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THE NUCLEAR-BOUND FORM OF THE PROGESTERONE RECEPTOR IS GENERATED THROUGH A HORMONE-DEPENDENT PHOSPHORYLATION

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The solubilized ("cytosolic") receptor present in the rabbit uterus in the absence of hormone and the chromatin-bound ("nuclear") receptor obtained after injection of a progestin were compared. Crude cellular extracts were analyzed by immunoblotting and receptors were purified by immunoaffinity chromatography. With both methods it was observed that the electrophoretic mobility of the "nuclear" receptor was slower than that of the "cytosolic" receptor. This difference in mobility appeared to be due to the existence of variably phosphorylated forms of receptor. The phosphorylation reaction was examined in uterine slices. In the absence of hormone the cytosolic receptor was phosphorylated. When hormone was added the phosphorylation of receptor was markedly enhanced and the electrophoretic mobility of the "nuclear" receptor was decreased. These experiments thus show that the receptor in its "cytosolic" form is a phosphoprotein. Under the effect of the hormone the receptor is further phosphorylated on some supplementary site(s). This polyphosphoprotein is the chromatin-bound, putatively active, form of the receptor. In this respect the intracellular progesterone receptor is similar to various membrane receptors for hormones and growth factors which are phosphorylated upon binding of their ligand.

Seventeen years ago a two step mechanism was proposed to explain the action of steroid hormones in their target cells (1). The first step consists in the binding of the hormone to a cytosolic receptor. The steroid-receptor complex is then activated to a form exhibiting an affinity towards chromatin and thought to be responsible for the modulation of the expression of specific genes. This mechanism has remained essentially accepted until now with the exception that the cytosolic receptor has recently been shown to be loosely bound to the nucleus and artefactually solubilized during cell homogenization (2,3).

The present work shows that in the case of the rabbit progesterone receptor, there exists a third step in the initial action of the steroid involving a hormone dependant phosphorylation of the receptor.

Abbreviation: R5020: 17,21-dimethyl-19-norpregna-4,9-diene-3,20-dione.

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METHODS

Animals: Prepuberal rabbits were primed with estrogen, their uteri were dissected and homogenized as described (4). In some cases the animals received 30 min before sacrifice a subcutaneous injection of R5020 (10 mg in 0.5 ml sesame oil).

Preparation of subcellular fractions: The homogenization conditions, the buffer (supplemented with EDTA 1.5 mM and NaF 50 mM) and the preparation of cytosol have been described (4). A crude nuclear pellet was prepared by centrifugation at 800 g for 10 min, and 3 washes in 5 ml of buffer/g uterus. Nuclei were extracted for 30 min at 4°C with the homogenization buffer supplemented with 0.4 M NaCl (1.2 ml/g uterus). The supernatant of a centrifugation of 30 min at 48,000 rpm in a Ti 50 rotor was used. When nuclear receptor was purified on the immunomatrix, the nuclear extract was diluted 5.3-fold with the low ionic strength homogenization buffer to increase antigen-antibody binding.

Procedures for receptor electrophoresis (in 9% polyacrylamide gels), immunoblotting and immunoaffinity purification have been described (4,5): In most experiments the elution conditions of the receptor from the immunomatrix were changed: 0.2 M glycine, 0.15 M NaCl pH 2.2 buffer was used instead of the alcaline buffer (5).
After elution, the pH was neutralized by adding 1/20th of volume of Tris 1M HCl pH 8. Alcaline buffer was omitted in the elution step and also in the washing of the immunosorbent to prevent any possible dephosphorylation of receptor. These changes in washing and elution conditions decreased the degree of purification of receptor. A leakage of protein A and immunoglobulins from the immunomatrix was also observed (see Fig.1B). However receptor could still be clearly detected by silver staining.

Phosphorylation of receptor in uterine slices. Uteri were cut into cubes of about 2-3 mm of length. Two parallel incubations were performed at 37°C, each with 10 g of uterine tissue in 20 ml of minimum essential Eagle medium, without phosphate and supplemented with 1 mM glutamine and 10 mM Hepes pH 7.4 under an atmosphere of 95% O_2 and 5% CO_2. ^{32}P orthophosphate, carrier free (2 mCi, Amersham) was added. After 20 min of incubation the uterine slices were transferred into fresh medium containing ^{32}P orthophosphate and in one of the two vials 0.1 µM R5020. Incubation was continued for 40 minutes.

Phosphorylation of receptor in vivo. Six rabbits received intraperitoneal injections of ^{32}P orthophosphate (1 mCi per rabbit per injection) at time 0, 60 and 120 min. Three of the rabbits were injected subcutaneously with R5020 (10 mg in 0.5 ml sesame oil) at time 120. Three other rabbits received sesame oil only. Animals were killed at time 150.

RESULTS

1) Differences in the electrophoretic mobility of "nuclear" and "cytosolic" forms of the progesterone receptor.

