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Hormone-Dependent Processing of the Avian Progesterone Receptor

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Avian progesterone receptor exists as two forms, A and B, with molecular weights of 79,000 and 110,000 daltons, respectively. The origin and significance of these two forms is an area of active investigation and debate. Monoclonal antibodies produced against these two forms were used to examine receptor stability in cytosol and changes in the receptor forms induced by hormone binding.

The lability of hormone binding at elevated temperatures is well documented. Analysis by Western blotting showed the receptor was stable in freshly prepared oviduct cytosol for 2 hr at 37°C, while hormone binding was lost within 30 min. However, loss of receptor through degradation was seen when cytosol was prepared from frozen tissue or when homogenization was excessive.

Progesterone was injected into diethylstilbestrol-stimulated chicks to examine, in vivo, effects of hormone treatment on receptor forms in the cytosol and nuclear fractions. Progesterone treatment caused a time- and dose-dependent conversion of the A receptor to a form (A′) with a slower electrophoretic mobility. The cytosolic progesterone receptor was divided equally between the B and A forms, while the nuclear receptor was predominantly A′. The amount of nuclear receptor was consistently less than cytosolic receptor. Receptor phosphorylation was analyzed by incubating tissue minces with [^{32}P]orthophosphate with or without progesterone followed by immune isolation of receptor forms. Progesterone treatment caused a time-dependent increase in cytosol receptor phosphorylation which was evident after 5 min of treatment. This phosphorylation was observed with both the A and B receptor forms. The results indicate that receptor phosphorylation is a very early event during progesterone action.

Key words: progesterone receptor, avian, processing, phosphorylation, degradation, antibody probes

The avian progesterone receptor has long been a model for studying steroid hormone action. In this system two receptor forms, A and B, are commonly found, with molecular weights of about 79,000 and 110,000, respectively. An early model held that these proteins were separate gene products and each played a distinct role in

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the action of progesterone [1]. While the model remains viable, it has been questioned in recent years. Peptide mapping analyses of the A and B receptors indicate that they are very similar [2,3]. Two receptor forms are also observed with mammalian progesterone receptors [4], but in the case of the rabbit receptor, this is thought to be caused by in vitro proteolysis [5]. However, studies on the human progesterone receptor indicate that the A receptor is not simply an in vitro degradation product of B [4].

We have used antibody probes to examine receptor stability, size, and heterogeneity during in vitro and in vivo conditions that promote receptor transformation. Western blotting procedures using monoclonal antibodies raised against avian progesterone receptor [6] allowed direct detection of the receptor forms in cytosolic or nuclear extracts without extensive purification. We found that the A and B receptor species are relatively stable proteins in vitro. In addition, both receptor forms appear to be modified by phosphorylation after in vivo treatment with progesterone.

MATERIALS AND METHODS

Immature chicks, stimulated for at least 2 weeks with daily injections of diethylstilbestrol (5 mg per injection) were used. In some experiments, chicks were treated with progesterone by s.c. injection of hormone in 200 μl sesame oil. The birds were killed and the magnum portion of the oviducts removed. The oviducts were homogenized in 4 vol of 50 mM Tris, 10 mM Na_2MoO_4, 10 mM thioglycerol, pH 7.4 (homogenization buffer). A Polytron PT-20 homogenizer regulated at a setting of 4 on a PCU-2 rheostat (Brinkmann Instruments) was used after precooling the generator in an ice bath. Tissue was homogenized in a 50-ml Sorvall centrifuge tube immersed in an ice bath; two or three bursts (3 sec each) were used with 20-sec cooling between bursts. After centrifugation at 800g for 5 min, the supernatant was centrifuged for 30 min at 100,000g. The low-speed pellet containing nuclear material was washed with 4 ml homogenization buffer, and any residual receptors extracted by incubation for 1 hr on ice in buffer containing 10 mM Tris, 1.5 mM EDTA, 0.4 M KCl, pH 8. The insoluble material was pelleted at 4,000g for 30 min. Saturated ammonium sulfate, adjusted to pH 7.4, was added to an equal volume of high-speed supernatant (cytosol) and to the soluble nuclear extract. After 30-min incubation at 4°C on a rocker platform, the precipitate was pelleted by centrifugation at 4000g for 30 min. The precipitate was redissolved in 300 μl of distilled water and mixed with 300 μl 2× sample buffer (0.25 M Tris, 2% (W:V) SDS, 20% (V:V) glycerol, 0.05% (W:V) bromphenol blue pH 6.8) containing 10% (V:V) mercaptoethanol. After 15-min incubation at room temperature with intermittent vortexing, the samples were heated for 2 min in a boiling waterbath.

Western Blotting Procedure

Proteins were resolved by electrophoresis on discontinuous polyacrylamide gels according to the method of Laemmli [7]. The resolving gel was 7.5% T, 0.3% C. Following electrophoresis, the proteins were transferred to nitrocellulose using a TE Series Transphor Electrophoresis Unit with Transphor Power-Lid, Model TE50 (Hoefer Scientific Instruments, San Francisco, CA) set at 1 A for 2 hr. The transfer buffer was 20 mM sodium phosphate, 20% (V:V) methanol, 0.2% (W:V) sodium dodecyl sulfate, pH 6.5. The nitrocellulose sheet was blocked with 20 mM Tris, 150

mM NaCl, 0.5% (V:V) polyoxyethelenesorbitan monolaurate (Tween-20), 1.0% (W:V) bovine serum albumin, pH 7.4 (Western buffer), for 30 min at 37C. The sheet was then incubated overnight in Western buffer containing the indicated monoclonal antibody at 10 μg/ml. The nitrocellulose was washed three times with Western buffer. Then alkaline phosphatase conjugated antimouse IgG (Southern Biotechnology Associates, Birmingham, AL), diluted 1:500 in Western buffer, was added for 4 hr at room temperature. The nitrocellulose was washed as above and the alkaline phosphatase complexes were detected by the method of Blake et al. [8] modified as follows: nitrocellulose sheets were rinsed for 2 min with substrate buffer (100 mM Tris, 100 mM NaCl, 50 mM $MgCl_2$, pH 9.5). Nitro blue tetrazolium (Sigma, St. Louis, MO) was added to substrate buffer to a final concentration of 330 μg/ml from a stock solution of nitro blue tetrazolium (75 mg/ml) in 70% dimethylformamide stored at −20°C. The reaction was initiated by addition of 3 bromo-4 chloro-5-indolyl phosphate (BCIP) to a final concentration of 166 μg/ml substrate buffer. BCIP was added from 2 mg/ml stock prepared just before use. In some cases the intensity of stained bands were quantitated by densitometry. The stained nitrocellulose sheet was first photographed to prepare a positive transparency. This was then scanned using a Helena Cliniscan with the gain set manually to allow comparison between lanes. Peaks of interest were selected and quantified in arbitrary integral units.

