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GRANT NUMBER DAMD17-94-J-4202

TITLE: Regulation of Agonist--and Antagonist--Mediated activation of Human Progesterone Receptors by Phosphorylation

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REPORT DATE: July 1996

TYPE OF REPORT: Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release;
distribution unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 1996	Annual (1 Jul 95 - 30 Jun 96)

4. TITLE AND SUBTITLE Regulation of Agonist--and Antagonist--Mediated Activation of Human Progesterone Receptors by Phosphorylation

5. FUNDING NUMBERS

DAMD17-94-J-4202

6. AUTHOR(S)

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)

Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 2170-25012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200*

The human progesterone receptor (hPR) in breast cancer cells (T47D) is phosphorylated on multiple serine residues. I have previously reported the identification of eight phosphorylation sites. Here I show the identification of a new site, Ser20. This site is hPR-B specific and contains a Ser-Pro consensus sequence. The role of phosphorylation in hPR and RU 486 antagonist/agonist switch has also been investigated. Using a yeast system, the effect of B-specific phosphorylation on AF3 transactivation has been studied. Mutation of Ser102 to Ala nearly depleted the activity of AF3, suggesting that phosphorylation is a specific and an important regulatory step for hPR activity. I have also compared the activity of the mutant Ala400 with the wild type hPR. Surprisingly, the mutant's activity is significantly higher than that of the wild type, implying that regulation of hPR by phosphorylation is complex. Recently, several co-regulators of steroid receptors have been cloned and characterized. I have just begun to study their roles in the RU 486 antagonist/agonist switch. Initial results show that the PKA may potentiate the hPR activity through SRC-1. Whether SRC-1 can mediate the switch is under investigation.

14. SUBJECT TERMS

Breast Cancer

15. NUMBER OF PAGES

16

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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Johan Vaam 8 7/26/96

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I. Introduction

Human progesterone receptor (hPR) belongs to a superfamily of ligand-activated nuclear transcription factors, and is a specific mediator of the activity of progesterone, an important regulator of growth and development of the human mammary gland. There are two forms of hPR, hPR-A (Mr = 94,000) and hPR-B (Mr = 120,000) resulting from differential transcription of a single gene (1,2). hPR-A differs from hPR-B in that hPR-A lacks the first 164 amino acids (a.a.) of hPR-B. Like other members of the family, hPR contains a highly conserved DNA binding domain, a ligand binding domain, and a variable amino-terminal region, which contains a transactivation domain (AF1). There is also a hormone dependent activation domain (AF2) in the carboxyl terminus. Sartorius et al. (3) recently showed that the first 164 a.a. of hPR-B is an independent transactivation domain which they named AF3. Progestin activation of hPR in target tissues is mediated via a series of yet to be learned events that include at least dimerization and phosphorylation of the receptor, receptor interacting with other factors, and receptor binding to PR responsive elements on target genes. The effect of progestin can be blocked by antiprogestins, which leads to the potential role of antiprogestin for treating breast cancer. However, like an estrogen antagonist tamoxifen, which is also a partial agonist for estrogen receptor, agonist activities of antiprogestin have been reported (4,5). In cultured breast cancer cell lines, RU 486 behaves as an agonist in the presence of a protein kinase A activator, 8-Br cAMP (6,7). Since the functional receptor is a prerequisite for the effectiveness of endocrine treatment for breast cancer, the focus of my study is to understand whether altered phosphorylation of hPR or factors associated with hPR causes the switch.

The goals of this research are to understand the role of phosphorylation in modulating hPR activity and the mechanisms underlying the agonist effect of RU 486 in the presence of 8-Br cAMP. To accomplish these goals, it is critical to first identify the phosphorylation sites in hPR and to investigate each individual site concerning its biological function and relation to this problem.

hPR is highly phosphorylated in T47D breast cancer cells upon hormonal stimulation (8,9). The rapid phosphorylation takes place in a few minutes after the addition of hormone. Prolonged treatment up to 60 min does not change the overall ^{32}P incorporation significantly, but decreases the mobility of hPR (upshift) on SDS gel electrophoresis (SDS-PAGE), indicating that phosphorylation can cause significant conformational changes within hPR. In the previous report, I demonstrated the identification of multiple phosphorylation sites; three are hormone inducible sites (Ser102, Ser294 and Ser345) and their phosphorylation correlates with the timing of the change in mobility on gel electrophoresis in response to hormone treatment (10). The other sites, Ser81, Ser162, Ser190, Ser400, Ser558 are basally phosphorylated and their phosphorylation exhibits a rapid increase in response to hormone (10,11).