In the absence of hormone most of the progesterone receptor is present in the soluble fraction of the cell homogenate. Thirty minutes after injection of a saturating dose (10 mg) of a potent synthetic progestin (R5020), only 10-20% of the receptor remains in the cytosol (data not shown). The majority of steroid-receptor complexes are present in the nucleus from which they can be extracted by high salt. The cytosol from control animals and the nuclear extract from progestin-treated rabbits were analyzed by polyacrylamide gel electrophoresis in denaturing conditions. The receptor was detected by the Western blot method. As shown in Figure 1A, the cytosolic receptor (lanes 1

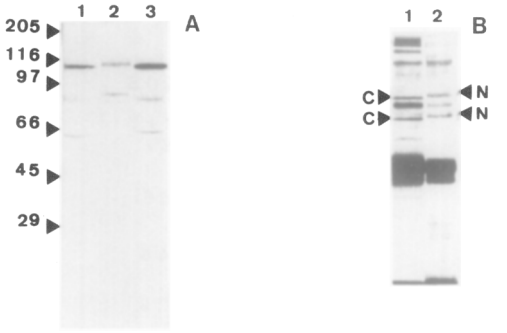

Fig.1. Differences in the electrophoretic mobility of "nuclear" and "cytosolic" forms of the progesterone receptor.
A : Immunoblot study of the progesterone receptor in crude cytosol (lanes 1 and 3) and crude nuclear extract (lane 2).
B :Receptor was purified by immunoaffinity chromatography from cytosol (lane 1) and from nuclear extract (lane 2). After electrophoresis, proteins were stained by silver. As stated in methods section the receptor is impure due to elution conditions by acidic pH. Arrows and letters N and C indicate protein bands of nuclear and cytosolic origin which react (as shown by immunoblot) with antireceptor monoclonal antibody. "Nuclear" receptor was prepared from progestin-treated rabbits. "Cytosolic" receptor was prepared from non-treated rabbits. Molecular weights (x 10^{-3}) of marker proteins are shown by arrows.

and 3) migrated clearly ahead of the nuclear receptor (lane 2). This difference of mobility was observed for the 110,000 dalton receptor and also for its smaller proteolytic fragment of 79,000 daltons (4,5). (It may also be remarked that the 65,000 dalton fragment was only present in the cytosol, and that the 79,000 dalton nuclear receptor contained a minor band of faster mobility).

Moreover, the small amount of receptor which was recovered in the cytosol after hormone injection also exhibited a slower migration, identical to that of the nuclear receptor (not shown). To further confirm this result and to eliminate the possibility of any artefacts related to the Western blot methodology and to the use of crude cellular extracts we purified by immunoaffinity chromatography the cytosolic and nuclear forms of receptor. Electrophoresis followed by silver straining again showed the same difference in mobility between both forms of receptor (Fig.2B).

2) Phosphorylation of the progesterone receptor: Effect of the hormone.

It has been observed for several proteins that phosphorylated forms have an electrophoretic mobility slightly slower than that of the dephosphoproteins (6). To test this explanation of the proceeding results we incubated uterine slices with ^{32}P in presence or in absence of the progestin R5020. Cytosolic and nuclear extracts were prepared, receptors purified by immunoaffinity chromatography and analyzed by SDS polyacrylamide gel electrophoresis. Cytosolic receptor prepared from uteri incubated in the absence of

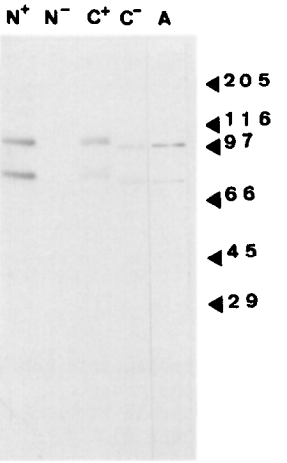

Fig.2. Phosphorylation in uterine slices of the progesterone receptor. Effect of the hormone.
Uterine slices were incubated with ^{32}P either in the absence or in the presence of the progestin R5020. "Cytosolic" and "nuclear" receptors were purified by immunoaffinity chromatography. The eluates of the columns were precipitated by trichloroacetic acid, washed and an aliquot (1/10th) was electrophoresed and autoradiographed.
N+ : nuclear receptor, incubation in the presence of hormone.
N- : nuclear receptor, incubation in the absence of hormone.
C+ : cytosolic receptor, incubation in the presence of hormone.
C- : cytosolic receptor, incubation in the absence of hormone.
Lane A: Western blot of an immunopurified "cytosolic" receptor.
Molecular weights (x 10^{-3}) of marker proteins are shown by arrows.
To obtain quantitative data the Kodak No-screen NS-2T film (Eastman Kodak, Rochester, NY) was used (5). Scanning of the autoradiograph gave the following values (in arbitrary units) for ^{32}P present in the 110,000 and 79,000 bands of the receptor : 49 in N+, 0 in N-, 35 in C+ and 21 in C-.
The same receptor aliquots were examined by Western blot (not shown) to quantitate the mass of receptor (5). The autoradiograph was scanned and the following values were observed (arbitrary units) : 58 in N+, 0 in N-, 12 in C+ and 67 in C-. ^{32}P incorporated into receptor in the presence or in the absence of hormone was computed (see Results chapter).

hormone was phosphorylated (Fig.2, lane C-). After administration of hormone the nuclear receptor (Fig.2, lane N+) was 2.7 -fold more phosphorylated and its electrophoretic mobility was decreased. The cytosolic receptor prepared from hormone treated uteri also exhibited an increased phosphorylation and a decreased mobility (Fig.2, lane C+).