Antibodies

Monoclonal antibodies have been prepared against avian progesterone receptor isolated from oviduct cytosol by steroid affinity chromatography. Their characterization has been described elsewhere [6]. In summary, αPR 11, 13, 16, and 22 recognize both the A and B forms of the progesterone receptor while αPR6 is specific for the B form.

Receptor Binding of [^{3}H]-Progesterone

Cytosol was prepared from oviducts homogenized in 4 vol of 50 mM Tris, 10 mM thioglycerol, pH 7.4. Cytosol was labeled with 10 nM [^{3}H] progesterone and 3 μM cortisol plus or minus 1 μM unlabeled progesterone. After 20 min on ice, 500 μl aliquots of each were exposed to 37°C for 0, 10, 30, 60, 120, or 180 min. Following heat treatment, samples were returned to ice for the remainder of the 180 min. Next, the samples were treated with 0.5% charcoal, 0.05% dextran T-70 (Pharmacia, Piscataway, NJ) for 10 min on ice. After the charcoal was pelleted by centrifugation for 10 min at 1,200g, 200-μl aliquots were removed for counting.

In a parallel experiment, unlabeled cytosol was divided into aliquots and incubated at 37°C for the times given above. Following the incubation, two 600-μl aliquots were removed for each time point and incubated on ice for 90 min with 10 nM [^{3}H]-progesterone, and 3 μM cortisol with or without 1 μM unlabeled progesterone. All samples were treated with charcoal for 10 min and 200 μl were removed from each for counting.

Hormone-Dependent Phosphorylation

Dulbecco's minimal essential medium without phosphate (Irvine Scientific, Santa Ana, CA) was reconstituted according to manufacturer's protocol and supplemented with 10 μM NaH_2PO_4.

A diethylstilbestrol-stimulated chick was killed and the oviduct removed to medium. It was divided into four 400-mg pieces. In each case, the tissue was minced to pieces approximately 2 mm^2. The mince was washed with several changes of medium by sedimentation at unit gravity. The tissue was suspended in 4 ml of medium; approximately 1 mCi of ortho [^{32}P] phosphate (NEN) was added and the mixture incubated at 37°C with agitation. After 20 min, progesterone dissolved in medium was added at varying times to the incubations to a final concentration of 1×10^{-7}M. The time points for progesterone exposure were 40, 20, and 5 min. The fourth incubation received medium without progesterone. After 60 min the medium was removed, the tissue was washed with 10 ml each ice-cold saline, ice-cold homogenization buffer (50 mM K_2HPO_4, 10 mM Na_2MoO_4, 10 mM EDTA, 50 mM NaF, 10 mM thioglycerol, pH 7.0) and resuspended in 1.6 ml (4 vol) of homogenization buffer. The tissue was homogenized with a glass/glass homogenizer. The homogenate was centrifuged at 800g for 5 min and the resulting supernatant was centrifuged at 100,000g for 30 min. Monoclonal antibody αPR13 (20 μg) was added to each sample. After 2 hr on ice, 50 μl (packed gel) of antimouse IgG agarose (Sigma) was added to each tube and incubation continued for 1 hr with intermittent vortexing. The resin was pelleted by centrifugation, washed three times with 4 ml of homogenization buffer, followed by two wash steps with 4 ml of homogenization buffer with 0.4 M KCl. The resin was transferred to clean tubes in 4 ml of 50 mM K_2HPO_4, pH 7.0. After pelleting, the resin was mixed with an equal volume of 2× sample buffer and boiled for 2 min. The supernatant containing approximately one-half of the immunoprecipitated material was subjected to electrophoresis. Following electrophoresis, the gel was incubated in a solution of 25% methanol, 10% acetic acid for 1 hr. After equilibration in distilled water, the gel was dried under vacuum. The ^{32}P-labeled proteins were visualized by exposure of film (Kodak X-Omat AR5) in a cassette with lighting plus intensifier screens (Dupont) at −70°C.

RESULTS

Unlike other steroid receptors, the receptor for progesterone in avian [3, 9–11] and mammalian [4] systems is observed as two proteins which both bind hormone. The origin of this heterogeneity is unresolved. Western blotting of chick oviduct cytosol with four different monoclonal antibodies against the progesterone receptor [6] have confirmed earlier observations [11] using hormone binding data that the proportion of A and B forms of receptor are roughly equivalent. Others [4] studying the progesterone receptor from mammalian systems have also found two forms, but laboratories working with the rabbit uterus [12,13] have reported that the appearance of the lower molecular weight form only occurred in vitro.

To determine if protease inhibitors could influence the formation of the smaller receptor form in the avian system, oviduct tissue was homogenized with or without protease inhibitors. Samples were taken from homogenate, low-speed supernatant, and high-speed supernatant (cytosol) and analyzed by Western blotting as shown in Figure 1. We found the abundance of B and A to be constant at each point of the cytosol preparation, regardless of the presence of protease inhibitors.