Interestingly, all the identified sites except Ser81 contain a Ser-Pro consensus sequence, a motif for proline-dependent kinases such as MAP kinases and Cdks. Using

purified baculovirus expressed hPR-B as a substrate, Ser190 and S400, as well as another basal site Ser162 were specifically phophorylated to a high specific activity *in vitro* by Cdk2 while Ser81 is specifically phosphorylated by casein kinase II (11). Thus *in vitro* phosphorylation studies suggest that hPR is phosphorylated by multiple kinases. The physiological significance of these findings needs to be assessed by future *in vivo* studies.

Phosphorylation is a basic event of cell regulation; numerous proteins have been characterized to be modulated by phosphorylation. In recent years, its importance in steroid receptors' functions has been recognized and demonstrated by both *in vitro* and *in vivo* approaches (12,13). I have shown that mutation of Ser81 to Ala caused significant decrease of hPR activity when compared with wild type, confirming that phosphorylation is important for hPR function. It is of profound interest to characterize the functional role of the remaining phosphorylation sites in hPR.

To address the question of whether RU 486 plus 8-Br cAMP can alter or induce new sites in hPR, we have also performed phosphotryptic mapping experiments. Our studies showed that there is no altered phosphorylation or any new site induced by RU 486 and 8-Br cAMP (my first annual report), suggesting that the agonist activity of RU 486 is not due to the phosphorylation of receptor itself. Therefore, we speculated that the agonist activity is mediated through hPR-associating proteins, whose activity is perhaps modulated by cAMP-mediated phosphorylation.

Recently, several lines of evidence indicate that steroid receptors interact with additional factors to achieve maximum activation or repression. Using genetic and biochemical approaches, several groups have recently identified different coactivators for steroid receptors, including CBP/P300, SRC-1, GRIP-1, RIP140, and others (14-16). The mechanisms by which these factors regulate the functions of receptors remain to be established. CBP was initially identified as a co-activator for the cAMP response element-binding protein (CREB) (14). Its potential effect on several members of nuclear receptor family has recently been illustrated (17). The activation of steroid receptors by CBP involves multiple protein-protein interactions including CBP binding to receptors and P160, a variant of SRC-1, and receptors binding to P160. It has been shown that the phosphorylation of CREB is required for interaction with CBP. In that respect, it is of interest to study how phosphorylation regulates the CBP/receptor/coactivator complex resulting in the activation of specific target genes. SRC-1 is a co-activator identified for its ability to activate hPR (15). Brian Rowan in our lab has shown in his preliminary labeling experiments that SRC-1 is a phosphoprotein. This result strongly supports the idea that phosphorylation is a potential regulatory step involved in steroid receptor-factor interactions.

Taken together, recent studies suggest to us that diverse signal transduction pathways lead to the activation steroid receptors by targeting their associated partners. Many of these factors are either phosphoproteins and/or potential targets of protein kinases. I believe that phosphorylation of steroid receptors and these factors are a key step to protein-protein interaction, which potentiates the hormone mediated functions. I

speculate that the antagonist/agonist switch may be due to the activation of these factors. Therefore, it is necessary to evaluate these newly identified factors with regard to their effect on the agonist activity of RU 486. Moreover, since the agonist activity of RU 486 has only been demonstrated for hPR-B in the presence of 8-Br cAMP, hPR-B-specific phosphorylation may play a critical role in this and I will test the possibility with all the hPR-B mutants.

In this report, I show: (1) the identification of a new phosphorylation site; (2) evidence that phosphorylation may be responsible for the transcriptional activity of AF3 using a yeast system; (3) preliminary results of the effect of SRC-1 on hPR activation in the presence of 8 Br cAMP; (4) mutation of Ser400 to Ala causes an increase in hPR activity when compared to wild type hPR.

II. Body

A. Materials and Methods

Materials

Minimum essential medium (MEM) was purchased from Irvine (Santa Ana, CA). Phosphate-free MEM was obtained from GIBCO BRL (Grand Island, NY). AB-52 mouse monoclonal antibody that recognizes both PR-A and PR-B is provided by Dr Dean Edwards. R5020 and carrier-free [^{32}P]H_3PO_4 were puchased from Dupont/New England Nuclear Products (Boston, MA). Protein-A Sepharose was purchased from Pharmacia LKB Biotechnology, Inc. (Piscataway, NJ). Tosylphenylalanyl chloromethy ketone-treated trypsin was purchased from Worthington Biochemical Corp. (Freehold, NJ). Sequencing grade endoproteinases Asp-N and Glu-C, and lipofectamine transfection reagent were purchased from Boehringer Mannheim (Indianapolis, IN). Phenylisothiocyanate and sequencing grade trifluoroacetic acid (TFA) and HPLC reagents were purchased from J. T. Baker Chemical Corp. (Phillipsburg, NJ). Triethylamine and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide) were purchased from Sigma (St. Louis, MO). Sequelon-AA membranes and Mylar sheets were obtained from Millipore Corp. (Milford, MA). Transformer^{TM} site-directed mutagenesis kit was obtained from Clontech (Palo Alto, CA). Sequenase version 2.0 DNA sequencing kit was obtained from USB (Cleveland, Ohio).

