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Comparison between Different Forms of Estrogen Cytosol Receptor and the Nuclear Receptor Extracted by Micrococcal Nuclease[1]

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Abstract

As an approach to the mechanism of the nuclear translocation of estrogen receptor, the estradiol nuclear receptor (RN) of lamb endometrium was extracted with micrococcal nuclease at 2–4° and compared to the "native" 8S and to the Ca^{2+}-transformed cytosol receptors. After extensive digestion of chromatin, giving up to 10% perchloric acid-soluble DNA and a majority of nucleosome monomers, up to 80% of the RN was extracted under low ionic strength. This RN was found to be completely different from the partially proteolyzed Ca^{2+}-transformed cytosol receptor. It migrated with a sedimentation constant of 4 and 6 S. The Stokes radius of the predominant form as determined by ACA 34 chromatography was 5.3 nm. The calculated apparent molecular weights were 130,000 and 90,000, respectively. The RN was able to bind DNA and was eluted from a diethylaminoethyl cellulose column at 0.23 and 0.30 M KCl. We conclude that the mechanism proposed by Puca *et al.*, according to which the Ca^{2+}-transformed cytosol receptor is split by a Ca^{2+} receptor-transforming factor into a smaller form able to cross the nuclear membrane, is very unlikely.

Introduction

The estrogen-dependent RC[3] translocation to the nucleus is one of the first steps that follow hormone binding and is probably involved with hormonal stimulation in the nucleus (16). However, the nature of the "activation" of the RC-estradiol complex that leads to its nuclear translocation through the nuclear membrane is unknown. Schematically, 3 series of hypotheses have been proposed: (*a*) the RC-estradiol complex is transformed into a heavier form by dimerization or interaction with other components (9, 15); (*b*) the RC-estradiol complex undergoes a conformational change without modifying its molecular weight; (*c*) finally, the RC-estradiol complex is transformed into a smaller receptor in order to pass through the pores of the nuclear membrane either by reversible dissociation of RC into subunits (21) or by an irreversible proteolysis. This last possibility was suggested by Puca *et al.* (16), who demonstrated the presence of an uterine Ca^{2+}-activated transforming factor that partially proteolyzed the native RC into a 4S Ca^{2+} RC. RC was also shown to be split by an endogenous serine protease in uteri (14) and in human breast cancer (22).

In order to choose between these 3 mechanisms, 2 approaches were essentially taken. The first was to evaluate *in vitro* in a cell-free reconstituted system the efficiency of different forms of RC in interactions with nuclei and DNA. It has thus been demonstrated that RC partially proteolyzed by exogenous trypsin did lose its ability to interact with nuclei (27) and DNA (2), while the Ca^{2+} RC did not interact with DNA (2) but was still binding to nuclei (27). Moreover, we have shown recently that the Ca^{2+} RC-nuclei interaction was not inhibited by intercalating drugs such as ethidium bromide contrary to the 8S RC-nuclei interaction (3). However, the nuclear translocation observed in cell-free conditions could be different from that observed *in vivo* or *in vitro* in the whole cells (7, 28).We now report another approach to compare different forms of RC with the RN formed in uteri and solubilized by DNA hydrolysis.

Actually, the contradictory results (6) concerning RN characterization may be due to several artifacts. RN has been mainly extracted by KCl (>0.3 M) which modifies the interaction of RC with other proteins or between subunits (5, 10, 18). The subsequent removal of salt leads to artifactual proteolysis and/or aggregations of the receptor (17, 19). In the previous attempts to extract RN by nuclease digestion, the receptor was either aggregated or bound to chromatin (8, 23).

