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HORMONE SPECIFIC PHOSPHORYLATION AND TRANSFORMATION OF CHICKEN OVIDUCT PROGESTERONE RECEPTOR

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SUMMARY. Phosphorylation of chick progesterone receptor (PR) was attempted by incubating tissue minces from estrogen-primed oviducts with ortho [^{32}P]phosphate in the absence and presence of different steroids. The phosphorylated PR was immunopurified from the cytosol using anti-PR monoclonal antibody αPR22 (Sullivan et al., 1986). Although all three known peptides of PR, peptides B (110K), A (79K) and the 90 kDa nonhormone binding peptide (heat shock protein, hsp-90), were phosphorylated, the presence of only progesterone increased the degree of phosphorylation of receptor peptides A and B and the dissociation of the hsp-90 from the PR heterooligomer. Other steroids, cortisol, estradiol and dihydrotestosterone (DHT) had no effect on the phosphorylation or on the dissociation of hsp-90 from the PR. Incubation of phosphorylated PR at 23°C or at 4°C with 0.3 M KCl or 10 mM ATP also caused dissociation of the hsp-90. Presence of progesterone in vitro increased dissociation of the hsp-90 and the subsequent PR binding to DNA-cellulose. Transformation in vivo or under cell free conditions did not alter the degree of phosphorylation of PR peptides A and B. Our results demonstrate that PR is phosphorylated in a hormone-specific manner and that its transformation by various agents leads to loss of the hsp-90 from the oligomeric structure without an apparent involvement of dephosphorylation. © 1989 Academic Press, Inc.

Steroid receptors (SRs) are known to function as trans-acting proteins regulating gene expression in hormone responsive cells (1). In cell-free systems, SRs are recovered in the high speed supernatant fraction (cytosol) where they can be incubated with hormonal ligands to form nontransformed or nonactivated complexes that exhibit very little binding to target cell nuclear sites (2). Treatment of the steroid-receptor complexes (SRc) with a variety of agents, causes alterations in their physicochemical properties collectively known as transformation or activation. The latter is accompanied by an increase in the affinity of SRc toward isolated nuclei or chromatin, DNA-cellulose and phosphocellulose, and ATP-Sepharose (3-6). The transformed SRc have altered kinetics of the dissociation of steroid from the complex (7,8) and sediment at slower rate in density gradients (9). Although the exact sequence of events leading from steroid binding to gene expression is not fully understood, transformation and phosphorylation of SRs have been suggested to be involved in the process of hormone action.

Results of a number of published studies had provided indirect clues to a possible involvement of phosphorylation in steroid hormone action (4,10). It is now believed that SRs are phosphoproteins, and phosphorylation of different SRs has been described under a variety of experimental conditions (11-21). The physiological role of phosphorylation in receptor function, however, still remains far from being completely understood. The

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first detailed analysis of phosphorylation of PR was reported by Toft and co-workers (14) who demonstrated that the non-transformed chicken PR exists in two 8 S forms: Type I contains steroid binding peptide A (79 kDa) plus an M_r 90,000 peptide which does not bind progesterone; Type II 8 S form is composed of peptide B (110 kDa) and the M_r 90,000 peptide. All three peptides were also shown to exist as phosphoproteins (13). Furthermore, the non-hormone binding 90 kDa peptide was found to be associated with nontransformed form of all steroid receptors (22). Recently, the 90 kDa peptide was identified as a heat-shock protein (hsp-90) (23,24). Although the significance of the association of hsp-90 with transformed SRs is not clear, hsp-90 may be involved in the regulation of active and inactive forms of SRs.

In the present report, we have examined the steroid-specific phosphorylation of chicken oviduct PR in vivo, and have investigated the influence of various experimental conditions which are known to transform SRs under cell-free conditions. We have employed phosphorylation as a means to observe molecular changes in the composition of PR to explore the relationship between phosphorylation and transformation processes.

