In Vitro Conversion of Estradiol-Receptor Protein to Its Nuclear Form: Dependence on Hormone and DNA

(DNA-cellulose chromatography/rat uterus)

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Communicated by Arthur B. Pardee, May 11, 1972

ABSTRACT Early events in the action of 17-β-estradiol can be studied in soluble extracts of rat uterus by exposure of the estradiol-receptor protein to a DNA-cellulose matrix. After complexing with [3H]estradiol, the 4S receptor protein binds to the DNA, and it can be eluted with buffer of high ionic strength as a more tightly binding, 5S form. This parallels the in vivo situation, where migration of the receptor to the nucleus follows addition of hormone and is concomitant with a similar increase in sedimentation rate to 5 S. In both cases, the formation of a 5S receptor requires the presence of 17-β-estradiol. The rate at which 5S receptor forms is sensitive to extraction concentration in a way that suggests that this receptor is a complex created by addition of a second subunit to the hormone-binding 4S component; physical studies on both in vivo and in vitro 5S receptors also support this view. These results are interpreted in terms of a model for action of estrogen in which the hormone potentiates binding of receptor to DNA, and in turn, the DNA-binding process triggers the cell response.

The earliest known event in the process of induction of uterine growth by the steroid hormone 17-β-estradiol is a strong interaction in the uterine cell cytoplasm between the entering hormone and its specific receptor protein (1). This interaction is immediately followed by migration of the hormone, presumably still complexed with receptor, into the cell nucleus (2). There, it appears to bind to chromatin (3, 4).

Soluble extracts of uterine tissue contain an estradiol-receptor protein that sediments at 4 S under suitable ionic conditions. After migration into the nucleus, the bound hormone can be solubilized by extraction of nuclear pellets with buffer of high ionic strength, and it now sediments with a protein at 5 S (2). This “conversion” from 4 S to 5 S can be observed as a temperature-dependent process in vitro by mixing labeled soluble extracts with isolated nuclei (2).

These observations suggest a possible mechanism of steroid hormone action whereby genes are activated by a specific receptor–chromosome interaction that is hormone-dependent. The chromosomal components involved in this proposed interaction are unknown. Since retention of the hormone–receptor complex in nuclei is abolished by treatment of the nuclei with DNase (4, 5), the DNA appears to play an important role. In addition, certain nuclear acidic proteins have been proposed as the chromosomal “acceptor” component (6–8). In the experiments described here, we use DNA–cellulose chromatography to characterize the binding of the “cytoplasmic” estradiol-receptor protein to purified DNA, and measure the specific effect of the hormone on this interaction.

MATERIALS AND METHODS

Buffers and Sucrose Gradients. All buffers contained 0.01 M Tris·HCl, pH 8.1 (20°)–1 mM Na2 EDTA (ethylene diaminetetraacetate)–1 mM 2-mercaptoethanol–1 mg/ml egg white lysozyme as a protective agent (Worthington Biochemical)–NaCl at the indicated concentration. Sucrose gradients (5–20%) contained the same buffer as the sample to be applied, and were prepared and calibrated with protein standards according to Martin and Ames (9).

Preparation of Uterine Extracts. Sprague–Dawley female rats (19 to 24 days old; Camm Research, Wayne, N.J.) were killed by cardiac puncture and perfused with 0.15 M NaCl. Uteri were stripped of fat, excised, and homogenized (1 uterus per 0.2 ml of buffer) at 0° in a Dauil ground-glass tissue grinder (Kontes Glass Co., Vineland, N.J.). The homogenate was centrifuged for 10 min at 41,500 × g in a Sorvall SE-12 rotor. The supernatant was made 6 mM in [2,4,6,7-3H]estradiol (95–100 Ci/mmol; New England Nuclear Corp.), or [2,4,6,7-3H]estrone (90 Ci/mmol; New England Nuclear Corp.), and 0.15 μM in testosterone (Sigma Chemical Co., St. Louis, Mo.). This 25-fold excess of unlabeled testosterone prevents labeling of nonspecific steroid-binding proteins. The labeled supernatant was then centrifuged for 35 min at 165,000 × g in a Spinco 50 Ti rotor. The clear supernatant, denoted “uterine extract,” was used immediately (within 2 hr after killing the rats). Unless noted otherwise, experiments were performed at 0–4°.

