Identification of a Phosphorylation Site in the Hinge Region of the Human Progesterone Receptor and Additional Amino-terminal Phosphorylation Sites*

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We have previously reported the identification of seven in vivo phosphorylation sites in the amino-terminal region of the human progesterone receptor (PR). From our previous in vivo studies, it was evident that several phosphopeptides remained unidentified. In particular, we wished to determine whether human PR contains a phosphorylation site in the hinge region, as do other steroid receptors including chicken PR, human androgen receptor, and mouse estrogen receptor. Previously, problematic trypsin cleavage sites hampered our ability to detect phosphorylation sites in large incomplete tryptic peptides. Using a combination of mass spectrometry and in vitro phosphorylation, we have identified six previously unidentified phosphorylation sites in human PR. Using nanoelectrospray ionization mass spectrometry, we have identified two new in vivo phosphorylation sites, Ser250 and Ser259, in baculovirus-expressed human PR. Ser250 is analogous to the hinge site identified in other steroid receptors. Additionally, precursor ion scans identified another phosphopeptide that contains Ser190-Pro211, a likely candidate for phosphorylation. In vitro phosphorylation of PR with Cdk2 has revealed five additional in vitro Cdk2 phosphorylation sites: Ser25, Ser213, Thr230, Ser254, and Ser257. At least two of these, Ser213 and Ser257, are authentic in vivo sites. We confirmed the presence of the Cdk2-phosphorylated peptide containing Ser213 in PR from in vivo labeled T47D cells, indicating that this is an in vivo site. Our combined studies indicate that most, if not all, of the Ser-Pro motifs in human PR are sites for phosphorylation. Taken together, these data indicate that the phosphorylation of PR is highly complex, with at least 14 phosphorylation sites.

Human progesterone receptor (PR),¹ a ligand-activated transcription factor and member of the steroid receptor superfamily (1), is expressed as two forms: the full-length PR-B and the shorter form, PR-A, which lacks the first 164 amino acids of PR-B (2, 3). These proteins differ in their relative ability to activate target genes and in the unique repressor activity of the PR-A isofrom (4–8). Recently, the physiological roles for the different isoforms have been examined by the generation of isofrom-specific knockout mice. Interestingly, knockout of the PR-A isofrom in mice demonstrated a strong tissue-specific role for PR-A in uterus not shared by PR-B (9). In addition, overexpression of either PR-A or PR-B results in aberrant mammary gland development, and the phenotypes of the two overexpressing lines differ (10, 11). Moreover, recent studies have suggested that differential coactivator recruitment may be responsible for the isofrom-specific differences observed in trans-activation assays (8).

In addition to regulation dependent on isofrom, PR is a phosphoprotein (12–17) whose activity can be regulated by phosphorylation. There is ample evidence that regulation of cell signaling pathways alters the activity and phosphorylation of PR (18–21) as well as other steroid receptors (22–30). Some of these changes are due to direct alterations in receptor phosphorylation, whereas others appear to affect associated proteins (29, 31–35). Additionally, the role of individual sites has not been examined for PR. Mutation of Ser190 results in decreased activity of PR (21). Since different kinases target unique subsets of sites and these phosphorylations may have opposing effects on receptor activity, it is important to know which sites are coregulated by each kinase for mutagenesis studies to determine the impact on receptor function.

We have previously identified seven phosphorylation sites in human PR isolated from 32P-labeled T47D breast cancer cells. Each of these sites is located in the amino-terminal (A/B) domain. Three of the previously identified sites, Ser81, Ser190, and Ser400, are unique to the B form (15). In vivo labeling of the endogenous PR in T47D cells showed that the PR is phosphorylated basally in the absence of hormone at serines 81, 162, 190, and 400. Upon hormone stimulation, the net phosphorylation of these basal sites is dramatically increased within 5 min of treatment. In addition, hormone induces the phosphorylation of three new phosphorylation sites, serines 102, 294, and 345, in a temporally delayed manner, requiring 2 h for maximal phosphorylation. These sites are referred to as the hormone-dependent phosphorylation sites (16). Recombinant human PR expressed in Sf9 insect cells displays the same phosphorylation pattern as PR from hormone-treated T47D cells; however, the hormone dependence is lacking in Sf9 cells (36). Additionally, we have shown that PR is a substrate for several kinases in vitro. We reported previously that three in vivo phosphorylation sites, Ser162, Ser190, and Ser400, are phosphorylated in

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† The abbreviations used are: PR, progesterone receptor; Cdk2, cyclin-dependent kinase-2; HPLC, high performance liquid chromatography; NTA, nitrilotriacetic acid; FeNTA, ferric ion nitrilotriacetic acid resin; PBS, phosphate-buffered saline.