Cell Culture, PR labeling, and Receptor Preparations T47D human breast cancer cells were maintained and grown in 75-cm^2 T-flasks with frequent changes of media as previously described. Cells were incubated for 24 h in MEM containing 5% fetal calf serum that has been stripped of steroid hormones by dextran-coated charcoal treatment. Steady state labeling with [^{32}P]orthophosphate was carried out in phosphate-free serum-free medium for 1 h at 37^{0}C and then incubated in phosphate-free MEM containing [^{32}P]orthophosphate (0.83 mci/ml) for 6 h at 37^{0}C. Cells were treated with 40 nM R5020 for 2 h before harvest.

Cells were harvested in 1 mM EDTA in Earle's balanced salt solution and homogenized at 40^{0}C in a Teflon-glass Potter-Elvenhjem homogenizer (Fisher, Pittsburgh, PA) in KPFM buffer [50 mM potassium phosphate (pH 7.4), 50 mM sodium fluoride, 1 mM EDTA, 1 mM EGTA, and 12 mM monothioglycerol] containing 0.5 M NaCl and a mixture of proteinase inhibitors as previously described. The whole cell extract was obtained by centrifuging the homogenates at 100,000 x g for 30 min, and dialyzed in KPFM to remove the salt before the immunoprecipitation step.

Immunoprecipitation and Gel Purification of PR. Monoclonal antibody AB-52 bound Protein-A Sepharose was prepared as previously described. Dialyzed whole-cell extracts containing PR were incubated with Protein-A Sepharose on an end-over-end rotator for 4 hours at 4^0 C. Protein-A Sepharose was washed three times with KPFM buffer containing 0.3 M NaCl to remove nonspecific protein. Bound receptors were then eluted with 2% SDS sample buffer and electrophoresed on a 7.0% discontinuous SDS

polyacrylamide gel. ^{32}P-labeled receptors were located by autoradiography of the gels, and PR-A and PR-B were retrieved by excising the corresponding gel pieces.

HPLC Analysis of Tryptic Peptides of PR. The gel slices containing PR were washed with 50% methanol for 1 hour followed by H_2O for 30 min and 50 mM ammonium bicarbonate for 5 min in a 1.5 -ml microfuge tube. 20 ug trypsin was added to the tube. After incubating the tube for 4 hours at 37^{0}C, another 20 ul trypsin was added, and this was repeated three more times. The digested peptides were dried in a Speedvac (Savant Instruments, Hicksville, NY), dissolved in 150 ul 50% formic acid, loaded on a Vydac (Hesperia, CA) C18 reverse phase column in 0.1% TFA in water, run at a flow rate of 1 ml/min, and eluted with a linear gradient form 0-45% acetonitrile over 90 min. The labeled peptides were detected with an on-line model IC Flo-One Beta-radioactivity flow detector (Radiomatic Instruments, Inc., Tampa, FL), and collected as 1 ml fractions.

Phosphorylation Site Identification. Fractions corresponding to each labeled peptide were dried and further separated by electrophoresis on a 40% alkaline gel. Labeled peptides were detected by autoradiography of the dried gel, excised, and eluted with H_2O as previously described [1919]].

To find the position of phosphoamino acids in the peptides, I used manual Edman degradation as described by Sullivan and Wong (18). In brief, the peptide to be analyzed was dissolved in 30 ul of 50% acetonitrile, and spotted on an arylamine-Sequelon disc, which was placed on a Mylar sheet on top of a heating block set at 50°C. After 5 min, the aqueous solvent was evaporated and the disc was removed from the heating block. 5 ul of EDAC solution (50 mM in Mes, pH 5.0) was added to the disc to allow the peptide to covalently link to the disc, and the disc was placed at RT for 30 min. The disc was then washed five times with water and five times with TFA to remove unbound peptide. The disc was then washed three times with methanol, and subjected to Edman degradation: The disc was treated at 50°C for 10 min with 0.5 ml coupling reagent (methanol:water:triethylamine:phenylisothiocyanate; 7:1:1:1, v/v). After five washes with 1 ml of methanol, the disc was treated at 50°C for 6 min with 0.5 ml TFA to cleave the amino terminal amino acid. The TFA solution was placed in a scintillation vial and the disc was washed with 1 ml of TFA and 42.5% phosphoric acid (9:1, v/v). The wash was combined with the TFA solution and the released [^{32}P] was determined by Cerenkov counting. The next cycle began after the disc was washed five times with 1 ml methanol.

