, 0.05% SDS, 0.5% Nonidet P-40, 50 mM NaF) in the mixture of protease and phosphatase inhibitors used above. Cells were collected by scraping and cleared at 100,000 × g for 1 h. The lysate was then filtered (0.2 μm) and loaded onto a 1-mL M2 (Sigma) affinity column previously equilibrated with 10 bed volumes of Triton lysis buffer. The absorbed column was washed sequentially with 10 bed volumes Triton lysis buffer and 10 bed volumes radioimmune precipitation lysis buffer with.
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out SDS. The column was eluted with 10 bed volumes of glycine, pH 3.5, into neutralizing Tris for a final pH of 7.0. Fractions were then analyzed by SDS-PAGE and Western blotting using anti-FLAG MS (Sigma).

**Protease Digestion of FLAG-tagged AR for MS**—FLAG-tagged AR (10 pmol) was digested with trypsin (Promega), Glu-C, Asp-N, pepsin, or elastase (Molecular Biochemicals) according to the manufacturer's instructions. In some experiments, AR was reduced by adding dithiothreitol (Sigma) to a final concentration of 10 mM and incubating at 51 °C for 45 min. Reduced AR was then alkylated by adding iodoacetamide (Sigma) to a final concentration of 20 mM and incubating for 45 min in the dark at room temperature. Digests (trypsin, Glu-C, Asp-N, elastase) were quenched by adding glacial acetic acid (Aldrich) until the pH was 3.5. Pepsin digestion was quenched by freezing.

**Immobilized Metal Affinity Chromatography Enrichment of Phosphorylated Peptides**—Immobilized metal affinity chromatography (IMAC) columns were constructed as described previously (28). Briefly, 360-μm outer diameter × 100-μm inner diameter-fused silica (Polymer Microtechnologies, Phoenix, AZ) was packed with 8-cm POROS 20 MC (Applied Biosystems, Foster City, CA). Columns were activated with 200 μl of 100 mM FeCl₃ (Aldrich) and rinsed with 20 μl of 0.1% acetic acid. Protein digests (1–2 pmol) were loaded at a flow rate of 1 μl/min. To remove non-binding peptides, the column was washed with a solution of 2 pmol) were loaded at a flow rate of 1 μl/min. To remove non-binding peptides, the column was washed with a solution of 20 mM NaCl (Aldrich) in acetonitrile (Mallinckrodt Chemical Works), Novato, CA) electrospray ionization emitter tip (2 pmol). Peptides were eluted with 10 bed volumes of glycine, pH 3.5, and separated on SDS-PAGE. In response to the synthetic analog androgen, R1881, there was a small but reproducible decrease in mobility and an apparent increase in overall AR phosphorylation (Fig. 1A). Treatment of cells with FSK, an activator of adenylyl cyclase, or EGF did not decrease the mobility of AR. A slight increase in the overall level of AR phosphorylation was observed. It has been reported that steroid treatment stabilizes AR (31). Therefore, whole cell lysates of LNCaP cells treated with different agonists were blotted with anti-AR antibody and 125I secondary antibody. The overall amount of AR increased with steroid treatment, consistent with increased AR stability, suggesting that the increase in AR phosphorylation is due at least in part to the increase in protein level. However, this does not address whether individual phosphorylation sites change in response to agonist. There was no significant change in AR protein levels with FSK or EGF treatment. Phosphoamino acid analysis demonstrated that endogenous LNCaP AR is phosphorylated solely on serine residues (Fig. 1B).

Two-dimensional tryptic phosphopeptide mapping was performed to determine whether phosphorylation of individual serine residues changed in response to different agonists (Fig. 1C). Analysis of AR isolated from LNCaP cells treated with either R1881 or FSK revealed differences in the relative labeling of specific phosphopeptides. In response to R1881, the relative amounts of phosphopeptides 1, 5, and 10 as well as some of the phosphopeptides with lower levels of phosphorylation increased (e.g. 6–9, Fig. 1C). FSK treatment led to an increase in the relative phosphorylation of phosphopeptide 5. A similar but significantly less pronounced increase in the phosphorylation of phosphopeptide 5 was also observed with EGF treatment.