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vitro by the cyclin A-Cdk2 complex (17) and that Ser\textsuperscript{81} is phosphorylated by casein kinase II (15).

Although many of the phosphorylation sites have been identified in PR, several phosphotryptic peptide peaks detected in the HPLC analyses of in vivo labeled PR have yet to be identified. Most of the phosphorylation sites in steroid receptors contain Ser/Thr-Pro motifs, and in the case of the chicken PR all four of these motifs are phosphorylated (37, 38). Human PR contains 15 of these sequences, several of which have already been identified as phosphorylation sites (15–17). We were particularly interested in determining whether the conserved Ser/Thr-Pro in the hinge region between the hormone and DNA binding domains is phosphorylated as has been described for chicken PR (37), mouse estrogen receptor (39), and human androgen receptors (26).

Initial analyses by Sheridan et al. had suggested that this site is not phosphorylated in human PR (13). Arg-Pro and Lys-Pro motifs are not efficiently cleaved by trypsin (40), and PR has an unusually large number of these sequences. Analyses of candidate Ser-Pro phosphorylation sites including Ser\textsuperscript{676} in the hinge region reveal that many are located within peptides bounded by these highly resistant cleavage sites. If left uncleaved, these phosphopeptides will be very large and difficult to recover by HPLC using a C\textsubscript{18} reversed-phase column. Using trypsin modified by reductive alkylation, which has decreased susceptibility to autolysis, we have increased the percentage cleavage at these problematic sites, allowing resolution of new phosphopeptides both in PR phosphorylated in vitro by Cdk2 and by mass spectrometry of phosphopeptides isolated from PR expressed in SF9 cells.

Here we describe the identification of six candidate phosphorylation sites identified either in vitro phosphorylation experiments using Cdk2 or by mass spectrometry of peptides derived from PR expressed in SF9 cells. These sites include Ser\textsuperscript{676} in the hinge region, which was identified as both an in vitro Cdk2 site and by mass spectrometry. Three additional sites, common to PR-A and PR-B, and two sites unique to the PR-B form have also been found. Finally, an additional site has been localized to a region between amino acids 107 and 159 in the portion of the receptor unique to PR-B. These studies suggest that most, if not all, of the Ser/Thr-Pro motifs in PR are phosphorylated.

**EXPERIMENTAL PROCEDURES**

**Materials**—Phosphate-free Dulbecco’s modified Eagle’s medium and Hanks’ balanced salt solution were purchased from Life Technologies, Inc., and fetal bovine serum was from HyClone (Logan, UT). Radioinert R5020 was obtained from Amersham Pharmacia Biotech. \(^{32}\text{P} \)H\textsubscript{3}PO\textsubscript{4} and \(^{32}\text{P} \)ATP were obtained from ICN (Irvine, CA). For affinity purification, nickel-NTA resin was purchased from Qiagen, Inc. (Valencia, CA), and glutathione-Sepharose 4B and protein A-Sepharose were obtained from Amersham Pharmacia Biotech. Rabbit anti-mouse antibody was obtained from Zymed Laboratories Inc. (South San Francisco, CA). Phospho-labeled gels were run for 90 min followed by an increase to 100% acetonitrile by 110 min at 85°C. The tryptic digest was performed using an online radioactively labeled PR as described previously (43) using the Sequelon-AA Reagent (Savant, Farmingdale, NY), and the phosphopeptides were separated on a C\textsubscript{18} reversed-phase column using a Beckman System Gold HPLC. For manual Edman degradation, 10% acetonitrile in acetic acid (1:9, vol/vol) was added to the beads and incubated at 4°C for 15 min. The supernatant containing the kinase complex was removed, and aliquots were frozen at –80°C.

**In Vitro Phosphorylation of His Tag PR—**Purified recombinant His tag PR-A or PR-B (1 μg) was incubated with 5–10 μl of purified Cdk2-cyclin A and 50 μl \(^{32}\text{P} \)ATP (37,000 cpm/pmol) in Cdk2 buffer (20 mM Tris-HCl, pH 7.5, 10 mM MgCl\textsubscript{2}, 1 mM Na\textsubscript{3}citrate, 10 mM NaF, 5 μg/ml leupeptin, 0.01% B-mercaptoethanol, 10% glycerol) plus 50 mM NaF and a mixture of phosphoamino acid analysis enzymes (Sigma). The disc was then subjected to additional cycles of Edman degradation. The disc was then subjected to additional cycles of Edman degradation.
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**Phosphoamino Acid Analysis**—Phosphoamino acid analysis was performed as described (44). In brief, phosphopeptide samples containing 4 μg of each phosphoamino acid standard (phosphoserine, phosphothreonine, phosphotyrosine, and phosphohistidine) were hydrolyzed in 6 M HCl and 0.5% (v/v) pyridine at 1300 V for 17 min. The negatively charged phosphoamino acids migrate toward the (+)-terminal during electrophoresis in both dimensions. The amino acid standards were visualized by spraying the plate with a 0.5% solution of ninhydrin in acetone. Phosphorylated amino acids were detected by autoradiography.