In negative controls of Western blots in which primary antibody was omitted, a faint band comigrating with A was observed in samples prepared directly from homogenate (not shown). This band is likely a protein which normally pellets with

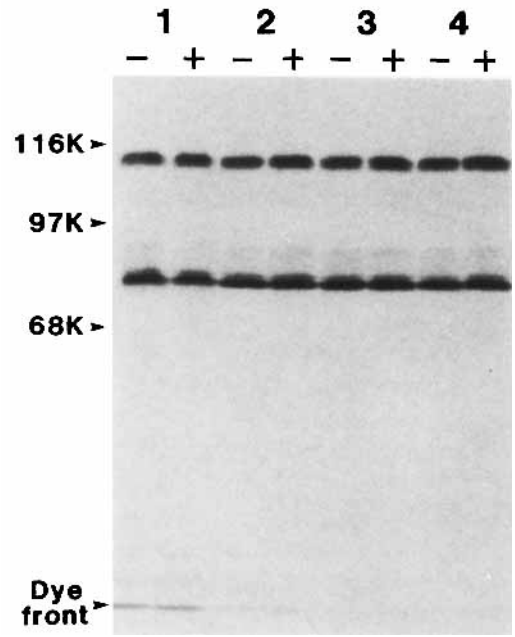

Fig. 1. Effect of protease inhibitors on receptor stability. Chick oviduct tissue was homogenized without (−) or with (+) a mixture of protease inhibitors as described in Materials and Methods. Samples were removed, adjusted to 2% (w:v) SDS, and 5% (v:v) mercaptoethanol and boiled for 2 min immediately after homogenization (**1**), low-speed centrifugation (**2**), or high speed centrifugation (**3**). Alternatively, each homogenate was incubated on ice for 1.5 hr before processing to cytosol (**4**). Loads equivalent to 1.6 μl of homogenate or supernatants were applied to gels. Western blotting was done with αPR13, which reacts with both the 110-kDa B receptor (**upper band**) and the 79-kDa A receptor (**lower band**). The molecular weight markers indicated by arrows are β-galactosidase, 116K; phosphorylase b, 97K; and bovine serum albumin, 68K. Where indicated, the following protease inhibitors were included during homogenization: leupeptin, 0.1 mM; bacitracin, 100 μg/ml; aprotinin, 77 μg/ml; pepstatin, 1.5 μM; and phenylmethylsulfonyl fluoride, 0.5 mM.

the particulate fraction during centrifugation. The only other non-specifically staining material migrated at the dye front (MW <20 kilodaltons).

Another aspect of receptor stability in vitro which might involve proteolytic degradation of receptor is the irreversible loss of hormone binding ability at elevated temperatures [1]. We examined this possibility using a time-course experiment comparing the loss of specific hormone binding at 37°C with the mobility of receptor bands in Western blots. Protease inhibitors were excluded from these samples. Shown in Table I is the specific binding data obtained using cytosol labeled prior to heat treatment with radioactive progesterone, plus or minus excess unlabeled progesterone, or cytosol labeled after heat treatment. As expected, specific binding of ligand to receptor was rapidly depleted with more than half the initial binding activity lost within the first 10 min. Aliquots for Western blots were removed from these and additional samples which were heated up to 3 hr. Figure 2 shows the results obtained by blotting with αPR16, an antibody which recognizes both forms of receptor. Identical results were obtained for hormone-bound or unbound receptor. There was no loss or shift in mobility of either form of the receptor after 3 hr at 37°C. The band migrating below 68 kilodaltons did react with αPR16, but is probably not related to

TABLE I. Stability of Hormone Binding by Receptor After Heat Treatment*

Time at 37°C (min)	Specifically bound [^{3}H]-progesterone (cpm/sample)	
	Prelabeled	Postlabeled
0	153,100	150,800
10	52,500	27,700
30	0	4,300

*200 μl aliquots of oviduct cytosol, labeled as described in Materials and Methods, were assayed for bound radioactivity after incubation at 37°C for the times shown. Specific binding was determined by subtracting bound radioactivity in the presence of excess unlabeled progesterone from radioactivity in the absence of unlabeled progesterone.

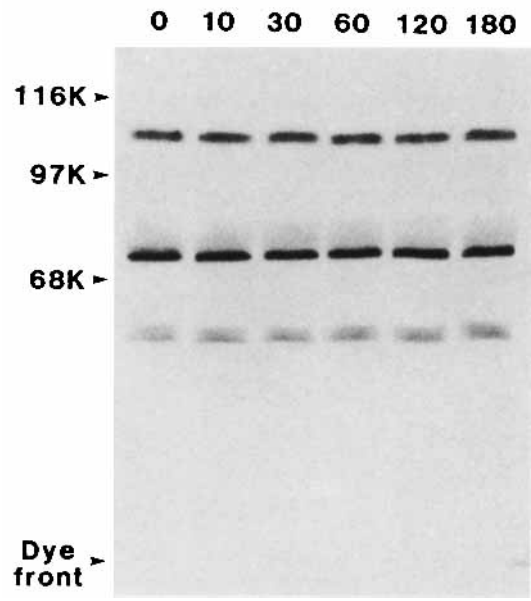

Fig. 2. Stability of progesterone receptor in cytosol during incubation at 37°C. Chick oviduct cytosol was prepared as described in Materials and Methods and incubated on ice for 20 min with 1 μM progesterone and 3 μM cortisol. Following the initial incubation, aliquots were incubated at 37°C for the times indicated (in min). At the completion of the 37°C incubation, the samples were placed on ice for the remainder of the 180 min. Samples equivalent to 2 μl of cytosol were applied to the gel. Following transfer to nitrocellulose, the receptor proteins were detected with αPR16 as the primary antibody.

receptor subunits A and B since it was not recognized by other antibodies against progesterone receptor and was also found in liver cytosol lacking progesterone receptor (not shown).

As is evident from Figure 2, progesterone receptor can display a surprising resistance to proteolysis in crude cytosol. However, we did find two circumstances in which receptor was much more susceptible to proteolysis. First, proteolytic degradation of receptor is very sensitive to homogenization conditions. Second, receptor in cytosol prepared from frozen oviduct was unstable. The results shown in Figure 3 illustrate the degradation of receptor, possibly by two distinct pathways, and the stabilization of receptor by protease inhibitors.

