Identification of Phosphorylation Sites Unique to the B Form of Human Progesterone Receptor

IN VITRO PHOSPHORYLATION BY CASEIN KINASE II*

Yixian Zhang, Candace A. Beck†, Angelo Poletti‡, Dean P. Edwards§, and Nancy L. Weigell

From the Department of Cell Biology, Baylor College of Medicine, Houston, Texas 77030 and the Department of Pathology and Molecular Biology Program, University of Colorado Health Sciences Center, Denver, Colorado 80262

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The human progesterone receptor (PR), a member of the steroid/thyroid receptor superfamily of ligand-activated transcription factors, is expressed in most tissues as two forms that exhibit differential transcriptional activation potentials, full-length PR-B and NH₂-terminally truncated PR-A. In human breast cancer cells (T47D) both forms of PR are constitutively phosphorylated but phosphorylation is increased in response to hormone treatment, suggesting that this modification has a role in regulating the activation state of the receptor. To more directly define the functional role of phosphorylation in the action of A and B receptors requires knowledge of the phosphorylated amino acid residues and the protein kinase(s) involved. Toward this end we have developed a strategy that combines isolation of PR phosphotryptic peptides by reverse phase high performance liquid chromatography, secondary analytical protease digestion, manual Edman degradation, and release of ³²P that resulted in identification of two major phosphorylation sites, Ser⁶⁵ and Ser¹⁶⁹. Both sites are located in the amino-terminal region unique to PR-B, and one of these sites (Ser⁶⁵) is encompassed in a casein kinase II (CKII) consensus sequence. Although human PR contains 11 potential CKII consensus sequences, CKII in vitro phosphorylated purified PR-B only at Ser⁶⁵ suggesting that this may be an authentic site for CKII in vivo.

Progestosterone receptors (PR)¹ belong to a large family of ligand-activated transcription factors (1). PR in most species are expressed as two isoforms, PR-A and PR-B. Both forms are produced from a single gene either from separate messenger RNAs that arise by alternate use of two promoters or by initiation of translation from a second start site on a single PR mRNA (2-4). PR-A is an NH₂-terminally truncated version of PR-B.

PR in chicken oviduct (5, 6), rabbit uterus (7), and human breast cancer cells (8-10) are phosphorylated in the absence of hormone and undergo an increase in phosphorylation upon hormonal stimulation. Treatment of T47D human breast cancer cells with a progestin induces a 2-fold increase in net phosphorylation of human PR (hPR), and a characteristic decrease (or upshift) of receptor mobility on SDS-gel electrophoresis (11). A time course study under steady-state labeling conditions elucidated a two-phase phosphorylation mechanism; a rapid phosphorylation that occurs between 5 and 10 min after addition of hormone, accounting for most of the net increase, followed by the PR upshift that begins at 20 min and requires 40-60 min for completion. Interestingly, the phosphorylation associated with PR upshifts occurs with little additional change in net ³²P incorporation (11). Additional data have suggested that this late phase of phosphorylation may be both hormone- and DNA-dependent (12, 13).

It is becoming increasingly evident that phosphorylation of PR, as well as other steroid receptors, plays a role in regulating the activity of the receptors. Modulators of protein kinases and phosphatases such as 8-bromo-cAMP; okadaic acid, calyculin, vanadate, and epidermal growth factor have been used to assess the role of phosphorylation in regulating the activity of PR (ePR) in transient transfection assays (14). These compounds are capable of activating receptor in the absence of hormone (14). This striking ligand-independent activation has also been reported for several other steroid receptors (15-18). In contrast to ePR, human PR does not appear to be susceptible to ligand-independent activation by modulators of protein phosphorylation. However, modulators such as cAMP, okadaic acid, and phorbol esters do potentiate hormone-dependent activation of human PR (11). Interestingly, the progesterone receptor antagonist RU486 can be converted to a partial but potent agonist by treating cells with 8-bromo-cAMP (19).

Whether this antagonist to agonist switch is the consequence of altered receptor phosphorylation or phosphorylation of another protein involved in PR-mediated transcriptional enhancement remains to be determined.

