Phosphorylation in Vivo of Chicken Oviduct Progesterone Receptor*

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Progesterone receptor from an oviduct tissue mince incubated with \( ^{32}P \) orthophosphate was purified using affinity chromatography, gel filtration, and DEAE-Sephadex chromatography. Two receptor peaks were eluted from the DEAE column. Peak I contained one major protein band with a molecular weight of 90,000 and a \( ^{32}P \) band at 90,000. Peak II also had a major 90,000-dalton protein band and a \( ^{32}P \) band at the same position. In addition, peak II contained a major protein band at 104,000 daltons and a \( ^{32}P \) band which appeared to be slightly larger. Peaks I and II from chickens injected with \( ^{32}P \) orthophosphate showed the same pattern of labeling. The 90,000-molecular weight proteins from peaks I and II were indistinguishably. Both contained labeled phosphate. Two-dimensional gels separated the 104,000-dalton peptide from the slightly larger \( ^{32}P \)-labeled protein which traveled close to it on a one-dimensional gel. The relationships of the 104,000-dalton protein and this phosphoprotein to the progesterone receptor are not known. The 90,000-dalton receptor form common to both peaks is a phosphoprotein.

Several recent studies have suggested that phosphorylation might significantly affect the functioning of steroid receptors. It has been known for some time that the steroid-binding ability of cells can be correlated with ATP levels. Munck and Brinck-Johnsen (1) showed that ATP levels and glucocorticoid-binding ability decreased in thymocytes incubated briefly under nitrogen. Glucocorticoid-binding ability was restored as ATP levels rose in cells shifted back to aerobic conditions. Lowering cellular ATP by treatment with 2,4-dinitrophenol also reversibly lowered glucocorticoid-binding ability (2). The protein synthesis inhibitor cycloheximide did not impede the recovery of specific binding which followed removal of 2,4-dinitrophenol or restoration of oxygen (2, 3). A similar relationship between glucocorticoid receptor and ATP levels has been observed in cultured human lymphoblasts (4). In addition, it is known that the specific glucocorticoid binding of mouse fibroblast cytosol decreases during in vitro incubation and can be partially restored by added ATP (5). Moreover, Aurrichio et al. (6) have partially purified an enzyme which can utilize ATP to restore the estrogen-binding ability of mouse uterine cytosol.

Low levels of ATP might control steroid binding by decreasing the phosphorylation of a steroid receptor or of some other cellular component. Supporting this hypothesis is the demonstration by Nielsen et al. (7) that incubating cytosol with alkaline phosphatase greatly decreased its ability to bind glucocorticoids. Furthermore, the phosphatase inhibitors, molybdate and fluoride, have been shown to stabilize the steroid-binding ability of cytosolic receptors (5, 8). Steroid-binding ability is not the only receptor function which may be affected by phosphorylation. The phosphatase inhibitor molybdate has been shown to reversibly inhibit the transformation of cytosolic steroid-receptor complexes to nuclear binding forms (9–11). Moreover, Weigel et al. (12) have recently shown that progesterone receptor can be phosphorylated in vitro by a cyclic AMP-dependent protein kinase.

Thus, there was substantial reason to believe that steroid receptors might be phosphoproteins. We have recently identified two 8 S forms of nontransformed progesterone receptor (10) and have purified these to near homogeneity (14). These methods have allowed us to directly test for receptor phosphorylation. We have purified progesterone receptor from oviduct minces treated with \( ^{32}P \) orthophosphate and from the oviducts of chickens injected with \( ^{32} \)PPO. Our results show that this receptor is phosphorylated in vivo.

EXPERIMENTAL PROCEDURES

Materials—[12-\( ^{3} \)H]Progesterone (55 Ci/mmol) and \( ^{32}P \) orthophosphate (carrier-free) were purchased from New England Nuclear. Affinity resin, seocxycorticosterone attached to agarose by an epoxide linker, was from G and K Biochemicals. PCS (Phase Combining System) was from Amersham. Butyl-agarose was from Miles Laboratories. High molecular weight standards for sodium dodecyl sulfate-polyacrylamide gel electrophoresis were purchased from Bio-Rad.