To characterize the peptides, the tryptic peptides were digested with the endoproteinases Glu-C and Asp-N. Glu-C cuts on the C-terminal side of Glu, except for Glu-Pro bonds. Moreover, Glu-X bonds within three residues of the end of a peptide are cleaved poorly (19). Asp-N cuts on the N-terminal side of Asp residues. Peptides, digested and undigested, were loaded to a peptide gel electrophoresis or manual Edman degradation. Glu-C digestion was performed in 200 ul 25 mM ammonium bicarbonate, pH 7.8, for 8 h at 37^{0}C. Asp-N digestion was perfomed in 200 ul 50 mM sodium phosphate buffer, pH 8, containing 0.2 ug Asp-N and incubated at 37^{0}C for 4 hours.

Site-directed Mutagenesis and Sequencing. Site-directed mutagenesis was perfomed using a kit from Clontech. Briefly, two primers were simultaneously annealed to one strand of the denatured double-stranded expression vector. The selection primer contains a mutation which changes a restriction site from Nde 1 to Afl II in the plasmid backbone. The mutagenesis primer changes Ser to Ala. After DNA elongation, ligation and a primary selection by digesting with restriction enzyme Nde 1, the mixture of mutated and unmutated plasmids were transformed into a mutS E. coli strain defective in mismatch repair. Plasmid DNA was enriched from the pool of transformants and and digested again with Nde 1 and transformed in DH5α bacterial strain. Transformants resistant to Nde 1 digestion were selected and plasmid DNA prepared and sequenced using a USB Sequenase Version 2.0 DNA Sequencing Kit based on chain-termination sequencing theory (20).

Plasmid construction. The $Gal4_{DBD}$-AF3 chimeric protein was constructed in the pAS1 yeast expression plasmid that contains a tryptophan marker (21). The hPR-B N-terminal BsPH1 fragement that codes the first 164 amino acids of wild type or individual B-specific phosphorylation mutants was fused in frame with the Gal4 (1-147) DNA binding domain by cloning into the NCoI site of pAS1.

Yeast culture, transformation and *B*-Gal assay. The yeast strain Y190 which contains an integrated Gal4 reporter was grown in YPD rich medium (22). Transformation was performeed by first culturing the yeast in YPD rich medium at 30^0C with shaking to 1 x 10^7 cells/ml and then pelleted by centrifugation. Cells were washed with LiTE [100 mM LiAC in TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0)] followed by LiSORB wash (LiSORB is LiTE containing 1M Sorbitol), resuspended in 5 ml LiSORB and incubated for another 15 min at 30^0C with shaking. Cells were pelleted and resuspended in 500 ul of LiSORB; 100 ul of resuspended cells were mixed with 200 ug of carrier DNA (salmon sperm DNA treated with sonication) and 2 ug of transforming plasmid DNA, and incubated at 30^0C without shaking. After 10 min of incubation, 900 ul LiPEG (LiTE containing 40% PEG3350) was added to the cells and the mixture was heat shocked in a 42^0C bath for 22 min. Cells were then plated on selection plates without Trp. Positive clones were picked and incubated in 5 ml selective media without Trp overnight at 30^0C with shaking. The cells were pelleted and washed twice in 1 ml of assay buffer (per liter, 16.1 g of $Na_2HPO_4.7H_2O$, 5.5 g $NaH_2PO_4.H_2O$, 0.75g of KCl, 0.246 g of $MgSO_4.7H_2O$ and 2.7 ml of 2-mercaptoethanol, pH 7.0) and then were disrupted by glass bead homogenization in 1.5 ml tubes. After the debris was removed by centrifugation, the protein concentrations were determined. The *B*–Gal activities were determined by incubating 100 ug protein, 200 ul of substrate, O-Nitrophenyl *B*-D-Galacto-pyranoside (4 mg/ml) at 37^0C. The assays were stopped by the addition of 500 ul of 1 M Na_2CO_3, and the OD was measured at 420 nM. The results were expressed as Miller units which are calculated as follows: Activity = OD420 X 100/ (T x P), where T represents the time of the assay in minutes, and P is the amount of protein used in mg.

Transient transfection and CAT assays. CV1 and Hela cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. The day before transfection, 1 x10^6 cells were plated in 24 well plates and 4-6 h later switched to serum-free medium supplemented with Nutridoma-SR (Boehringer Mannhein). Cells were transfected with mammalian expression vectors encoding hPR or other factors as indicated in figure legends along with 1 ug of CAT reporter using the Lipofectamine method (GibcoBRL). 24 hr later, medium was replaced with fresh medium, and treated with hormone or other reagents as indicated in the figure. Cells were harvested and proteins prepared by three cycles of freeze-thaw lysis in the buffer (25 mM Tris-HCl, pH 8.0) 48 hr after transfection. The CAT activity was determined using a liquid CAT assay method (23).