There is increasing evidence that PR-A and PR-B have distinct functional properties that are dependent on the cell type and target genes with which they interact. PR-B when expressed alone in HeLa cells was reported to be able to mediate partial agonist activity of RU486 (20, 21), whereas PR-A alone was not capable of doing this. In addition, PR-A has been reported to be capable of exerting dual functional roles; to serve as a positive transcriptional activator or as a repressor (21, 22). In cells and target genes where PR-A exhibits no transcriptional activation properties, it has been reported to act as a repressor of PR-B-mediated transcription. Surprisingly, PR-A but not PR-B, can also act as a trans-repressor of glucocorticoid, androgen, and mineralocorticoid receptor-mediated transcription in a cell type and promoter-dependent manner (22). The mechanism for these distinct functional properties of PR-A and PR-B is not known. Clearly, the different NH₂-terminal se-

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† To whom correspondence should be addressed. Tel.: 713-798-6234; Fax: 713-790-1275.

‡ The abbreviations used are: PR, progesterone receptor; hPR, human PR; ePR, chicken PR; MEM, minimal essential medium; EDAC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; CKII, casein kinase II; HPLC, high performance liquid chromatography; Mes, 4-morpholineethanesulfonic acid; MAP, mitogen-activated protein.

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quences present in PR-A and PR-B must play a role in directing the distinct activities of the two PR isoforms.

Although the phosphorylation is thought to have a regulatory function, the role of specific phosphorylation sites in PR function is still poorly understood. One of our laboratories has identified four phosphorylation sites in cPR (23, 24). All four sites are on serine residues that are common to both cPR, and cPR. Two sites (Ser<sup>167</sup> and Ser<sup>206</sup>) are hormone-dependent phosphorylation sites. The phosphorylation of human PR is not as well characterized. Initial phosphopeptide mapping analysis by Sheridan et al. (8) showed that hPR phosphorylation is more complex than cPR. They reported that there are at least five constitutive phosphopeptides common to PR-A and PR-B and a sixth one is unique to PR-B. One additional phosphopeptide, and possibly a second, were detected only after hormone treatment. In addition, all phosphorylations are on serine residues. The exact location of hPR phosphorylation sites, however, has not been reported.

In the present study, we have identified two major phosphopeptides which are unique to the B form of hPR and have developed a simple strategy that should be applicable to other phosphorylation studies. We have searched for phosphopeptides which exhibit a minimum amount of [<sup>32</sup>P]-labeled protein to identify phosphorylation sites. Using this strategy, we have identified Ser<sup>81</sup> and Ser<sup>167</sup> as the major PR-B specific phosphorylation sites. In addition, we have performed in vitro phosphorylation studies with casein kinase II (CKII) and purified PR-B showing that CKII preferentially phosphorylates Ser<sup>81</sup>, which is part of a consensus sequence for CKII.

**EXPERIMENTAL PROCEDURES**

**Materials**

- R5020 and carrier free [<sup>32</sup>P]H<sub>3</sub>P0<sub>4</sub> were obtained from DuPont-NEN. (Y-<sup>32</sup>P]ATP was purchased from ICN (Irvine, VA). Probin A-Sepharose was obtained from Pharmacia Biotech Inc. (Piscataway, NJ). Alkaline phosphatase, a phosphodiesterase, and a 5'-nucleotidase were purchased from Miles (Elkhart, IN). Casein kinase II was purchased from Promega (Madison, WI). Minimum essential medium (MEM) was purchased from Irvine Scientific (Santa Ana, CA). Phosphate-free MEM was obtained from Life Technologies, Inc. All other chemicals were reagent grade.

**Purification of Bovine Hormone PR B-Form—Human PR-B was used in protein sequencing experiments and for in vitro phosphorylation. It was purified and fractionated as a full-length recombinant protein from the baculovirus expression system as described previously (26, 27). Purification was by single-step monoclonal antibody affinity chromatography using the B-30 antibody specific for PR-B (25). As judged by SDS-polyacrylamide gel electrophoresis on 7.0% discontinuous SDS-polyacrylamide gels as described previously (11), [<sup>32</sup>P]-Labeled peptides were identified on-line with a model IC Flo-One radioactive flow detector (Radiomatic Instruments, Inc., Tampa, FL) (23).