MATERIALS AND METHODS

Chemicals - All reagents were of analytical grade and were prepared in deionized water. RU486 was gifted from Roussel Uclaf (Romainville, France). Nucleotides, progesterone, cortisol, Na_2MoO_4, glycerol, EDTA, monothioglycerol were from Sigma Chemical Co. (St. Louis, MO). [^{3}H]R5020 (70-87 Ci/mmol) and R5020 were purchased from New England Nuclear (Boston, MA). Ortho[^{32}P] phosphate (285 Ci/mg P) was from ICN Radiochemicals (Invine, CA).

Buffers - The following Buffers were used: Buffer A, 50 mM sodium phosphate, 10 mM EDTA, 12 mM monothioglycerol, and 50 mM NaF, pH 7.4, at 23°C; Buffer B, Buffer A plus 20 mM Na_2MoO_4. The nucleotide solutions were prepared in 10 mM Tris-HCl, pH 7.5, and the pH was adjusted to 7.5 at 23°C.

Phosphorylation in vivo - Oviducts were removed from immature female chicks treated with DES for 2 to 4 weeks as described previously (9). The tissue was rinsed in ice cold saline (0.9% NaCl), and minced to pieces approximately 2mm^2 with razor blades. The mince was washed with several changes of the phosphorylation buffer (25 mM Hepes, 128 mM NaCl, 6.3 mM KCl, 2.8 mM $CaCl_2$, 1.3 mM $MgCl_2$, 10 mM glucose, 0.5 mM pyruvate, 0.5 mM glutamate, 0.5 mM succinate, 10 μM NaH_2PO_4 pH 7.4) (21). The tissue mince (0.5 g) was suspended in 5 ml of phosphorylation buffer; approximately 1 mCi of ortho [^{32}P]phosphate (ICN) was added and the mixture was incubated at 41°C with agitation for 1 h. Various hormones (100 nM) dissolved in 5% DMSO/phosphorylation buffer were added to the incubations 20 min after the onset of the reaction. To obtain cytosol the tissue was homogenized in 2 ml (4 vol) of Buffer B + 0.3 mM PMSF using a Tissuemizer (Tekmar, Model SDT). Typically, 1 ml cytosol containing ^{32}P-incorporated PR was incubated with 20 ug of αPR22 (25) for 2 h at 4°C. Subsequently, PR-antibody complexes were adsorbed by incubation with 10 mg of protein A-Sepharose (Pharmacia) for 1 h at 4°C, which had been preequilibrated with Buffer B. The resin bound PR was washed 4 times with 4 ml Buffer B and 2 times with 4 ml Buffer B + 0.4 M KCl. Then the resin was transferred to clean tubes containing 4 ml of 50 mM sodium phosphate, pH 7.4. After pelleting, the resin was mixed with 100 μl of SDS sample buffer, boiled 5 min, and 50 μl of the extract were subjected to electrophoresis. Following electrophoresis, the gel was fixed in a solution of 25% methanol, 10% acetic acid for 1 h. After equilibration in distilled water, the gel was dried with BioGEL-WRAPs (Biodesign Inc., Carmel, NY), and exposed to Kodak X-Omat AR film in a Kodak x-ray cassette with intensifying screens for 3-4 days at -70°C. After developing, the film was scanned using a scanning densitometer (CS-9000, Shimadzu, Columbia, MA) with the gain set manually to allow comparison between lanes. Peaks of interest were selected and quantified in arbitrary integral units.

Polyacrylamide Gel Electrophoresis - For Western Blotting, SDS-PAGE was performed on precast PhastGels (Pharmacia P-L Biochemicals, 43x50x0.45 mm, 7.5% homogenous

polyacrylamide) (26). Immunopurified extracts (1 μl) were applied to the gel. The separations took approximately 30 min to run to completion at the running condition of 250 V, 10 mA, 3 W, 15 C, 90 Vh. Prestained SDS-PAGE Standard proteins (Biorad, Richmond, CA) were phosphorylase b (130K), bovine serum albumin (75K), ovalbumin (50K), carbonic anhydrase (39K), soybean trypsin inhibitor (27K), and lysozyme (17K).