B. Results

1. Identification of a fourth hPR-B specific phosphorylation site. Fig. 1 shows a reverse phase HPLC map of phosphotryptic peptides of hPR phosphorylated *in vivo* in the presence of hormone and a diagram of hPR structure. The location of phosphorylation sites and peptide peaks containing the sites are indicated by lines that connect both. Each phosphopeptide peak has been numbered based on the order of its retention time. In order to identify phosphorylation sites in each peak, HPLC fractions corresponding to each peak were collected and subjected to a 40% alkaline gel for further separation. Fig. 2 shows that fractions of peak 5 contains two phosphopeptides (p5-1 and p5-2) indicated by two distinct bands; p5-2 is much less abundant compared to p5-1. I have previously reported the identification of Ser558 in p5-1. To identify the phosphorylation site in p5-2, I extracted p5-2 from the gel and digested it with specific proteinases Asp-N and Glu-C, which cut, respectively, at the N-terminal side of Asp residues and C-terminal side of Glu. The digested and undigested p5-2 were subjected to 40% alkaline gel electrophoresis to determine if it contains a cleavable Asp or Glu based on changes in

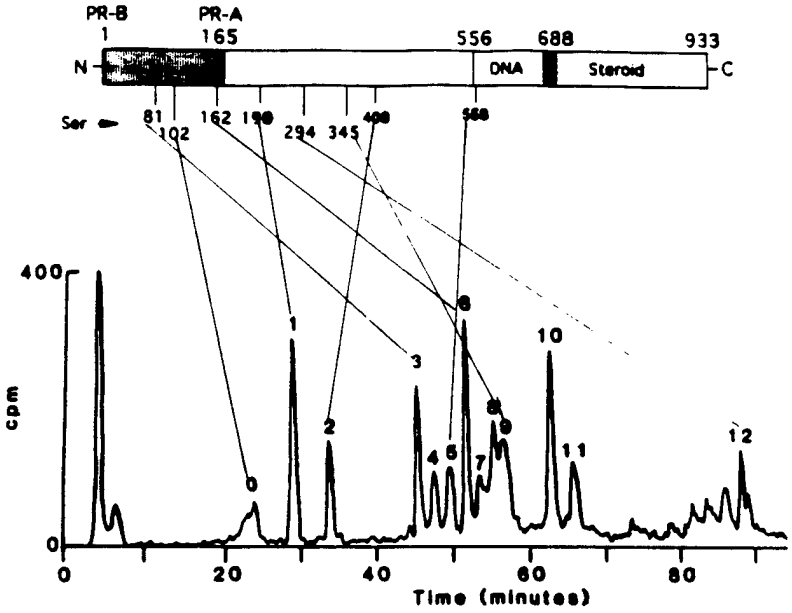

Figure 1. Summary of Human PR Phosphorylation Sites. The top panel shows a diagram of human PR. The lower panel represents the reverse phase HPLC profile of the tryptic phosphopeptides. Phosphorylation sites and their corresponding peaks are indicated by lines connecting the two. Two of the hormone-inducible sites (Ser294 and Ser345) are common to the A and B forms of PR, while Ser102 is unique to hPR-B. Ser81 and Ser162 are previously identified sites that reside in the unique N-terminal segment of hPR-B and both are characterized as basal sites whereas Ser190, Ser400 and Ser558 are basal sites common to both hPR-A and hPR-B.

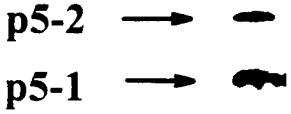

Figure 2. Fractions of peak 5 contain two phosphotryptic peptides. HPLC fractions corresponding to peak 5 were collected and dried in a speedvac. Dried sample was separated by a 40% alkaline gel and peptides were detected by autoradiography.

mobility. Fig. 3 shows that p5-2 can be cleaved by Glu-C but not by Asp-N, suggesting the presence of Glu but not Asp-N. A manual Edman degradation experiment determined that the phosphorylated residue is located at the tenth position in p5-2 as shown in Fig. 4. Because all phosphorylation sites in hPR are serine, I listed in table 1 all the potential candidates containing Ser in position 10. There is only one tryptic peptide that satisfies these criteria. Therefore, I have determined that Ser20 is phosphorylated in vivo. Interestingly, it is a B-specific phosphorylation site containing a common Ser-Pro motif. I plan to confirm the identification by automated a.a. sequencing using baculovirus expressed hPR as a carrier as described previously.

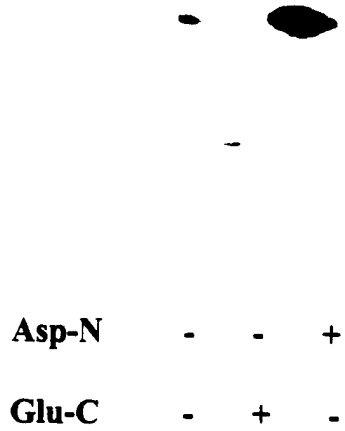

Figure 3. Characterization of tryptic phosphopeptide p5-2 by redigestion with additional proteases. p5-2 extracted from the alkaline gel was treated with Asp-N (cuts on the N-terminal side of Asp) or Glu-C (cuts on the C-terminal side of Glu provided it isn't within three amino acids of the carboxyl terminal) and analyzed by 40% alkaline polyacrylamide gel electrophoresis. The gel was dried and peptides detected by autoradiography.