The phosphorylation of receptor was quantified by autoradiography and scanning, the amount of receptor was measured by Western blot and scanning of the corresponding autoradiography. This allowed us to compare the extent of receptor phosphorylation (^{32}P in cytosolic receptor + ^{32}P in nuclear receptor/mass of cytosolic receptor + mass of nuclear receptor) in the uterine slices incubated either in the presence or in the absence of hormone. This method showed that there was a 4-fold increase in phosphorylation of the receptor after incubation with the hormone . Moreover, it must be empha-

sized that the difference was not only quantitative but also qualitative since migration of the phosphorylated receptor before and after hormone administration was different. Thus phosphorylation on some supplementary site(s) has occured. Addition of the progestin did not change total protein phosphorylation (measured by precipitation with trichloroacetic acid) in the cytosol and in the nuclear extract of uterine slices.

The phosphorylation of receptor was also studied *in vivo*: ^{32}P was administered intraperitoneally and the animals were injected with the progestin R5020 or not treated by hormone. Receptors were purified by immunoaffinity chromatography from uterine nuclear extracts and cytosol, respectively. The labeling was very weak and long exposure of the autoradiograms was necessary for the detection of radioactive phosphate only in the nuclear receptor (data not shown).

DISCUSSION

It is generally admitted that steroid hormones act by converting their receptors into activated (DNA and chromatin-binding) forms which in turn regulate gene transcription. In the present work we show that *in vivo* the activation of receptor (conversion into a DNA-binding form) is followed or accompanied by a hormone-dependent phosphorylation.

Moreover, electrophoresis experiments show that the totality (or nearly the totality) of "cytosolic" and "nuclear" receptors formed *in vivo* migrate as distinct species. Thus the hormone-dependent phosphorylation is not a partial reaction involving only a fraction of hormone-receptor complexes.

If the "nuclear" receptor is the active form then all acellular studies of genetic regulation by receptors should involve this form: interaction with specific regions of hormone-regulated genes (7,8) should be reassessed using the nuclear form of receptor. This form should also be used for *in vitro* gene transcription experiments (9).

The succession of events leading to hormone action may be summarized as follows: Receptor (newly synthesized) → Phosphoreceptor ("cytosolic") $\xrightarrow{\text{Hormone}}$ Polyphosphoreceptor ("nuclear") → gene regulation.

Most studies on steroid receptor phosphorylation published to date (see review in 10) have examined cytosolic receptor and thus have probably analyzed the first reaction and not the second one which is hormone-dependent and leads to the formation of the "nuclear" receptor.

In acellular conditions it is possible to separate receptor activation (defined as the generation of a DNA-binding form) from hormone-dependent phosphorylation. For instance, the cytosolic immunopurified receptor binds to DNA (5) but has an electrophoretic mobility identical to that of the non-activated receptor present in crude cytosol.

Experiments on the enzymatic dephosphorylation of the nuclear receptor and its effect on receptor electrophoretic mobility are now being performed.

The hormone-regulated phosphorylation may also explain the mechanism of receptor "processing" or "down regulation", i.e.. the decrease of receptor concentration following steroid administration (11,12). For several proteins it has been shown that phosphorylation increases the turn-over rate (13).

It is usually considered that the mechanism of action of steroids is completely different from that of hormones acting through membrane receptors. We show here that this is not the case for all the steps of progestin action and that the hormone provokes, upon binding to the receptor, its phosphorylation. In this respect, it acts similarly to several polypeptidic hormones and growth factors. It remains to be established if the hormone-dependent phosphorylation described here for the progesterone receptor exists for other steroid hormone receptors and for other nuclear receptors (receptors for thyroid hormones and metabolites of vitamine D_3). The similarity in the general features of their mechanism of action suggests that this may be the case.

The analogy with the polypeptidic hormones and growth factors (14,15) raises the question: is the receptor itself a kinase ? We have found a kinase activity in highly purified preparations of progesterone receptor (F. Logeat, M. Le Cunff and E. Milgrom unpublished observations) but until now we have been unable to show an activation of this kinase activity by hormone. It is thus possible that either we have lost some regulatory element(s) during purification, or that the kinase which copurifies with the receptor is not the one which phosphorylates in vivo only progestin-receptor complexes.

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