For autoradiography and silver staining, conventional slab gels (14 x 13 x 0.15cm, 7.5% acrylamide) were prepared (11). Different receptor preparations were electrophoresed for 6 h at 125 V/slab. For autoradiography, ^{14}C-methylated protein markers (NEN) were used, which were myosin (200K), phosphorylase B (97.4K), albumin (69K), ovalbumin (46K). For silver staining, the calibration kit used (MW-SDS-200 Kit, Sigma) consisted of myosin (205K), B-galactosidase (116K), phosphorylase (97K), bovine serum albumin (66K), ovalbumin (45K), and carbonic anhydrase (29K). Gels were first stained with Coomassie Blue for 4 h (0.2% Coomassie Brilliant Blue R in 50% methanol, 10% acetic acid), destained for 1 h in the destaining solution (50% methanol, 10% acetic acid), followed overnight with occasional changes of the destaining solution (5% methanol, 7% acetic acid). Then the gels were silver-stained by the method described by Wray et al. (26). Western blotting was performed as described (21,25) and the immunoreactive bands were visualized by incubation with substrate buffer containing 0.33 mg/ml nitroblue tetrazolium (Sigma, St. Louis, MO) and 0.17 mg/ml 3-bromo 4-chloro 5-indolyl phosphate (27).

Preparation of DNA-cellulose and DNA Binding Analysis - DNA-cellulose was prepared according to the methods of Alberts and Herrick (28). Calf thymus DNA (type III) was linked to Cellex-410 and these preparations contained 2 mg DNA/g of dry DNA-cellulose as quantitated by the methods of Burton (29). The binding of SRs to DNA-cellulose was determined as described previously (30).

RESULTS AND DISCUSSION

Figure 1A illustrates the ligand specificity of the effects of different hormonal steroids on the phosphorylation of chick oviduct PR. In the absence of any exogenous steroid, the three known PR peptides were all seen to be phosphorylated. In our studies we have employed anti-PR antibodies, αPR22 (25), which recognize both PR-B and PR-A peptides but not the hsp-90. The receptor-associated hsp-90, however, is precipitated along with PR peptides in the nontransformed cytosolic preparations. Therefore, PR appeared to exist in phosphorylated form at a basal level. When the phosphorylation reaction was carried out in the presence of different steroids, only progesterone appeared to enhance the extent of phosphorylation of both PR-B and PR-A peptides. The increase in the extent of phosphorylation was accompanied by the loss of the 90 K

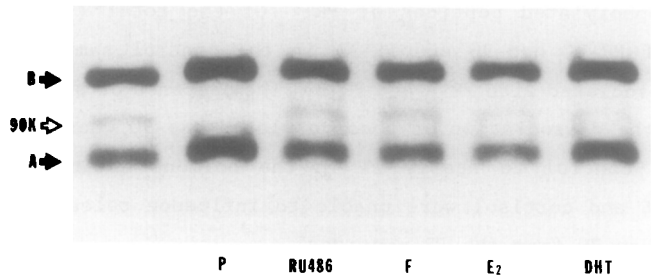

Fig. 1. Effects of different steroids on the phosphorylation in vivo of chicken PR: hormone specificity. DES-stimulated chick oviducts were excised, minced, and 0.5 g of tissue mince was exposed to various hormones (100 nM) during the incubation with 1 mCi [^{32}P] orthophosphate at 41°C. After phosphorylation, cytosol aliquots containing PR were immunopurified with anti-PR monoclonal antibodies αPR22. Purified PRs were analyzed by SDS-PAGE and autoradiography, as described in Materials and Methods. P, progesterone; RU 486, RU 38486 (mifepristone); F, cortisol; E_2, estradiol; DHT, dihydrotestosterone. Left lane, control - non hormone-stimulated.