2. Effect of phosphorylation on the activity of AF3. In addition to the two well characterized transactivation domains AF1 and AF2, the first 164 amino acids of hPR-B has recently been characterized as an activation domain, which has been termed AF3 (3). To date, four phosphorylation sites have been located in this short stretch of peptide. To determine the role of phosphorylation in AF3 function, I fused the AF3 with or without Ser to Ala mutation in frame to the yeast Gal4 DNA binding domain and

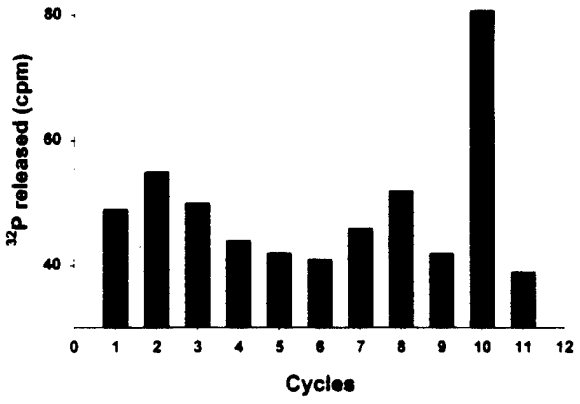

Figure 4. Phosphopeptide p5-2 contains a phosphoserine at 10th position. p5-2 was covalently coupled to arylamine membrane discs using carbodiimide and subjected to manual Edman degradation. The radioactivity in the released amino acid was determined after each cycle using a scintillation counter. The background counts (24 $\pm$ 4, n >10) were not subtracted from the counts. The cycle containing the released [^{32}P] is the cycle containing the phosphoamino acid.

transformed yeast strain Y190 that has a stably integrated Gal4 reporter gene. This strategy allows me to investigate the effect of specific phosphorylation on individual domain of the hPR. Moreover, the effect of phosphorylation may be more profound than it is when using full length protein due to the complexity of the global structure and interactions. Plasmids which express the DNA binding domain and the chimeras were transformed into the strain and *B*-Gal activity determined. Fig. 5 shows that wild type AF3 is active but not the DNA binding domain, confirming that AF3 is indeed a transactivation domain in yeast. Surprisingly, mutant Ala81 and Ala162 are as active as the wild type whereas the mutant Ala102 activity is almost undetectable. It is not clear why the Ala81 activity is not decreased in this system compared to a significant drop of activity observed in co-transfection experiments when full length hPR was used. Although other possibilities can not be ruled out, a simple explaination is perhaps that Ser81 is not responsible for the activation of AF3 but the overall activity of the receptor. This result suggests that phosphorylation of specific sites may be important for the activity of certain domains of hPR as demonstrated by this experiment. However, it is difficult to detect the protein expression of these chimeras by western analysis using anti-$Gal4_{DBD}$ antibody. Although there is no reason to believe that the changes in activity are due to altered expression level of the mutants Ala102, I am currently using a yeast strain that is deficient in proteases to overexpress these chimeras to check their expression level.

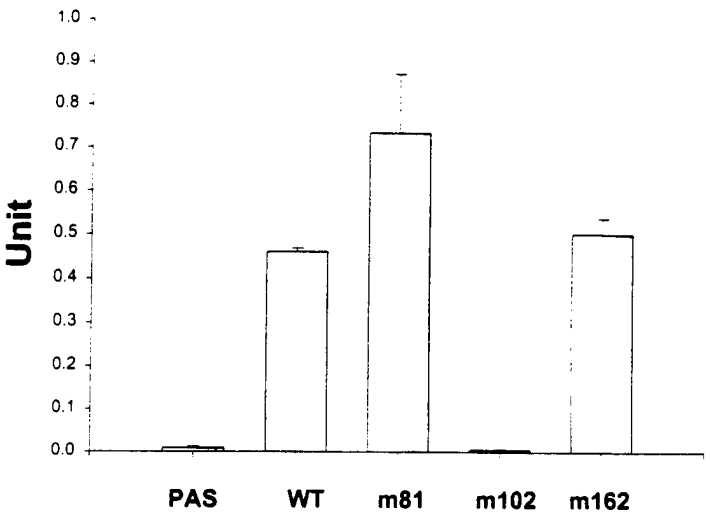

Figure 5. Transcriptional activity of wild type and mutant chimeras. The detail of the transformation and assay was described in the methods. PAS is a plasmid expresses the DNA binding domain of Gal4 protein. WT is the wild type of 164 amino acids of hPR-B. m81, m102, and m162 are mutants in which Ser is mutated to Ala. The results are expressed as Miller units.