Characterization of Tryptic Phosphopeptides by Protease Digestion and Manual <sup>32</sup>P Release—Fractions containing tryptic phosphopeptides resolved by reverse phase HPLC were further separated by electrophoresis on a 40% alkaline polyacrylamide gel (28). The gel was dried and autoradiographed. Bands containing tryptic phosphopeptides were excised and eluted with H<sub>2</sub>O overnight, and dried in a Speedvac. Eluted peptide samples were subsequently analyzed for tryptic phosphopeptides by reverse phase HPLC using a 50% formic acid and 50% ammonium bicarbonate solution. All phosphopeptides were subjected to digestion with the endopeptidase Lys-C. Eluted fractions were analyzed by manual Edman degradation and sequencing-grade trypsin (27).

**In Vitro Phosphorylation of PR-B with Casein Kinase II**—Purified PR-B and purified [<sup>32</sup>P]atriophosphate in KPFM buffer were incubated at 37 °C for 6 h at 37 °C. Cells were then incubated with [<sup>32</sup>P]atriophosphate in KPFM buffer and the supernatant (whole cell extract) was dialyzed against KPFM to reduce salt concentration before immunoprecipitation.

**Immunoprecipitation and Gel Purification of PR—**Protein A-Sepharose was prebound with the receptor-specific monoclonal antibody, AB-52 (25) as described previously (11). Receptors were eluted with SDS sample buffer and electrophoresed on 7.0% discontinuous SDS-polyacrylamide gels as described previously (11). [<sup>32</sup>P]-Labeled receptors were detected by autoradiography of wet gels and the gel pieces corresponding to the PR-A and PR-B isoforms were excised and incorporated radioactivity was measured by Cerenkov counting of the gel pieces.

**HPLC Analysis of Trypsin-digested PR—**SDS gels containing PR were cut and placed in 1.5 ml microcentrifuge tubes, washed with 50 mM Tris-<sub>HCl</sub>, pH 8.0, for 10 min, and then with 50 mM Tris-<sub>HCl</sub>, pH 8.0, for 10 min. Twenty µg of trypsin were added to the gels followed by 500 µl of 50 mM ammonium bicarbonate and incubated at 37 °C; four additional aliquots of trypsin were added at 1.5-h intervals. Tryptic phosphopeptides were separated by reverse phase HPLC using a microbore, C<sub>18</sub>, reverse phase column maintained at 37 °C. The column was eluted with a linear gradient of acetonitrile in 0.1% trifluoroacetic acid in water, run at a flow rate of 1 ml/min, and eluted with a linear gradient from 0% to 45% acetonitrile over 90 min. [<sup>32</sup>P]-Labeled phosphopeptides were identified on-line with a model IC Flo-One radioactive flow detector (Radiomatic Instruments, Inc., Tampa, FL) (23).

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forms were separated by SDS-gel electrophoresis and eluted.

Phosphopeptides were metabolically labeled to steady-state in T47D cells as described previously (11). Radiolabeled receptors were immunopurified and separated by C18 reverse phase HPLC on a C18 reverse phase column. Shown in Fig. 1 are tryptic phosphopeptide maps of PR-A and PR-B, which were separated by HPLC and detected with an on-line radioactivity detector. The majority of the 32P in peptide 3 and peptide 6 was released at cycle 8 and cycle 3, respectively.

In addition, to determine the position of the phosphoamino acid in tryptic peptides 3 and 6, manual Edman degradation was performed. Since HPLC peaks 3 and 6 each contain only one phosphopeptide as judged by alkaline gel electrophoresis (Fig. 2), samples from the HPLC analysis were used for this experiment. Fig. 4 shows the cycle at which 32P was released. Therefore, we decided to test CKII for its ability to phosphorylate peptide 3 and to confirm the deduced identity of peptide 6, we have performed amino acid sequencing of peptide 3 and 6 using purified baculovirus PR-B as the source of receptor. After tryptic digestion, peptides were separated by HPLC and fractions which had the same retention time as either [32P]phosphopeptide 3 or peptide 6 of PR-B from T47D cells were collected, and the amino acid sequence was determined by an automated microsequencer. The sequencing results shown in Table II reveal that peptide 3 contains the sequence beginning with residue 74. However, these sequences did not match with the 32P release and endoprotease digestion results. Thus, the secondary protease digestion, 32P release, and amino acid sequencing results collectively provide unambiguous identification that Ser102 is the only possibility for phosphorylation of peptide 6.