DNA–Cellulose Chromatography. DNA–cellulose columns were prepared and run according to Alberts and Herrick (10). They contained denatured calf-thymus DNA at a concentration of about 1 mg per packed ml. Preliminary experiments with columns containing native calf-thymus DNA gave similar results; however, these columns behave as if about 3% of the DNA is single stranded (unpublished results of L. Moran, our laboratory), so that the significance of such experiments is unclear. Columns of 1.5-(packed) ml bed volume were loaded with 0.6 ml of uterine extract. Flow rates were regulated by pumping at 2 ml per hr.

Protein-Coated Glassware. Estradiol-receptor protein, especially after purification on DNA–cellulose columns, tends to adsorb strongly onto glass surfaces. This was avoided by two treatments of all glassware, according to a procedure devised by Dr. U. Laemmli (personal communication): each vessel was filled with an aqueous solution of bovine
serum albumin (2 mg/ml; crystalline; Miles Lab.), warmed to 50° for 30 min, drained, and dried in the oven.

RESULTS

Intracellular form of soluble receptor

When uteri are extracted in 0.15–0.25 M NaCl or KCl and labeled with [3H]estradiol, sucrose-gradient centrifugation at the same ionic strength reveals a homogeneous peak of receptor-bound hormone sedimenting at about 4S. Although a minor peak at about 6 S is occasionally found, the heterogeneous 7–9S peak observed at a concentration of ≤0.05 M NaCl is not detected (e.g., Fig. 1A). This suggests that the true intracellular receptor is the 4S form, rather than the “8S” aggregate previously assumed (1). However, we do not rule out the possibility that the 8S form has biological significance.

Binding of soluble receptor to DNA-cellulose columns

Labeled 4S receptor binds to DNA–cellulose columns, but not to columns of plain cellulose or DNA–cellulose from which the DNA has been removed with pancreatic DNase. Thus, the receptor binds to the DNA and not to the cellulose matrix. Free [3H]estradiol does not bind to DNA–cellulose.

With extracts prepared in 0.15 M NaCl, at least 80% of the input 4S receptor can potentially bind to DNA–cellulose at 4°, given sufficient time and DNA–cellulose. Moreover, at least 90% of the receptor becomes bound at elevated temperatures. The residual fraction of 4S receptor that does not bind could be explained by inactivation during preparation or by suboptimal binding conditions. However, heterogeneity of the receptor could also account for this incomplete binding.

Conversion from 4S to 5S on DNA-cellulose columns

All of the DNA-bound receptor can be eluted from a DNA–cellulose column with 0.40 M NaCl. Sucrose-gradient sedimentation of this eluate (Fig. 1A and B) reveals that an appreciable fraction of the labeled estradiol has switched from the original 4S receptor peak to a second distinct peak at 5S. Subjection of the 4S receptor in unfractonated extracts to 0.40 M NaCl has no effect on its sedimentation rate. Likewise, the breakthrough material from DNA–cellulose columns that have been overloaded with extract remains entirely 4S. Therefore, this conversion of the receptor from 4S to 5S accompanies actual DNA binding, and is not due simply to the shift in ionic strength or to the exposure to DNA–cellulose. It should be noted that others have reported that the 5S receptor can be formed by warming DNA-free extracts containing the 4S receptor to 25° for 1 hr (12). Thus, while DNA clearly accelerates the rate of conversion from 4S to 5S, it may not be absolutely required.

The 4S and 5S forms of the receptor, which elute together at a concentration of 0.40 M NaCl, can be partially separated by use of intermediate NaCl concentrations. For example, as shown in Fig. 1C, a rinse with 0.21 M NaCl elutes most of the 4S receptor, while the 5S form and the remaining 30% of the 4S form are eluted with a subsequent 0.40 M NaCl rinse. Similarly, when the concentration is raised from 0.15 to 0.25 M NaCl, all of the 4S and about 40% of the 5S receptor is eluted; the remaining 5S receptor is eluted with 0.40 M NaCl (Fig. 1D). Therefore, the 5S form of the receptor appears to bind more strongly to DNA.

