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PHOSPHORYLATION OF CALF UTERINE PROGESTERONE RECEPTOR BY cAMP-DEPENDENT PROTEIN KINASE

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SUMMARY: We have examined the potential for using calf uterine progesterone receptor (PR) as a substrate for phosphorylation by cAMP-dependent protein kinase (cAMP-PK). PR was found to interact with anti-PR monoclonal antibody αPR6 (Sullivan et al., 1986), which was used to immunopurify the receptor. Protein staining of the purified preparation revealed the presence of two major bands corresponding to 114 kDa and 90 kDa peptides; only 114 kDa peptide could be photoaffinity-labeled with R5020. The 90 kDa peptide co-migrated with 90 kDa heat shock protein (hsp-90) precipitated by anti-hsp-90 monoclonal antibody AC88 (Riehl et al., 1985). Incubation of the immunopurified protein-A-Sepharose-adsorbed PR with the catalytic subunit of cAMP-PK in the presence of γ-[^{32}P]ATP and divalent cations resulted in a Mg^{++}-dependent incorporation of ^{32}P-radioactivity into both the 114 kDa and the hsp-90 peptides. Small ^{32}P-incorporation was also seen in the 114 kDa peptide in the presence of Mn^{++}. A 60°C preincubation of immunopurified PR increased the extent of phosphorylation of the hsp-90 peptide. A pre-treatment with alkaline phosphatase reduced the ability of PR to act as a substrate while the steroid occupancy of PR appeared to enhance the phosphorylation of the 114 kDa peptide. The differential cation requirement for the phosphorylation of 114 kDa and hsp-90 peptides and a selective hormone-dependent increase in the phosphorylation of the 114 kDa peptide suggest a possible role of phosphorylation in mediating progesterone action in the calf uterus.

Steroid receptors (SR) are intracellular *trans*-acting gene regulatory proteins which mediate steroid hormone-induced responses. Following binding of hormone to receptor, the steroid-receptor complex (SRc) is known to undergo a process called transformation which is thought to precede binding of SRc to hormone responsive elements in the target cell genome (1). A growing body of evidence indicates that in their untransformed state, SR are associated with hsp-90. This association is thought to mask the region on SR which interacts with gene regulatory sites (2). Dissociation of the receptor and hsp-90 is, therefore, thought to be required for the nuclear binding. The exact molecular details of the processes of hormone binding and receptor transformation, however, remain obscure.

Phosphorylation and dephosphorylation of proteins have been shown to regulate a wide variety of intracellular metabolic processes. It has been shown that SR are phosphoproteins and that phosphorylation may regulate their biological activity (3). Phosphorylation of receptors for Progesterone (4), glucocorticoid (5,6) and estradiol (7) has been demonstrated under *in vivo* conditions. It has also been shown that avian PR (8,9), and rat hepatic glucocorticoid receptor (GR) (6) act as substrates for phosphorylation by

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cAMP dependent protein kinase (cAMP-PK) in vitro. Calf uterine estradiol receptor also serves as a substrate for phosphorylation by an endogenous, although independent kinase (10).

Over the past five years, we have developed an in vitro system utilizing calf uterus to study the molecular regulation of PR (11-13). In this report, we demonstrate the potential for using immunopurified calf uterine PR as a substrate for the catalytic subunit of cAMP-PK as a means to further elucidate the role of phosphorylation in steroid hormone action. Our results show that in the untransformed state, both the steroid binding subunit and the associated hsp-90 of calf uterine PR are phosphorylated by cAMP-PK and that phosphorylation of these peptides is differentially affected by the particular cation present in the reaction mixture.

MATERIALS AND METHODS

Reagents: All reagents were of analytical grade and were prepared in deionized water. Sodium molybdate, phenylmethylsulfonyl fluoride (PMSF), ATP, monothioglycerol, dithiothreitol, catalytic subunit of cAMP-dependent protein kinase, alkaline phosphatase from bovine intestinal mucosa and ammonium persulfate were purchased from Sigma Chemical Co. (St. Louis, MO). Tris was from Schwarz/Mann Co. (Cambridge, MA). Bis and acrylamide were purchased from Bio-Rad (Richmond, CA). Protein-A sepharose was from Pharmacia, (Upsulla, Sweden). The antibody preparations αPR22 and AC88 were gifts from Dr. David Toft (Mayo Clinic, Rochester, MN). γ-[^{32}P]ATP (2-10 Ci/mmol), [^{3}H]R5020 (87 Ci/mmol) and R5020 were obtained from New England Nuclear, (Boston, MA).