To distinguish between the two likely possibilities for peptide 3 and to confirm the deduced identity of peptide 6, we have performed CKII assays we decided to test CKII for its ability to phosphorylate PR-B in vitro. As a substrate for in vitro kinase assays we utilized a highly purified preparation of human PR-B expressed as a full-length recombinant protein in a baculovirus system (27). The receptor was phosphorylated as described under "Materials and Methods" and separated by SDS-gel electrophoresis. Phosphorylated PR-B was detected by autoradiography and quantified by counting the radioactivity in the band containing labeled PR-B. The stoichiometry of receptor phosphorylation (>25%) was calculated based on the total amount of PR-B used and moles of phosphate incorporated. Fig. 5 shows that PR-B was readily phosphorylated only in the presence of CKII, indicating that there is no endogenous kinase activity.
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**FIG. 2.** Alkaline polyacrylamide gel electrophoresis of tryptic phosphopeptides. The phosphopeptides isolated by HPLC were separated by electrophoresis on a 40% polyacrylamide gel and detected by autoradiography. The PR-A (A) and PR-B (B) peptides with the same retention times on HPLC were run in parallel.

**FIG. 3.** Characterization of tryptic phosphopeptides by redigestion with additional proteases. The indicated tryptic peptides were treated with Asp-N which cleaves on the amino-terminal side of aspartic acid or with Glu-C which cleaves on the carboxyl-terminal side of glutamic acid and analyzed by polyacrylamide gel electrophoresis.

**FIG. 4.** Identification of the cycle containing phosphoserine by manual Edman degradation. Peptides 3 and 6 were covalently coupled to arylamine membrane discs using carbodiimide and subjected to manual Edman degradation. The radioactivity in the released amino acid was determined after each cycle using a scintillation counter. The background count (24 ± 4, n > 10) was not subtracted from all the counts. The cycle containing the released 32P is the cycle containing the phosphoamino acid.

**TABLE I**

| Potential tryptic phosphate candidates for peptides 3 and 6 |
|----------------------------------------------------------|
| The number preceding the peptide indicates the position of the first amino acid of the peptide within the sequence of PR-B. |

| Phosphoserine in eighth position |
|---------------------------------|
| 63 PCQGOQPDSDEK |
| 74 TQDQOSLDVEGAYSR |
| 95 GAGGSSSPPEK |
| 371 DDAYPYSDFQPAPLK |
| 547 PDSEAQSPPQYSFESLFPK |
| Phosphoserine in third position |
| 160 VLPCEPLMSR |
| 188 GLSPAR |
| 271 EDSR |
| 547 PDSEAQSPPQYSFESLFPK |
| 770 HVSPQHLYFAPDLILLNEDQR |
| 791 ESSFYSLCLETMWOQPQFEPFK |
| 900 ALSVEFPESMSEVIAAQLPK |

associated with purified PR-B, and that it is an excellent substrate for CKII. The tryptic phosphopeptide map of the in vitro phosphorylated PR-B shown in Fig. 6 reveals one major 32P peak which corresponds to peptide 3 isolated from in vivo phosphorylated PR. The fraction was collected and dried, digested with Asp-N and Glu-C and run on a peptide gel. The mobility of this peptide treated or untreated with endoproteinase was the same as that of peptide 3 (data not shown). To further confirm the identity of this single major phosphopeptide, we performed manual Edman degradation. As shown in Fig. 7, 32P was released in cycle 8 confirming that Ser11 was preferentially phosphorylated by CKII in vitro. In contrast, purified PR-A was not phosphorylated in vitro by CKII (data not shown), further confirming the preference of this enzyme for the PR-B specific Ser11.