To determine the kinetics of AR phosphorylation in response to different agonists, comparative phosphopeptide maps of immunoprecipitated LNCaP AR treated with FSK or R1881 for different times were quantified on a PhosphoImager. The counts of each phosphopeptide were standardized to the total counts on the TLC plate. The fold increase in phosphorylation over the untreated phosphopeptide was then determined, yielding a minimum estimate of the increase in peptide phosphorylation (Fig. 2). The phosphorylation of peptide 5 increased 5-fold, reaching near maximal phosphorylation at 1 h and peaking at 2 h post-FSK treatment. No other phosphopeptide consistently changed in response to FSK.

Phosphopeptide 1 is one of the higher stoichiometric phosphorylations to occur in response to hormone. However, this peptide does not resolve well on tryptic peptide maps. Therefore, we digested metabolically labeled and immunoprecipitated AR from LNCaP cells with the endoproteinase Glu-C in addition to trypsin. This double peptidase digestion significantly improved the resolution of the major stoichiometric phosphorylation site induced by hormone (designated p2*) and was used to assess the kinetics of AR phosphorylation in response to R1881. Multiple phosphopeptides exhibited a change in kinetics and magnitude in response to R1881 (Fig. 3). All of the peptides followed one of three patterns as follows. Phosphorylation increased throughout the 6-h experiment, phosphorylation was maximal at 2 h and remained elevated, or phosphorylation reached maximum and then decreased by 6 h. For example, phosphopeptide 2* continued to increase throughout the 6-h treatment, reaching a 6-fold increase over untreated and becoming the highest stoichiometric phosphorylation in response to hormone. Phosphopeptide 3* reached a maximal phosphorylation of 4–5-fold at 2 h and remained elevated at 6 h. At 2 h, phosphopeptide 1* reached a maximal phosphorylation of 3-fold, with phosphorylation decreasing to 2-fold at 6 h. These data suggest complex regulation of AR phosphorylation in response to both steroid and PKA signaling.

**Identification of AR Phosphorylation Sites**—To identify the major endogenous AR phosphorylation sites in the LNCaP prostate cancer cell line, we used a combination of Edman
degradation, phosphoamino acid analysis, mass spectrometry, and mutational analysis. For Edman degradation and phosphoamino acid analysis, spots on TLC plates from analysis on LNCaP AR and FLAG-AR expressed in COS-1 cells (see below) were scraped and eluted in pH 1.9 buffer. Table I displays the data from Edman degradation and phosphoamino acid analysis of the phosphopeptides. As with the total AR phosphoamino acid analysis, the individual phosphopeptides all displayed phosphorylation only on serine. From these data, a list of candidate AR phosphorylation sites was generated; however, the number of candidates for each peptide was large. Furthermore, trypsin often cleaves at atypical sites, yielding unpredictable peptides. Therefore, we used tandem mass spectrometry to identify the specific peptides and phosphorylation sites on affinity-purified AR.

LNCaP cells express relatively low levels of AR, making purification of sufficient ARs for mass spectrometric analysis difficult. Therefore, we overexpressed FLAG-tagged human AR by transient transfection in COS-1 cells (see below) were scraped and eluted in pH 1.9 buffer. Table I displays the data from Edman degradation and phosphoamino acid analysis of the phosphopeptides. As with the total AR phosphoamino acid analysis, the individual phosphopeptides all displayed phosphorylation only on serine. From these data, a list of candidate AR phosphorylation sites was generated; however, the number of candidates for each peptide was large. Furthermore, trypsin often cleaves at atypical sites, yielding unpredictable peptides. Therefore, we used tandem mass spectrometry to identify the specific peptides and phosphorylation sites on affinity-purified AR.

LNCaP cells express relatively low levels of AR, making purification of sufficient ARs for mass spectrometric analysis difficult. Therefore, we overexpressed FLAG-tagged human AR by transient transfection in COS-1 cells. To be certain this would yield information reflective of endogenous AR in prostate cells, we tested whether exogenously expressed AR has the same phosphorylation pattern as endogenous LNCaP AR. Phosphopeptide maps of FLAG-AR exogenously expressed in COS-1 cells displayed the same phosphorylation pattern as endogenous LNCaP AR when digested with trypsin and Glu-C (Fig. 4A). This suggests that FLAG-tagged AR from COS-1 cells is phosphorylated on the same residues as endogenous LNCaP AR. This is also suggested from Edman cycling data; the residue of phosphorylation was the same for a given peptide in every case where Edman data was obtained from both endogenous LNCaP AR and exogenous FLAG-AR. Thus, identifying AR phosphorylation sites on exogenous FLAG-AR from COS-1 cells is a valid surrogate for identifying the phosphorylation sites from endogenous LNCaP AR.