**In Vivo Labeling of T7D Cells and Extraction**—T7D cells were plated at a density of 3.5 × 10^6 cells in 75-cm^2^ flasks in 5% charcoal-stripped fetal calf serum in Dulbecco’s modified Eagle’s medium. Prior to labeling, the medium was replaced with serum-free phosphate-free Dulbecco’s modified Eagle’s medium for 1.5 h at 37 °C. [32P]H_3PO_4 (0.833 mCi/mL) was added to the phosphate-free medium, and the cells were incubated overnight at 37 °C. 2 h prior to harvest, the cells were treated with hormone at a final concentration of 10 nM R5020. The medium was aspirated, and the cells were washed once with calcium and magnesium-free Hank’s balanced salt solution. TEN buffer (40 mM Tris-HCl, pH 8.0, 1 mM EDTA, 150 mM NaCl) was added to the cells and allowed to incubate for 5 min. Cells were dislodged by shaking, and the suspension was pooled and transferred to 50-mL conical tubes. The cells were pelleted by centrifugation at 3000 rpm. Cell pellets were washed with Hank’s balanced salt solution and then KPFM (50 mM potassium phosphate, pH 7.4, 50 mM sodium fluoride, 1 mM EDTA, 1 mM EGTA, and 12 mM monothioglycerol), with centrifugation between washes. To make whole-cell extract, the cells were suspended in KPFM containing 0.5 M NaCl plus a mixture of protease and phosphatase inhibitors (18) and homogenized by 50 strokes in a glass-Teflon homogenizer. The homogenates were diluted 1:1 with KPFM buffer to reduce the salt concentration, transferred to Tip95 tubes, and centrifuged at 40,000 rpm for 30 min. The extract supernatant was directly added to the prepared antibody-coupled Sepharose beads.

**Immunoprecipitation and Gel Separation of PR**—Protein A-Sepharose was preswollen in water and washed with PBS. Per six-flask pool, 200 μl of bead suspension in PBS (3:1 beads/buffer) were incubated with 25 μg of rabbit anti-mouse for 4 h on a rocking platform. The beads were then washed twice with PBS, 40 μg (per six-flask pool) of the antibody-bead suspension was added to the bead suspension and incubated overnight at 4 °C on a rocking platform. The antibody-conjugated beads were washed once with PBS and twice with TEG buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 10% glycerol). The cell extract was then added to the prepared beads and incubated overnight at 4 °C on a rocking platform. The beads were washed once with PBS and twice with TEG and buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 10% glycerol) with centrifugation at 40,000 rpm to remove nonspecific proteins. PR was extracted from the beads by heating at 100 °C in 1× SDS-sample buffer and loaded on a 6.5% SDS-polyacrylamide gel. Labeled PR was detected by autoradiography.

**Protease Digestion of His Tag PR for Mass Spectrometric Analysis**—Purified His tag PR was dialyzed for 8 h against 50 mM ammonium bicarbonate in a Tube-O-Dialyzer (Research Products International Corp., Mount Prospect, IL). PR (40 μg) was digested with 1 μg each of trypsin, Asp-N, and Glu-C for 4 h at 37 °C. An additional 1 μg of each enzyme was added after 4 h, and incubation continued for an additional 4 h. The digest was then dried in a SpeedVac.

**FeNTA Isolation of Phosphopeptides**—FeNTA columns were prepared and used essentially as described (46) by sequentially washing a 0.5-mL Ni^2+^-NTA-Sepharose column with 2 mL of H_2O, 2 mL of 0.1 M EDTA, pH 7.4, 2 mL of H_2O, 2 mL of 0.1 M HOAc, 2 mL of 0.1 mM ferric chloride in 0.1 M HOAc and 2 mL of 0.1 M HOAc. Dry digests were dissolved in 0.1 M HOAc and loaded on the column. The column was sequentially washed with 2 mL of 0.1 M HOAc, 2 mL of H_2O, and 2 mL of 0.1 M Fe(II)EDTA, pH 8.0. The phosphopeptides were eluted with 2 mL of 0.1% ammonium acetate, pH 9.5. Samples were desalted using C_{18} resin prior to analysis.

