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PHOSPHORYLATION OF HEN PROGESTERONE RECEPTOR BY cAMP DEPENDENT PROTEIN KINASE

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SUMMARY: Progesterone receptor A and B subunits from laying hen oviducts were highly purified and their phosphorylation by cAMP-dependent protein kinase from bovine heart was studied. Both proteins are phosphorylated by the kinase using physiological or subphysiological concentrations of the enzyme. This result indicates that the receptors are good substrates. The reaction is dependent upon exogenous enzyme; no phosphorylation is seen in the absence of protein kinase.

INTRODUCTION: Phosphorylation of proteins is an important mechanism for the regulation of many biological processes (1). A number of investigators have suggested that phosphorylation plays a role in the mechanism of steroid hormone action (2,3), but phosphorylation of steroid hormone receptors has not been directly demonstrated. There is, however, indirect evidence that phosphorylation affects hormone binding. Numerous studies with steroid receptors (4,5), have shown that non-specific phosphatase inhibitors such as molybdate and fluoride prevent loss of hormone binding. Moreover, treatment with phosphatase decreases binding (6). Finally, treatment of crude receptors with ATP enhances binding (4). Because these experiments were done with receptors in cytosol, it is not possible to determine directly whether or not these effects are due to phosphorylation reactions.

Using the purified hen progesterone receptor A and B subunits, we have observed that both A and B appear as more than one spot on two dimensional gel

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ABBREVIATIONS: cAMP, cyclic 3':5' adenosine monophosphate; [^{3}H]progesterone, [1,2,$^3H_2$] progesterone; R5020, trade name (Roussel-UCLAF) for 17 α,21-dimethyl-19-nor-pregn-4,9-diene-3,20-dione.

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electrophoresis (7). In each case, the minor spots are more acidic, but of the same molecular weight suggesting that the protein was either partially phosphorylated or perhaps, deaminated. Therefore, we decided to directly examine the phosphorylation of the hen receptor proteins.

MATERIALS AND METHODS:

Purification of Receptor Proteins: Receptor A was purified by a modification of the procedure of Coty et al. (8). Two kg of the magnum portion of hen oviducts were homogenized in 2.5 volumes of 10 mM Tris pH 7.4 containing 1 mM Na_2EDTA and 12 mM 1-thioglycerol (Buffer A), centrifuged in a Beckman J-6 centrifuge at 4200 rpm, and the supernatant fluid was passed through cheesecloth to remove floating fat. The receptor complexes were then precipitated at a final concentration of 10% polyethylene glycol 6000, centrifuged to collect the precipitate, redissolved in Buffer A with the aid of a tissue homogenizer and re-centrifuged to remove insoluble material. The receptor complexes were passed through a 700 ml phosphocellulose column (Whatman P-11) equilibrated with Buffer A and then through a 500 ml DNA cellulose column (8) equilibrated with Buffer A containing 50 mM KCl. The intact receptor A-B complexes do not bind to either of these columns (8). The receptors were then labeled with 10^{-8} M [^{3}H]progesterone (42 Ci/mmole, Amersham-Searle), precipitated and dissociated into subunits A and B with ammonium sulfate at a final concentration of 40% and dialyzed to remove excess salt. The receptors were next applied to a 40 ml DNA cellulose column equilibrated with Buffer A containing 0.1 M KCl and eluted with a 500 ml KCl gradient in Buffer A. The receptor A peak eluting at 0.19 M KCl was applied to a 10 ml DEAE cellulose column equilibrated with Buffer A containing 50 mM KCl. The receptor was eluted with a 150 ml KCl gradient in Buffer A; receptor A eluted at 0.1-0.15 M. Finally, the receptor pool was applied to a 1 ml heparin Sepharose column equilibrated with 0.1 M NH_4HCO_3 and eluted with a 20 ml linear gradient (0.1 M NH_4HCO_3 to 1.0 M NH_4HCO_3). Receptor A eluted at 0.34 M buffer.

The B protein was obtained from the non-adsorbed fraction of the second DNA column and purified by chromatography on DEAE cellulose, phosphocellulose and hydroxylapatite as described previously (9).

Catalytic Subunit of cAMP-Dependent Protein Kinase: The catalytic subunit of cAMP dependent protein kinase was purified from bovine heart by the method of Tash et al. (10). The specific activity of the enzyme preparation used in these experiments was 2.7 x 10^5 pmole ^{32}P incorporated into histone (Sigma Type II) per min per mg of enzyme as assayed by the procedure of Fakunding and Means (11).