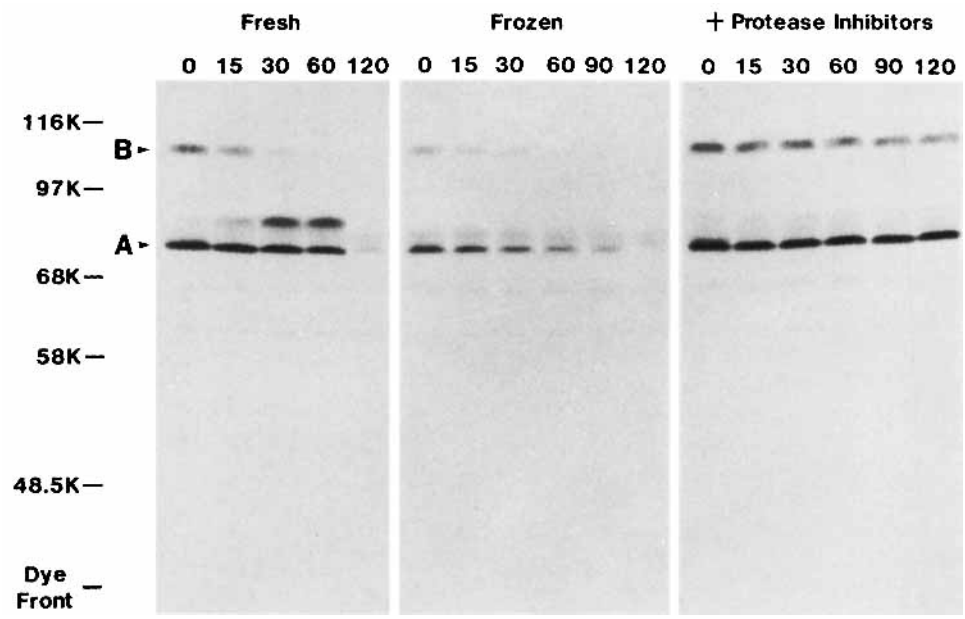

Fig. 3. Conditions enhancing in vitro proteolysis of receptor subunits. Cytosol, plus or minus protease inhibitors, was prepared from oviduct which was excessively homogenized (**Fresh**) or which was homogenized normally from oviduct frozen for 4 days at −70°C (**Frozen**). The cytosol was then incubated at 37°C for the number of minutes indicated and treated as in Figure 2. Receptor bands were detected with αPR13. The samples in lanes marked +**Protease Inhibitors** were prepared from frozen oviduct cytosol, but identical results were obtained from fresh, overhomogenized cytosol with inhibitors present or from fresh tissue homogenized normally with no inhibitors. The molecular weight markers were as described for Figure 1 plus pyruvate kinase, 58K; and fumarase, 48.5K.

During tissue homogenization, much care is taken to minimize homogenization time, aeration and temperature elevation. We have found that the conditions need not be very harsh to produce cytosol in which the receptor is readily degraded. In the example given in Figure 3 ("Fresh"), the homogenization time was only increased by 50%, but using a relatively small volume which provided more agitation of the sample than usual. When this cytosol was incubated at 37°C, a prominent band of around 85 kilodaltons appeared concomitant with the disappearance of the B form of receptor at 110 kilodaltons. This band reacted with five monoclonal antibodies against progesterone receptor, including αPR6, which does not recognize the A form. At 60 minutes, when B was no longer seen, the intensity of the A band was little reduced from its initial value, but was almost completely gone by 2 hr. It is possible that the A band was being degraded at the same rate as B but was replenished by a stepwise degradation of B to a form comigrating with A. Forms smaller than A were not seen owing either to the loss of the five antibody epitopes which are all located in the amino-terminal half of the receptor (B.W. O'Malley, personal communication) or to the rapid degradation of fragments smaller than 79 kilodaltons.

Cytosol was also prepared, plus or minus protease inhibitors, by mild homogenization but using oviduct tissue which had been frozen at −70°C. With protease inhibitors present (Fig. 3, "+ Protease Inhibitors"), there was almost no degradation of receptor forms during heat treatment. In the absence of protease inhibitors (Fig. 3,

"Frozen"), there was essentially complete degradation of receptor forms after 2 hr of heat treatment. This degradation was distinct from that seen after excessive homogenization (Fig. 3, "Fresh") in that there was no appearance of the 85-kilodalton fragment. Also, the loss of A and B was more rapid, with significant losses, compared to samples containing inhibitors, even in cytosol kept on ice throughout. It is possible in this case, as above, that B was degraded to a form comigrating with A, but it is also possible that A was simply degraded more slowly.

Since Western blotting provided a consistent and sensitive means for receptor measurement, this method was used to study the distribution of receptor forms following progesterone treatment in vivo. Graded doses of progesterone in sesame oil ranging from 0.02 mg to 4 mg (or vehicle alone) were injected into diethylstilbestrol-primed chicks for 30 min. The dose range is consistent with previously published studies [14–16]. Cytosolic and nuclear receptors were prepared as described in Materials and Methods. Because previous studies (not shown) indicated the nuclear receptor was less abundant than the cytosolic receptor, the nuclear extract samples were loaded onto gels at four times the volume of cytosolic receptor preparations. Figure 4 shows the results of Western blotting with monoclonal αPR13. Lane 1 represents a control injection of sesame oil alone. Lanes 2 through 7 show the effect of increasing doses of progesterone from 0.02 mg to 4 mg per bird. There was a low level of receptor present in the nuclear extracts of the control. It is not clear at this point whether this small proportion of receptor was tightly associated with nuclear material or if it remained with the nuclear material due to incomplete washing. There are two significant observations. First, the cytosolic receptors, while decreasing

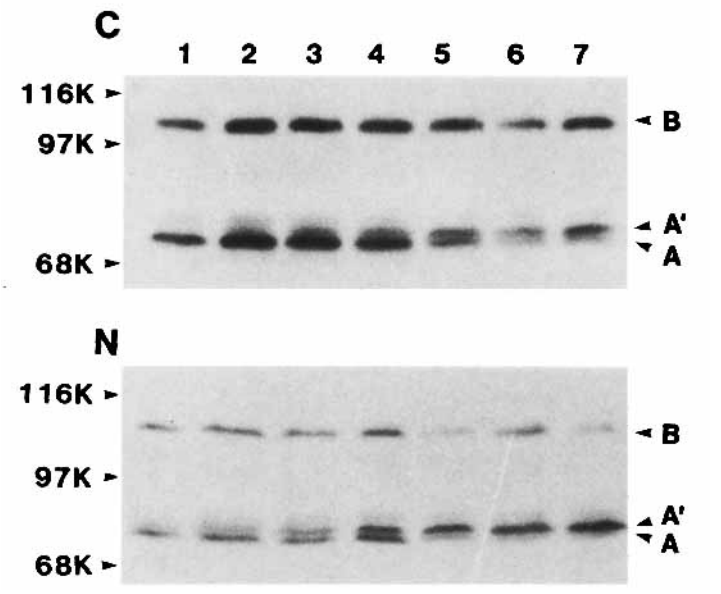

Fig. 4. Effect of increasing in vivo dose of progesterone on the distribution of receptor forms in cytosolic and nuclear extracts. Chicks were injected s.c. with the following doses of progesterone: 0, oil alone (**lane 1**), 0.02 mg (**lane 2**), 0.05 mg (**lane 3**), 0.2 mg (**lane 4**), 0.5 mg (**lane 5**), 2 mg (**lane 6**), or 4 mg (**lane 7**). After 30 min the birds were killed and the oviducts were removed. Cytosolic (C) and nuclear (N) extracts were prepared from each as described in Materials and Methods. Western blotting was done with αPR13 as the primary antibody.