Tissue Mince—Oviducts were removed from white leghorn chicks treated with diethylstilbestrol for 2 to 4 weeks as described previously (15). The oviducts were minced on a paraffin block with a Bio-Rad gel slicer with razor blades 1 mm apart. The mince was washed with 10 volumes of incubation buffer by spinning for 3 min at 650 \( 	imes \) g. The incubation buffer used was 25 mM 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid, 128 mM NaCl, 6.3 mM KCl, 2.8 mM CaCl\(_2\), 2 mM MgCl\(_2\), 10 mM glucose, 0.5 mM succinate, 0.5 mM pyruvate, 0.5 mM glutamate, pH 7.4, at 39 °C.

Minces were incubated for 1 h at 39 °C in an orbital shaker set at 120 rpm. Ehrlemeyer flasks (250 ml) were used containing a maximum of 4 g of minced oviduct in 40 ml of buffer. Buffer was pre-equilibrated in the orbital shaker for \( rac{1}{2} \) h prior to use. (\( ^{32} \)P)Orthophosphate (10 \( \mu \)Ci) was added to the flask just prior to the 1-h incubation.

Progesterone Receptor Purification—Receptor was purified using a modification of the method of Puri et al. (14). Following incubation at 39 °C, the tissue mince was pelleted for 1 min at 650 \( 	imes \) g. All subsequent operations were performed at 0–4 °C. Receptor was resuspended three times in 10 volumes of homogenization buffer and pelleted in the same manner. Prior to homogenization, the mince was mixed with 2–4 w/v unlabeled oviduct. Homogenization buffer was 50 mM potassium phosphate, 10 mM sodium molybdate, 50 mM sodium fluoride, 1 mM EDTA, 10 mM thiglyglycol, pH 7.0, at 22 °C. The homogenization buffer and the buffer used to wash the affinity resin contained 0.5 mM phenylmethylsulfonyl fluoride and 1 \( \mu \)g/ml of pepstatin A. Including 10 mM EDTA in the homogenization buffer did not alter the pattern of labeling of the purified components.
Homogenization was done in 3.1 v/w homogenization buffer using an Ultra-Turrax homogenizer (IKA, Janke and Kunkel). The homogenate was spun for 10 min at 20,000 × g. The supernatant was treated with 2.5 μM cortisol and centrifuged for 60 min at 110,000 × g. Lipid was removed by aspiration and the supernatant was mixed with a volume of affinity resin consisting of deoxycorticosterone linked to agarose by a spacer arm (16). The suspension was stirred for 2 h on ice. The resin was then pelleted and washed successively with 3 × 5 volumes of ethyl acetate:2.5% (v/v) acetic acid:100 mM potassium chloride in buffer C, then with 2.5% (v/v) acetic acid:100 mM potassium chloride in buffer C, and then with 1% (v/v) acetic acid:100 mM potassium chloride in buffer C. Two peaks of affinity resin were eluted from the DEAE, form I (175 mM KCl) and form II (225 mM KCl).

Gel Electrophoresis—Polyacrylamide gel electrophoresis was performed on slab gels following the procedure of Laemmli (17) with 7% or 8% acrylamide. Gels were fixed and stained for 2 h in 25% acetic acid:10% acetic acid:0.05% Coomassie blue and shaken overnight. They were then shaken for at least 2 h in 10% acetic acid. For autoradiography, gels were dried on a Bio-Rad gel slab dryer. The dried gel was put with Kodak X-Omat AR film in a cassette with Kodak X-Omat regular enhancer screens and placed in a −70°C freezer.