**Mass Spectrometry**—Analyses were performed on a PE Scieix (Foster City, CA) API 3000 tandem quadrupole mass spectrometer equipped with a Protana (Odense, Denmark) nanoelectrospray source. The samples were dissolved in an aqueous solution of 50% methanol and 1% formic acid, and 2–3 μl were deposited in the gold/palladium-coated glass nanoelectrospray capillaries. The samples were analyzed for phosphorylated peptide identification using negative ion precursor ion scanning for m/z 79.1 with nitrogen as the collision gas and a collision energy of 100 eV. Positive ion full scanning spectra were then recorded using ultraviolet light at 200 nm as the mass filter and a cone potential of 70 V. Product ion spectra of the appropriate positively charged precursor ions were then recorded to identify the site of phosphorylation with nitrogen as the collision gas and collision energies of 20–40 eV.

**RESULTS**

**Identification of Sites Phosphorylated by Cdk2**

**HPLC Analysis**—Baculovirus-expressed His tag human PR (A or B) (1 μg) was phosphorylated by cyclin A-Cdk2 as described under “Experimental Procedures” and isolated by SDS-PAGE, and the PR was in-gel digested using modified trypsin. The tryptic digest was loaded on a C_{18} reversed-phase HPLC column that yielded the phosphopeptide patterns seen in the two lower panels in Fig. 1. Comparing the phosphopeptide profiles of PR-B and PR-A obtained with the new digestion procedure (panels III and IV) to the previous Cdk2 profile obtained with the original procedure (panel II), we observed several novel late eluting phosphopeptide peaks (Fig. 1, peaks A–D) as well as the previously identified peaks 1 (Ser^{186}), 2 (Ser^{400}), and 4/6 (Ser^{162}). These new peaks (A–D) are specific to Cdk2, since inclusion of the Cdk2 inhibitor roscovitine in the phosphorylation reaction blocks their induction (data not shown). One of these peaks, peak C, is clearly found only in PR-B (arrow). Comparing the new Cdk2 phosphopeptides, peaks A–D (shown in panel III), with the previously published profile of phosphopeptides from in vivo labeled PR-B treated with R5020 (panel I), we observe that the Cdk2 sites represent only a subset of the total sites found in vivo. Several of the major phosphopeptide peaks from the in vivo profile have not yet been identified. Interestingly, although the elution times vary slightly due to a change in the HPLC apparatus used, peaks with elution positions comparable with peaks A–D are detected in the in vivo profile, suggesting that they are previously unidentified authentic sites. As demonstrated below, even the modified cleavage procedure is insufficient to produce complete cleavage of resistant sites; thus, the magnitude of the peaks cannot be used as an indication of the extent of phosphorylation of these sites.

**Phosphopeptide Analysis**—To identify these new phosphopeptides, we used a combination of manual Edman degradation, secondary endoproteinase digestion, and phosphoamino acid analysis. Table I displays the data from the manual Edman degradation of the phosphopeptides in peaks A–D. Peak A and B both release in cycle 2, with peak B having an additional smaller release in cycle 8. Peak C has a weak release in cycle 15, and peak D releases in cycle 4. Weaker releases are expected in late cycles due to gradual loss of yield due to incomplete coupling and cleavage as well as the low efficiency of manual Edman degradation when the reactions are not performed under an inert atmosphere (40).

To further characterize these phosphopeptides, secondary digests were performed with the endoproteinases Asp-N and Glu-C followed by separation on a 40% acrylamide peptide gel. Glu-C digests were also split for manual Edman degradation and peptide gel analysis. Table II summarizes the manual Edman degradation data and secondary digest analysis.