FLAG-tagged AR was expressed in COS-1 cells and purified to near homogeneity in a single affinity anti-FLAG chromatography step for mass spectrometry analysis (Fig. 4B). Peptides resulting from digestion of AR with several different proteases were analyzed by nanoflow-HPLC/microelectrospray ionization/MS. MS/MS spectra were acquired and correlated to AR peptide sequences with the data base-searching program SEQUEST (30). Peptides corresponding to 81% of the AR and containing 88% of all serine residues were sequenced by this approach (Fig. 5A). Mass spectrometry covered all of the Ser-Pro motifs.

IMAC was employed to detect phosphopeptides present at trace levels in protease digests of AR; peptide fragments were enriched with IMAC before MS analysis. IMAC enrichment was performed in line with MS analysis to avoid losses due to additional sample handling. Phosphopeptides were identified with SEQUEST and by screening MS/MS spectra for neutral loss of phosphoric acid or phosphoric acid and water from the precursor mass. Such neutral losses are commonly observed in MS/MS spectra of phosphopeptides (32). IMAC consistently enriched phosphorylated peptides by a factor of 10 when compared with analyses without IMAC (Fig. 5B). For example, using C18 chromatography alone, the peptide LQEEGAST-TpSPTEETTQK (p represents phosphorylated serine) was the 10th most abundant peptide species in the full scan mass spectrum recorded at its elution height. After IMAC enrich-
This peptide was the most abundant peptide observed in the full scan mass spectrum. Fragment ions in the MS/MS spectrum of this peptide (y8 and y9) specify the site of phosphorylation (Fig. 5C). Note that the most abundant peak in this spectrum ($m/z$ 1059.3) is due to neutral loss of phosphoric acid and water [M$^{2-}$H-H$_3$PO$_4$-H$_2$O] from the precursor mass (M$^{2-}$H$^{2+}$; $m/z$ 1117.3).

Although IMAC facilitated the detection of several phosphorylated peptides, it is not suited for identifying phosphorylation sites present on hydrophilic peptides such as those containing polyglutamine tracts. Moreover, because IMAC enriches for phosphopeptides, it does not provide maximal coverage of peptides from a given protein. Thus, we also analyzed AR phosphorylation by MS/MS using only C18 chromatography without prior IMAC enrichment. The phosphorylated peptide LLQ$^{18}$ETpSPR, generated by trypsin digestion of AR, was detected by this analysis. Note that this peptide results from an atypical tryptic cleavage and was only detected by SEQUEST when searching with no-enzyme specificity. An additional atypical tryptic-phosphorylated peptide was detected (YGDLASL-HGAGAAGPSGSpSPSAAS). This underscores the importance of not biasing spectral analysis and data base searching toward a particular enzyme specificity.

Seven phosphorylation sites were identified during analysis of trypsin-digested AR by mass spectrometry (Fig. 5D). Fragment ions in MS/MS spectra verified the location of the phosphate group in each peptide. To maximize coverage of the AR, we did MS analysis of Glu-C, Asp-N, pepsin, and elastase digests of the AR. However, this did not reveal new phosphorylation sites. Consistent with the phosphoamino acid analysis of metabolically labeled AR, only serine phosphorylation was observed by MS analysis.

Mutational analysis of the phosphorylation sites determined by mass spectrometry was used to identify which phosphorylation sites change in response to steroid or PKA signaling. Phosphorylation sites on FLAG-tagged AR were mutated to the
non-phosphorylatable residue, alanine, and exogenously expressed in COS-1 cells. Tryptic phosphopeptide maps of the first phosphopeptides identified by MS/MS are presented in Fig. 6A. Trypsin alone yields better resolution of the majority of phosphopeptides; however, the migration of the major hormone-induced phosphorylation (phosphopeptide 1) from exogenous FLAG-AR is different than for endogenous LNCaP AR. This may be due to the different number of polyglutamine repeats in the exogenous FLAG-AR when compared with the endogenous LNCaP AR (see below).

Mutation of Ser-16 to Ala resulted in the loss of phosphopeptide 8, a low stoichiometric phosphorylation. Edman degradation resulted in the loss of radioactivity at position 7, consistent with phosphopeptide 8 containing Ser-16 (Table I).