The conversion from 4S to 5S seems to be a complex reaction, sensitive to time, temperature, and the protein concentration of the extract. At 4°, the receptor in extracts prepared in 0.15 M NaCl binds to DNA and converts to the 5S form of receptor slowly. In one experiment, an extract was divided into four equal parts, loaded onto separate DNA–cellulose columns, and eluted after different times of exposure to the column. The results shown in Fig. 2 reveal that the 5S:4S ratio increases from about 0.8 after 30 min to about 3 after 150 min of contact between the extract and the DNA. In addition, the total binding of labeled receptor to the DNA increases, going from 40% of the total receptor bound after 30 min to 80% after 150 min of contact. At higher temperatures, both the binding and the conversion to 5S are markedly accelerated. As shown in Fig. 2D, 30 min of contact at 18° is sufficient to bind 90% of the receptor to DNA, and virtually all of it is converted to the 5S form. For determination of whether the conversion from 4S to 5S involves interaction of the 4S receptor with another

\[ \text{Fig. 1. Sucrose-gradient sedimentation of uterine extracts labeled with [3H]estradiol before or after fractionation on DNA–cellulose: conversion from 4S to 5S forms and partial separation of the two forms. Extracts were prepared at 0.15 M NaCl; sedimentation was as described in Methods for 18 hr at 234,000 x g. Bacterial alkaline phosphatase (EC 3.1.3.1; Worthington Biochemical; 2 µg per gradient) was run as a 6.2S internal standard in all gradients, and assayed according to Garen and Levinthal (11). (A) O --- O, unfractonated extract; • --- •, unfractionated extract, adjusted to 0.40 M NaCl; △ --- △, breakthrough from DNA–cellulose column overloaded with extract. (B) 0.40 M NaCl eluate from extract loaded onto DNA–cellulose at 0.15 M NaCl. (C) O --- O, 0.21 M NaCl eluate from extract loaded onto DNA–cellulose at 0.15 M NaCl; • --- •, 0.40 M NaCl eluate after 0.21 M NaCl step. (D) O --- O, 0.25 M NaCl eluate from extract loaded onto DNA–cellulose at 0.15 M NaCl; • --- •, 0.40 M NaCl eluate after 0.25 M NaCl step. These gradients contained different NaCl concentrations, adjusted to match that of the sample applied.} \]
molecule in the extract, the effect of extract concentration on the rate of conversion was investigated. Such experiments (not shown) show a 16-fold decrease in the rate of formation of 5S receptor upon a 4-fold dilution of the extract. Thus, it appears that the rate-limiting step in the conversion to 5 S is a bimolecular reaction between two substances in the extract.

Physical properties of 4S and 5S receptors

Although estradiol could in theory be passed from the 4S receptor to a different protein upon entry into the nucleus, this is unlikely since the purified 5S form decays to the 4S form during prolonged storage (unpublished results) or manipulation (13). If we assume that estradiol remains bound to the same protein, the 4S to 5S shift could be due to a conformational change of the protein to a more compact form; alternatively, the protein could acquire additional mass.

If the 5S receptor is merely a more compact form of 4S protein, it should elute later than the 4S receptor from a gel permeation column. If instead, the 5S receptor results from addition of a new subunit, it would have larger dimensions, and elute ahead of the 4S receptor from such a column. In fact, the elution position from a gel-permeation column, together with the sedimentation rate on a sucrose gradient, enables calculation of both the molecular weight and axial ratio of a protein (14). By these techniques, an "in vitro" 5S

![Fig. 2. Sucrose-gradient sedimentation of 0.40 M NaCl eluates from DNA-cellulose columns loaded with uterine extracts labeled with [3H]estradiol: time and temperature dependence of receptor binding and conversion from 4 S to 5 S. Extracts were prepared at a concentration of 0.15 M NaCl, except (D) was prepared at 0.21 M NaCl. After loading onto DNA-cellulose columns at 4°, column flow was stopped, leaving the entire extract exposed to the DNA-cellulose until pumping was resumed in each column at different times. The 0.40 M eluates were sedimented as in Fig. 1. (A) 30-min "contact time" of sample with DNA-cellulose before rinsing and eluting; 40% of input receptor bound; (B) 90-min "contact time"; 53% bound; (C) 150-min "contact time"; 90% bound; (D) extract chromatographed at 18°; all other procedures were performed at 0–4°; 30-min "contact time"; 90% bound. Warming the extract for this period without exposure to DNA was not sufficient to convert 4S receptor to 5S.