Buffers: The following buffers were prepared at 23°C and pH was adjusted to 7.5. The buffers were stored at 4°C before use. Buffer A, 20 mM Tris-HCl, 12 mM monothioglycerol, 20 mM sodium molybdate; Buffer B, 20 mM Tris-HCl, 12 mM monothioglycerol; Buffer C, 20 mM Tris-HCl, 20 mM sodium molybdate; Buffer D, 20 mM Tris-HCl, 20 mM sodium molybdate, 0.4 M KCl; Buffer E, 20 mM Tris-HCl, Buffer F, 50 mM sodium phosphate.

Preparation of Cytosol: Cytosol was prepared as described previously (11-13). The uterine tissue was homogenized on ice with a precooled (0°C) homogenization probe and tissuemizer (Teckmar, model SDT) using four, 5-second bursts at medium speed in 4 vol (v/w) of buffers A or B. PMSF (0.3 mM) was added just after the first homogenization burst.

Formation of PR-antibody complex (PR-Ab): Cytosol containing PR was incubated 3 h with the monoclonal antibody, αPR6 (14), in the ratio of 0.02 mg of Ab to 1 ml of cytosol. Samples (1 ml) of PR-Ab were added to 10 mg pellets of protein-A that were prewashed 3 times with 4 ml of buffer B. After 1 h incubation with a gentle resuspension of the pellet at 10 min intervals, samples were then pelleted and the supernatant removed by aspiration. The sediments were then washed 4-5 times with 4 ml of buffer C and then 2-3 times with 4 ml of buffer D. The pellet was then transferred to clean tubes containing 4 ml of buffer E or F, pelleted and the supernatant aspirated.

Phosphorylation Reaction and Analysis of ^{32}P Incorporation: Protein-A-bound PR-Ab complexes were incubated with 0.1 mM γ-[^{32}P]ATP (10^{5} cpm/pmole), in the presence or absence of 20 units of the catalytic subunit of cAMP-PK, and 5 mM Mg^{++}, Mn^{++} or Ca^{++}. The reaction mixture was incubated for 30 min at 23°C. Pellets were then washed 3 times with 4 ml of buffer F. The resins were mixed with 0.1 ml of SDS sample buffer (125 mM Tris 4% SDS, 10% β-mercaptoethanol, 20% glycerol, (pH 6.8), boiled 5 min and the extracts (50 μl) were subjected to SDS-PAGE and autoradiographic analyses (6). In some cases, the migration of peptides in the SDS-gels was visualized by silver staining (6).

Western Blotting Procedure: Following electrophoresis, the gel was equilibrated in transfer buffer (20 mM sodium phosphate, 20% methanol, 0.2% SDS, pH 6.5) for 30 min. The gel was removed from the plastic backing by sliding a 0.25 mm wire between them. Then the proteins were transferred to nitrocellulose using a Trans-Blot Cell with Bio-Rad Model 200/20 power supply (Bio-Rad Laboratories, Richmond, CA) set at 50 V for 45 min. The nitrocellulose was blocked with Western buffer (20 mM Tris, 150 mM NaCl, 0.5% Tween-20, 1% non-fat dry milk, pH 7.4) for 30 min at 37°C. The sheet was then incubated overnight in Western Buffer containing 1 ug/ml αPR6 at 4°C. After washing 3 times with Western buffer, the nitrocellulose was incubated at room temperature for 2 h with alkaline phosphatase conjugated anti-mouse IgG (Fisher Scientific, Orangeburg, NY) at

a 1/500 dilution in Western buffer. Unbound antibody was removed by washing 3 time with Western buffer, and followed by rinsing for 2 min with substrate buffer (100 mM Tris, 100 mM NaCl, 50 mM $MgCl_2$, pH 9.5).