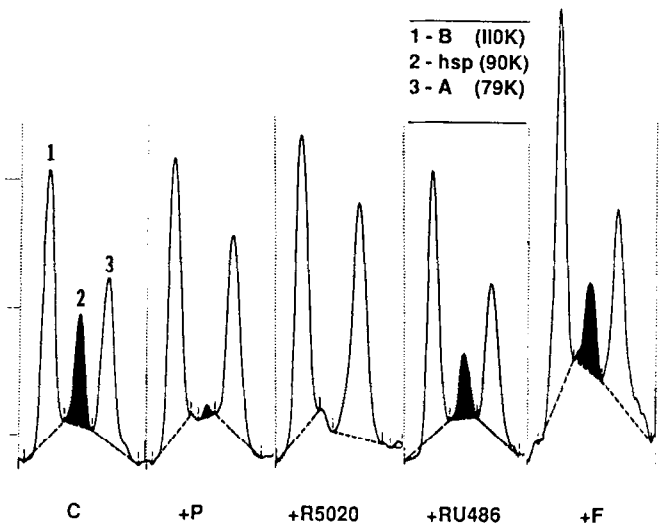

Fig. 2. Effects of various hormones on transformation of chicken oviduct PR during phosphorylation in vivo. DES-stimulated chick oviducts were excised, minced, and the tissue mince was exposed to various steroids (100 nM) during the incubation with 1 mCi [^{32}P]orthophosphate. Following phophorylation, cytosol aliquots containing PR were immunopurified with anti-PR monoclonal antibodies PR22. Purified PR was analyzed by SDS-PAGE and autoradiography. The autoradiogram was scanned using a scanning densitometer (CS-9000, Shimadzu, Columbia, MA) at 550 nm wave length. Peaks of interest were selected and quantified in arbitrary integral units. C, control - hormone; + P, progesterone; + R5020, promegestone; + RU486, RU38486 (mifepristone); + F, cortisol; 1, PR-B (110 kDa); 2, hsp-90 kDa; 3, PR-A (79 kDa).

peptide. Estradiol and DHT were partially influential in causing the loss or dissociation of the hsp-90 peptide. The known antiprogestin, RU486, had no effect on phosphorylation or the dissociation of hsp-90. These effects are expected since RU486 does not interact with chick PR (31). Immuno-blot and protein staining revealed that the increase in phosphorylation was not due to an increase in the amount of receptor (not shown). The results described in figure 1 suggest that PR exists in a phosphorylated form and that it is a substrate for additional hormone-specific phosphorylation.

Figure 2 shows the densitometric analysis of the effects of various steroids on the proportion of phosphorylated peptides of PR. Of the total (100%) ^{32}P-radioactivity (taken as a sum of PR-B, hsp-90 and PR-A) in the control sample, equal to variable proportions were associated with PR-A and PR-B. The presence of progestins (progesterone and R5020) caused the dissociation of phosphorylated hsp-90 whose proportion decreased from 17.4% in the control to nearly 1% in the hormone administered samples. The antiprogestin RU486 and cortisol were unable to influence release or the dissociation of phosphorylated hsp-90 from the PR peptides.

Fig. 3A illustrates effects of salt, heat and ATP on the transformation in vitro of PR phosphorylated in vivo in the absence of progesterone. In this and subsequent experiments, the term transformation has been used operationally to indicate loss of hsp-90 from the PR structure and to show increased binding of PR to DNA-cellulose. Incubation of cytosol aliquots with 0.3M KCl caused a complete disappearance of hsp-90 as examined by autoradiography following the SDS-PAGE. The loss of phosphorylated hsp-