**DISCUSSION**

We have identified two PR-B-specific phosphorylation sites, Ser51 and Ser16 located within the 164-amino acid amino terminus of PR-B. We have also found that casein kinase II preferentially phosphorylates Ser51 in vitro. Analysis of phosphopeptide maps of PR from hormone-treated T47D cells was previously reported by Sheridan (8) who indicated that there might be at least five common phosphopeptides between PR-A and PR-B with a single site unique to PR-B in the absence of hormone and one or two more additional ones after hormone treatment. Our study has shown that hPR phosphorylation may be more complex than initially reported. We find at least 12 phosphopeptides including the B-specific peptides. The difference in the total number of peptides may be due to our use of an on-line radioactive flow detector, which gives higher resolution than counting individual fractions after HPLC.

Analysis of phosphorylation of steroid receptors has been hampered by both the complexity of the phosphorylation (30, 31) and the low abundance of receptor both of which make the use of conventional protein chemistry techniques to identify the sites both very expensive and difficult. Phosphorylation sites in chicken progesterone receptors, isolated from 32P-labeled oviduct tissue minces (23) and in mouse glucocorticoid receptors overexpressed in Chinese hamster ovary cells, have been identified by HPLC isolation of phosphopeptides followed by amino acid sequencing. Some of the phosphorylation sites in...
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**TABLE II**

Peptide sequence analysis

Peptide 3 and 6 were sequenced as described in the text.

| Cycle | Amino acid | Position
|-------|------------|-----------
| 1     | Thr        | 74        | 29.8 pmol |
| 2     | Gln        | 75        | 34.3 pmol |
| 3     | Glu        | 76        | 18.2 pmol |
| 4     | Gln        | 77        | 32.1 pmol |
| 5     | Glu        | 78        | 39.2 pmol |
| 6     | Ser        | 79        | 7.9 pmol  |
| 7     | Leu        | 80        | 28.1 pmol |
| 8     | Ser        | 81        | 6.5 pmol  |
| 9     | Glu        | 82        | 13.3 pmol |
| 10    | Val        | 85        | 28.0 pmol |

Peptide 6

| Cycle | Amino acid | Position
|-------|------------|-----------
| 1     | Val        | 160       | 11.7 pmol |
| 2     | Leu        | 161       | 24.7 pmol |
| 3     | Ser        | 162       | 1.7 pmol  |
| 4     | Pro        | 163       | 5.5 pmol  |
| 5     | Leu        | 164       | 7.8 pmol  |
| 6     | Met        | 165       | 2.5 pmol  |
| 7     | Ser        | 166       | 1.4 pmol  |
| 8     | Arg        | 167       | 1.1 pmol  |

* Amino acid position within the PR-B.

**Fig. 5.** In vitro phosphorylation of PR-B by casein kinase II. Phosphorylation of PR-B was done under the conditions described in the text of Methods. After incubating at 37 °C for 30 min, the reaction was terminated by addition of sample buffer, heated at 90 °C for 5 min, separated by SDS-gel electrophoresis, and autoradiographed. *Lane 1,* reaction without casein kinase II; *lane 2,* reaction with casein kinase II. Position of PR-B is indicated.

**Fig. 6.** Phosphopeptide map of PR-B phosphorylated in vitro with CKII. Phosphorylated PR-B was isolated by SDS-gel electrophoresis, digested with trypsin, and separated by reverse phase HPLC. The elution time of the major peak corresponds to that of peptide 3. The elution time differs somewhat from that in Fig. 1 but was confirmed by comparison with known phosphopeptides.

**Fig. 7.** Manual 32P release of peptide CKII. Peptide CKII was subjected to nine cycles of manual 32P release using the procedure described under "Materials and Methods."