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 (15). After the desired band intensity was achieved, the nitrocellulose was washed with distilled water and photographed.

Photoaffinity labeling: Cytosol was prepared in buffer E and incubated for 2 h with 20 nM [^{3}H]R5020 in the presence or absence of 2 μM R5020. Aliquots were then irradiated with ultra-violet light (300 nm) for 2 min, mixed with an equal volume of SDS treatment buffer and heated in a boiling water bath for 3 minutes. Subsequently, samples were analyzed by SDS-PAGE. Each lane of the gel was sliced into 2 mm fractions, treated with 0.5 ml 30% hydrogen peroxide and heated at 50°C for 16 h to extract radioactivity.

RESULTS AND DISCUSSION

The monoclonal antibody αPR6, raised against avian PR (14), was used to immuno-purify calf uterine PR and subsequently analyze the extent to which calf uterine PR complexed with αPR6 acts as a substrate for phosphorylation in vitro by the catalytic subunit of cAMP-PK. Recent data from this lab has demonstrated that αPR6 interacts with calf uterine PR and can be used to immunopurify the latter (16).

Figure 1 illustrates an analysis of the effect of ligand, cAMP-PK, phosphatase and heat on phosphorylation of PR in the cytosol immunoprecipitated with αPR6 under nontransforming conditions. The presence of 114 kDa and 90 kDa peptides, corresponding to the hormone binding subunit and the SR-associated hsp-90 respectively, was clearly evident. Both peptides appeared to be good substrates for phosphorylation by cAMP-PK in the presence of Mg^{++}. The presence of the progestin ligand, R5020, in the reaction mixture appeared to slightly enhance phosphorylation of the 114 kDa band. In the absence of exogenous kinase, no detectable level of phosphorylation was observed either in the presence or absence of the ligand. Heat treatment (60°C, 10 min) prior to the phosphory-

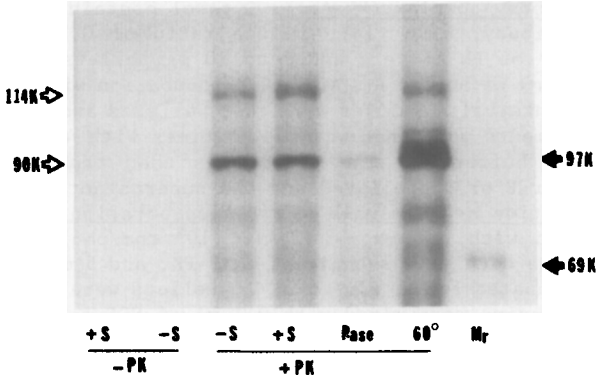

Fig. 1. Phosphorylation of calf uterine PR by cAMP-dependent kinase: Effects of ligand, heat and phosphatase. Samples of calf uterine cytosol (1 ml) were incubated in the presence (+S) or absence (-S) of 100 nM R5020, prior to immunopurification with αPR6, the anti PR monoclonal antibody. Subsequently, the samples were incubated at 23°C for 30 min with 10 mM Mg^{++}, and 0.1 mM γ-[^{32}P]ATP, in the presence (+PK) or absence (-PK) of 20 units of the catalytic subunit of cAMP-PK. One immunopurified sample (P-ase) was incubated with phosphatase (20 ug, 20 units) for 30 min at 23°C and then with γ-[^{32}P]ATP, Mg^{++} and kinase as above. Another sample was heat treated (60°C, 10 min) prior to the addition of γ-[^{32}P]ATP, Mg^{++} and kinase. All samples were subjected to SDS-PAGE and autoradiographic analyses. M_r, molecular weight standards. The arrows point to the approximate molecular weight of the peptides.

