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# Phosphorylation Sites in Ligand-Induced and Ligand-Independent Activation of the Progesterone Receptor<sup>†</sup>

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**ABSTRACT:** Steroid hormone receptors are phosphoproteins that undergo hyperphosphorylation upon binding of hormone. The mechanism and the role of this reaction remain poorly understood. Two-dimensional analysis of ligand-free progesterone receptor (PR) tryptic digests showed the existence of seven main phosphopeptides. Incubation of the cells with the progestin R5020 led to a global increase in the levels of PR phosphorylation. However, the same phosphopeptides were seen, and their levels of labeling relative to each other were unchanged. A similar result was observed after incubation of the cells with the antiprogestin RU486. The antiprogestin ZK98299 demonstrated only half of the activity of RU486 in terms of receptor hyperphosphorylation, but the same phosphopeptides, proportionally labeled to the same extent, were observed by chromatography electrophoresis. Ligand-induced DNA binding did not play a role in receptor hyperphosphorylation since the mutant  $\Delta 547-592$ , which is devoid of the first zinc finger region, exhibited the same phosphopeptides, labeled to the same extent, as did wild-type receptor after incubation of cells with hormone. These results suggest that the same kinase(s) act *in vivo* on ligand-free and on agonist or antagonist-bound progesterone receptor. Binding of different ligands produces different conformational changes in the ligand binding domain of the receptor which enhance, to varying extents, affinity of the receptor for the kinase(s). The DNA binding region also plays a role in the interaction with the kinase(s), although binding to DNA *per se* is not necessary for the hyperphosphorylation of the receptor to take place. Similar phosphorylation patterns were induced by agonists and antagonists, suggesting that receptor hyperphosphorylation was not directly related to its transactivation properties. This conclusion was further supported in a cell-free transcription assay using purified receptor which had or had not undergone hyperphosphorylation *in vivo*. In conditions where their phosphorylation state was not changed during the incubation, the two receptor species produced the same enhancement of transcription. Receptor phosphorylation patterns were also shown to be unchanged during cAMP-induced, PR-mediated increase of transcription. Thus, both ligand-dependent and ligand-independent enhancement of PR biological activity is unrelated to a change in its phosphorylation state.

The phosphorylation of transcription factors and activators has been extensively studied [review in Hunter and Karin (1992); Meek & Street, 1992]. In many cases, phosphorylation has been shown to modulate transcription factor activity, either by changing their subcellular localization (Kessler & Levy, 1991; Moll et al., 1991; Mosialos et al., 1991), by modifying their DNA binding properties (Lüscher et al., 1990; Boyle et al., 1991a), or by regulating their transactivation efficiency (Binétruy et al., 1991; Gonzalez et al., 1991; Smeal et al., 1991). Steroid hormone receptors are ligand-dependent transcription regulators. They have been shown to be phosphoproteins that undergo a hyperphosphorylation event under the effect of the hormone (Logeat et al., 1985b; Pike & Sleator, 1985; Moudgil, 1990; Orti et al., 1992). With the exception of one series of results obtained with the estrogen receptor (Migliaccio et al., 1986), serines have been shown to be the major substrate of

phosphorylation. Studies have localized these modified residues to the N-terminal half of the progesterone (Sheridan et al., 1988; Sullivan et al., 1988; Chauchereau et al., 1991), estrogen (Le Goff et al., 1994), glucocorticoid (Hoeck & Groner, 1990; Bodwell et al., 1991), and androgen receptors (Kuiper et al., 1993). However phosphorylation sites outside this region have been described for the chicken progesterone receptor (Denner et al., 1990; Poletti & Weigel, 1993) and for the mouse (Lahooti et al., 1994) and human estrogen receptor (Ali et al., 1993). In all cases, multiple phosphorylation sites have been reported.

Based upon *in vivo* experiments (Takimoto et al., 1992) and *in vitro* transcription studies (Bagchi et al., 1991, 1992), it has been proposed that the hyperphosphorylated receptor is the biologically active species. Binding of the hormone to the receptor provoked receptor dissociation from a complex involving several heat shock proteins and the subsequent binding of receptor to DNA. At both of these steps, specific kinases were supposed to modify the receptor in several successive reactions. Furthermore, it appears that enhancement of transcription can occur through receptors and hormone responsive elements in the absence of ligand (Power et al., 1991a; Sartorius et al., 1993). This was achieved by treatment of cells with cyclic AMP or with

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adenylate cyclase activators. These experiments thus agreed with the hypothesis which suggests that hyperphosphorylation may be involved in the acquisition of biological activity by the receptor.

We have previously shown that heterologous cells transfected with an expression vector encoding the rabbit progesterone receptor (PR) performed basal and hormone-induced phosphorylation in a manner similar to that seen with the natural target cells for progesterone (Chauchereau et al., 1991). Using this same system, we have further analyzed several of the features of receptor phosphorylation. However, due to the existence of multiple phosphorylation sites, it was possible that one or a small number of sites were involved in the ligand-induced or ligand-independent activation of the receptor. To answer this question, we have performed phosphopeptide analysis of the receptor in ligand-free conditions, after binding to DNA and after ligand-dependent and ligand-independent activation. We have also analyzed the transcriptional activity of purified, hyperphosphorylated or non-hyperphosphorylated receptor in a cell-free transcription assay.

## EXPERIMENTAL PROCEDURES

**Cell Culture and Transient Expression.** COS-7 cells were cultured at 37 °C and 5% CO<sub>2</sub> in DMEM medium (Gibco) supplemented with 10% fetal calf serum. Transfection of the cells was performed using the calcium phosphate method as previously described (Chauchereau et al., 1991) except that the charcoal-stripped fetal calf serum was replaced by charcoal-stripped calf serum (BioMérieux) 1 day before the transfection. For studies of the hormonal induction of a reporter gene, the cells were co-transfected with an expression vector encoding PR (5 µg/10 cm<sup>2</sup> plate) and the reporter gene PRE<sub>2</sub>-tk-CAT (5 µg/plate). The pCH110 β-galactosidase expression vector (5 µg/plate) was used as an internal control, and Herring sperm carrier DNA was added to a total of 20 µg of DNA/plate. The steroids were added in ethanol 24 h before harvesting the cells.

