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Phosphorylation and Progesterone Receptor Function

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Four phosphorylation sites have been identified in the chicken progesterone receptor. Two of these sites exhibit basal phosphorylation which is enhanced upon treatment with hormone and two of the sites are phosphorylated in response to hormone. Mutation of one of these hormone dependent sites, Ser^{530} to Ala^{530}, causes a decrease in transcriptional activation at low concentrations of hormone, but the activity is unaffected at high concentrations. However, the hormone binding of the mutant is unaffected suggesting that phosphorylation of Ser^{530} plays a role in facilitating the response of the receptor to low concentrations of hormone. The chicken progesterone receptor can be activated by modulators of kinases in the absence of hormone. The finding that signals initiated by tyrosine phosphorylation (through treatment with EGF) or through the dopamine receptor suggests that there are multiple means of activating chicken progesterone receptor. In contrast, the human progesterone receptor does not exhibit ligand independent activation; however, its activity in the presence of the agonist R5020 is enhanced by treatment with 8-Br-cAMP, an activator of protein kinase A, and treatment with 8-Br-cAMP causes the antagonist, RU486, to act as an agonist.

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INTRODUCTION

The progesterone receptor is a member of the ligand-activated superfamily of transcription factors [1]; this family of nuclear receptors includes not only the steroid receptors, vitamin D, retinoid and thyroid receptors, but also many other proteins, termed orphan receptors, whose function and/or ligands are unknown [2]. The chicken and human progesterone receptors are each expressed as two forms (PR_A, M_r = 72,000, and PR_B, M_r = 86,000, in chicken; PR-A, M_r = 90,000, and PR-B, M_r = 120,000, in humans) which are derived from a single gene either from separate mRNAs [3] or by alternate initiation of translation [4]. In each case the A form is essentially a truncated version of the B form lacking either the amino-terminal 128 amino acids of cPR_B or the amino-terminal 165 amino acids of hPR-B. Recent evidence demonstrates that the two forms of the receptor have different biological functions although their precise roles *in vivo* have not been elucidated. Not only do the two forms exhibit promoter preference in activating target genes [5], but several studies have demonstrated that PR-A has a significant repressor function and can repress the activity not only of PR-B, but also of other steroid receptors [6].

IDENTIFICATION OF PHOSPHORYLATION SITES IN PROGESTERONE RECEPTORS

All of the nuclear receptors examined thus far are phosphoproteins [7–11]. Most of the steroid receptors exhibit basal phosphorylation and this phosphorylation is enhanced in response to hormone treatment suggesting that phosphorylation plays a role in receptor activation. The phosphorylation of the chicken progesterone receptor (cPR) has been most completely characterized. Figure 1 shows the locations of the four phosphorylation sites which have been identified in cPR. By convention, the numbering of the sites refers to the numbers in the cPR_B sequence. The corresponding amino acids in cPR_A are 128 amino acids less. Denner *et al.* [12], using oviduct tissue mince as the source of cPR, showed that two of the sites, Ser^{211} and

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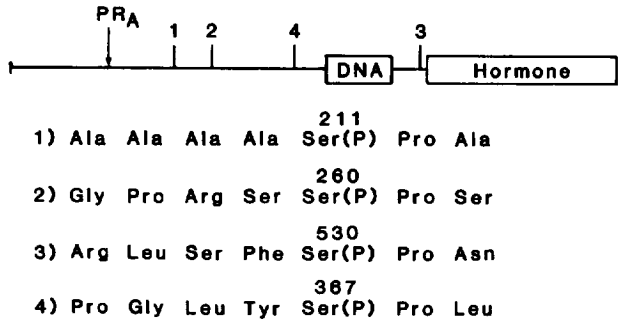

Fig. 1. Location of the four phosphorylation sites in cPR. The numbering is based on the sequence of the full-length PR_B, PR_A is a smaller form of the receptor, which is produced from the same gene and is essentially a truncated form of PR_B. In this figure, PR_A represents the amino-terminal end of PR_A. DNA is the DNA-binding domain, and Hormone is the hormone-binding domain. The sequences surrounding the phosphorylation sites as well as the amino acid numbers of the phosphoserines are shown. Reproduced from [13]. Copyright, Endocrine Society.

Ser^{260}, are phosphorylated in the absence of hormone but that their phosphorylation is increased about 2-fold in response to progesterone. The other two sites are phosphorylated in response to hormone treatment [12, 13]. Although the Ser^{530} phosphorylation site in the hinge region is in a Ser/Thr–Pro motif which has been conserved in all of the steroid receptors [12], this site has not been shown to be phosphorylated in other receptor family members. Whether this phosphorylation site is unique to cPR remains to be determined. All of the phosphorylation sites in cPR contain Ser–Pro motifs and the four sites identified represent all of the Ser–Pro motifs in cPR.