slowly with time, maintained rough equivalence between the A and B forms independent of the dose. However, in nuclear fractions, there was a dose-dependent shift in the A/B ratio in favor of the A form so that A became the dominant receptor form extracted from the nucleus. Second, there was a small but significant increase in apparent molecular weight of the A form that was dose-dependent. This transition in electrophoretic mobility to form A′ was seen in both cytosol and nuclear extracts.

A time-course experiment was performed in which chicks were injected s.c. with 2 mg of progesterone in oil at times ranging from 15 min to 4 hr. In Figure 5, lanes 1 and 8 represent control injections of oil alone at 15 min and 4 hr, respectively. Lanes 2 through 7 correspond to treatment with progesterone for 15 min, 30 min, 1 hr, 1.5 hr, 2 hr, and 4 hr. Receptor bands from control birds treated with oil alone for 15 min or 4 hr were identical. At either time point there was equivalence between A and B forms of the receptor in both cytosolic and nuclear extracts. The proportion of A and B receptor in the cytosol remained about the same during progesterone treatment. However, this was not the case in the nuclear extract where A′ was the predominant form. Evidence of progesterone action could be detected as early as 15 min after treatment by the appearance of the A′ form in both the cytosol and nuclear extracts. The maximum accumulation of nuclear receptor was reached after 30 to 60 min. However, a marked decrease of receptor in both fractions occurred at later times. After 4 hr, the cytosol receptor level had decreased to about 30% of the control value as determined by densitometry (see Materials and Methods). Nuclear receptor increased to about 160% of control by 30 min, but then decreased to 60% of control by 4 hr. Thus, two types of receptor processing were observed during hormone treatment; the appearance of the A′ receptor, and a progressive loss of total receptor.

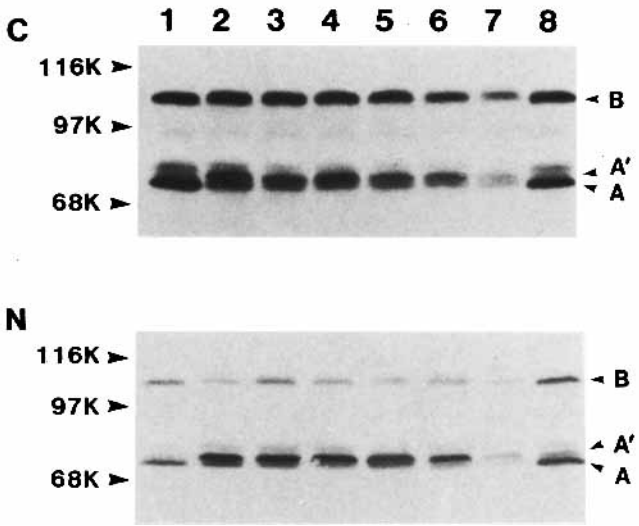

Fig. 5. Time course of changes after in vivo administration of progesterone. Chicks were injected s.c. with either sesame oil alone (**lanes 1** and **8**) or with 2 mg of progesterone in oil (**lanes 2–7**) for the following times: 15 min (lanes 1 and 2), 30 min (lane 3), 60 min (lane 4), 90 min (lane 5), 2 hr (lane 6), or 4 hr (lanes 7 and 8). At the end of the time course, chicks were killed and their oviducts removed. Cytosolic (C) and nuclear (N) extracts of each were prepared as described in Materials and Methods. Western blotting was done with αPR13 as the primary antibody.

The time-course experiment was repeated using the synthetic hormone R5020. It was thought that the time course with R5020 may differ from that of progesterone since the former is more resistant to degradation [17]. This experiment, using a dosage of 2 mg, is shown in Figure 6. A parallel comparison using 2 mg progesterone was also conducted (results not shown). The times of injection were 30 min, 60 min, 2 hr, 4 hr, and 20 hr. Controls were injected with vehicle alone at 30 min and 4 hr. The maximum nuclear accumulation was at 30 min to 60 min, as found in the previous experiment; but the increase was 250% of the control (by densitometry). The loss of cytosol and nuclear receptors was noticeable at 2 hr (lane 4), and minimal levels were observed at 4 and 20 hr after treatment. At 4 hr, the cytosol receptor level was about 10% of the control. The nuclear receptor bands, while rather diffused, represented about 70% of the control value. The maximum increase in nuclear receptor and the loss of cytosol receptor are more dramatic than shown in the experiment with progesterone in Figure 5. However, this is due primarily to experimental variation since a match progesterone control (not shown) gave results equivalent to the R5020 results in Figure 6.