**DNA Constructs.** The reporter gene PRE<sub>2</sub>-tk-CAT contains two copies of the GRE/PRE II element of the TAT gene (Strähle et al., 1987; Tsai et al., 1989) inserted into the previously described TK-CAT vector (Klein-Hitpass et al., 1986).

The plasmic PRE<sub>2</sub>-pLov contains two copies of the same GRE/PRE element inserted at the BgIII site of the pLovTATA plasmid (Klein-Hitpass et al., 1990; Elliston et al., 1990) upstream of the HSV thymidine kinase promoter sequences (-41/-29) (McKnight & Kingbury, 1982) and the chicken ovalbumin promoter (-33/-21, including the TATA box), which is linked to a G-free cassette of 377 bp (Klein-Hitpass et al., 1990). The pMLcas9 control plasmid is a derivative of pMLC<sub>2</sub>AT19 (Sawadogo & Roeder, 1985) and contains the adenovirus major late promoter (AdML) (-400/+10) linked to a 180-bp G-free cassette. All the constructs were verified by sequencing.

**Receptor Purification.** The receptors were purified essentially as previously described (Logeat et al., 1985a,b), but with some minor modifications. Briefly, hyperphosphorylated and non-hyperphosphorylated receptors were prepared in parallel from uteri of prepubertal rabbits that had been treated daily with diethylstilbestrol (100 µg) for a total of 9 days. Hyperphosphorylated receptor was obtained by sub-

cutaneous injecting of 10 mg of R5020 into animals 30 min before sacrifice. Uteri were homogenized in 10 mM Tris, pH 7.4, 1.5 mM EDTA, 10% glycerol, and 300 mM NaCl in the presence of a cocktail of protease inhibitors (Logeat et al., 1985a) and of phosphatase inhibitors (50 mM NaF, 20 mM disodium pyrophosphate, 25 mM β-glycerophosphate, 50 nM okadaic acid). A total of 3 mL of buffer was used per gram of tissue. R5020 (10<sup>-8</sup> M) was added in the homogenization buffer for the preparation of the hyperphosphorylated receptor. Tissue extracts containing receptors were obtained after centrifugation at 100000g for 65 min and were diluted to obtain a final NaCl concentration of 80 mM. The extracts containing the non-hyperphosphorylated receptor were incubated for 2 h at 0 °C with 10<sup>-7</sup> M [<sup>3</sup>H]-R5020 (3 Ci/mmol, NEN Dupont) and were then chromatographed through two successive monoclonal antibody-coupled protein A-Sepharose columns as described (Logeat et al., 1985a). The first column contained a nonspecific antibody, while the Mi 60 antireceptor antibody was coupled to the second column. Phosphatase inhibitors were added during the first washes of the Mi 60 column. Receptors were eluted as described (Logeat et al., 1985a) and were concentrated in a 20 mM Tris, pH 7, 10% glycerol, 50 mM NaCl, and 2 mM DTT buffer using the Centricon-30 system (Amicon). The final receptor concentration was determined by immunoblot using the Mi 60 antibody.

**In Vitro Transcription.** The *in vitro* transcription assay, which uses template plasmids containing G-free cassettes and HeLa cell nuclear extract, has been previously described (Klein-Hitpass et al., 1990; Bagchi et al., 1990; Elliston et al., 1990). Transcription assays were performed in a 30-µL volume and contained 25 mM HEPES, pH 7.9, 21 mM Tris, pH 7.5, 27 mM KCl, 25 mM NaCl, 3 mM DTT, 5 mM MgCl<sub>2</sub>, 10% glycerol, 2.5 mM creatine phosphate, 0.6 mM ATP, 0.6 mM CTP, 20 µM UTP, 12.5 µCi [α-<sup>32</sup>P]UTP (NEN Dupont), 0.45 mM 3-*O*-methyl-GTP, 10 units of T1 RNase, 0.05 mM EDTA, 0.05 mM PMSF, 100 ng of the test template PRE<sub>2</sub>pLov, 50 ng of the internal control template pMLcas9, 1 µg of sonicated herring sperm DNA, and 75 µg of a HeLa nuclear extract (Promega). Purified receptor (non-hyperphosphorylated or hyperphosphorylated) was added at a concentration of 13 pmol/mL. Incubations were carried out for 20 min at 30 °C and were stopped by the addition of 70 µL of a solution containing 25 mM Tris, pH 7.5, 10 mM EDTA, 0.5% SDS, 200 µg/mL yeast tRNA (BRL), and proteinase K (400 µg/mL). After an incubation of 30 min at 37 °C, 200 µL of a solution of 7 M urea in 10 mM Tris, pH 8, and 1 mM EDTA was added. Two extractions in phenol/chloroform/isoamyl alcohol (25/24/1) and one in chloroform/isoamyl alcohol (24/1) were performed before RNA was precipitated in 0.3 M sodium acetate and cold ethanol (2.5 vol). The pellets were washed, dried, and solubilized in 80% formamide, 10 mM NaOH, and 1 mM EDTA. The samples were then boiled 5 min and loaded onto a 7 M urea, 6% acrylamide gel. The dried gel was autoradiographed using direct-exposure Kodak DEF-5 films, and the transcript quantification was achieved by measuring integrated optical densities using an Imager system (Millipore Corp.). Values obtained for the transcripts originating from the PRE-driven test template were corrected for the values obtained for the transcripts derived from the control template.