We used milder conditions allowing us to analyze the RN under low-salt conditions and without aggregations. Our results strongly suggest that RN does not derive from the Ca^{2+}-transformed RC.[4]

Materials and Methods

Preparation of Extracts Containing RN and RC. Whole lamb uteri were incubated for 1 hr at 37° in Eagle's medium with 20 nM [^{3}H] estradiol (specific activity, 60 Ci/mmol) and with or without 2 μM nonradioactive estradiol for nonspecific uptake. The endometrium nuclei were purified with a 40% yield (1) in 10 mM Tris-HCl-0.25 M sucrose buffer, pH 7.4, containing 3 mM $MgCl_2$ and 0.2 mM phenylmethylsulfonyl fluoride. They were 90% pure as checked by light and electron microscopic examination, and their weight proportions for proteins, DNA, and RNA were, respectively, 3, 1, and 0.4. The purified nuclei were washed and resuspended

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[3] The abbreviations used are: RC, cytosol estradiol receptor; Ca^{2+} RC, RC partially proteolyzed by a Ca^{2+}-transforming factor; RN, nuclear estradiol receptor; DCC, dextran-coated charcoal.

[4] This paper is a preliminary one; a detailed report on the characterization of the RN solubilized by micrococcal nuclease digestion will be published elsewhere.

in 10 mM Tris-HCl-0.25 M sucrose buffer, pH 7.4, containing 1 mM $CaCl_2$. The suspension (1 to 3 × 10^8 nuclei/ml) was incubated for 1 hr at 2–4° with micrococcal nuclease Worthington (EC 3.1.4.7) at 750 units/mg DNA. The reaction was stopped by adding 10 mM EDTA. The suspension was then centrifuged to separate the first nuclear extract from the pellet; the extract was then suspended in 10 mM Tris-HCl-1.5 mM EDTA buffer, pH 7.4, to lyse the nuclei. This suspension was centrifuged to separate the second nuclear extract from the final pellet. The cytosol containing the 8S RC was prepared directly from nonincubated lamb uteri (18). The Ca^{2+} RC and the trypsin RC were prepared from crude cytosol according to the methods of Puca *et al.* (16) and Erdos and Fries (5), respectively.

Analysis of RN and RC. Ultracentrifugation in a 5 to 20% sucrose gradient was performed after adsorption of free estradiol by DCC. Sedimentation coefficients were determined (13) with ^{14}C-labeled bovine immunoglobulin (7S), [^{14}C]ovalbumin (3.8S), and the monomer subunit of the chromatin (11S) as internal standards. DNA-receptor interactions were measured by sucrose gradient centrifugation (2). Gel filtration chromatography with Ultrogel ACA 34 (LKB Instruments, Rockville, Md.) was performed with a filtration flow rate of 15 to 25 ml/hr (25). The Stokes radius of receptors were determined according to K_{ii}'s after calibration of the column with standard proteins. DEAE-cellulose chromatography was performed as in Ref. 21, with a 10 mM Tris-HCl-1.5 mM EDTA buffer, pH 7.4, containing 12 nM thioglycerol and a linear 0 to 0.5 M KCl elution gradient. KCl molarity was evaluated by refractometry.

Miscellaneous. The saturable estradiol-RN complexes were evaluated by direct counting of the radioactivity incorporated into nuclei with or without isotopic dilution. The estradiol-receptor complexes contained in nuclear and in cytosol extracts were measured by the dextran charcoal (18) or the protamine sulfate (4) assays. Radioactivity was counted in a 3-ml ethanol-10-ml toluene-POPOP-PPO scintillating mixture with a constant efficiency of 20% evaluated by external standard. Calf thymus DNA was purified according to the method of Marmur (12). Other techniques were described previously (3).