To be sure that all progesterone receptor forms were recovered in the fractions routinely assayed, homogenate was prepared from the oviduct of a chick injected for 30 min with 2 mg progesterone and processed as described in Methods except that the low speed pellet was washed twice. Each wash supernatant was retained separately

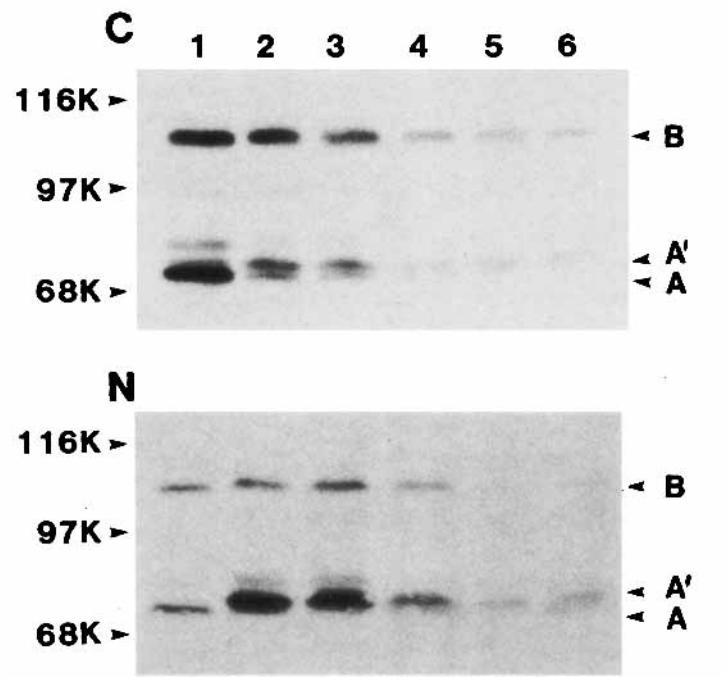

Fig. 6. Time course of changes after in vivo administration of R-5020. Chicks were injected s.c. with either sesame oil alone (**lane 1**) or with 2 mg of R5020 in oil (**lanes 2–6**) at the following times: 30 min (lane 2), 60 min (lane 3), 2 hr (lane 4), 4 hr (lanes 1 and 5), or 20 hr (lane 6). At the end of the time course, chicks were killed and their oviducts removed. Cytosolic (C) and nuclear (N) extracts were prepared from each as described in Materials and Methods. Western blotting was done with αPR13 as the primary antibody.

as well as the supernatants from the ammonium sulfate precipitations. Any proteins in supernatant samples were precipitated by additions of TCA to 20% (final volume) and all samples were redissolved in a constant volume of distilled water. Western blotting with PR13 showed immunostaining only in the ammonium sulfate precipitates of cytosol and the nuclear extracts.

Monoclonal antibody αPR13 was used in most of these experiments. To validate this choice all of our antibodies to progesterone receptor were screened against a portion of the sample used in lane C2 of Figure 5. This sample was chosen because it contained B, A, and A′ forms of the receptor. The antibody binding patterns in Figure 7 show that αPR 11 13, 16, and 22 each recognized the progesterone-dependent A′

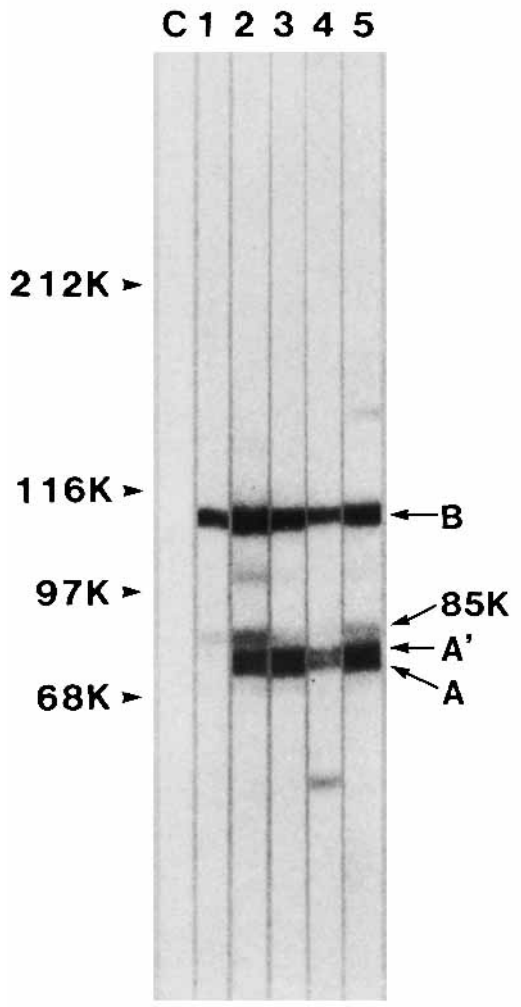

Fig. 7. Assay of antiprogesterone receptor monoclonal antibody reactivity with forms of progesterone receptor. A proportionally larger volume of sample from C2 Figure 5 was applied to an 8-cm-wide well of SDS gel. After electrotransfer of proteins, the nitrocellulose was cut into strips. The strips were incubated either without antibody (**lane c**) or with αPR6 (**lane 1**), αPR11 (**lane 2**), αPR13 (**lane 3**), αPR16 (**lane 4**), or αPR22 (**lane 5**).

form as well as the A and B forms of the receptor. αPR6 was specific for the B form of the receptor and did not recognize either the A′ or A forms. This observation reenforces the interpretation that the A′ species is related to the A receptor form. There was a weak interaction at approximately 85 kilodaltons that was seen with all antibodies including αPR6. This band appears to be related to the B form of the receptor. Although its significance is unknown, it is probably the same 85-kilodalton proteolytic product reported above (Fig. 3).

Detection sensitivity to the B, A′, and A forms was assayed on Western blots with αPR13 (Fig. 8). This experiment was done to ensure a linear response to these three receptor forms with this antibody. Typically, 20 μl of sample was applied to the gels. In this experiment the sample loaded was equivalent to 2× (lane 1), 1× (lane 2), 0.25× (lane 3), 0.1× (lane 4), or 0.05× (lane 5). As seen in Figure 8, the antibody stain appeared to vary in proportion to the sample load. For reference, the standard 20-μl sample was equivalent to 6.7 μl of cytosol from a 4-volume homogenate which would correspond to about 170 μg protein or 70 fmol receptor.