**Receptor Phosphorylation and Phosphopeptide Tryptic Mapping.** The day after transfection, cells were preincubated

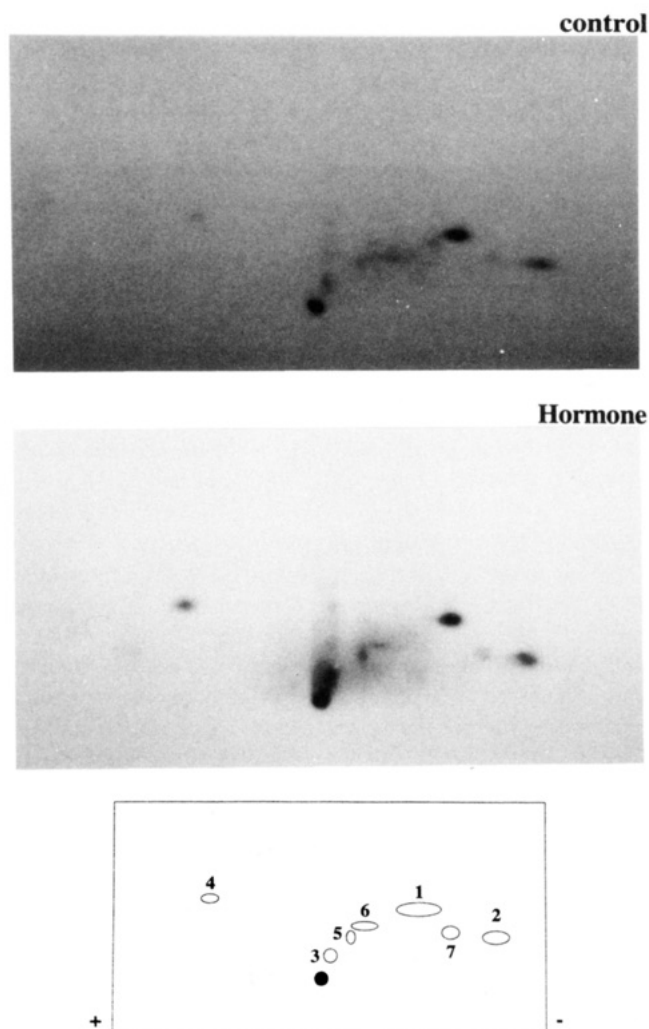
for 4 h in a phosphate-free DMEM medium containing 10% dialyzed charcoal-stripped fetal calf serum and were then incubated for 24 h at 37 °C with 0.167–0.5 mCi/mL [ $^{32}$ P]orthophosphate (carrier free; 10 mCi/mL) in 3 mL of the same medium. The progestin R5020 ( $10^{-8}$  M) or the antiprogestins RU486 ( $10^{-8}$  M), ZK 98299 ( $10^{-7}$  M), or 8-Br-cAMP ( $10^{-3}$  M) were added with [ $^{32}$ P]orthophosphate. Cells were lysed in the presence of phosphatase inhibitors as described above, and the receptors were immunoprecipitated using the monoclonal antibody Let 126 as previously described (Chauchereau et al., 1991). The phosphorylated receptors were solubilized with SDS sample buffer and were resolved by electrophoresis on a 7.5% polyacrylamide gel.

The phosphorylated receptors were visualized by gel autoradiography, extracted from the gel slices, and digested with trypsin (trypsin sequencing grade, Boehringer) according to the procedure described by Boyle et al. (1991b). The resultant phosphopeptides were separated in two dimensions on cellulose thin-layer plates (Merck) by electrophoresis at pH 1.9 for 45 min using the HTLE-7000 system and then by ascending chromatography in acetic acid/1-butanol/pyridine. Autoradiography was performed with Kodak no-screen DEF-5 films, and quantification was achieved by measuring integrated optical density of the spots using an Imager system (Millipore Corp.).

**Assay of Reporter Gene Activity.** Transfected cells were lysed in PBS buffer containing 5 mM EDTA by five cycles of freezing/thawing. The cellular extracts were obtained by centrifugation, and protein concentrations were determined using the BCA assay (Pierce Laboratories). The CAT activity was measured according to the method described by Gorman et al. (1982), using 2  $\mu$ g in the protein of COS-7 cellular extracts and 0.5  $\mu$ Ci of [ $^{14}$ C]chloramphenicol. The reaction products were resolved by thin-layer chromatography in chloroform/methanol (90:10) for 45 min and autoradiographed with Kodak X-OMat films. Quantitative determination was obtained by the excision and counting of the radioactivity present in the spots.

## RESULTS

**Basal and Hormone-Induced Phosphorylation Occur in the Same PR Phosphopeptides.** We have previously shown that hormone treatment caused a  $\sim 7$ -fold increase in the phosphorylation level of the progesterone receptor which had been transiently expressed in COS-7 cells. However, it was not clear if this increase corresponded to the phosphorylation of new residues in the protein or to an increase of the stoichiometry of incorporation of phosphates into basally phosphorylated sites. We thus used two-dimensional phosphopeptide analysis to investigate differences in the phosphorylation state of individual peptides as produced by the binding of the hormone to the receptor. PR, transiently expressed in COS-7 cells, was labelled *in vivo* to steady state with [ $^{32}$ P]orthophosphate in the presence or absence of R5020. Progesterone receptors were immunoprecipitated and submitted to SDS-PAGE. Phosphorylated receptors were then excised from the gel and treated with trypsin, and the phosphopeptides were resolved by two-dimensional electrophoresis and chromatography. Since the phosphopeptide pattern was reproducible and did not change with longer incubation with trypsin, we assumed that we had reached the limit of digestion and that all possible cleavages had occurred.