Phosphopeptides 2 and 3 were lost when Ser-94 was mutated to Ala; phosphopeptide 2 also had a cycle release at position 11, consistent with Ser-94 phosphorylation. No Edman cycling data were obtained on phosphopeptide 3. Phosphopeptide 3 most likely represents a partial tryptic digest of a Ser-94 containing phosphopeptide. This interpretation is consistent with the observation that up to 10% of total AR peptides analyzed by MS were generated from atypical tryptic cleavages.

Mutating Ser-308 to Ala resulted in the loss of two phosphopeptides (6 and 7). Interestingly, only phosphopeptide 7 had a cycle-9 release. Edman degradation also showed releases at cycles 8 and 12 for phosphopeptide 7. A mixture of phosphopeptides in this area of the plate may account for the additional release of radioactivity at these cycles. Atypical tryptic cleavages of a phosphopeptide containing Ser-308 may also account for the loss of phosphopeptide 6 when Ser-308 is mutated to Ala. Alternatively, phosphorylation of phosphopeptide 6 may be on a residue different than Ser-308, with this phosphorylation conditional on phosphorylation of Ser-308.

Mutation of Ser-424 to Ala led to the loss of peptide 10. Edman degradation shows phosphate release at cycle 19, consistent with phosphopeptide 10 containing Ser-424. There was also a release at cycle 5; however, the number of counts released relative to the amount analyzed by Edman degradation was minor. The counts released at position 19 were slightly lower, but this site represents more phosphorylation than at position 5; based on estimates of Edman cycling efficiency, position 19 represents 5-fold more phosphorylation than position 5. This suggests that release from position 5 comes from a second phosphopeptide in this area. Interestingly, mutating Ser-424 to Ala causes a change in a second phosphorylation site; there is either a mobility shift in phosphopeptide 1 or the loss of phosphopeptide 1 with the phosphorylation of a new site. No phosphorylation peak was observed in 20 cycles with Edman degradation of this phosphopeptide. We also never observed a peak of phosphorylation in phosphopeptide 1 by Edman. This may be due to phosphorylation beyond position 20 or a modification to the NH$_2$ terminus of the peptide that prevents Edman degradation. The inability to obtain a release of radioactivity by Edman degradation on these two phosphopeptides is consistent with the shifted phosphopeptide in the S424A map being the same as phosphopeptide 1 although now with the loss or gain of an additional modification when Ser-424 is mutated to Ala.

FIG. 3. LNCaP AR phosphorylation changes in response to R1881. A, LNCaP cells were labeled metabolically with $^{32}$P for 6 h in the absence or presence of 5 nM R1881 for various times. Endogenous LNCaP AR was purified by immunoprecipitation and digested with trypsin and then Glu-C. The resulting phosphopeptides were resolved by two-dimensional thin-layer electrophoresis and ascending chromatography. B, the amount of $^{32}$P incorporated into phosphopeptides relative to total counts was determined using the Image Quant program. The counts of each phosphopeptide at time 0 were set to 1.

Mutation of Ser-650 to Ala resulted in the loss of phosphopeptide 5, consistent with the Edman degradation of this peptide showing the release of radioactivity at position 12. Phosphopeptide 4 was also lost with the mutation of Ser-650 to Ala. Edman degradation of phosphopeptide 4 showed a release at position 8. This may indicate a phosphorylation conditional on phosphorylation of Ser-650 phosphorylation or an atypical tryptic peptide. Interestingly, mutating Ser-81 to Ala resulted in the loss of two phosphopeptides (6 and 7). Interestingly, only phosphopeptide 7 had a cycle release. Edman degradation also showed releases at cycles 8 and 12 for phosphopeptide 7. A mixture of phosphopeptides in this area of the plate may account for the additional release of radioactivity at these cycles. Atypical tryptic cleavages of a phosphopeptide containing Ser-308 may also account for the loss of phosphopeptide 6 when Ser-308 is mutated to Ala. Alternatively, phosphorylation of phosphopeptide 6 may be on a residue different than Ser-308, with this phosphorylation conditional on phosphorylation of Ser-308.

Subsequent MS/MS analysis revealed AR phosphorylation on Ser-256. Mutation of Ser-256 to Ala resulted in the loss of phosphopeptide 9 (Fig. 6B). Loss of phosphopeptide 9 with the S256A mutant was observed in multiple experiments.