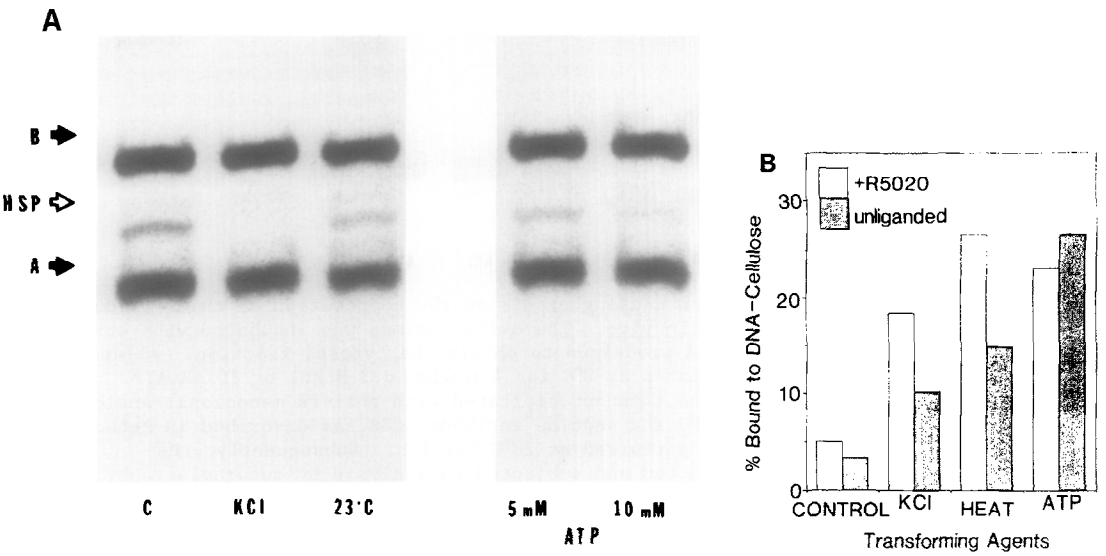

Fig. 3A. Transformation in vitro of chicken PR phosphorylated in vivo. Tissue minces (2.0 g) from DES-stimulated chick oviducts were incubated with 4 mCi [^{32}P] orthophosphate in the absence of progesterone. Cytosol fraction was prepared in Buffer A and 1 ml cytosol aliquots were used for transformation. C, + 20 mM Na_2MoO_4; KCl, 0.3 M KCl at 4°C for 1 h; 23°C, room temperature for 1 h; ATP, 5 or 10 mM ATP for 1 h at 4°C. After transformation, 20 mM Na_2MoO_4 was added to stop the reaction. Following purification with PR22 and protein A-sepharose, ^{32}P-incorporated PR peptides were analyzed by autoradiography. B, PR-B receptor; HSP, 90 K hsp; A, PR-A receptor.
Fig. 3B. DNA-cellulose binding of chicken PR after in vitro transformation. The cytosol from DES-stimulated chicken oviducts was prepared in Buffer A and divided in two aliquots. The first one was labeled with 20 nM [^{32}P] R5020 for 2 h on ice and subjected to various methods of transformation. The receptor in the second aliquot was transformed unliganded, then labeled with 20 nM [^{3}H]R5020. After the addition of 20 mM Na_2MoO_4, 0.2 ml cytosol aliquots were used for DNA binding assays.

90 was also evident when cytosol was exposed to 23°C for 1h. This is consistent with our earlier observation (9) that the rate of thermal transformation of PR is enhanced significantly in the presence of hormone. Treatment of cytosol with 5-10 mM ATP caused a progressive loss of hsp-90. This observation also agrees with our published results that 10 mM ATP and 0.3 M KCl can effectively transforms SRc in the absence of hormonal ligands (6,9,30,32). Treatment of PR under all three transformation conditions increased the DNA-cellulose binding, the latter was significantly higher in the presence of hormone (Fig. 3B). Under all three conditions, no loss of ^{32}P-radioactivity occurred in PR-A and PR-B peptides. Silver staining, once again, confirmed that equal amounts of receptor were present in the samples under the three transforming conditions (not shown).