**FIGURE 1:** Phosphorylation sites of the progesterone receptor in the absence of ligand and after binding of hormone. COS-7 cells were transfected with a vector encoding the wild-type rabbit progesterone receptor (Loosfelt et al., 1986). The cells were incubated with [ $^{32}$ P]orthophosphate in the absence (control) or in the presence of  $10^{-8}$  M progestin R5020 (hormone). The receptors were immunoprecipitated with the monoclonal antibody Let 126 as previously described (Chauchereau et al., 1991) and separated on a 7% acrylamide gel before being digested with trypsin as described in Experimental Procedures. The tryptic phosphopeptides were separated on thin-layer cellulose plates by electrophoresis at pH 1.9 and by ascending chromatography and visualized by autoradiography (3-day exposure). A scheme shows the seven major phosphopeptides numbered from 1 to 7. The black spot represents radioactivity remaining at the origin. The same peptides were observed in four separate experiments. Scanning and measuring the integrated optical densities of all the spots has shown a parallel change in labeling of all the peptides under the effect of the hormone.

The phosphopeptide map of the receptor which had been phosphorylated in basal conditions (absence of hormone) showed the existence of seven major phosphopeptides (Figure 1). One of these peptides was more extensively phosphorylated than the others and was referred to as no. 1. Another was extremely acidic as shown by its migration toward the anode and was named no. 4. Comparison with the hormone-treated hyperphosphorylated PR phosphopeptide map showed the same number and the same relative localization of all the phosphopeptides. The relative increase in phosphorylation was identical in all the peptides (some minor differences in the intensity of labeling could be observed from different phosphopeptides in individual experiments but were

not reproducible). These experiments thus indicated that treatment with the hormone did not provoke PR phosphorylation at any major new site but rather increased the phosphorylation at all of the sites which were phosphorylated in the basal conditions. It should be noted that phosphorylation of another serine residue on a peptide already bearing one or several phosphoserines would have modified its electrophoretic migration and that this would have been detectable.

**Antagonists and Agonists Induce Phosphorylation at the Same Sites.** RU486 and ZK98299 inhibit the transactivation properties of the progesterone receptor (Philibert et al., 1985; Horwitz, 1985; Guiochon-Mantel et al., 1988; Wiechert & Neef, 1987; Henderson, 1987; Delabre et al., 1993). In these studies, they both increased the phosphorylation of PR. Whereas RU486 had an activity similar to that of the agonist R5020, ZK98299 provoked only about 40% of the hyperphosphorylation seen with the agonist (Figure 2A). The latter phenomenon has been observed previously (Takimoto et al., 1992) and was ascribed to the fact that ZK98299–receptor complexes were supposed to be unable to bind to DNA. It was hypothesized that these complexes did not undergo phosphorylation mediated by the DNA-dependent kinase, in contrast to the remaining phosphorylations, which could be accomplished by the other kinases acting on the receptor. To verify this hypothesis, we analyzed the receptor phosphopeptides obtained after treatment of the cells by both antagonists. As shown in Figure 2B, the same phosphotryptic pattern was observed for the two antagonists and for the agonist.

Previous experiments have shown receptor hyperphosphorylation to occur in the presence of RU486 (Sheridan et al., 1988; Chauchereau et al., 1991). This was an evidence against the hypothesis according to which the hyperphosphorylated protein was the active form of the receptor. However, the possibility remained that a specific serine exists whose phosphorylation gives rise to the active form of the receptor and whose phosphorylation is masked by the occurrence of several other activity-unrelated phosphorylations. The comparison of the phosphopeptide maps of agonist-bound and antagonist-bound receptor has allowed us to dismiss this hypothesis.

**Phosphopeptide Analysis of Non-DNA-Binding Mutant of Progesterone Receptor.** The suggested involvement of the DNA-dependent kinase in receptor phosphorylation *in vivo* was based on two lines of evidence: the decrease in hyperphosphorylation due to ZK98299 (see above) and the decreased phosphorylation of some receptor mutants unable to bind DNA (Takimoto et al., 1992). We have also observed a decrease in hormone-provoked hyperphosphorylation of several mutants of the zinc finger region (Chauchereau et al., 1991). However the  $\Delta 547$ –592 mutant receptor, in which the first zinc finger region is deleted, is unable to bind DNA but undergoes normal hyperphosphorylation including the previously described upshift in electrophoretic mobility (Figure 3A). However the possibility remained that only one (or a small number) of the serines underwent a DNA-dependent phosphorylation. As shown in Figure 3B, this hypothesis was not supported by the phosphopeptide analysis, which showed similar patterns for the DNA-binding and the non-DNA-binding receptor species.