Phosphoamino Acid Analysis—Protein bands were extracted from a stained and destained gel after the method of Beemon and Hunter (18). The bands were crushed in 15-ml Corex tubes using a glass rod. Each band was suspended in 2.5 ml of 50 mM ammonium bicarbonate, 0.1% sodium dodecyl sulfate, and 5% β-mercaptoethanol, pH 8.5, and heated for 5 min in a boiling water bath. The gel pieces were shaken overnight in the buffer at 37°C. The gel was then centrifuged for 10 min at 3000 × g. The supernatant was transferred to a 15-ml Corex tube containing 50 μg of bovine serum albumin as carrier. The pellet was extracted again in 1.5 ml of the same buffer for 2 h at 37°C and the second supernatant was pooled with the first. Samples were adjusted to 15% trichloroacetic acid and precipitated for 1 h at 0°C. Samples were washed with 2 × 2 ml of ethanol/ether (1:1) at −20°C and lyophilized. The precipitate was dissolved in 1.5 ml of 8 N HCl by heating at 100°C for 1 min and transferred to a glass tube. This was evacuated and sealed. The samples were hydrolyzed for 3 h at 110°C and the HCl was removed by lyophилиsation in a flask containing NaOH pellets. Samples were dissolved in water containing 2.5 μg each of phosphoserine and phosphothreonine and spotted on an Eastman cellulose thin layer plate. The electrophoresis buffer was acetic acid, 88% formic acid, H₂O (78:26:896), pH 1.9. Electrophoresis was performed toward the anode for 90 min at 1 kV in a Savant thin layer electrophoresis tank at 0°C. The sheet was then dried for 15 min at 50°C, stained with ninhydrin, and heated at 50°C to locate the phosphohamo acids. 32P was detected by autoradiography.

Two-dimensional Electrophoresis—This was performed according to the method of O'Farrell et al. (19). In some experiments, samples were separated in the first dimension by nonequilibrium pH gradient electrophoresis. In other experiments, focusing was done for 10,000 volt-hours to ensure that acidic proteins had focused at their isoelectric points.

Scintillation Counting—Samples were counted in PCS/xylene 2:1.

RESULTS

We first investigated the ability of a tissue mince to incorporate phosphate into progesterone receptor. Minced oviducts were incubated with [32P]orthophosphate for 1 h at 39°C in the 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer. The mince was washed into phosphate buffer containing fluoride and molibdate and homogenized with carrier oviduct. Following a low speed spin, 2.5 μM cortisol was added and a high speed supernatant was prepared. This was equilibrated with affinity resin and then washed as indicated under "Experimental Procedures." Receptor was eluted overnight with 3 μM [3H]progesterone and chromatographed on Bio-Gel A-0.5m. It was then chromatographed on DEAE-Sephadex A-25, as indicated in Fig. 1. Two peaks of progesterone-binding material were seen. These have been seen previously and designated form I and form II in the order of elution from DEAE. They have been shown to be two 8.5 forms of progesterone receptor (13, 14). These progesterone-binding peaks coeluted with peaks of 32P-containing material. The purification method used here is similar to that described previously (14), but the ammonium sulfate precipitation step was omitted. This modification allowed a more rapid isolation of receptor with only a minor loss of final purity. Receptor is routinely obtained in greater than 50% purity with a recovery of about 20% (14).

Electrophoresis in gels containing sodium dodecyl sulfate was used to investigate whether 32P was covalently linked to receptor proteins. Aliquots of peak I and peak II were precipitated with 10% trichloroacetic acid and washed with 10% trichloroacetic acid and with 1% ethanol ether. Samples were applied to slab gels containing 0.1% sodium dodecyl sulfate and separated by gel electrophoresis as shown in Fig. 24. Peak I showed one major protein band with a molecular weight of 90,000. Peak II showed two major components, one at 90,000 daltons and one at 104,000. These are the receptor components previously identified by Puri et al. (14). Autoradiograms of these gels showed a similar but not identical pattern. Peak I had a major band at 90,000 daltons and peak II had two major bands, one at 90,000 daltons and a second which appeared to be slightly larger than the 104,000-dalton protein band. Similar results were seen when gels were sliced and 32P was detected by liquid scintillation counting (data not shown). Thus, the 90,000-dalton major component of peak I and the 90,000-dalton band of peak II apparently contained covalently attached 32P.