To examine the relative proportion of phosphorylated PR peptides with respect to the total phosphorylated cellular hsp-90, the cytosol was first incubated with KCl to presumably remove phosphorylated hsp-90 from the transformed PR peptides, and was then immunoprecipitated with αPR22. Another portion of cytosol was incubated with anti-hsp-90 monoclonal antibody AC88 (33). Samples from both groups were subsequently subjected to SDS-PAGE and autoradiography. The extent of phosphorylation of the total cellular hsp-90 was seen to be much greater than that of PR-B and PR-A peptides (Fig. 4). It is

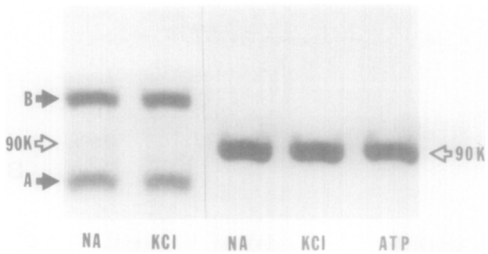

Fig. 4. Effect of transformation causing agents on the composition of chick oviduct cytosol proteins phosphorylated in vivo. The oviduct mince was incubated with ortho [^{32}P]phosphate and the tissue was processed to obtain the cytosol fraction. Aliquots (1 ml) of the cytosol were incubated at 0°C for 1 h with 0.3 M KCl or 10 mM ATP. Following this, the aliquots were immunoprecipitated with anti-PR monoclonal antibody PR-22 (left two lanes) or anti-90 kDa peptide antibody AC88, as described in Methods except incubation with AC88 was performed at 23°C for 1 h. Subsequently, the precipitated peptides were extracted and subjected to analysis by autoradiography. NA, control-nontransformed; B, PR-B (110 kDa); A, PR-A (79 kDa).

thus clear, that although hsp-90 is present in molar excess over PR and much of it can be phosphorylated in vivo, only a small part of the total hsp-90 associates with PR.

In order to examine the influence of hormone on the transformation of PR in vitro, cytosol containing phosphorylated preparation was either complexed with steroid and subjected to transformation or was incubated with the transforming agents first and then complexed with 20 nM R5020. Results of figure 5 show that although KCl, ATP and temperature were all able to decrease the concentration of hsp-90 in the unoccupied phosphorylated receptor, ATP effects appeared to be more pronounced in the presence of hormone. Silver staining of the preparation revealed the presence of hsp-90 in molar excess over PR peptides B and A and that it dissociated from the unoccupied receptor under the influence of transforming agents, all of which were more effective in the presence of hormone.

Previous work from this laboratory had suggested that transformation of chick PR involves separation of subunits as indicated by 8S to 4S alteration in the sedimentation rate (9). Furthermore, it was observed that the thermal transformation of glucocorticoid receptor (GR) was hormone dependent (34) and that the extent of transformation of chick PR was significantly increased in the presence of the ligand (9). It has also been shown that transformation of steroid hormone receptors involves a general process of separation of hsp-90 from the oligomeric structure of the untransformed receptors (35). Results of many recent reports have shown the influence of hormone on phosphorylation of SRs (15,18-21,36-38). Our results (Figs. 1,2) have shown that the hormone-dependency of phosphorylation and loss of hsp-90 are selective processes. Other steroids, estradiol, DHT and cortisol were relatively less effective or were ineffective.