**Effect of Receptor Hyperphosphorylation on Cell-Free Transcriptional Activation.** Beside the *in vivo* evidence

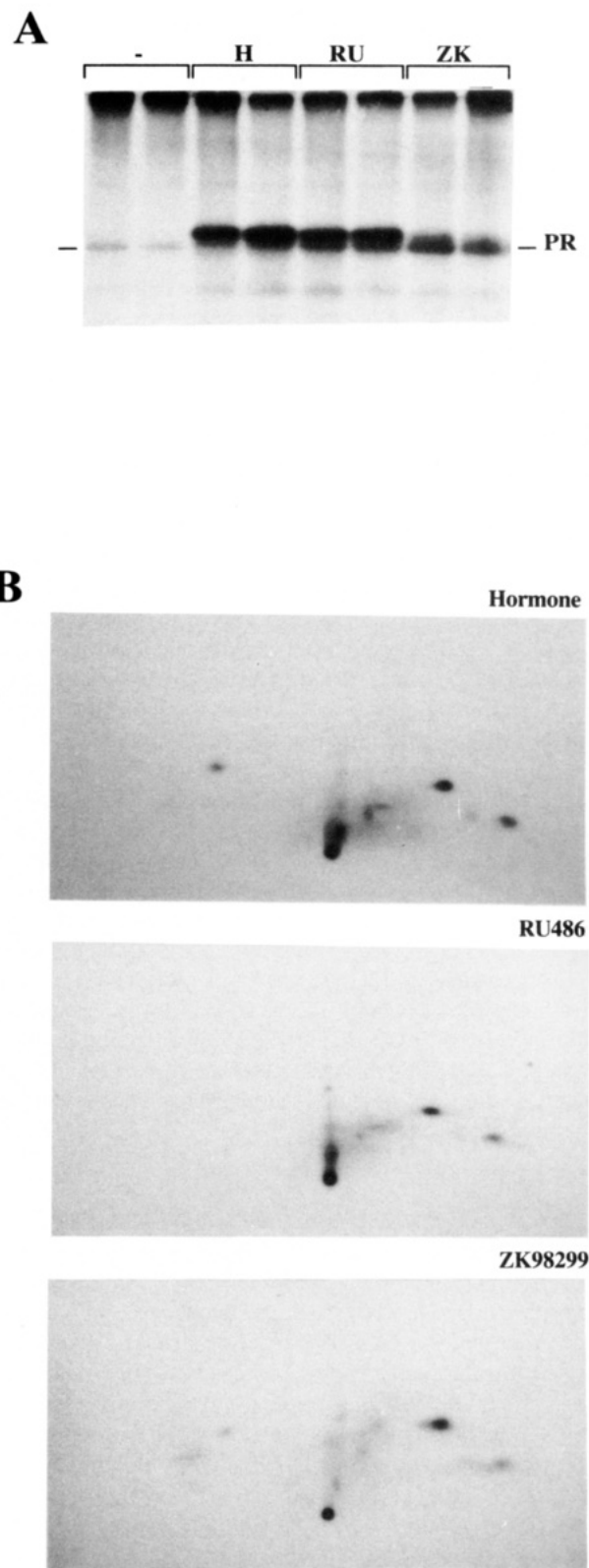


FIGURE 2: Phosphorylation sites of the antagonist-bound progesterone receptors. (A) Wild-type receptor was expressed in COS-7 cells, in the absence of hormone (–) or in the presence of  $10^{-8}$  M R5020 (H),  $10^{-8}$  M RU486 (RU), or  $10^{-7}$  M ZK98299 (ZK). The receptors were immunoprecipitated with the monoclonal antibody Let 126 as previously described (Chauchereau et al., 1991). [ $^{32}$ P]-Orthophosphate incorporation was detected by autoradiography of the nondried gel for 2 h. Position of PR is indicated. (B) The tryptic phosphopeptides were obtained as described in the legend of Figure 1 and visualized by autoradiography for 3 days. One of four experiments is shown. Scanning and measuring the integrated optical densities of all the spots showed parallel changes in labeling of all the peptides under the effect of the different ligands.



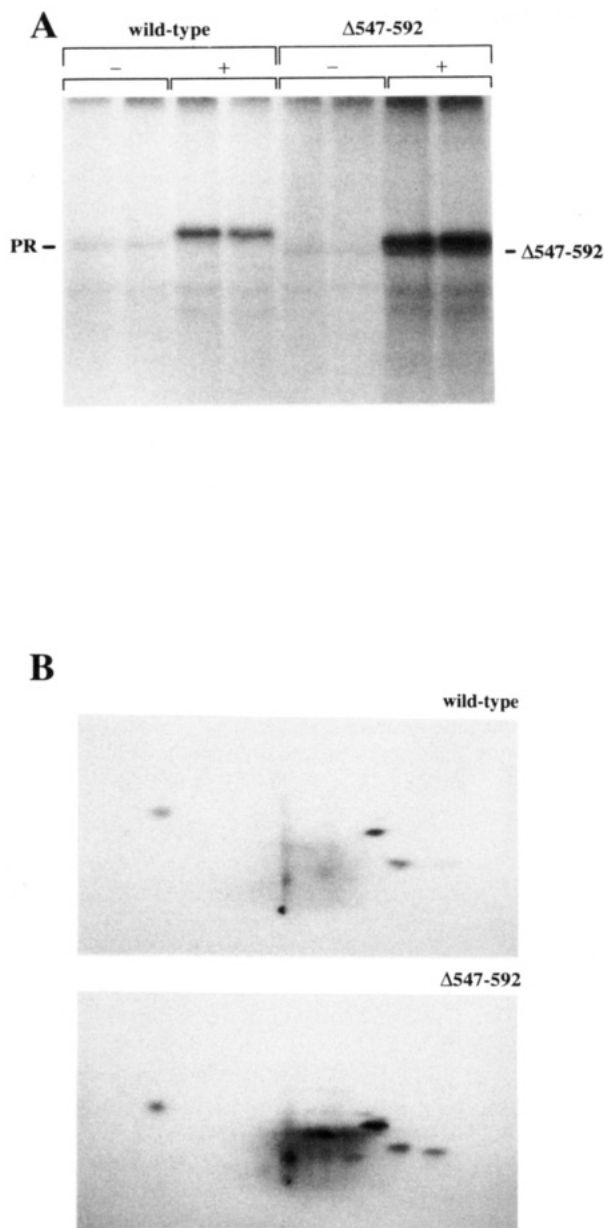


FIGURE 3: Receptor hyperphosphorylation is independent of DNA binding. (A) The wild-type receptor (PR) and the mutant  $\Delta 547-592$ , in which the first zipper of the DNA-binding domain was deleted, were expressed in COS-7 cells in the absence (-) or in the presence of  $10^{-8}$  M R5020 (+). The receptors were immunoprecipitated with the monoclonal antibody Let 126. [ $^{32}$ P]Orthophosphate incorporation into the receptor was detected by autoradiography of the nondried gel for 2 h. (B) Tryptic phosphopeptide maps of the mutant  $\Delta 547-592$  and of the wild-type receptor (prepared from hormone-treated cells) were obtained as described in Experimental Procedures. One of three separate experiments is shown.

discussed above, *in vitro* experiments using cell-free transcription systems have also been used to support the concept that receptor hyperphosphorylation is responsible for its biological activity (Bagchi et al., 1991, 1992; Weigel et al., 1992). This conclusion was mainly based on the change in electrophoretic mobility (upshift) that occurs during activation of transcription by the basally phosphorylated receptor. We devised an experiment to verify this hypothesis: the receptor was purified from the uteri of rabbits treated or not treated by the progestin R5020. We could thus add a receptor which has been hyperphosphorylated *in vivo* (Logeat et al., 1985b) or a control receptor to cell-free transcription

systems. If the incubation was carried out for a time which has been shown to be insufficient to modify the phosphorylation status of the receptor, the difference in transactivation properties between the two states of the receptor should be detectable.