The Ser-81 phosphorylation site was identified using standard chromatography without IMAC enrichment. The non-phosphorylated analogue was not observed, suggesting that Ser-81 was a high stoichiometric phosphorylation; phosphopeptide 1 was the last unidentified high stoichiometric phosphorylation. Because the combination of trypsin and Glu-C provides better resolution of phosphopeptide 1, it was used to identify Ser-81. Mutation of Ser-81 to alanine resulted in the loss of the phosphorylation of peptide 2*. Furthermore, Edman degradation on phosphopeptide 2* showed a release at cycle 2, consistent with Ser-81 phosphorylation. The tryptic phosphopeptide containing
and labeled metabolically with 32P or in the absence or presence of NH2-terminally FLAG-tagged human AR was expressed in COS-1 cells. The same phosphopeptide pattern as endogenous LNCaP AR. A, analyzed by mass spectrometry. Bottom panel of 7% SDS-PAGE of lysate and column fractions. Fractions 5 and 6 were resolved by two-dimensional thin-layer electrophoresis and ascending chromatography. B, FLAG-AR was expressed in COS-1 cells and purified over an anti-FLAG column. The column was washed with 10 bed volumes of lysis buffer, and FLAG-AR was eluted with 100 mM glycine-HCl, pH 3.5, into neutralizing Tris buffer.

The polyglutamine tract from the phosphopeptide containing Ser-81 that was observed by MS analysis contains a polyglutamine tract.

Ser-81, resulting in phosphopeptides with similar mobilities. This may explain the different mobilities of Ser-81 versus Ser-791 shown).

TABLE I

| Peptide | Endopeptidase | Hydrolysis | Counts at cycle of release |
|---------|---------------|------------|----------------------------|
| 2       | Trypsin       | S          | 35 81 42 43 40 35 37 52 66 70 650 158 82 61 52 ND ND ND ND |
| 4       | Trypsin       | S          | 28 25 26 19 29 39 31 102 43 35 41 54 37 35 35 |
| 5       | Trypsin       | S          | 35 36 60 73 73 115 83 71 157 78 74 909 252 134 ND ND ND ND ND |
| 6       | Trypsin       | S          | 36 35 41 43 102 85 43 280 52 45 44 40 33 36 40 30 26 28 28 ND |
| 7       | Trypsin       | S          | 34 45 36 59 67 70 62 268 378 152 90 161 70 59 54 51 41 38 38 ND |
| 8       | Trypsin       | S          | 35 33 34 29 41 29 102 56 53 43 55 50 ND ND ND ND ND ND ND ND ND ND ND ND ND |
| 9       | Trypsin       | S          | 37 35 61 42 117 54 51 33 52 39 49 32 39 39 40 44 58 54 102 68 ND |
| 10      | Trypsin       | S          | 37 35 61 42 117 54 51 33 52 39 49 32 39 39 40 44 58 54 102 68 ND |
| 2*      | Trypsin/Glu-C | S          | 30 224 74 21 10 15 13 11 3 8 ND ND ND ND ND ND ND ND ND ND ND |

**Candidate AR Kinases**—Previous studies suggest that both MAPK and Akt can phosphorylate the AR (20–22). However, these kinases do not appear to directly phosphorylate endogenous LNCaP AR in living cells. Like FSK, EGF induced the phosphorylation of Ser-650 (phosphopeptide 5) but to a significantly lower stoichiometry (Fig. 1C). To further test for a role of MAPK in AR phosphorylation, we treated LNCaP cells with R1881 or FSK in the presence or absence of pharmacologic inhibitors of MEK (PD98059; U0126). The stoichiometric phosphorylations induced by R1881 and FSK are preserved under conditions where MAPK activity is inhibited (data not shown). Furthermore, the tryptic peptide containing the candidate MAPK site on the AR, Ser-515, was covered by MS analysis; only the non-phosphorylated form of this peptide was observed. Collectively, these data suggest that MAPK does not directly phosphorylate endogenous LNCaP AR on Ser-515.

LNCaP cells have a PTEN mutation, leading to constitutive activation of Akt (33). Therefore, to determine whether Akt phosphorylates endogenous AR in LNCaP cells, we treated cells with R1881 or FSK in the absence or presence of the PI3K inhibitor, LY294002. Under conditions where Akt activity is inhibited, all of the AR phosphorylation sites are preserved, and the stoichiometric phosphorylations induced by R1881 and FSK are maintained (data not shown). Furthermore, the non-phosphorylated tryptic peptide of the Akt consensus phosphorylation site, Ser-213, was observed by MS analysis. We did not observe the peptide containing the Ser-791 Akt consensus site by MS analysis. Collectively, this suggests that Akt does not phosphorylate endogenous LNCaP AR in cells.