Whether there are additional phosphorylation sites in cPR is unknown. The experiments described above are limited in that oviduct tissue minces can only be maintained for brief periods (about 1 h) so that constitutive sites which turn over slowly or sites which are phosphorylated slowly in response to activation might not be detected. *In vitro* studies using HeLa nuclear extracts show that cPR is an excellent substrate for DNA-dependent kinase and that the receptor is rapidly phosphorylated during *in vitro* transcription assays [14]; these studies suggest that cPR may be phosphorylated *in vivo* by the DNA-dependent kinase.

An analysis of the phosphorylation of cPR produced in a heterologous yeast expression system revealed that the same four phosphorylation sites were phosphorylated and that the hormone-dependence of the Ser^{367} and Ser^{530} phosphorylations was conserved [15]. Receptor expressed in yeast (*Saccharomyces cerevisiae*) exhibits normal hormone binding affinity and specificity and is transcriptionally active [16, 17]. Because the yeast studies were done under conditions of uniform labeling with [^{32}P]phosphate, the failure to detect any phosphorylation in the hormone binding site or in any other region which affects hormone binding indicates that phosphorylation is not required for ligand binding. An analysis of the phosphorylation of a receptor which lacks a portion of the DNA binding domain (and as a result does not bind to DNA) revealed that the basal Ser^{260} and hormone-dependent Ser^{530} sites were still phosphorylated, whereas the Ser^{367} and the Ser^{211} sites were not phosphorylated [15]. Therefore, although the phosphorylation of Ser^{530} is hormone-dependent, this phosphorylation does not require binding of the receptor to DNA. The finding that Ser^{211} was not phosphorylated was unexpected in that Ser^{211} exhibits basal phosphorylation [12] and presumably, does not require the DNA binding activity of the receptor for this basal phosphorylation. However, the DNA binding mutant also displayed weaker nuclear binding as assessed by subcellular fractionation subsequent to progesterone treatment, and it may be that the enzyme which phosphorylates Ser^{211} resides in the nucleus.

The phosphorylation of the human progesterone receptor (hPR) is much more complex. Phosphopeptide mapping reveals that there may be as many as a dozen phosphorylation sites [18a, b] A time-course of receptor phosphorylation of endogenous hPR in T47D breast cancer cells uniformly labeled with [^{32}P]phosphate reveals that the phosphorylation of hPR is a multistage process [19]. Although the receptor is phosphorylated in the absence of hormone, treatment with R5020, a progesterone analog, causes a rapid increase in phosphorylation (approx. 2-fold) within the first 5–10 min. A second slower round of phosphorylation does little to change the net phosphorylation, but is characterized by a decrease in the mobility of the receptor on SDS gels [19]. This final phosphorylation requires DNA binding [20].

In contrast to cPR, the hPR exhibits PR-B specific phosphorylation. This phosphorylation causes the PR-B to appear as a triplet when isolated from control T47D cells and analyzed by SDS–gel electrophoresis whereas the corresponding PR-A appears as a single band. Both forms exhibit reduced mobility in response to treatment of T47D cells with R5020 [19]. The region of hPR unique to hPR-B contains four Ser–Pro motifs which is the sequence found in the sites in cPR as well as in many of the sites reported for the glucocorticoid [21] and estrogen receptors [22, 23]. In addition there are numerous potential phosphorylation sites which are consensus sites for other kinases. Zhang *et al.* have identified one of the hPR-B phosphorylation sites as a casein kinase II phosphorylation site [24].

PHOSPHORYLATION AND PROGESTERONE RECEPTOR FUNCTION

That progesterone receptors contain multiple phosphorylation sites which are phosphorylated by different kinases and at different times in the activation process indicates that phosphorylation plays multiple roles in receptor function. Several studies have correlated

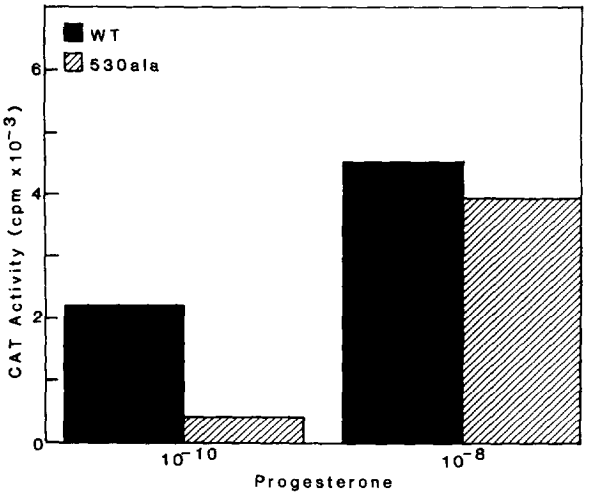

Fig. 2. The Ala^{530} mutant receptor is transcriptionally less active than the wild type at low concentrations of hormone. Each 10 cm dish of HeLa cells was transfected with 0.2 μg of either the wild type or mutant receptor DNA and 5 μg of PREtkCAT reporter DNA, treated with either 0.1 or 10 nM progesterone, and assayed for CAT activity 48 h after transfection as previously described [33].