To test this hypothesis, the purified receptors were incubated for 20 min with a HeLa nuclear extract in an *in vitro* transcription reaction mixture including the PRE<sub>2</sub>-pLov test template. This synthetic gene contains two copies of a PRE placed 20 bp upstream of a TATA box linked to a 377 bp long G-free cassette (see Experimental Procedures). As shown in Figure 4A, no detectable initiation from the PRE-linked promoter (marked by an arrow) occurred in the absence of PR, whereas the control adenovirus major late promoter was active. Addition of PR enhanced, in a dose-dependent manner (not shown), the transcription from the PRE-linked promoter without changing the level of transcription from the control promoter. In Figure 4A, it can be seen that both purified hyperphosphorylated and non-hyperphosphorylated receptors induced a similar increase in transcription from the PRE-linked promoter (scanning of the autoradiogram confirmed the visual impression: relative values of the PR-induced transcripts versus the control adenovirus promoter transcripts were 27% and 23% for the non-hyperphosphorylated and the hyperphosphorylated receptors, respectively). We then verified the electrophoretic mobility of the receptors recovered from the cell-free transcription incubations by immunoblotting. As shown in Figure 4B, no obvious change in electrophoretic mobility had occurred with the receptor that had been hyperphosphorylated *in vivo* giving the characteristic upshift, which was not observed for the control receptor. The only change that was observed was a decrease in intensity of the receptor bands. This was probably due either to adsorption or proteolysis of the receptor which occurred during the incubation. Thus, no evidence of a link between receptor hyperphosphorylation and its transactivation properties could be observed in cell-free transcription conditions.

#### Progesterone Receptor Phosphopeptide Analysis in Ligand-Independent, cAMP-Dependent Activation of Transcription.

A number of studies, in particular those concerning the chicken PR, have suggested that the cAMP-dependent protein kinase (PKA) is involved in the regulation of the steroid receptor function. It has been shown that treatment with 8-bromo-cyclic AMP (8-Br-cAMP) enhances progesterone receptor-mediated transcription in the absence of the hormone. Based on these results, it has been suggested that PKA activates PR-mediated transcription by phosphorylation and that PR phosphorylation is a mechanism by which PR transcriptional activity is regulated (Denner et al., 1990).

To establish if the PKA signaling pathway is involved in the PR-mediated transcriptional activation and if, as proposed, PR phosphorylation is the underlying mechanism, we have analyzed the effects of elevated cAMP levels on both PR-mediated transcriptional activation and on PR phosphorylation.

COS-7 cells were cotransfected with the PRE<sub>2</sub>-tk-CAT construct (see Experimental Procedures) and the rabbit progesterone receptor expression vector. Treatment of these cells with R5020 or 8-Br-cAMP led to equivalent increases in PR-mediated transcription (Figure 5A); a combined treatment resulted in a 2–3-fold enhancement of the progestin induction. The effect of 8-Br-cAMP was observed in

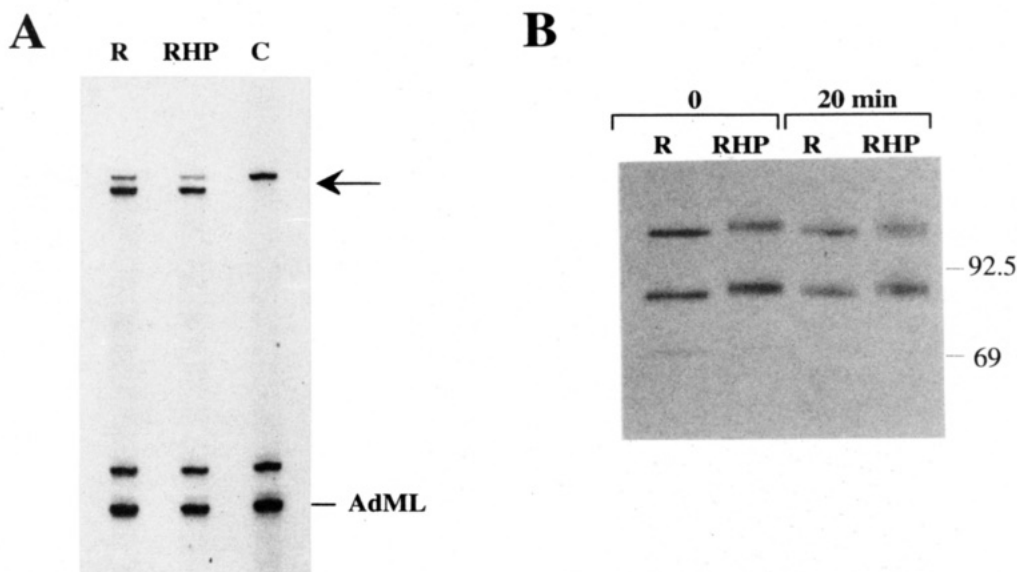


FIGURE 4: Transcriptional activity of hyperphosphorylated and non-hyperphosphorylated receptors in a cell-free system. (A) *In vitro* transcription was performed for 20 min with the PRE<sub>2</sub>-pLov test template as described in Experimental Procedures in the absence of progesterone receptor (C) or in the presence of purified receptors (R = receptor from control rabbits, RHP = receptor from progestin-treated rabbits). The arrow indicates the transcripts correctly initiated from the test template PRE<sub>2</sub>-pLov. The transcripts initiated from the AdML control test template are also indicated. (B) The phosphorylation state of the purified receptors used for the *in vitro* transcription assay was analyzed by electrophoresis and immunoblotting. 0, receptors before incubation in the *in vitro* transcription conditions; 20 min, receptors after 20-min incubation in the *in vitro* transcription conditions. The basally phosphorylated (R) or hyperphosphorylated (RHP) receptors were visualized with the monoclonal antibody Mi 60, which recognizes the full-length receptor and the 79-kDa species (Loosfelt et al., 1984). Molecular weight markers (kDa) are indicated.

the presence or the absence of serum (not shown) (Power et al., 1991a; Aronica & Katzenellenbogen, 1993). Similar results were obtained with forskolin.