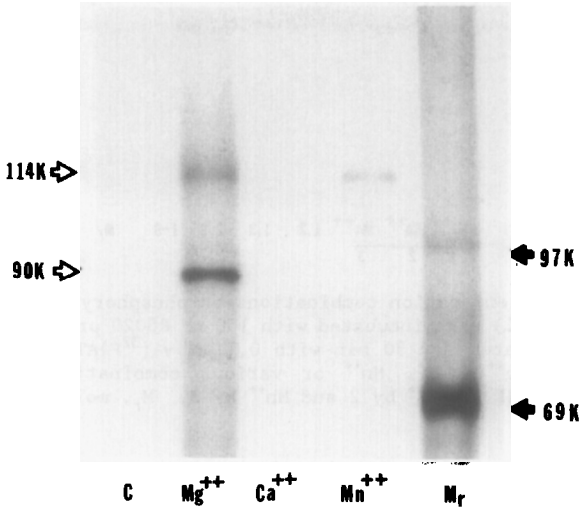

Fig. 2. Effects of divalent cations on phosphorylation of PR. Samples of calf uterine cytosol (1 ml) were incubated with 100 nM R5020 prior to immunopurification and then incubated for 30 min with 0.1 mM γ-[^{32}P]ATP, in the presence of 20 units of the catalytic subunit of cAMP-PK and Mg^{++}, Ca^{++} or Mn^{++}. In the control sample, no cation was added (C). M_r, molecular weight markers.

lation reaction appeared to expose more phosphorylation sites on hsp-90. In contrast, there was no increase in the extent of phosphorylation of the 114 kDa subunit under the same conditions. This observation suggests that additional sites for phosphorylation are exposed selectively on the hsp-90 molecule at elevated temperatures. It is possible that the phosphorylation sites on the 114 kDa subunit are located on the surface of the molecule and are maximally exposed to cAMP-PK in the absence of heat treatment. Treatment with alkaline phosphatase, prior to phosphorylation, resulted in the loss of the ability of the 114 kDa subunit to act as a substrate for cAMP-PK. Similarly, the extent of phosphorylation of hsp-90 was significantly reduced, but not completely obliterated. Decreased phosphorylation of both subunits after phosphatase treatment suggests that either the employed phosphatase concentration was unphysiological or *in vitro* phosphorylation by cAMP-PK may be influenced by the phosphorylation state of both hsp-90 and the 114 kDa peptides prior to phosphorylation reaction.

Phosphorylation *in vitro* of SR has been shown to be dependent on the presence of specific divalent cations (9,17,18). Results shown in Fig. 2 reveal that the presence of Mg^{++} enhanced phosphorylation of the 114 kDa subunit and appeared to be required for phosphorylation of hsp-90. The steroid binding subunit of PR is phosphorylated in the presence of Mn^{++} as well, but not to the same extent as in the presence of Mg^{++}. No phosphorylation was observed in the absence of Mg^{++} and Mn^{++} or in the presence of Ca^{++}. The Mn^{++} specific phosphorylation of the 114 kDa subunit of PR and the absence of hsp-90 phosphorylation in the presence of this cation demonstrates a specificity in the cation requirement for phosphorylation of the two peptides. There are a number of plausible explanations for this observation. The activity of cAMP-PK is Mg^{++}-dependent as illustrated by phosphorylation of hsp-90 and 114 kDa peptides. The lower level of

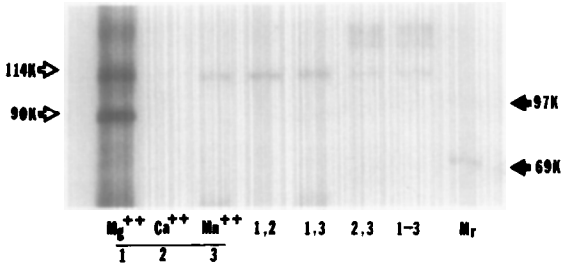

Fig. 3. Effects of divalent cation combinations on phosphorylation of PR. Samples of calf uterine cytosol (1 ml) were incubated with 100 nM R5020 prior to immunopurification with αPR6 and then incubated for 30 min with 0.1 mM γ-[^{32}P]ATP, in the presence of 20 units of cAMP-PK and Mg^{++}, Ca^{++}, Mn^{++} or various combinations of these. Mg^{++} is represented by the numeral 1, Ca^{++} by 2 and Mn^{++} by 3. M_r, molecular weight markers.

phosphorylation of the 114 kDa subunit seen in the presence of Mn^{++}, may reflect the heterogeneity of phosphorylation sites, some of which are Mn^{++}-dependent. Alternatively, Mn^{++} may also contribute to the reaction by acting as a specific cofactor for phosphorylation of the 114 kDa subunit on sites that are distinct and not available or present on hsp-90.