Peptide gel analysis of peak A indicated the presence of a phosphopeptide that did not cleave with Asp-N but cleaved well with Glu-C. There was no change in the cycle of release after Glu-C digestion (cycle 2). Since the possible candidates did not include a Ser-Pro motif (a Ser-Thr-Pro sequence is required for phosphorylation by Cdk2), phosphoamino acid analysis was performed. Threonine was the predominant residue phosho-
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Fig. 1. Detection of new phosphopeptide peaks in PR phosphorylated by Cdk2 in vitro by cyclin A-Cdk2 as described under “Experimental Procedures.” PR was separated from the kinase complex on a 6.5% SDS-polyacrylamide gel, and the phosphorylated band corresponding to PR was excised and subjected to trypsin digestion. The phosphopeptides were separated by reversed-phase HPLC and detected by an online radioactivity detector. The phosphopeptide profiles are shown here. Panel I shows the previously published phosphopeptide profile of PR-B from in vivo labeled T47D cells, which shows all of the in vivo phosphopeptides identified to date (reproduced and modified with permission of the Endocrine Society from Fig. 2 of Zhang, Y., Beck, C. A. Poletti, A., Clement, J. P., IV, Prendergast, P., Yip, T.-T., Hutchens, T. W., Edwards, D. P. and Weigel, N. L. (1997) Phosphorylation of Human Progesterone Receptor by Cyclin-dependent Kinase 2 on Three Sites That Are Authentic Basal Phosphorylation Sites in Vivo. Mol Endocrinol. 11, 823–832. These in vivo Cdk2 phosphorylation sites, Ser108, Ser162, and Ser250, have also been identified in vivo (see panel I). Panels III and IV show the new phosphopeptide profiles obtained by Cdk2 phosphorylation of His tag PR-B (III) and His tag PR-A (IV) after digestion with trypsin. Several new peaks were detected (peaks A–D) after utilizing the new trypsinization method (compare panels II and III). Comparison of the new PR-B (panel III) and PR-A (panel IV) profiles identifies a peak (C) that is not present in the PR-A profile (arrow). Additionally, these new peaks A–D (panel III) elute in similar positions to smaller peaks from the in vivo profile (panel I), consistent with peaks A–D containing authentic in vivo sites. The peak preceding peak 1 in the PR-A profile has not yet been identified.

Peaks B and C elute early from the HPLC column. Peak B contains two phosphopeptides as detected by peptide gel analysis. Phosphoamino analysis indicates that serine is the predominant modified residue (data not shown). By collecting individual fractions under the peak, the two phosphopeptides could be partially separated. In this case, the fraction containing the phosphopeptide with the cycle 2 release (B2) cleaved with both Asp-N and Glu-C. Of the three peptide candidates with a cycle 2 release that also contain both Asp and Glu residues, only two peptides in PR contain a serine in position 2 followed by a proline (Fig. 3A). Furthermore, since the first candidate peptide (containing Ser108) is from the region unique to PR-B, the candidate can be eliminated because the phosphopeptide was observed in both PR-A and PR-B profiles. Additionally, the Ser345-containing peptide is not a likely candidate, since we have previously shown that this phosphopeptide elutes much earlier than Peak B (see Fig. 1I). Examination of the remaining candidate peptide containing Ser213 reveals that digestion with Glu-C would greatly change its mobility, whereas the Ser345 peptide would be resistant to cleavage due to the location of the glutamic acid. Glu-Pro motifs are inefficiently cleaved, especially at the carboxyl terminus of a peptide (40). Glu-C digestion of this peptide results in a faster migrating phosphopeptide (data not shown); thus, the phosphorylated peptide can only be Ser213. In the case of the phosphopeptide with a cycle 8 release (B8), digestion of the entire peak 8 region with Glu-C prior to manual Edman degradation converts the cycle 8 to a cycle 4 release, while the cycle 2 release remained unchanged as expected (Fig. 3C). These data conclusively identify the peak B8 phosphopeptide from the candidates (Fig. 3B). Only the peptide containing Ser554 would cleave to produce a peptide with a cycle 4 release. Therefore, the two phosphorylated peptides in peak B induced by Cdk2 in vitro correspond to the peptides containing Ser213 and Ser554.

Peak C, observed solely in the PR-B profile, exhibits a weak release on cycle 15 by manual Edman degradation. Only two of the three candidate peptides with cycle 15 releases are located in the B-specific region of PR (Fig. 4A). Of these, only Ser25 is contained within a Ser/Thr-Pro motif. Examination of the sequence of the tryptic peptide containing Ser25 shows that digestion of the tryptic peptide with Glu-C should convert the cycle 15 release to a cycle 3 release. Indeed, we observe a cycle 3 release after Glu-C digestion (Fig. 4B), whereas the other B-specific peptide candidate (which does not contain the required Ser-Pro motif) would produce a release in cycle 4. These results identify the phosphorylated site in peak C as Ser25.

Peak D elutes very late in the profile, suggesting a large and hydrophobic peptide. Manual Edman degradation data show that the phosphoamino acid is in position 4. Of the eight candidates with Ser in position 4, only one contains a Ser-Pro motif. Secondary digestion with either Asp-N or Glu-C causes a significant change in mobility of the peptide observed by peptide gel analysis (Fig. 5B). Of the two peptides with a serine in position 4 that contain both Asp and Glu residues (Fig. 5A), only the Ser676 site is consistent with these results. First, Ser676 is the only site located within a Ser-Pro motif, and second, although the peptide containing Ser711 contains a gua-
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Identification of Additional in Vivo Phosphorylation Sites in PR Expressed in Sf9 Cells