To identify candidate AR kinases, we used GCG to look for sequence motifs by searching through the AR for phosphorylation sites defined in the PROSITE Dictionary of Protein Sites and Patterns. Four of the seven phosphorylation sites correspond to known kinase consensus sites. Serines 256 and 650 are candidate casein kinase II sites. Ser-16 is a candidate PKA/calcium calmodulin II kinase site, and Ser-81 is a candidate MAPK site. It is unlikely that Ser-16 is a PKA site; Ser-16 (phosphopeptide 8) is not up-regulated on endogenous LNCaP AR in response to FSK (Fig. 2).

PKC signaling has been implicated as both a positive and negative regulator of the androgen and AR-responsive PSA gene (34, 35). We investigated whether Ser-81 (phosphopeptide 2*) is phosphorylated by PKC in LNCaP cells. Cells were either treated with R1881 or left untreated in the absence or presence of the pan-PKC inhibitor, bisindolylmaleimide I (Bis I), or the negative control compound Bis V at concentrations known to inhibit PKC activity in LNCaP cells. The induction of Ser-81 by R1881 was not inhibited by Bis I (data not shown). This suggests that PKC does not phosphorylate AR on Ser-81. Interestingly, phorbol 12-myristate 13-acetate treatment increased phosphorylation of Ser-650 (phosphopeptide 5; data not shown).

3 J. Carson, personal communication.
Role of AR Phosphorylation in Transactivation—To determine the role of AR phosphorylation in AR transactivation activity, we analyzed AR phosphorylation mutants in a transient co-transfection reporter assay. CV1 cells were co-transfected with AR phosphorylation site mutants along with pGL3-PSA, a PSA luciferase reporter, and a tkRL reporter as an internal control (data not shown). All mutants were transfected to equal expression (data not shown). At a dose of 1 nM R1881, no consistent differences in transcriptional activity were observed among the AR phosphorylation site mutants. This included mutant proteins in which the phosphorylation sites were changed to alanine as well as mutant proteins in which the high stoichiometric phosphorylation sites, serine 81 and 650, were changed to aspartic acid. Moreover, the double mutants, S81A/S650S or S81D/S650D also had no effect on AR transcription. It is worth noting that we often found increased transcriptional activity of the S308A mutant. However, this result was not consistent, and we have been unable to identify the uncontrolled variable that makes these results irreproducible.

DISCUSSION

We initiated a comprehensive study of the in vivo AR phosphorylation sites in the LNCaP prostate cancer cell line as a first step toward understanding how peptide growth factor and steroid hormone-signaling cross-talk contribute to prostate cancer progression. Previous studies have inferred candidate phosphorylation sites on the AR by identification of consensus sequences for phosphorylation by specific kinases and/or by in vitro phosphorylation reactions with purified enzymes. These analyses have identified serines 81, 94, 213, 515, 650, and 791 as candidate sites of phosphorylation (18–22). However, these determinations were made indirectly. Most recently, Ser-308 was directly identified as a phosphorylation site using mass spectrometry (23). We have completed the most comprehensive analysis of AR phosphorylation to date, covering 81% of the protein and 88% of the serine residues by mass spectrometric analysis. All of the Ser-Pro motifs were covered by mass spectrometry. We observed in vivo phosphorylation at serines 81, 94, 308, and 650, as previously proposed (18, 19, 23). In addition, we have shown that serines 16, 256, and 424 are phosphorylated, that serine 650 phosphorylation is increased by forskolin, EGF and phorbol 12-myristate 13-acetate, and that all the sites of serine phosphorylation except for serine 94 are regulated by androgen. All of these phosphorylation sites are in the NH2-terminal domain of the AR with the exception of Ser-650, which is located in the hinge region (Fig 7). Other studies suggest that Ser-515 is phosphorylated by MAPK and that Akt phosphorylates serines 213 and 791 (20–22). However, our study does not corroborate direct phosphorylation of the LNCaP AR by either MAPK or Akt in vivo, although it is possible that these sites become phosphorylated under physiologic conditions that we have not investigated. The study by Yeh et al. (20) used in vitro kinase reactions to show that a fragment of the AR containing Ser-515 could be phosphorylated by MAPK. We found that EGF treatment of LNCaP cells did not lead to phosphorylation of Ser-515.