Decreased mobility during gel electrophoresis, like that of the A′ receptor, has been shown to occur with several proteins after their phosphorylation [18,19]. Thus, it was thought possible that A′ was a more highly phosphorylated form of the A receptor. This possibility was supported by recent studies showing that hormone treatment promotes phosphorylation of the mammalian progesterone receptor [20,21]. This was tested using a tissue incubation system similar to that used by Longeat et al [20]. Oviduct tissue was incubated in low phosphate buffer containing [^{32}P]-orthophosphate. Progesterone (1×10^{-7}M final) was added to individual incubations for 5, 20, and 40 min. As a control, one incubation was not exposed to progesterone. The total incubation time was the same for all samples (60 min). Cytosol was prepared from each tissue, and the progesterone receptor was immunoprecipitated using αPR13 and antimouse IgG-agarose. The precipitated proteins were extracted, resolved by electrophoresis, and visualized by autofluorography. This immune-isolation is rapid and provides sufficient purification for a clear identification of receptor bands after gel electrophoresis, as shown in Figure 9. The avian progesterone receptor from untreated tissue is known to be a phosphoprotein [22], and ^{32}P labeling of the A and B receptors was observed in the sample without progesterone (Fig. 9). However, progesterone treatment clearly increased the extent of ^{32}P labeling. This was noticea-

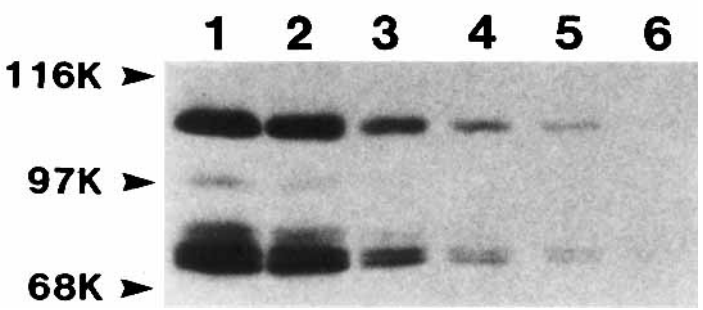

Fig. 8. Sensitivity of αPR13 Western blotting procedure. Varied volumes of sample from C2 Figure 5 were applied to SDS gel. Volumes were proportional to standard load (20 μl in sample buffer, equivalent to 6.7 μl cytosol) as follows: 2× (**lane 1**), 1× (**lane 2**), 0.25× (**lane 3**), 0.1× (**lane 4**), 0.05× (**lane 5**), and 0.025× (**lane 6**). Western blotting was done with αPR13 as the primary antibody.

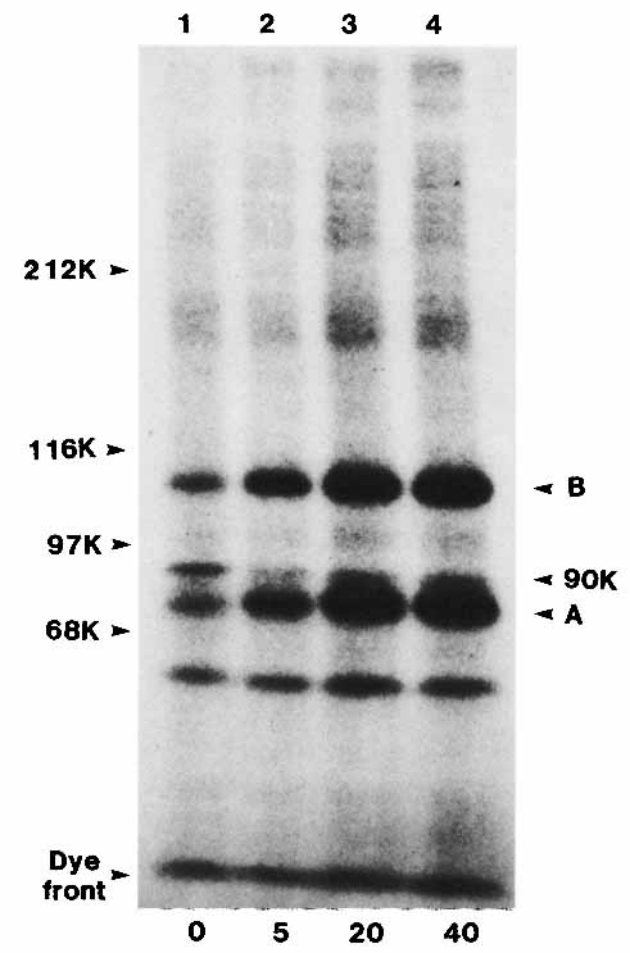

Fig. 9. Hormone-dependent phosphorylation of progesterone receptor in vitro. For each lane, minced oviduct tissue (400 mg) from diethylstilbestrol-treated chick was incubated at 37°C for 60 min in 4 ml of medium containing [^{32}P]-orthophosphate. During the incubation, medium without progesterone (**lane 1**) or with progesterone (to 1×10^{-7}M final concentration) was added for 5 min (**lane 2**), 20 min (**lane 3**), or 40 min (**lane 4**). Following the incubation, the tissue was processed to cytosol, immune precipitated with αPR13, and resolved by SDS gel electrophoresis as described in Materials and Methods. Shown is the autoradiogram of the dried gel.

ble after 5 min with hormone and appeared maximal at 20 min. High levels of phosphorylation were maintained at 40 min with little, if any, increase. Although the Western blotting experiments did not indicate a modification of the B receptor, the results in Figure 9 show a hormone-dependent phosphorylation of both A and B receptors to about the same extent. Under these conditions, the slower migrating A′ form of receptor was not obvious owing to lower resolution of the autoradiograms compared to Western blotting. When aliquots of these incubations were transferred to nitrocellulose, immunostaining with PR13 showed a shift to A′ depending on time of exposure to progesterone (results not shown). No immune reactivity was observed with the phosphoprotein that migrates at about 60K in Figure 9, and this protein is believed to be a contaminant. However, a phosphorylated band at 90 kilodaltons

(kDa) was precipitated with progesterone receptor that was not exposed to progesterone (lane 1). Western blotting indicates that this is hsp90, a phosphoprotein, which has been found complexed with nontransformed receptors in vitro. This band was apparently reduced in incubations with progesterone (lanes 2–4); however, further studies are needed to establish any hormone-dependent change in the appearance of this protein.

DISCUSSION

In attempting to show the mechanisms by which steroid receptor activity is regulated within cells, one hypothesis has been that receptors undergo covalent modifications to alter their activity. One such irreversible modification would be partial proteolysis of receptor subunits. However, one must be cautious in ascribing biological significance to heterogeneous receptor forms seen in crude tissue extracts. Certainly, many of these forms are artifacts resulting from the gross mixing of cellular components by tissue homogenization.