![Fig. 3. Physical characterization of 4S receptor, in vivo 5S receptor, and in vitro 5S receptor. "In vivo" 5S receptor was prepared essentially according to Shyamala and Gorski (15): intact uteri were incubated in Dulbeco Modified Eagle's Medium containing [3H]estradiol for 1 hr at 37°; the pellets obtained by low-speed centrifugation (29,000×g) of the uterine homogenate were thoroughly washed, then extracted with 0.40 M NaCl-Tris buffer (pH 8.1). After high-speed centrifugation (165,000×g), the supernatant was denoted "in vivo" 5 S. All columns and gradients in this experiment were run at 0.40 M NaCl. Receptor molecular weights were calculated by combination of data from gel-permeation chromatography and sucrose gradient sedimentation (14). Sedimentation rates of 4.1 S and 5.3 S were calculated for "4S" and "5S" receptors, respectively. (A) Gel-permeation chromatography on Bio-Gel A 0.5 M (Bio-Rad Laboratories). 1 × 40-cm columns were eluted at 4° at a rate of 4–5 ml/hr. Void volume (V0) was determined with [3H]DNA, sheared to 5–10 × 10⁶ molecular weight. Total volume (Vt) was determined with [14C]thymidine. Protein standards shown were selected because both their molecular weights and shapes are known: 1, beef-liver catalase (16); 2, rabbit-muscle lactate dehydrogenase (17); 3, yeast hexokinase (18); 4, bovine hemoglobin (19); 5, sperm-whale myoglobin (20). ○—○, in vivo 5 S; Δ—Δ, in vitro 5 S; •—•, 4 S. (B) Sucrose-gradient sedimentation of peak fractions from Bio-Gel A 0.5 M columns eluting at 23–24 ml. ○—○, in vivo 5 S; Δ—Δ, in vitro 5 S. (C) Sucrose-gradient sedimentation of peak and shoulder fractions from Bio-Gel A 0.5-M columns eluting at about 27 ml. Δ—Δ, in vitro 5 S; •—•, 4 S. These gradients confirm that the elution peaks from the gel columns correspond to the 5 S and 4 S sedimenting forms, and not to aggregation or breakdown products arising during chromatography.
receptor purified by elution from DNA-cellulose, an "in vitro" 5S receptor purified from labeled intact uteri, and an unfractonated 4S preparation were compared under identical conditions. The results are shown in Fig. 3. It can be seen that the 5S receptor behaves as if it is larger than the 4S form on gel columns as well as in sucrose gradients, thus ruling out a simple change in shape as the cause of conversion from 4 S to 5 S. Moreover, the fact that the 5S form produced in vivo behaves identically to the 5S form produced on DNA-cellulose shows that the molecular weight and shape of the two species are the same. By reference to protein standards (see legend to Fig. 3), the molecular weights of the 4S and 5S receptors were estimated as 60,000 and 104,000, with approximate axial ratios for prolate ellipsoids of 3.8:1 and 5.4:1, respectively. (The values for the 4S receptor agree well with those reported by Puca et al., ref. 3.) On this basis, we infer that the 5S receptor produced in vivo is identical to that produced in vitro, and that it is more asymmetric and of higher molecular weight than the 4S form.

Hormone requirement for receptor-DNA interaction

Results with intact uteri reveal that the receptor remains in the cell cytoplasm in the absence of hormone, and is depleted from the cytoplasm upon addition of estradiol (2). An obvious explanation for migration to the nucleus is that only the estradiol-receptor complex can bind to DNA in a physiologically significant manner. Two types of experiments were performed to investigate this possibility.

The first experiment examines the affinity of the receptor protein for DNA in the absence of hormone. Aliquots of a uterine extract, one with and another without [3H]estradiol, were chromatographed on DNA-cellulose columns, and the bound receptor eluted with 0.40 M NaCl. Only then were the fractions from the hormone-free receptor preparations labeled by addition of excess [3H]estradiol. The breakthroughs and elutes from both columns were analyzed for receptor content in sucrose gradients. The sedimentation patterns of the bound receptors, shown in Fig. 4A, reveal that estradiol greatly increases the affinity of receptor for DNA. The analysis of the column breakthroughs confirmed this result, demonstrating that without hormone the vast majority of receptor passes directly through the column (not shown).