Preparation of [γ-^{32}P]ATP: [γ-^{32}P]ATP (8-9 Ci/ μ mole) was synthesized by a modification of the method of Schendel and Wells (12). ATP was separated from other nucleotides with a 0.01 to 0.4 M ammonium carbonate gradient applied to DEAE-Sephadex A-25 (Pharmacia). The specific activity of the ATP used to phosphorylate receptor was 0.5 to 2.1 x 10^4 cpm/pmole.

Product Analysis: Phosphorylated proteins were analyzed by SDS gel electrophoresis as described by Laemmli (13), and stained with Coomassie Blue. Autoradiography was performed using Kodak XAR-5 film; usually bands were visible after 1 to 6 hours.

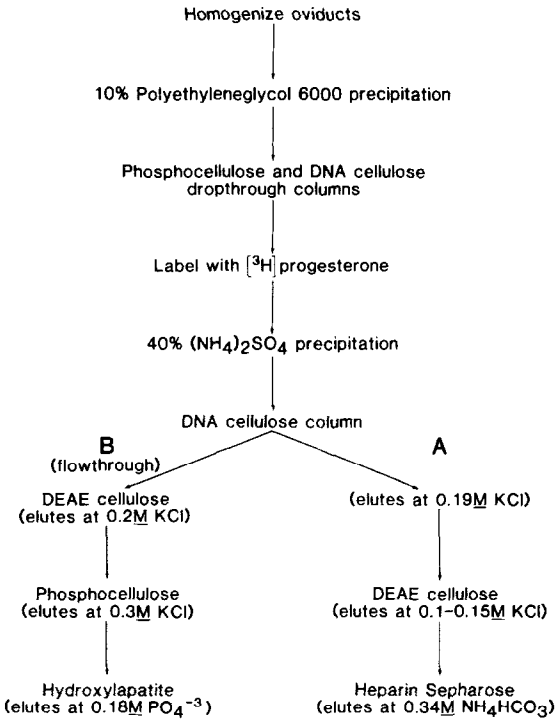

Fig. 1: Flow Chart for Receptor Subunit Purification. Hen receptor A and B for the phosphorylation studies were purified as shown in the flow chart. A, purification steps for receptor A not used for the B receptor; B, separate purification steps for the B protein.

RESULTS: The protocol for bulk purification of receptors A and B from laying hen oviducts is outlined in Figure 1. Details of the steps are presented in Methods. Yields of the proteins are about 150 µg of each from a 2 Kg batch of fresh oviducts. Purity has been assessed by both one- and two-dimensional gel electrophoresis, the latter by the method of O'Farrell (14). Using this procedure, both are about 80% pure by these criteria; in addition, the major contaminant of B has been shown by affinity labeling (data not shown) to be a proteolytic fragment of the receptor and not an unrelated protein.

Figure 2 shows the phosphorylation of purified subunit B under the conditions described in Methods. Lane 1 shows the Coomassie blue staining of the B preparation containing the bovine heart kinase. Protein B appears at 108,000 g/mole (15); kinase catalytic subunit migrates at 40,000 g/mole. Lane 2 shows

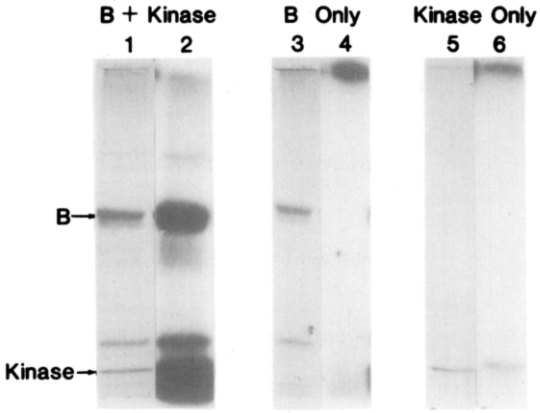

Fig. 2: Phosphorylation of Receptor B by cAMP Dependent Protein Kinase. Incubations were performed at 30° for one hour and stopped by addition of Laemmli SDS sample buffer (13). The incubation mixtures (20 µl) contained 10 mM K_xPO_4, pH 7.0, 3 mM $MgCl_2$, and 0.15 mM ATP (10^4cpm/pmol) and the indicated additions. For each incubation, the Coomassie blue stain (lanes 1,3,5) and the corresponding autoradiograph (lanes 2,4,6) are shown. Lane 1, 4 µg receptor B and 0.5 µg protein kinase; Lane 3, 4 µg receptor B; Lane 5, 0.5 µg protein kinase.

the autoradiogram of this gel lane. The protein B band is phosphorylated extensively. Also visible is another phosphorylated band at M_r = 45,000; this is the proteolytic fragment of B termed Form IV containing the hormone binding domain (16). The fragment is present in low amounts in the B preparation, and can be seen as a stained band in Lane 1.