FIG. 5. Mass spectrometric analysis of AR. A, total coverage of AR by MS. Amino acid residues from peptides identified by SEQUEST (cross-correlation coefficient > 2) are shown in black. Note 71 of 81 serines were observed. B, IMAC enrichment of an AR-derived phosphopeptide. Full scan mass spectra shown were recorded during MS analysis of tryptic AR peptides with (bottom) or without (top) before IMAC enrichment at the elution height of doubly charged LQEEGEASSTTpSFPSTEETQK (m/z 1117.5; labeled with an asterisk). HPLC gradient conditions were identical in each analysis. C, MS/MS spectrum of the peptide LQEEGEASSTTpSFPSTEETQK. Peaks corresponding to ions of type y are labeled. Fragment ions in the MS/MS spectrum of the above peptide (y8, y9) define the site of phosphorylation. D, tryptic fragments of the seven phosphorylation sites identified by MS. Phosphorylated serine residues are indicated as S.
not yield new phosphorylation sites, and our mass spectrometric analysis of hormone-stimulated AR in cells did not show phosphorylation of Ser-515. This suggests that Ser-515 is not phosphorylated in LNCaP cells. The studies by Wen et al. (22) and Lin et al. (21) show that AR is an Akt substrate in vitro. Lin et al. (21) show phosphorylation of exogenous AR in COS-1 cells stimulated with IGF-1. However, both studies examined only overall AR phosphorylation of wild type and Ser to Ala mutants, not the phosphorylation of individual residues. Our mass spectrometric analysis of in vivo hormone-stimulated AR did not show phosphorylation of Ser-213. However, our mass spectrometric analysis did not cover the peptide containing Ser-791.

Most importantly, the phosphorylation pattern of the AR was preserved when we inhibited the constitutive Akt activity in LNCaP cells. This suggests Akt does not phosphorylate endogenous LNCaP AR.

There are conflicting reports as to whether AR phosphorylation is regulated by steroid (31, 36, 37). We did observe an increase in the overall phosphorylation of the AR in response to steroid and also an increase in the amount of AR, presumably due to receptor stabilization. However, it is clear from our results that steroid treatment increases the relative phosphorylation of specific residues on the AR. The kinetics of these phosphorylations were slow; for most sites, phosphorylation

**Fig. 6. AR is phosphorylated on serines 16, 81, 94, 256, 308, 424, and 650.** A, COS-1 cells expressing FLAG-wtAR or with Ser-16, 94, 308, 424, 650 to Ala mutations were labeled metabolically with $^{32}$P for 6 h in the presence of 5 nM R1881. Wild type (WT) and mutant FLAG-AR was purified by immunoprecipitation and digested with trypsin. The resulting phosphopeptides were resolved by two-dimensional thin-layer electrophoresis and ascending chromatography. The circles denote phosphopeptides that disappear with mutation of specific residues to Ala. The arrow in the wild type panel indicates alternative migration of phosphopeptide 1 in tryptic map of FLAG-AR. The arrow in the $S424$A panel indicates the appearance of new phosphopeptide with a mutation of Ser-424 to Ala. B, tryptic phosphopeptide map of wild type and Ser-256 to Ala mutant in the presence of 5 nM R1881. C, tryptic and Glu-C phosphopeptide map of wild type and Ser-81 to Ala mutant in the presence of 5 nM R1881.
was maximal at 2 h after hormone treatment. The exceptions are Ser-94, which did not change in response to hormone, and Ser-81 whose phosphorylation was still increasing at 6 h. The slow kinetics parallels the temporally delayed hormone-dependent phosphorylations in the PR (2). Accumulation of the AR in the nucleus in response to hormone was rapid; nuclear AR was observed by 15 min after hormone addition and was maximal by 30 min (38, 39). The rapidity of nuclear accumulation relative to phosphorylation suggests that the AR phosphorylations are not involved in chaperone binding, nuclear translocation, or initiation of transcription. Rather, it suggests that the AR phosphorylations play a role in a late phase of transcriptional regulation or receptor recycling. The only mutant that displayed a change in transcriptional activity was Ser-308 to Ala, which often gave increased transcription of a PSA reporter. However, the inconsistence of this effect and our inability to identify the uncontrolled variable makes it difficult to interpret this result. No effect on AR transactivation was observed with any of the other phosphorylation site mutants. However, this is possibly due to the limitations of reporter experiments to assay the function of steroid receptor phosphorylation sites (40).