pressed receptors. Use of secondary protease digestion of tryptic phosphopeptides isolated from HPLC as well as release of 32P by manual Edman degradation, enabled us to identify one of the major phosphorylation sites located in the unique NH2 terminus of PR-B (Ser160) and narrowed the possibilities for the second (Ser161) to two sites. Amino acid sequencing of corresponding tryptic peptides isolated from baculovirus expressed PR-B provided confirmation of Ser160 and identified Ser91 as the second site. However, if the carrier protein were unavailable we could have performed manual Edman degradation on the Asp-N digested peptide 3 to distinguish between the two remaining possibilities. Phosphopeptide mapping has shown that baculovirus expressed hPR is correctly phosphorylated on all sites but one (peptide 9) and thus is a suitable carrier protein for identification of all other sites by amino acid sequencing of phosphopeptides, including peptides 3 and 6 in this study. Hilliard et al. (33) successfully used vitamin D receptor isolated from a yeast expression system as a carrier protein for amino acid sequencing to identify a phosphorylation site in the human vitamin D receptor, and chicken PR expressed in yeast was found to be correctly phosphorylated on all of the sites that have been identified in the endogenously expressed receptor (34). Thus, correct phosphorylation of steroid receptors expressed as a recombinant protein in heterologous eukaryotic systems, such as baculovirus or yeast, may be more the rule than the exception. Therefore, the approach developed in our

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study of using endogenously expressed \(^{32}\)P-labeled protein as a tracer combined with unlabeled carrier protein purified from an overexpression system for amino acid sequencing may be a generally effective means to identify phosphorylation sites on steroid receptors and other proteins.

The identification of the major phosphorylation sites located in the unique NH\(_2\)-terminus of hPR-B is a potentially important finding that may help to explain the distinct functional properties of the two hPR isoforms that appear to be dependent upon cell type-specific factors and on target promoter context (20–22). Of particular interest is the recent discovery that PR-A can under certain circumstances act as a repressor of transcription mediated by PR-B as well as other steroid receptors in the closely related glucocorticoid receptor subfamily (22). The mechanism of the repressor activity of PR-A is unknown. It is possible that these S/I/P motifs may play a role in PR regulation of transcription and translation (36). CKII may also be involved in transcription, a CKII site in the human vitamin D receptor has been identified (37). It will now be possible to determine to what extent phosphorylation of Ser\(^{81}\) and Ser\(^{162}\) in PR-B lacking phosphorylation of Ser\(^{81}\) and Ser\(^{162}\) plays a role in PR interaction with other transcription factors or B-specific transcriptional factors (22).

We therefore tested the ability of CKII to phosphorylate PR in vitro, a CKII site in human vitamin D receptor has been identified (37). It was reported that the thyroid hormone receptor encoded by the chicken c-erbA\(_a\) gene is phosphorylated at a single site by CKII (37). In addition, a CKII site in human vitamin D receptor has been identified as a phosphorylation site in vivo and in vitro (33, 38). We therefore tested the ability of CKII to phosphorylate PR in vitro, finding that CKII preferentially phosphorylated Ser\(^{81}\) without phosphorylating any of the other potential CKII sites in hPR.

Ser\(^{162}\) is one of 15 potential phosphorylation sites in hPR that fit the consensus motif (X-Ser/Thr-Pro-X) of the proline-directed kinases. The ST/P motifs are largely located in the amino-terminal region of PR, the region considered important for interaction with other transcription factors. Interestingly, five Ser-Pro sites are located in the B specific amino terminus of PR. It is possible that these ST/P motifs play a role in PR interaction with other transcriptional factors and B specific functions. It is intriguing to note that many of the identified phosphorylation sites in steroid receptors such as chicken and human estrogen receptor, and mouse glucocorticoid receptor also contain Ser-Pro motifs which are part of the consensus sequences for mitogen-activated protein (MAP) kinases and cyclin-dependent kinases. MAP kinases, present in both cytoplasm and nucleus, are important intermediates in signal transduction pathways that are initiated by many types of cell surface receptors. There is evidence that MAP kinases play a key role in the transduction of signals through both protein kinases and protein phosphatases. Whether MAP kinases regulate the activity of PR remains to be established. Ser-Pro is also a minimum consensus for cyclin-dependent kinases; cyclins and cyclin-dependent kinases are key regulators of cell cycle progression in eukaryotic cells. There is also evidence that progesterone receptor regulates expression of cyclin genes in T47D cells (39, 40). The data suggest that the activity of human PR may be regulated by different kinases that are actively involved in either signal transduction or cell cycle regulation and that, in turn, PR may regulate proteins involved in cell cycle control.

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