Most of the phosphorylation sites identified in steroid receptors are in Ser/Thr-Pro motifs. Human PR contains 15 of these motifs, of which six have been shown previously to be phosphorylated (15–17). An analysis of the remaining sites reveals that conventional tryptic digests would produce peptides ranging from about 30 to 70 amino acids in length, too long for optimal binding and elution from a C18 column. Our previous radiolabeling studies had indicated that PR expressed in Sf9 insect cells exhibited the same phosphopeptide map as PR isolated from T47D cells (36). We therefore took advantage of the selectivity of mass spectrometers to look for additional phosphorylation sites in baculovirus-expressed PR isolated from Sf9 cells. Initial analysis of a tryptic digest of purified recombinant PR-B in precursor ion mode revealed a number of potential phosphorylated candidates, but the spectrum in positive ion mode was too complex to successfully isolate phosphorylated peptides for sequence analysis. Fractionation of the digest into 15 fractions by C18 reversed-phase HPLC revealed a novel, large phosphopeptide eluting at 50% acetonitrile. Negative ion precursor ion scan of the mass deconvoluted spectrum displayed a charge distribution series corresponding to [M + H]+, [M – 1H]+, [M – 2H]2+, [M – 3H]3+, and [M – 4H]4+. The mass deconvoluted spectrum displayed a peptide of mass 5481.30, corresponding to amino acids 107–159, which contained a single phosphorylation. This peptide contains multiple serines including Ser136, which resides in a Ser-Pro motif (data not shown). Since none of the serines in this region of PR have been previously identified as phosphorylation sites, this must represent a novel site. Phosphopeptides with masses consistent with peptides containing previously identified sites, Ser162 and Ser345, were also detected.

In subsequent analyses, ferric ion affinity resin was used to preferentially absorb the phosphopeptides from digests. Purified recombinant PR-B was digested with trypsin, Asp-N, and Glu-C, and the phosphopeptides were purified on the ferric ion affinity resin as described under “Experimental Procedures.” Fig. 6A shows the full scan of the doubly charged peak at m/z 598 yielded a peptide of mass 5481.30, corresponding to amino acids 107–159, which contained a single phosphorylation. This peptide contains multiple serines including Ser136, which resides in a Ser-Pro motif (data not shown). Since none of the serines in this region of PR have been previously identified as phosphorylation sites, this must represent a novel site. Phosphopeptides with masses consistent with peptides containing previously identified sites, Ser162 and Ser345, were also detected.

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reveals that it corresponds to 672–679 produced by a double trypsin and Asp-N cut, which contains phosphoserine at position 676 (Fig. 7B). Previously identified phosphopeptides detected in this analysis included the doubly charged peptide at m/z 492 identified as the phosphotryptic peptide containing Ser162, the doubly charged peptide at m/z 571 identified as the phosphotryptic peptide containing Ser102, and the phosphopeptide at m/z 680 as the phosphopeptide containing Ser190 (data not shown).

Analysis of T47D Receptor for Newly Identified Sites
To determine whether any of the newly detected sites phosphorylated by Cdk2 in vitro are in vivo phosphorylation sites, we performed in vivo labeling of endogenous PR from T47D cells in the absence and presence of hormone. PR was purified by immunoprecipitation, separated by SDS-PAGE, and processed as previously indicated for the in vitro samples. HPLC peaks with identical retention times from in vivo and in vitro samples were analyzed simultaneously by alkaline peptide gel electrophoresis. Most of the analyses were inconclusive, presumably due to the poor efficiency of cleavage and extraction from gel slices and the background from additional peptides. However, comparison of the HPLC fractions from in vivo labeled PR with the same retention time as the fractions containing Ser213 phosphorylated by Cdk2 reveal that they contain a phosphopeptide with the same mobility on alkaline gels as the phospho-Ser213 peptide (Fig. 8). To determine whether phosphorylation of this site is strictly hormone-dependent or whether the site is basally phosphorylated, its phosphorylation was compared in the presence and absence of hormone with that of the Ser162 phosphopeptide, which has previously been shown to be a basal phosphorylation site and whose phosphorylation is increased upon treatment with hormone. Fig. 8 shows that the relative change in intensity of Ser213 phosphorylation as a result of hormone treatment is similar to that of Ser162. These data suggest that Ser213 is phosphorylated in vivo in T47D cells and has the characteristics of a basal phosphorylation site.