Analysis of the phosphorylation state of the progesterone receptor was performed after its incubation in the presence or in the absence of 8-Br-cAMP. The labeled receptors were immunoprecipitated with Let 126 monoclonal antibody and resolved by polyacrylamide gel electrophoresis. As we had previously shown, PR transiently expressed in COS-7 cells was phosphorylated in the absence of hormone (Figure 5B). Hormone treatment led to a strong increase in phosphorylation, which was accompanied by a characteristic gel retardation (upshift). Treatment of the cells with 8-Br-cAMP alone did not produce any enhancement of phosphorylation, nor did 8-Br-cAMP increase the progestin-induced hyperphosphorylation when combined with hormone. The transcriptional effect of the 8-Br-cAMP could not be attributed to the modification of a particular phosphorylation site since the two-dimensional maps of receptors isolated from hormone-treated or nontreated cells were not changed by the addition of 8-Br-cAMP (not shown). Taken together, these results show that 8-Br-cAMP modifies neither the basal nor the hormone-dependent phosphorylation levels of the PR, suggesting that the effect of 8-Br-cAMP on PR-mediated transcriptional activity is not directly related to a change in the phosphorylation of the receptor.

## DISCUSSION

We have identified seven peptides in the progesterone receptor that can be strongly phosphorylated. Similar results have been obtained for the glucocorticoid receptor (Bodwell et al., 1991). The existence of multiple sites of phosphorylation makes the interpretation of mutagenesis studies difficult, since multiple substitutions have to be made in the protein to prevent its phosphorylation. This has been shown

to often lead to a relatively limited change in transcriptional activation (Mason & Housley, 1993; Le Goff et al., 1994). In addition, it is difficult to interpret such an effect since it may be related to the change in the conformation of the receptor provoked by the introduction of multiple mutations. Furthermore, it has been shown that mutagenesis of a specific serine can lead to a compensatory phosphorylation of a proximal site (Hilliard et al., 1994). These observations have made it especially important to identify the specific amino acids whose phosphorylation may be related to changes in the biological activity of the receptor. This identification could then allow very limited but effective mutagenesis to be performed. Such residues would be expected to be those which are specifically modified after administration of agonists but not of antagonists.

However, in this study, two-dimensional analyses showed that hormone treatment did not provoke the appearance of new phosphopeptides and that the increase of phosphorylation was proportional for all the peptides. Furthermore, administration of antihormones led to a similar pattern. These results suggest that the same serines are phosphorylated in the absence of hormone or in the presence of agonists or antagonists. Only the extent of phosphorylation varies. The method which was used for these studies would have detected the addition of a new phosphorylation site on an already phosphorylated peptide since this would have led to a change in its electrophoretic mobility. The only possible technical pitfall is the inability to detect the exact replacement of one phosphoserine by another in the same peptide. Such a mechanism seems highly unlikely.

These observations are in contrast to reports of studies performed with the chicken progesterone receptor where two serines have been suggested to be specifically phosphorylated after hormone administration (Denner et al., 1990; Poletti & Weigel, 1993). In the case of the estrogen receptor, it