3. Does Co-activator SRC-1 Have Any Potentiating Effect on hPR Activity ?
SRC-1 is a co-activator of several steroid receptors (15). When co-expressed in either CV1 or HeLa cells along with hPR and reporter, it potentiates the hPR activity in the presence of progestin. How exactly SRC-1 works is under intensive investigation. Although my ultimate interest is to know how the PKA pathway causes the agonist activity of RU 486, it is interesting to first test if the PKA pathway would activate hPR through SRC-1. I have just began to establish the conditions in which SRC-1 can activate

hPR and to test whether the activation can be potentiated by the presence of 8-Br cAMP. As Shown in Fig. 6, SRC-1 in both CV1 and HeLa cells acts as a co-activator in the presence of hormone compared to the same expression vector without the coding sequence. Additionally, overexpression of SRC-1 enhances the 8-Br cAMP mediated activation of hPR in the presence of R5020, suggesting that the SRC-1 is a potential candidate for the PKA mediated activation of hPR. I will next examine the effect of SRC-1 on the agonist activity of RU 486 in the presence of 8-Br cAMP.

4. Site-directed Mutagenesis and Transcriptional Activity of Mutant PR.

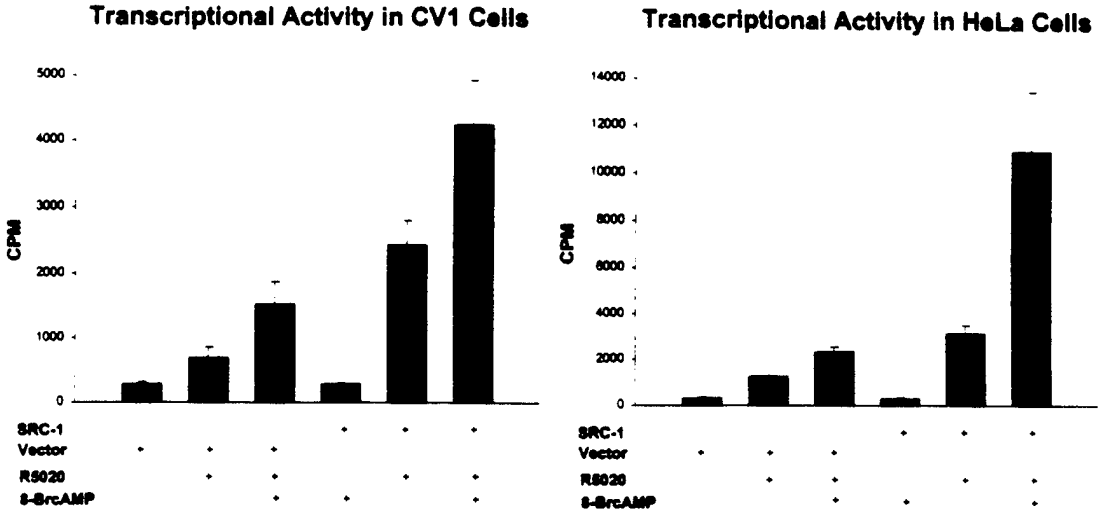

Figure 6. Effect of SRC-1 on the activity of hPR. Cells (2 x 10^5 cells/well) were transfected with lipofectamine. 0.1 ug of receptor, 0.05 ug of SRC-1 expression vector, 0.05 ug of vector without coding sequence, and 0.1 ug $GRE_2E1bCAT$ were used as marked in the figures. After 24 hrs, R5020 (10^{-8} M), RU 486 (10^{-8} M) and 1 mM 8-Br cAMP were added as indicated in the figure, cells were harvested 24 hrs later and CAT activity determined by a liquid assay procedure. **Left Panel:** CV1 cells.**Right Panel:** HeLa cells.

To ellucidate the functional role of phosphorylation in vivo, we have began to mutate individually all the identified sites either to Ala (mimicking the unphosphorylate state oi receptor) or Glu (mimicking the negatively charged receptor) to investigate the transcriptional activity of the mutants. Transcriptional activity of wild type and mutant were tested by transfecting the plasmids into CV1 or HeLa with a $GRE_2E1bCAT$ reporter, and subsequently determining the CAT activity. I previously reported that mutation of Ser^{81} to Ala significantly reduced the CAT activity whithin the linear range, suggesting that phosphorylation of this site is very important for the transcriptional activity of hPR. I next tested the mutant Ala400 (Ser400 mutated to Ala) because Ser400 is phosphorylated by Cdk2 in vitro and the site is near the AF1 domain. To best compare the activity of wt and the mutant, I purified the plasmids of wt and the mutant simultaneously, and two batches of wild type and mutant were evaluated. Interestingly, Fig. 7 shows that the activity of the mutant is significantly higher than that of the wild type. However, we do not know whether the difference is due to phosphorylation or protein expression level. I am in the process of determining the expression levels of both

wt and the mutant by western analysis using our newly developed polyclonal antibody which has a much higher sensitivity than the AB52 previously used in our lab.