DISCUSSION
The presence of unidentified peptides in our phosphopeptide maps (17) and the failure to identify Ser676 (the conserved Ser-Pro that is homologous to the Ser530 hinge site in chicken PR (37)) as a phosphorylation site led us to develop new means to identify phosphorylation sites in PR. An analysis of PR sequence revealed that the Ser-Pro sequences that had not been identified as phosphorylation sites are likely to reside in large peptides produced by incomplete digestion at resistant trypsin cleavage sites. Using trypsin modified to prevent au-
Results

marked with arrows in positive ion mode. The eluate from the affinity column was resuspended and analyzed either in negative ion mode (A) or using negative ion precursor ion scanning for m/z 79.1 (B) as described under “Experimental Procedures.” Peaks marked with arrows represent phosphopeptides identified (see “Results”).

tolysis as well as other proteases, we have identified Ser\textsuperscript{676} as a phosphorylation site in PR by mass spectrometry and have shown that it is a substrate for Cdk2. Using these techniques, we have also identified five other candidate phosphorylation sites. We previously identified three phosphorylation sites, Ser\textsuperscript{162}, Ser\textsuperscript{190}, and Ser\textsuperscript{400}, which were phosphorylated by Cdk2 in vitro (17). Using the specially modified trypsin that cleaves Lys-Pro and Arg-Pro sequences at a low but detectable efficiency, we have detected four small, late eluting peaks in the HPLC profile and have identified five additional Cdk2 sites in these peaks. Our studies here underscore the difficulty in detecting phosphorylation sites in large peptides produced by incomplete digestion. Comparison of the trypsin we used in our previous studies with the new modified trypsin showed that there was a significant difference in the phosphorylpeptide peaks detected by HPLC. Although the new peaks appear to be minor compared with those previously identified, Arg/Lys-Pro motifs will still be poorly cleaved relative to other trypsin cleavage sites (40). Evidence that we are only achieving partial cleavage is shown in Fig. 2. Redigestion of the purified peptide with excess Arg-C cleaves the resistant bond. Therefore, peak size cannot be considered representative of the extent of phosphorylation for peptides bounded by these difficult cleavage sites. This problem probably contributes to our failure to unambiguously identify most of these new peptides in digests of T47D receptor. The increased difficulty in releasing large peptides from gel slices coupled with a higher background as a well as phosphorylation by other kinases in vivo greatly increases the complexity of the analyses. For example, although some of the new peptides elute in positions similar to peaks 10 and 11, our previous characterization of peptides derived from PR from T47D cells suggests that there is at least one additional phosphopeptide in this region that has not been identified.

Employing the techniques of mass spectrometry to analyze peptides derived from PR expressed in SF9 cells and in vivo labeling of T47D breast cancer cells, we have identified two new phosphorylation sites (Ser\textsuperscript{20} and Ser\textsuperscript{676}) and two novel phosphopeptides (a peptide containing Ser\textsuperscript{130} and a peptide that comigrates with the Cdk2-phosphorylated Ser\textsuperscript{213}-containing peptide). The mass spectrometric analyses have allowed us to identify additional phosphorylation sites in PR as well as to confirm previously identified sites. However, mass spectrometric methods also have limitations, requiring purified protein in larger quantities than the analyses following peptides only by radiolabel. For our studies, we used His tag PR amplified by baculovirus infection of SF9 cells and purified by affinity column to achieve the amount and quality needed for analysis. Although we could detect phosphopeptides in a negative ion precursor ion scan, we found that some form of fractionation of peptides was required to enrich for phosphopeptides for sequencing by tandem mass spectrometry. FeNTA chromatography allowed us to preferentially obtain the phosphopeptides. Although this approach was useful in obtaining phosphopeptides of moderate length, we did not detect the very large phosphopeptides arising from incomplete digestion with trypsin in the FeNTA column eluate.

Previous studies from our laboratory have shown that in vivo phosphorylation patterns of PR from hormone-treated T47D and baculovirus-infected SF9 cells are indistinguishable, and no aberrant phosphorylation was observed in SF9 cells (36). Therefore, we expect that the new phosphorylation sites identified by mass spectrometry are also present in receptor expressed in T47D cells. The poor yield of these peptides suggests that it will be difficult to detect the sites in T47D PR either by radiolabeling or by mass spectrometry of purified protein. Phosphorylation site-specific antibodies have been produced to the Ser\textsuperscript{190}...
and Ser\textsuperscript{294} sites, and we have utilized them to study hormone-
dependent phosphorylation of PR in T47D cells (45). Therefore,
in the case of many of these new phosphorylation sites, gener-
ation of phosphorylation site-specific antibodies will be useful
in confirming their phosphorylation
\textit{in vivo} and studying their
regulation by hormone and cell signaling pathways.