In these studies, antibody probes were used to measure directly the appearance and stability of different progesterone receptor forms under a variety of conditions. Mammalian cytosolic progesterone receptors, like the avian receptor, have been found to contain two forms. Horowitz and co-workers [4,21] have described 120- and 94-kilodalton forms of receptor from the human T47D cell line. These forms were stable in crude cytosolic extracts for up to 1 hr at 37°C. In contrast, Milgrom and co-workers [5], working with rabbit uterine progesterone receptor, found two forms (110 and 79 kilodaltons), but were able to block the formation of the smaller form by careful homogenization and the inclusion of a mixture of protease inhibitors in the homogenization buffer. Also, Lamb et al [13] were able to demonstrate the formation of the 79-kilodalton form from isolated, photoaffinity-labeled 110-kilodalton receptor. In chick oviduct cytosol, we found (Fig. 1) that forms B and A were present at approximately a 1:1 ratio in unfractionated homogenate in the presence of protease inhibitors. Further, after a 3-hr incubation of cytosol at 37°C, in the absence of protease inhibitors, the amounts and proportions of forms B and A remained unchanged. It is improbable that such a stable ratio of the two forms could be established so quickly if A is derived from B after homogenization.

However, similar to the findings of Milgrom and co-workers [5,12], excessive homogenization of tissue did lead to a marked increase in proteolysis of receptor, including the appearance of a form migrating at 85 kilodaltons. We have previously noted [6] the presence of small amounts of this form in some of our cytosol preparations and speculated that it might be a degradation product of B. This now seems to be the case. Initial studies indicate that this form retains the ability to bind to DNA-cellulose (results not shown), but we have not examined if it can still bind hormone. We also do not know if the 85 kilodalton form is degraded to a form similar to A. It is possible that the 79-kilodalton receptor form seen in rabbit uterus is analagous to the 85-kilodalton form and not analagous to chick progesterone receptor A. That is, it may be that in some systems two progesterone receptor forms normally exist in vivo; whereas, in other systems only a single receptor form exists in vivo which is readily degraded to other forms after homogenization.

As has been proposed by others [23–25], the use of frozen tissue to prepare cytosol can lead to increased levels of artifactual proteolysis of steroid receptors. We

have presented direct evidence for induced degradation of progesterone receptor from frozen oviduct tissue (Fig. 3). This degradation was significant even in cytosol maintained at 0–4°C. In light of these and other findings [23–25], the use of frozen tissue, as is common with tumor biopsies, to assay for steroid receptor levels and heterogeneity must be carefully considered.

The loss of hormone-binding activity by steroid receptors in vitro at elevated temperatures is quite possibly due to covalent modifications of the receptor. However, from our results, this loss cannot be correlated with any measurable degree of proteolysis of receptor. Still, a very limited clipping of receptor might not be detected by SDS gel electrophoresis. Altered phosphorylation of receptor [26,27] or oxidation of sulfhydryls [28–31] is a more likely covalent modification responsible for the loss of hormone binding.

A most interesting aspect of this study is the identification of a new receptor state, A′, after in vivo treatment with progesterone. This appears to result from a modification of receptor A which decreases its mobility during electrophoresis on SDS gels. This modification most likely involves receptor phosphorylation since progesterone treatment greatly enhances the incorporation of [^{32}P] orthophosphate into the receptor. A similar mobility shift after hormone treatment has been shown to accompany receptor phosphorylation with the rabbit uterine progesterone receptor [20], the human progesterone receptor [21], and the receptor for vitamin D [32]. Also, several other proteins such as the SII factor that stimulates RNA polymerase [18] and the cardiac membrane protein, phospholamban [19], show a decreased mobility in SDS gels after phosphorylation. The reason for this mobility change after phosphorylation remains unclear, and there is no reason to believe that it must occur in all cases. Thus, while a mobility shift in receptor B is not observed, our results show that progesterone treatment increases the phosphorylation of this receptor species as well. It is still possible that additional modifications (ADP-ribosylation, acetylation, disulfide rearrangement, etc.) occur which contribute to the change in mobility of the receptor. Additional studies are needed to analyze fully receptor composition before and after hormone treatment.

The predominance of the A′ receptor in the nuclear fraction suggests that this may be the major "active" species that is involved in gene regulation. However, this suggestion should be viewed cautiously. Recent immunocytochemical studies indicate that the major portion of progesterone receptor is nuclear, both before and after progesterone treatment [33,34]. Thus, "cytosolic" receptor is an operational term for receptor that is readily removed from the nuclear fraction. The A′ receptor may simply have a somewhat higher affinity for nuclear sites than does the transformed B receptor. Preliminary experiments indicate that nuclear progesterone receptor is also highly labeled with ^{32}P after hormone treatment. However, additional studies are needed to quantitatively compare the phosphorylation of cytosol and nuclear receptors.

Although the level of receptor in nuclear extracts increased somewhat after progesterone treatment, it was substantially less than the cytosolic receptor level during early hormone treatment. This is consistent with observations made previously by Baulieu et al. [14]. In a series of experiments they studied in vivo transformed receptor by a nuclear exchange progesterone binding assay. They reported that nuclear receptor levels were maximal at 60 min and accounted for approximately 20% of initial cytosolic levels. They offered the plausible explanation of a limited number of specific binding (acceptor) sites in the nucleus.

After progesterone treatment, a second form of receptor processing was observed resulting in a loss of all receptor species. This down-regulation was very evident after 4 hr of treatment and persisted for at least 20 hr after treatment. From the present results one cannot determine whether loss of all receptor forms occurs simultaneously or if one particular form of the receptor becomes targeted for degradation. In either case, degradation appears to be rapid and complete since no immunoreactive degradation products were observed.

The present results are consistent with a model where receptors A and B are phosphorylated soon after hormone binding. Thus, progesterone may cause a conformational change in receptor making it a substrate for phosphorylation. This may then lead to a receptor that binds tightly to acceptor sites on the chromatin, but also becomes sensitive to a degradation process. Thus, there are two hormone-dependent steps of receptor processing that require further definition; phosphorylation of receptor, and receptor degradation. In preliminary experiments we have found that neither of these steps occurs upon the simple incubation of cell-free extracts. Therefore, they are likely to require specific proteins and cofactors that must yet be identified and characterized.

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