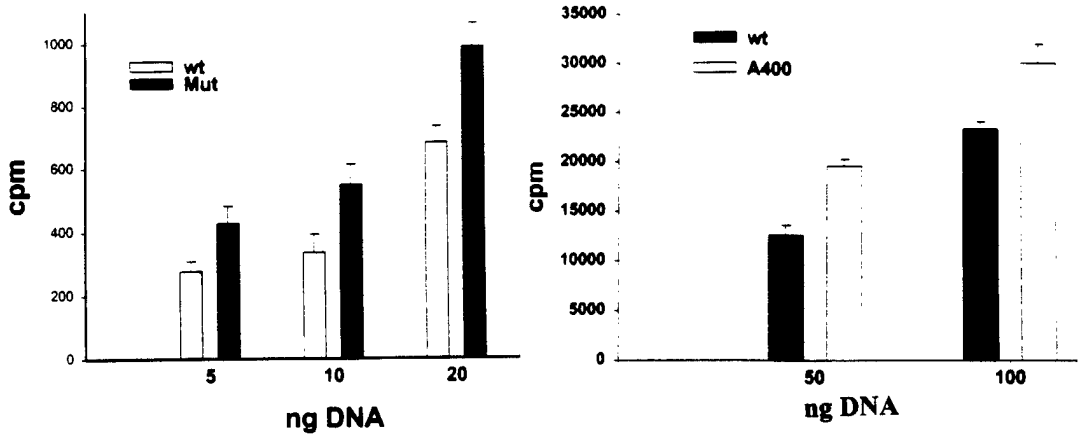

Figure. 7. Mutation of the hPR-B phosphorylation site, Ser400 to Ala increases the transcriptional activity of the receptor. Cells (2 x 10^5 cells/well) were transfected with lipofectamine bearing the indicated amount of receptor DNA and 0.1 ug $GRE_2E1bCAT$. After 24 hrs, R5020 (10^{-8} M) was added, cells were harvested 24 hrs later and CAT activity determined by a liquid assay procedure. **Left Panel:** CV1 cells. **Right Panel:** HeLa cells.

III Conclusion

Phosphorylation of the hPR is complex and involves multiple serine residues. The role of phosphorylation in modulating hPR functions and RU 486 antagonist/agonist switch has been investigated. In this report, I have identified of a hPR-B specific phosphorylation site, which brings the total number of phosphorylation sites in hPR to nine. This site, together with all the other identified sites except Ser81, contains a proline-directed kinase motif, Ser-Pro, strongly suggesting the involvement of MAP kinases or cyclin-dependent kinases.

Based on our phosphotryptic mapping studies, we hypothesized that hPR-B specific phosphorylation sites may be responsible for the B-specific functions and RU 486 antagonist/agonist switch. To support this idea, B-specific mutants have been made and their function characterized. Previously, I showed that the transcriptional activity of Ala81 is significantly lower than that of the wild type. Using a yeast system in this study, I illustrate that phosphorylation of Ser102 is critical for the AF3 transcriptional activity. The effect of phosphorylation appears to be both specific and dramatic. My future studies will address whether phosphorylation of B-specific sites is important for the switch using full length hPR.

We also concluded the switch is not caused by the PKA mediated phosphorylation of hPR. Therefore, other hPR associating factors need to be examined with regard to their effect on the switch. The recent demonstration of co-regulators of steroid receptors by our group and others allows me to test whether these co-regulators play any part in the switch. I chose to first test SRC-1 and CBP because they are capable of activating several members of the steroid receptor family and both are available to me. The preliminary results indicate that SRC-1 potentiates the PKA mediated activation of hPR in the presence of R5020. Whether or not it also affects the switch will be tested.

I have completed a major portion of Specific Aim #1, which was to identify all the phosphorylation sites. Based on the results shown in Fig. 1, site(s) in p10 and p11 remains to be identified. My initial results indicate that p10 and p11 may contain the same site(s). I have almost completed Specific Aim #2 in which I show that there is no altered or new phosphorylation site in hPR caused by treatment with RU 486 and 8-Br cAMP. I also demonstrate that hPR-B is responsible for the switch. As proposed, I will complete Aim #2 by testing the effect of several other modulators of kinases on the switch in the course of the next year. To date, the role of phosphorylation in hPR function still remains largely unknown. Despite the fact that multiple sites have been identified, only a few hPR phosphorylation mutants have been characterized. It is important to analyze other mutants once they are made. I believe that the progress on this project is ahead of the proposed schedule. I anticipate that I will complete the project on time including the work on co-activators in relation to the RU 486 antagonist/agonist switch.

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