Including the three sites previously identified, our laboratory
has identified a total of eight sites phosphorylated by Cdk2
\textit{in vitro}; five of these have been confirmed
\textit{in vivo} (162, 190, 213, 400, and 676). Notably, only subsets of the 15 Ser-Pro motifs in
PR are substrates for Cdk2 phosphorylation, illustrating the
site-specific discrimination by Cdk2. In fact, we observed that
Cdk2 phosphorylates Ser\textsuperscript{25}, but not Ser\textsuperscript{20}, despite the fact that
both phosphorylation sites are located within the same tryptic
peptide (Table I and Fig. 4). Seven of the sites phosphorylated
by Cdk2 \textit{in vitro}, Ser\textsuperscript{25}, Ser\textsuperscript{162}, Ser\textsuperscript{190}, Ser\textsuperscript{213}, Ser\textsuperscript{400}, Thr\textsuperscript{430},
and Ser\textsuperscript{676}, are situated within the phosphorylation-rich A/B
domain (Fig. 9). Interestingly, both Thr\textsuperscript{430} and Ser\textsuperscript{554} border
the ligand-independent activation domain AF-1. Perhaps most
significantly, Ser\textsuperscript{554} is located within only 13 amino acids of
the DNA binding domain. The proximity of these sites to the func-
tional domains suggests their potential ability to regulate or be
regulated by activation or DNA binding.

Only one Cdk2 site, Ser\textsuperscript{676}, was identified outside of the A/B
domain, located in the hinge region between the DNA and
ligand binding domains (Fig. 9). Ser\textsuperscript{676} is analogous to the
\textit{in vivo} phosphorylation sites Ser\textsuperscript{530} identified in chicken PR (37)
and Ser\textsuperscript{650} in human androgen receptor (26). Ser\textsuperscript{676} was not
detected as a phosphorylation site in a CNBr digest of radiola-
beled PR (13). However, this peptide would have been rather
small (about 6500 kDa) for detection by SDS-polyacrylamide
gel electrophoresis followed by nitrocellulose transfer and
would have contained only one site, whereas the other peptides
detected contain multiple (up to five) sites. In androgen recep-
tor and chicken PR, mutation of the serine to alanine resulted
in decreased transactivation (26); however, for chicken PR, the
effect was only observed at subsaturating hormone conditions
(47). Interestingly, this site in human PR has been mutated,
and the effect of an alanine substitution was examined. De-

\begin{table}[h]
\centering
\caption{New \textit{in vivo} phosphorylation sites identified by
\textit{mass spectrometry (MS)}}
\begin{tabular}{lll}
\hline
Peptide & m/z & Identity \\
\hline
Peptide 1 & 598 & APHVAGGPP(S)PE\textsuperscript{a} Ser\textsuperscript{20} \\
Peptide 2 & 863 & FTF(S)PGQ Ser\textsuperscript{676} \\
\hline
\end{tabular}
\textsuperscript{a}pS, phosphoserine.
\end{table}
creases in activity range from 20 to 50% depending upon the cell and promoter context, suggesting that this is an important site in human PR (21). We have confirmed by mass spectrometry that Ser<sup>676</sup> is an in vivo phosphorylation site. Due to its proximity to the LBD, it is tempting to speculate that agonist-and antagonist-induced conformational changes could dramatically alter the phosphorylation of Ser<sup>676</sup>.

The role of Cdk2 phosphorylation in receptor function has been examined for several steroid receptors. In the case of the human estrogen receptor, overexpression of cyclin A, a regulatory partner of Cdk2, increases estrogen receptor-mediated transactivation (28). Furthermore, two serine residues, Ser<sup>104</sup> and Ser<sup>106</sup>, have been shown by in vitro phosphorylation and site-directed mutagenesis studies to be involved in this pathway (30). Rat glucocorticoid receptor has also been shown to be a substrate for Cdk2. In vitro, Cdk2 complexes modify two serines, Ser<sup>224</sup> and Ser<sup>392</sup> (27). Analysis of glucocorticoid receptor transactivation in yeast reveals that hormone-induced transactivation is greatly diminished by perturbation of the Cdk pathway (27). Since Cdk2 and its cyclin partners are key cycle-dependent (48). All of this evidence points to a role for Cdk2-cyclin complexes in regulating steroid receptor function via the Cdk pathway (27).

In summary, five new phosphorylation sites have been identified in PR (Fig. 9). Five of these are phosphorylated in vitro by Cdk2. One Cdk2-induced site, Ser<sup>676</sup>, as well as two B-specific phosphorylation sites, Ser<sup>30</sup> and a peptide containing the putative site Ser<sup>130</sup>, were identified as in vivo phosphorylation sites by mass spectrometric methods. The phosphopeptide containing the newly identified Cdk2 site Ser<sup>131</sup> exhibited characteristics of a basal phosphorylation site in vivo. Identification of these additional phosphorylation sites in PR argues that we have only begun to understand the complex nature of PR phosphorylation.

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