The stoichiometry of phosphorylation varies for the different residues. Estimates from mass spectrometric analysis and band-shift on SDS-PAGE gels suggest at least half of the ARs are phosphorylated on Ser-81, the highest stoichiometric phosphorylation relative to phosphorylation suggests that the AR phosphorylation is not involved in chaperone binding, nuclear translocation, or initiation of transcription. Rather, it suggests that the AR phosphorylations play a role in a late phase of transcriptional regulation or receptor recycling. The only mutant that displayed a change in transcriptional activity was Ser-308 to Ala, which often gave increased transcription of a PSA reporter. However, the inconsistence of this effect and our inability to identify the uncontrolled variable makes it difficult to interpret this result. No effect on AR transactivation was observed with any of the other phosphorylation site mutants. However, this is possibly due to the limitations of reporter experiments to assay the function of steroid receptor phosphorylation sites (40).

A search of the AR for kinase consensus sites suggested that Ser-81 is a PKC site. Therefore, we looked at the role of PKC in AR phosphorylation using both inhibitors and activators of PKC signaling. The pan-PKC inhibitor, Bis I, inhibits PKC isoforms α, β, γ, δ, and ε. LNCaP cells express PKC isoforms α, γ, δ, ε, μ, and ζ (41). We did not see a change in the induction of Ser-81 phosphorylation or the phosphorylation of the other AR phosphorylation sites in the presence of Bis I. This suggests that PKC α, γ, and δ do not mediate the phosphorylation of the AR. It is still possible that PKC ε, μ, or ζ are involved in the regulation of AR phosphorylation. Phorbol 12-myristate 13-acetate treatment did increase the phosphorylation of Ser-650, suggesting that activation of PKC signaling can affect AR phosphorylation.

It has been suggested that FSK decreases AR phosphorylation in LNCaP cells (42). Although this study did not identify the down-regulated phosphorylation sites, the authors did suggest Ser-650 as one candidate. We find the opposite effect of FSK on AR phosphorylation; Ser-650 phosphorylation is dramatically increased in response to FSK. A role for this phosphorylation site in AR transactivation was previously suggested by Zhou et al. (18) who show a decrease in AR transactivation of the mouse mammary tumor virus promoter with a S650A mutant when sub-optimal levels of steroid were used. We did not observe a similar effect with the PSA promoter; however, we did not restrict the levels of hormone as was done by Zhou et al (18). Moreover, Zhou et al. (18) used a mouse mammary tumor virus reporter. The phosphorylation site in the hinge region is conserved in many of the steroid receptors (4). It is also phosphorylated in PR, and mutation of the hinge region phosphorylation site to alanine decreases transcription 20–50% depending on the cell and promoter context (43). In addition to its conservation among the steroid receptors, our observation that phosphorylation of the residue within the hinge region is up-regulated in response to hormone, PKA, EGF, and PKC signaling suggests it mediates a fundamental aspect of steroid receptor function. However, this function is yet to be determined.

The observation of regulated AR phosphorylations has direct relevance to human disease. In the normal prostate, paracrine production of growth factors by stromal cells appears important for epithelial cell growth and function (44). During prostate cancer progression toward an androgen-independent state, there is increased expression of growth factors and their cognate receptors that is believed to promote enhanced paracrine and autocrine stimulation of cell growth and survival. In addition to the increase of peptide growth factor signaling, the balance of neuropeptides in the prostate becomes altered during prostate cancer progression. There is an increase in neuroendocrine cells in more advanced prostate tumors, and these neuroendocrine cells are reportedly found in proximity to prostatic neuroendocrine cells in more advanced prostate tumors. Moreover, Zhou et al. (18) used a mouse mammary tumor virus reporter. The phosphorylation site in the hinge region is conserved in many of the steroid receptors (4). It is also phosphorylated in PR, and mutation of the hinge region phosphorylation site to alanine decreases transcription 20–50% depending on the cell and promoter context (43). In addition to its conservation among the steroid receptors, our observation that phosphorylation of the residue within the hinge region is up-regulated in response to hormone, PKA, EGF, and PKC signaling suggests it mediates a fundamental aspect of steroid receptor function. However, this function is yet to be determined.

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Acknowledgments—We thank Dr. Debra K. McMahon for a critical review of the manuscript and members of the Weber laboratory for helpful discussions.

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Androgen Receptor Phosphorylation: REGULATION AND IDENTIFICATION OF THE PHOSPHORYLATION SITES

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J. Biol. Chem. 2002, 277:29304-29314.
doi: 10.1074/jbc.M204131200 originally published online May 15, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M204131200

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