phosphorylation with increased DNA binding. Denner *et al.* [25], using isocratic elution of DNA cellulose columns to characterize the DNA binding of cPR, found that receptor isolated from progesterone treated tissue and consequently more extensively phosphorylated, bound more tightly to DNA than did receptor isolated from control tissue. In more recent studies, Beck *et al.* [19] demonstrated that hPR isolated from T47D cells treated with R5020 for as little as 1 min exhibited dramatically enhanced binding to a progesterone response element (PRE) in electrophoretic mobility shift assays (EMSA) when compared to receptor treated with R5020 *in vitro*. Again, the enhanced DNA binding correlates with increased phosphorylation of hPR, but the effect of phosphorylation on DNA binding has not been directly demonstrated.

An alternate approach to studying the role of receptor phosphorylation has been to mutate the phosphorylation sites and to examine the function of the mutated receptor. Bai *et al.* [26] have mutated the hormone dependent phosphorylation site Ser^{530} in cPR_B (Ser^{402} in cPR_A) to alanine to block phosphorylation at this site. Analysis of the activity of this mutant using transient co-transfection of HeLa cells with cPR and a reporter gene $GRE_2E1bCAT$ showed that at saturating hormone, there was no difference in the activities of the Ala^{530} and wild type receptors when the activity was measured as a function of the amount of receptor DNA plasmid transfected [26]. However, as shown in Fig. 2, the activity of the mutant was substantially decreased relative to wild type at 10^{-10} M progesterone. This phosphorylation site is in the region between the hormone binding and DNA binding domains. Although this region is not required for hormone binding, its proximity to the ligand binding domain suggests that mutation might alter the ligand binding affinity. However, analysis of the hormone binding affinity using conventional *in vitro* ligand binding assays showed that the affinity is unchanged. Moreover, a whole cell assay performed under conditions in which the wild type receptor would be phosphorylated reveals no differences in hormone binding. Since this assay was performed at 37°C, the experiment also eliminates the possibility of a temperature-dependent change in hormone binding affinity. What then is the explanation for the change in activation as a function of hormone concentration? The purified cPR can bind to DNA in the absence of hormone and is active in an *in vitro* transcription assay. Moreover, this receptor can be activated in the absence of hormone by treating cells with activators of protein kinases or inhibitors of protein phosphatases [27], so hormone is not absolutely required for receptor activity. In the absence of hormone the receptor is associated with a heat shock protein (hsp) complex which includes hsp90 as well as a number of other hsps [28,29]. This complex is in a dynamic equilibrium with free receptor. Smith has shown that although hormone does not accelerate the dissociation of this complex, binding of hormone does prevent reformation of the complex [30]. Ser^{530} is in the hinge region between the ligand binding and hormone binding domains. Using an antibody directed against a peptide containing this site, Weigel *et al.* showed that this region is occluded in the receptor–hsp90 complex [31]. A likely possibility is that just as the hormone causes a conformational change which blocks reassociation, so too does the phosphorylation at Ser^{530}. Therefore, at saturating hormone the hormone binding site is always filled and both the phosphorylated and unphosphorylated forms of the receptor are equally dissociated from hsps and are equally active in the transient transfection assay. At low concentrations of hormone, some of the receptor will be unoccupied. In the case of the wild type receptor, if the receptor has already bound progesterone, it will still be phosphorylated, it would not reassociate with hsps and it would be available to dimerize and to activate transcription. On the other hand, the mutant receptor will return to its initial conformation upon dissociation of hormone and would reassociate with hsps. An alternative explanation for the activity of the mutant is that hormone is sufficient to cause appropriate conformational changes to produce good transcriptional activation in the absence of the phosphorylation and that the phosphorylated receptor maintains the conformation when the hormone dissociates whereas the mutant returns to a less active conformation.