![Fig. 1. Chromatography of progesterone receptor on DEAE-Sephadex A-25.](image-url)
We also checked whether receptor labeled with $^{32}$P could be detected following the injection of ($^{32}$P)PO$_4$-$^3$. Two chicks were injected intraperitoneally with 2.5 mCi each of ($^{32}$P)orthophosphate. They were injected with the same dose 90 min later and were sacrificed after an additional 90 min. Oviducts were homogenized with carrier oviducts and a high speed supernatant was prepared and exposed to affinity resin. Oviducts which had not been exposed to ($^{32}$P)PO$_4$-$^3$ were similarly prepared and exposed to affinity resin. After washing in the normal fashion, both batches were eluted with [$^3$H]progesterone and chromatographed on DEAE-Sephadex. Peaks I and II from both batches were subjected to gel electrophoresis, stained, and examined by autoradiography (Fig. 2B). The samples from oviducts not exposed to ($^{32}$P)PO$_4$-$^3$ showed no bands on autoradiography. Thus, radioactivity from the tritiated progesterone is not a significant source of error. Peaks I and II from the oviducts of chickens injected with ($^{32}$P)PO$_4$-$^3$ showed patterns like those seen in the tissue mince experiments. Peak I had a major phosphopeptide at 90,000 daltons coinciding with its major protein band. Peak II had two major phosphorylated components, at 90,000 daltons and 109,000. As in the peak II from the tissue mince, the 90,000-dalton band detected by autoradiography coincided with one dense protein band and the 109,000-dalton band appeared to be slightly larger than the other dense protein band. In this experiment, the gel filtration step was omitted and the final preparations thus contained a number of contaminants.

We investigated the phosphoamino acid content of the major phosphorylated components of peaks I and II. Receptor was labeled in a tissue mince, purified as indicated under "Experimental Procedures," and electrophoresed in a slab gel. Bands representing the 90,000-molecular weight component of peak I and the 90,000 and 109,000-molecular weight components of peak II were excised from the gel, crushed, and protein was extracted. The protein was subjected to partial acid hydrolysis, and the products were separated by thin layer chromatography. As is shown in Fig. 3, phosphoserine was the only phosphoamino acid detected.

We next examined the components of peaks I and II by two-dimensional gel electrophoresis. The primary reason for doing this was to investigate the relationship between the 104,000-molecular weight protein in peak II and the highly phosphorylated component which had an apparent molecular weight of about 105,000. It has been reported that phosphorylation can cause a substantial increase in the apparent molecular weight of proteins estimated by sodium dodecyl sulfate-gel electrophoresis (21, 22). We wished to learn whether the 109,000-dalton form might be a more heavily phosphorylated version of the 104,000-dalton peptide. Two-dimensional electrophoresis also offered a chance to further check the relationship between the major protein band at 90,000 daltons and the phosphoprotein of the same apparent molecular weight. It was possible that the phosphorylated material might represent a more highly phosphorylated subpopulation of the 90,000-dalton protein. This could show up as a spot or series of spots close to the position of the 90,000-molecular weight protein and on the acidic side of it. It was also conceivable that the 90,000-dalton protein spot and the $^{32}$P label which co-migrated on sodium dodecyl sulfate-gel electrophoresis represented two unrelated proteins which could be well separated by isoelectric focusing.

A tissue mince was labeled and the receptor was purified on affinity resin and by chromatography on A-0.5m and DEAE-Sephadex. Peaks I and II were first subjected to isoelectric focusing in the presence of urea and then to gel electrophoresis in the presence of sodium dodecyl sulfate (Fig. 4). Staining with Coomassie blue showed that the 90,000-molecular weight...
components from I and II were indistinguishable by isoelectric focusing. Moreover, autoradiography showed \(is^3P\) centered at the position of the 90,000-molecular weight protein bands in both gels. Thus, the label appears to be on the receptor itself rather than on a contaminant with the same size but different charge. Since the positions of \(\is^3P\) labeling and of protein staining are the same, it appears that the bulk of the 90,000-dalton receptor form which we have purified is phosphorylated.

Two-dimensional electrophoresis separated the larger of the two major protein components of peak II from the highly labeled material seen near it on one-dimensional gels. The \(\is^3P\)-labeled material focused to the basic side of the 104,000-molecular weight component. As in one-dimensional gels, it appeared slightly larger than that component. Since the labeling with \((\is^3P)PO_4\) was done under nonequilibrium conditions, one cannot make definitive statements about total levels of protein phosphorylation. Phosphate-binding sites which turned over slowly might not have incorporated detectable amounts of \(\is^3P\) during the labeling period. Still, the component which is more highly labeled focuses to the basic side of the major 104,000-dalton protein. Thus, it seems quite unlikely that this \(\is^3P\)-labeled component represents a more highly phosphorylated form of the 104,000-dalton peptide.