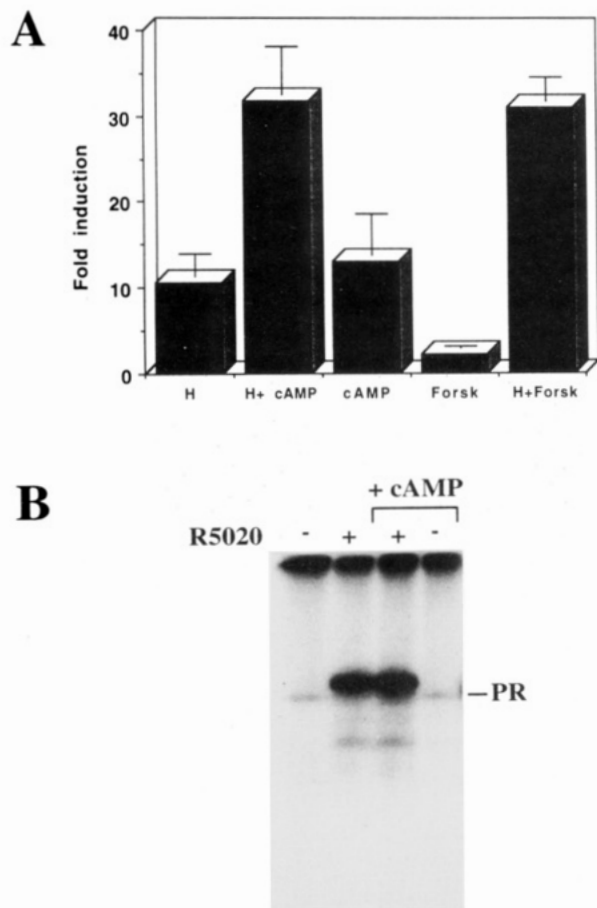


FIGURE 5: Effect of cAMP on the biological activity and the phosphorylation of PR *in vitro*. (A) Effect on transcriptional activation by PR. COS-7 cells were co-transfected with the wild-type PR expression vector and the PRE<sub>2</sub>-TK-CAT construct and were treated with  $10^{-8}$  M R5020 and/or  $10^{-3}$  M 8-Br-cAMP and/or  $10 \mu$ M forskolin. After 24 h, the cells were harvested and lysed, and CAT activity was measured as described in Experimental Procedures. The fold induction shown in the figure was calculated as the ratio of CAT activity in hormone- or/and cAMP-treated or/and forskolin-treated cells over CAT activity in untreated control cells. Each assay was performed in triplicate, and each experiment has been done three times. (B) Cyclic AMP does not modify the phosphorylation of PR. COS-7 cells were transfected with the vector encoding the wild-type rabbit progesterone receptor (PR) and incubated with [ $^{32}$ P]orthophosphate in the presence or in the absence of the progestin R5020 ( $10^{-8}$  M), of 8-Br-cAMP (1 mM), or of both compounds. The receptors were immunoprecipitated with the monoclonal antibody Let 126, separated on a 7% acrylamide gel, and visualized by autoradiography.

has been reported that the same sites are phosphorylated in basal and hormone- and antihormone-induced conditions (Le Goff et al., 1994). In the case of the glucocorticoid receptor, hyperphosphorylation has been only observed for the agonist-bound receptor (Hoeck et al., 1989; Orti et al., 1989). In addition, a two-dimensional analysis suggested that the phosphorylation of at least one phosphopeptide was specifically increased by agonist treatment (Somers & DeFranco, 1992). We only observed minor differences in the intensity of labeling of individual phosphopeptides, and these differences were not reproducible.

The role of DNA binding in receptor hyperphosphorylation has also been the subject of debate. Weigel et al. (1992) have shown that the chicken progesterone receptor may be phosphorylated by a DNA-dependent protein kinase *in vitro*. By mutating a single cysteine of the first zinc finger region,

Horwitz and co-workers have produced a receptor species unable to bind to DNA and in which hormone-induced hyperphosphorylation was markedly reduced (Takimoto et al., 1992). The same group has correlated the decreased phosphorylation due to ZK98299 with the lack of *in vitro* binding to DNA of ZK98299-receptor complexes. However, as shown in the present work, a mutant deleted of the first zinc finger region, and thus unable to bind DNA, is still hyperphosphorylated and exhibits the same profiles of phosphopeptides as the wild-type receptor. In a previous study, we have shown that deletion of the second zinc finger region markedly diminishes the hyperphosphorylation (Chauchereau et al., 1991). It is thus probable that the DNA-binding region is involved in the interaction with the kinase(s) but that the binding to DNA is not essential for the reaction to take place. Similarly, the change of electrophoretic mobility of the receptor (upshift) after hyperphosphorylation has been ascribed to the action of the DNA-dependent kinase (Weigel et al., 1992; Takimoto et al., 1992). This is not substantiated by the demonstration of an upshift in mutant  $\Delta 547-592$  (devoid of the first zinc finger) and its absence in mutant  $\Delta 663-930$  (Chauchereau et al., 1991), which does bind to DNA. It is thus possible that the upshift is linked to the multiplicity of phosphorylation sites which modify the accessibility of SDS to the protein.

Takimoto et al. (1992) have proposed the existence of three steps in receptor phosphorylation, which are probably catalyzed by different enzymes: basal phosphorylation, hormone-dependent phosphorylation, and hormone-dependent DNA-dependent phosphorylation. This succession of steps is predicted to result in the production of a transcriptionally active receptor. Our experiments do not concur with this hypothesis. The same phosphopeptides are produced in the absence of hormone, in its presence, in the presence of an antagonist, and irrespective of DNA binding. It is thus likely that the same protein kinase(s) act on both ligand-free and ligand-bound receptor. However, the affinity for the kinase(s) is increased by the conformational change which follows the binding of ligand. Several different conformational states can probably result from the binding of different ligands, the affinity of the kinase(s) for the ZK98299-bound receptor being lower than its affinity for the R5020- or RU486-bound receptor. Interactions of the kinase(s) with the receptor probably involve the steroid-binding and the DNA-binding domains (Chauchereau et al., 1991). Some sites of phosphorylation of the receptors have been determined. They correspond mainly to consensus sequences for proline-directed protein kinase, MAP kinase, and Cdc2 kinase (Bodwell et al., 1991; Le Goff et al., 1994; Poletti & Weigel, 1993). However, the actual involvement of these enzymes in the *in vivo* phosphorylation of receptors has yet to be proven.

An increase in cyclic AMP concentration has been shown to enhance transcriptional activity of the progesterone (Power et al., 1991a; Sartorius et al., 1993; Beck et al., 1992, 1993) and various other receptors (Power et al., 1991a,b; Rangarajan et al., 1992; Nordeen et al., 1993; Aronica & Katzenellenbogen, 1993; Huggenvik et al., 1993). It has been noted that such an effect can even be observed in the absence of ligand (Power et al., 1991a) or can turn an antagonist-bound receptor into a positive regulator (Sartorius et al., 1993; Beck et al., 1993; Nordeen et al., 1993). These experiments were interpreted as indicating that various means of enhancing



receptor phosphorylation could lead to the production of an active transactivator (Denner et al., 1990; Power et al., 1991a; Beck et al., 1993; Aronica & Katzenellenbogen, 1993). Experiments correlating the activity of the receptor *in vitro* with its phosphorylation state also concurred with this hypothesis (Weigel et al., 1992; Bagchi et al., 1992). Conversely, we show here that a change in the phosphorylation of the receptor is not involved in the transcriptional activation by cyclic AMP and that transcription activation in the acellular system also occurs with a non-hyperphosphorylated receptor. The biological role of hyperphosphorylation is probably more subtle than the transformation of a nonactive receptor into an active transcription regulator. If indeed hyperphosphorylation is linked to transcriptional activation, it may be restricted to some promoters and to some cell types.

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