LIGAND INDEPENDENT ACTIVATION OF THE cPR

Although the conventional model of steroid receptor activation assumes that the cognate hormone is

absolutely required for activation of the receptor, a number of studies in the last few years, beginning with the report by Denner *et al.* [27], have shown that at least under some circumstances, some of the steroid receptors can be activated in the absence of ligand. Denner *et al.* [27] showed that cPR transiently cotransfected into CV1 cells with a reporter gene PREtkCAT, can be transcriptionally activated by 8-Br-cAMP, an activator of protein kinase A. This activity was strictly dependent upon the presence of the progesterone receptor. Okadaic acid, an inhibitor of protein phosphatases 1 and 2A, can also activate the receptor. Whether this activation occurs through phosphorylation of the receptor is as yet unknown. None of the known phosphorylation sites in cPR are substrates for protein kinase A. However, *in vitro* phosphorylation studies show that protein kinase A can phosphorylate Ser^{528} immediately adjacent to the critical hormone dependent phosphorylation site described above. Alternatively, protein kinase A may act through a kinase cascade to cause phosphorylation of cPR at other sites or even to cause phosphorylation of receptor associated proteins allowing receptor activation.

More recent studies have shown that the cPR can be activated by other pathways as well. Power *et al.* [32] showed that the receptor can be activated by treatment of transfected cells with dopamine. The specificity of the dopamine agonists indicate that a receptor of the D1 type is responsible for this activation. Zhang *et al.* [33] have shown that, similar to the estrogen receptor [34, 35], the cPR can be activated by treatment of cells with epidermal growth factor (EGF) or by treatment with vanadate which blocks the activity of phosphotyrosine phosphatases. Clearly, this receptor can be activated by multiple signals initiated at the plasma membrane including those which are initiated by phosphorylation of tyrosines. The pathways by which these signals are transduced to activate the progesterone receptor remain to be elucidated. To date, phosphotyrosine has not been detected in cPR suggesting that if treatment with EGF causes phosphorylation of cPR it is through a cascade which causes serine or threonine phosphorylation.

The number of activators which activate cPR implicate multiple pathways in receptor activation. Alternatively, the signals may converge to activate only one or two kinases. The finding that there is a unique receptor mutant containing threonine in the place of Ser^{756} (Ser^{628} of cPR_A) which responds to hormone or to okadaic acid, but not to dopamine, demonstrates that there are at least two separate mechanisms by which the cPR can be activated in the absence of hormone.

MODULATORS OF KINASES AND ACTIVATION OF THE hPR

Although cPR [27], rat progesterone receptor [36] and the estrogen receptor from several species [34, 35, 37] all exhibit ligand independent activation, the results obtained with hPR isolated from T47D cells suggest that the hPR does not respond in the same way. Beck *et al.* [19] have shown that the activity of endogenous hPR in T47D breast cancer cells stably transformed with a reporter plasmid, MMTVCAT, is enhanced by treatment of the cells with activators of kinases such as 8-Br-cAMP, okadaic acid, or TPA in the presence of R5020. However, the receptors are not activated in the absence of ligand. This finding is consistent with the finding that the receptor isolated from T47D cells will not bind to DNA or enhance transcription *in vitro* in the absence of hormone. If, indeed, the ligand independent response is a biologically important activity, it is surprising that the hPR is not responsive. This phenomenon has only been examined in T47D cells or using expressed receptor whose sequence was determined from receptor DNA isolated from T47D cells. In the case of the estrogen receptor, the first estrogen receptor isolated contained a mutation and this mutant receptor also does not exhibit ligand independent activation although the wild type estrogen receptor does [37]. Whether the T47D receptor is, in fact, wild type hPR or contains a mutation remains to be examined.

Although the hPR does not exhibit ligand independent activation, its activity is affected by modulators of kinase activity. Of particular interest is the finding that treatment with 8-Br-cAMP in the presence of the antagonist RU486 causes activation of the hPR [38, 39]. Thus, a modulator of kinase activity can cause this antagonist to acquire agonist activity. RU486 promotes binding of receptor to DNA. When another progesterone antagonist, ZK98299, which does not promote strong DNA binding was used, 8-Br-cAMP was unable to activate the receptor; the ZK98299 blocked both the activity of the R5020 and of the RU486. The antagonist to agonist switch also exhibits specificity with respect to activators of kinases. Although treatment with TPA, which activates protein kinase C, is more effective in enhancing the R5020 dependent activity than is 8-Br-cAMP [19], treatment with TPA in the presence of RU486 does not cause activation of the receptor. This antagonist–agonist switch has important implications in considering treatments for breast cancer. Since oncogenes (which are frequently kinases) are activated in tumors, the use of an antagonist such as RU486 to block progestin action may not be effective whereas use of a compound such as ZK98299 would block PR activity.

In summary, although much remains to be learned, the recent studies of progesterone receptor as well as of the other members of the steroid receptor family indicate that phosphorylation plays a major role in receptor function and that there is substantial crosstalk between signal transduction pathways and the steroid receptors.

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