In a further attempt to analyze the effects of cations on kinase activity, cations were added in combinations to the phosphorylation reaction mixture. Phosphorylation of hsp-90 occurred only in the presence of Mg^{++} (Fig. 3). Furthermore, the presence of other cations in combination with Mg^{++} inhibited the extent of 114 kDa peptide phosphorylation over the sample which contained only Mg^{++}. The presence of Ca^{++} alone completely inhibited phosphorylation of PR peptides. All other combinations of cations resulted in the phosphorylation of only the steroid binding subunit of PR although to a lesser extent than in the presence of Mg^{++} alone. These data suggest that Mn^{++} competes with Mg^{++} during cAMP-PK activity, thus, inhibiting Mg^{++}-dependent phosphorylation of hsp-90.

Phosphorylation of the 114 kDa peptide does not appear to be due to autokinase activity or enzyme activity intrinsic to PR, since no phosphorylation was evident in PR preparations incubated in the absence of cAMP-PK (Fig. 1). The use of phosphate buffer instead of Tris in the reaction mixture appeared to selectively inhibit phosphorylation of hsp-90 but not the 114 kDa peptide (data not shown).

In order to establish the structural composition of the uterine PR, affinity labeling and western blot analysis was performed. Figure 4A shows that only a 114 kDa peptide was recognized by αPR6 in the uterine cytosol, where two major bands pertaining to the known peptides of avian PR were identified in the chick oviduct cytosol using αPR22 (14). These results were confirmed by a steroid affinity-labeling analysis (Fig. 4B). Although the photo-active steroid ligand [^{3}H]R5020 associated with different peptides, association of the steroid with the 114 kDa peptide was competable with radioinert R5020 (not shown).

Results of many studies have suggested that phosphorylation and transformation of SR may be coupled or related events. Administration of progesterone to estrogen-primed immature chicks leads to increased phosphorylation of PR with a concomitant loss of hsp-

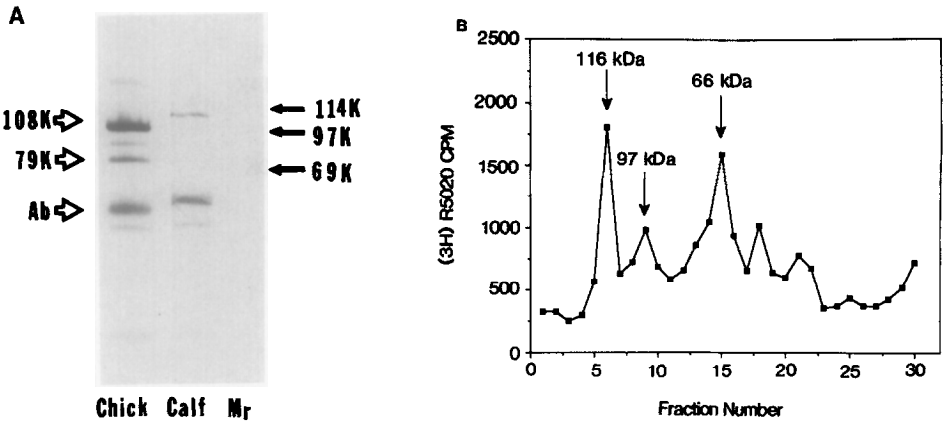

Fig. 4. A. Comparative Western blot analyses of PR from the calf uterus and chick oviduct. Calf uterine and chick oviduct PR were immunopurified with αPR6 and αPR22, respectively. Samples were electrophoresed on a Pharmacia Phast gel system and Western blotted using αPR6 (for Calf) or αPR22 (for Chick) according to the materials and methods. M_r, molecular weight markers.