Lane 3 and its autoradiograph (lane 4) show the receptor B alone, and the phosphorylation obtained in the absence of added kinase enzyme. No bands are seen; therefore the phosphorylation depicted in lane 2 is dependent upon the added kinase.

We conclude from these results that the receptor B preparation does not contain an endogenous protein kinase contaminant, nor is the receptor B itself a kinase. Finally, lanes 5 and 6 show the stained gel of kinase preparation alone (lane 5) and the resultant autoradiograph (lane 6). The kinase can phosphorylate itself, as shown here and also in lane 2.

Figure 3 shows the phosphorylation of the A protein, whose molecular weight is 79,000 (8,15). Lane 1 shows the Coomassie blue staining of the A prepara-

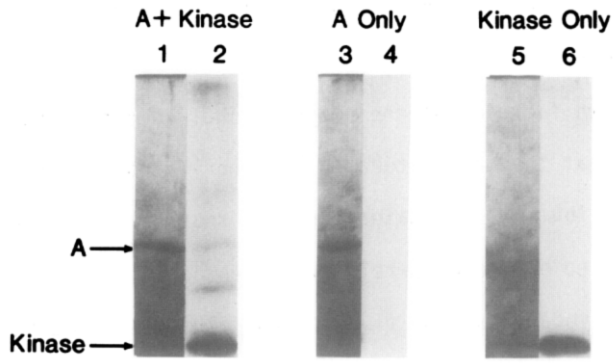

Fig. 3: Phosphorylation of Receptor A by cAMP Dependent Protein Kinase. Incubations were performed exactly as described in the legend of Fig. 2. For each incubation the Coomassie blue stain (lanes 1,3,5) and the corresponding autoradiograph (lanes 2,4,6) are shown. Lane 1, 2 µg receptor A and 0.5 µg-protein kinase, Lane 3, 2 µg receptor A, Lane 5, 0.5 µg protein kinase.

tion containing the bovine heart kinase. Lane 2 shows the autoradiogram of the complete reaction. Minor contaminants in the A preparation are also phosphorylated. This is perhaps not surprising since all of the proteins are DNA-binding proteins and many proteins of this type are phosphoproteins. Moreover, the smaller proteins may be proteolytic fragments of A (16) although this has not yet been demonstrated. This phosphorylation is again dependent on kinase as shown in lanes 3 and 4. Lanes 5 and 6 are kinase autophosphorylation controls, as shown above in Figure 2.

DISCUSSION: We conclude from the results presented here that both progesterone receptor subunits A and B are substrates for cyclic AMP-dependent protein kinase *in vitro*. When physiological concentrations of the kinase are used (0.5 µ M), phosphorylation of B protein is 50% complete by 5 minutes, the earliest time point tested (data not shown). A good correlation between the ease of phosphorylation *in vitro* and a protein's functional phosphorylation *in vivo* has been demonstrated for a variety of known substrates for cAMP-dependent protein kinase (1). The ability to rapidly phosphorylate the receptor subunits at the concentrations of enzyme used in these experiments is

consistent with, but does not prove, a role for phosphorylation of the receptor in regulation of its function.

Two other known facts about the progesterone receptor subunits corroborate the probability that they are naturally phosphorylated. First, both A and B show typical distributions of stained protein spots at discrete isoelectric points when analyzed by the O'Farrell two-dimensional gel system (7,14). Second, sodium molybdate, sodium fluoride and glucose-1-phosphate all have been shown by us to protect the crude receptor from alterations of the hormone-binding site (5). Since these compounds can act as phosphatase inhibitors, their mechanism of protection may indeed involve stabilization of phospho-receptors, as others have speculated (6).

It is noteworthy to point out that in studies to be published elsewhere we have observed using the radiolabeled synthetic progestin R5020 (7) that R5020 is bound equally well to all of the receptor spots seen on two-dimensional gels; therefore, we do not expect at present that receptor phosphorylation either precludes or produces high-affinity hormone binding directly, at least under the conditions that have been tested so far. More detailed studies of ligand binding kinetics are in progress.

In the present study, we have shown directly that a steroid receptor protein can be phosphorylated. No functional tests have yet shown what this covalent modification might do to the biologic activity of these interesting gene-regulatory molecules.

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