A second experiment takes advantage of the fact that although estrone binds to the receptor in vitro (21), it displays little, if any, estrogenic effect in vivo (22). Thus, half of a uterine extract was labeled with [3H]estradiol, and the other half with [3H]estrone. The two extracts were then fractionated on separate DNA-cellulose columns, and the column breakthroughs and 0.40 M NaCl eluates were analyzed for receptor content on sucrose gradients. The gradient patterns obtained in this experiment (0.40 M NaCl eluates are shown in Fig. 4B) are similar to those seen for free receptor, demonstrating again that the conversion from 4 S to 5 S requires estradiol.

We conclude that under the conditions used in our experiments, the ability of the receptor to convert to the 5S form in the presence of DNA is drastically reduced if there is no steroid bound to the 4S receptor, or if estradiol is replaced by estrone.

Discussion

Our observations in vitro appear to correlate closely with the events that occur in vivo. First, the conversion from 4 S to 5 S observed on DNA-cellulose columns yields a product indistinguishable in both molecular weight and shape from the "nuclear receptor" observed in vivo. Second, the 5S receptor is eluted from DNA by buffers of the same ionic strength as those used to extract "nuclear receptor" from preparations labeled in vivo. Third, both binding to DNA and conversion to 5 S are strongly enhanced at elevated temperatures, just as formation of "nuclear receptor" is a temperature-dependent process in uterine homogenates (2). Fourth, both binding to DNA and conversion to 5 S are dependent on the presence of the estradiol-complexed form of the receptor protein. Moreover, the hormone requirement is not satisfied by the presence of estrone, a closely related steroid that binds to the receptor, but has little or no estrogenic effects.

In order to mimic in vivo observations, we found it necessary to use buffers of relatively high ionic strength. At a concentration of 0.21 M NaCl, most of the receptor bound to DNA and was converted to the 5S form only when the receptor was also complexed with estradiol. At a concentration of 0.15 M NaCl, however, the free 4S receptor bound to DNA nearly as well as the hormone-complexed form, although conversion of free receptor to the 5S form was still blocked (unpublished experiments). Whether the receptor-DNA interaction involves a specific DNA sequence remains to be investigated. Recent reports from other laboratories suggest that additional factors could be involved in determination of binding specificity (6-8; 29-27).

The results reported here are consistent with a generalized model of steroid hormone action similar in some respects
to the scheme recently elucidated for cyclic AMP in the release of catobolite repression in bacteria (reviewed in ref. 28). The model is based upon the observation that both receptor–hormone interaction and nuclear migration appear to be required for full expression of the hormone effects (29, 30). We propose that receptor-protein resides primarily in the cell cytoplasm in the absence of the hormone, since it has only a low affinity for DNA. In fact, free receptor in the cytoplasm might serve an important function there, as proposed by Ohno (31). Upon entry of the hormone into the cell, and its concomitant binding to the receptor, the receptor–hormone complex undergoes a change that increases its affinity for specific sites on the DNA. In the case of estradiol receptor, this change appears to be associated with binding of a second subunit. The DNA-binding sites could be specified by DNA sequence, DNA structure, chromosomal proteins, or any combination of these. Beyond this point, the mechanisms for transcriptional control and amplification of the hormone response remain open questions (32–34). Induction of specific proteins (35) or stimulation of RNA polymerase as first observed by Gorski (36) are intriguing possibilities.

A similar model has independently been proposed by Baxter et al. (29), who observed an analogous binding of the glucocorticoid receptor protein to DNA.

We thank Dr. R. Lisk and Mrs. Clara Schroeder for advice and assistance in the early stages of this work, and also Dr. J. R. Fresco for a critical reading of the manuscript. Supported by grants from the National Institutes of Health (GM-14927 and Training Grant (GM-00962-10)) and the American Cancer Society (E-510). An abstract of this work has been published [Fed. Proc. 31, 429 (1972)].