B. Photoaffinity labeling of calf uterine PR with [^{3}H]R5020. An aliquot of calf uterine cytosol was incubated 2 h in the prsence of 20 nM [^{3}H]R5020. Labeled sample was irradiated with ultra-violet light (300 nm) for 2 min and analyzed by SDS-PAGE. The gel lane was sliced and the radioactivity was measured in each fraction. The arrows show the position of migration of known markers.

90 (19,20). In this study, uterine cytosol was incubated at 23°C or 0°C and fractionated with AC88 (anti-hsp-90 monoclonal antibody) (21) and with PR6. The antibody-precipitated samples were subjected to phosphorylation reaction. Figure 5 shows that AC88 interacts with a major 90 kDa peptide that is phosphorylated by cAMP-PK. The migration of this peptide is identical to the lower molecular weight phosphorylated peptide from preparation fractionated by αPR6. Heat treatment of cytosol causes loss of hsp-90 from the PR6-precipitated preparation suggesting that the nontransformed calf uterine PR is composed mainly of two peptides, both of which are good substrates for phosphorylation.

This report represents the first study exploiting the potential of calf uterine PR to act as a substrate for phosphorylation by cAMP-PK. Our results, together with those reported previously by others, suggest that phosphorylation *in vitro* by cAMP-PK is a general characteristic of receptors for various steroids (6,8,9). The selectivity of cation requirement for phosphorylation reaction is indicative of the heterogeneity of phosphorylation sites on the 114 kDa steroid binding peptide. Although no phosphorylation of hsp-90 could be accomplished in the presence of Mn^{++}, the 114 kDa peptide could be phosphorylated but to a lesser extent than when Mg^{++} is the cation. Furthermore, hsp-90 appears to contain additional phosphorylation sites that are selectively exposed upon heat treatment. Affinity labeling (Fig. 4B) and immunoprecipitation suggest, that although the major steroid binding peptide in the calf uterine cytosol is a 114 kDa peptide (22), the presence of other small peptide(s) cannot be ruled out. During immunoprecipitation αPR6 might have recognized only the 114 kDa peptide and, therefore, the presence of another smaller steroid binding subunit of PR (23,24) remains a

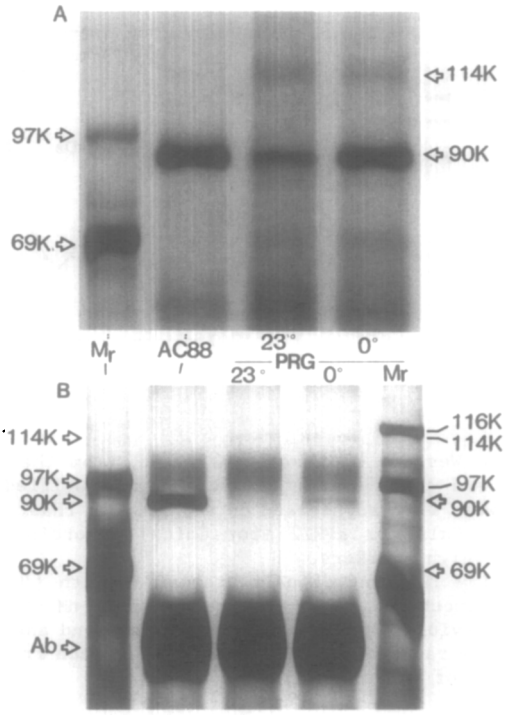

Fig. 5. Immunopurification and phosphorylation of PR and hsp-90. Samples of calf uterine cytosol (1 ml) were immunopurified with AC88 (an anti hsp-90 monoclonal antibody) or αPR6. Samples immunopurified with αPR6 were heat treated (23°C) or maintained on ice (0°C) prior to purification. Purified samples were then incubated for 30 min with 0.1 mM γ-[^{32}P]ATP, in the presence of 20 units cAMP-PK and Mg^{++}, and analyzed by SDS-PAGE with silver staining (B) and autoradiographic analysis (A).

possibility (Fig. 4). Regardless, the small increase in the extent of phosphorylation of 114 kDa peptide noted in the presence of R5020 (Fig. 1) is significant, since it has been difficult to observe the effects of hormonal ligands on phosphorylation in vitro.

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