Fig. 4 shows proteins which had been focused for 10,000 volt-hours to allow acidic proteins to reach equilibrium. The two major phosphorylated components and the two major protein components of peak II all focused in the acidic region, as did the major component of peak I. To estimate the pH profile of the two-dimensional gels, isoelectric focusing gels containing peak I and peak II were sliced into 1-cm lengths and placed in tubes containing degassed distilled water for pH measurement. All the proteins mentioned above showed apparent pi values between 4.5 and 5.5.

We also used nonequilibrium isoelectric focusing to check for the presence of proteins with isoelectric points outside the 4-10 region, which can migrate off the gel during standard isoelectric focusing. Labeled receptor from a tissue mince was purified on affinity resin, A-0.5m, and on DEAE A-25. Samples of peak I and peak II were focused for 1000 and 2000 volt-hours and then electrophoresed in gels containing sodium dodecyl sulfate. No additional components of either peak were detected by Coomassie blue staining or by autoradiography (data not shown).

**DISCUSSION**

Previous work from this laboratory has shown that 8 S forms of chicken oviduct progesterone receptor can be purified several thousand-fold in the presence of molybdate. The highly purified receptor eluted from DEAE-Sephadex as two peaks. On gel electrophoresis in the presence of sodium dodecyl sulfate, a 90,000-molecular weight peptide was the sole major component of peak I. Peak II contained a polypeptide of the same size and a second major peptide of about 104,000 molecular weight. The 90,000-dalton protein of the first peak clearly was a form of progesterone receptor. No contaminants were present in large enough amounts to account for the level of progesterone binding seen (14). Whether one or both of the
two components of peak II bound progesterone was not known. This is still not known with certainty. However, the 90,000-dalton component of peak II does appear quite similar to the 90,000-dalton component of peak I. The two are indistinguishable by isoelectric focusing as well as by electrophoresis in the presence of sodium dodecyl sulfate. Moreover, both show similar patterns of $^{32}$P/orthophosphate incorporation. It seems to us very likely that the 90,000-dalton peptide of peak II will also prove to be a form of progesterone receptor.

Peak II contains two other prominent components. A 104,000-molecular weight peptide is present in amounts roughly equivalent to the 90,000-molecular weight peptide. A second peptide slightly larger than 104,000 daltons is present in minor amounts but becomes highly labeled with $^{32}$P under the conditions of these experiments. The relationships of these components to the progesterone receptor are unclear. Both might simply be contaminants which happened to copurate with peak II receptor. However, it seems likely that the 90,000-dalton component of the second peak may be bound to something other than itself when it is in an 8 S form. The first 8 S peak apparently has one major component. The second 8 S peak contains an apparently identical component as well as a second major component. If the 90,000-dalton peptide of peak II and the 104,000-dalton peptide of that peak did form an 8 S complex together, this could explain why two 8 S peaks separate on DEAE-Sephadex. It had seemed likely to us that the 104,000-dalton peptide of peak II might be a precursor of the 90,000-dalton form. This may still be possible but it seems less likely since the smaller peptide rapidly incorporated $^{32}$P in these experiments while the larger did not. Receptor has been additionally purified by exposure to butyl-agarose prior to affinity chromatography, and by chromatography on sulfopropyl-Sephardex prior to DEAE chromatography. These resins did not bind the receptor, but they removed some minor contaminants. They did not remove the 104,000-dalton component of peak II or the 109,000-dalton phosphoprotein.

We have shown that the 90,000-dalton peptide is a progesterone receptor (14) and that it is phosphorylated in vivo. Thus, the cytoplasmic or nontransformed form of progesterone receptor is a phosphoprotein. When the 90,000-dalton components are examined by autoradiography of two-dimen-
sional gels, most of the radioactivity appears at the same spot as most of the protein. If a minor portion of the 90,000-dalton component were phosphorylated, one might expect to find the radioactivity shifted to the acidic side of the protein spot.

However, though most of the receptor which we have isolated appears to be phosphorylated, it is quite possible that our purification methods may have selected for phosphoryl-
ated receptor. There is considerable evidence suggesting that receptors which are not phosphorylated may bind steroids poorly (5-8). If so, such receptors might be lost during purification on the affinity resin.

In future studies, it should be highly interesting to learn how many sites on the receptor are phosphorylated and how phosphorylation at these sites affects receptor function.

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REFERENCES