In summary, we have examined the steroid specific phosphorylation of chick oviduct PR in vivo, and the influence of hormone on the transformation of PR in vitro. All three peptides representing PR are good substrates for endogenous kinase(s) and exhibit a basal level of phosphorylation. Phosphorylation of PR peptides A and B can be selectively amplified in the presence of hormonal ligands. Occupancy of PR by progestins causes the

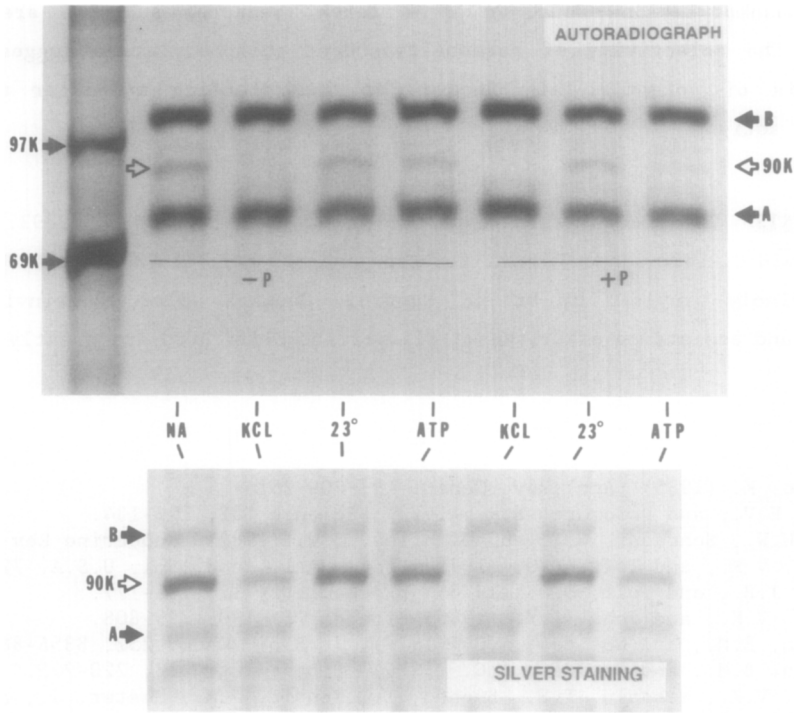

Fig. 5. Effects of hormone on the transformation of PR in vitro by salt, temperature and ATP. The cytosol from ^{32}P-incorporated oviducts was prepared in Buffer A and divided into two groups. Aliquots in one cytosol group (+P) were incubated with 100 nM progesterone for 2 h at 4°C and then incubated at 0°C with or without 0.3 M KCl and 10 mM ATP, or at 23°C for 1 h. Aliquots in the group (-P) were incubated with the above agents first and then complexed with 100 nM progesterone. After the addition of 20 mM Na_2MoO_4, the samples from both groups were immunopurified and analyzed by SDS-PAGE, followed by autoradiography and silver staining. NA, nonactivated.

dissociation of hsp-90, a phenomenon that can also be observed in vitro upon incubation of PR preparations at 23°C or at 0-4°C with salt and nucleotides. Since additional phosphorylation occurs with time after the dissociation of hsp-90, the latter event may unmask phosphorylation sites of hormone binding peptides of PR. While hsp-90 may suppress phosphorylation of PR-A and B peptides, additional phosphorylation of A and B peptides after its dissociation may facilitate their interaction with hormone responsive elements in the target cell genome. Although the observed effects of hormone on phosphorylation in vivo of PR are consistent with those reported by Toft and co-workers (21), the specificity of this effect seen in our studies provide additional support to the proposal that phosphorylation of PR represents a physiologically important event. Hormone presence also appears to enhance the in vitro transformation and DNA binding. Transformation or loss of hsp-90 does not appear to result in the dephosphorylation of receptor. This observation is consistent with the finding (20,39) that activation of GR in WEHI-7 cells does not dephosphorylate the steroid binding protein. Although it has been suggested that polyphosphorylation of human PR may occur after DNA binding (36), it is not clear from our results whether polyphosphorylation occurs subsequent or prior to DNA binding by PR and whether the enzymes responsible for the basal and hormone-

dependent phosphorylation of PR or of different receptor subunits are similar or different. The selectivity of hormone-dependent phosphorylation suggests that the phenomenon is of potential significance in understanding molecular mechanism of progesterone action.

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