Results

Solubilization of RN-Estradiol. An extensive digestion of nuclei with micrococcal nuclease followed by nuclear lysis solubilized a mean of 75% of nuclear [^3H]estradiol. A total of 58% of the [^3H]estradiol incorporated into nuclei was bound to RN as shown by DCC adsorption or protamine sulfate precipitation. This binding was thermolabile and saturable. These conditions corresponded to the solubilization of 60 to 80% of the total DNA and to the liberation of 10% of the DNA in the perchloric acid-soluble fraction.[4]

Sucrose Gradient Analysis. In order to specify whether the extracted RN's were bound to chromatin or free, we compared the radioactivity and the absorbance patterns of the nuclear extracts after ultracentrifugation. We defined as free the receptor labeled by [^3H]estradiol that migrated slower than the mononucleosome peak (11S) even though we could not exclude the possibility of an interaction of the

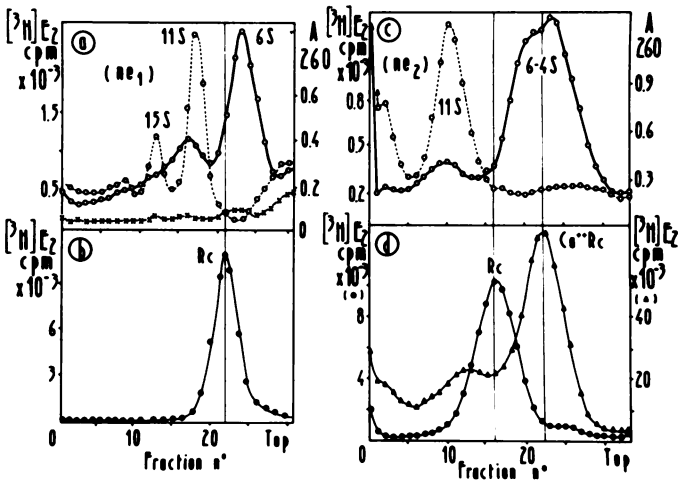

Chart 1. Sucrose gradient ultracentrifugation. The 2 nuclear extracts (ne_1 and ne_2) and the cytosol containing the native RC (●) or the Ca^{2+} RC (○) were prepared as indicated in "Materials and Methods." All extracts were treated by DCC for 5 min at 2° and then ultracentrifuged in a 5 to 20% sucrose gradient at 38,000 rpm for 14 hr at 0–2°. Absorbance at 260 nm (– – –) and radioactivity (——) were measured in each fraction. Results of 2 different experiments. In *a* and *b*, the extract (ne_1) contained 54 and 67% of RN and DNA, respectively. The nonspecific interaction of [^3H]estradiol ([^3H]E_2) (×) was obtained by isotopic dilution during incubation of uteri. The cytosol containing the 8S RC was prepared and analyzed in parallel (*b*). In *c*, the extract (ne_2) contained 62 and 56% of RN and DNA, respectively. The Ca^{2+} RC and 8S RC were prepared and analyzed in parallel (*d*).

receptor with other proteins or nucleic acids. After micrococcal digestion the absorbance pattern (Chart 1*a*) showed 3 peaks at about 11, 15, and 19S corresponding to the S values of the mono-, di-, and trimers of the chromatin subunit (11). When the amount of monomers was predominant, 60 ± 10% (S.D.) of the layered [^3H]estradiol was bound to 4 and 6S peaks or to a 6S peak alone (Chart 1). The first nuclear extract that contained diffusible material displayed light chromatin. In the second nuclear extract, the proportion of heavier chromatin was higher. The 6 and 4S peaks were absent when the uterus was labeled in the presence of a 100-fold excess of nonradioactive estradiol or when the nuclear extracts prepared from unlabeled uterus were subsequently incubated with 3 nM [^3H]estradiol and treated by DCC for 10 min before sucrose gradient analysis. We concluded that the [^3H]estradiol 4 to 6 S peaks represented the RN-estradiol complex which was either free or interacting with nondefined molecules. The sedimentation constant of the RN-estradiol complex was not significantly different from the Ca^{2+} RC (≃4.5S) but was always inferior to that of the 8S "native" RC (Chart 1). The small 10 to 12S peak sometimes observed could represent the RN-estradiol bound to some chromatin material such as mononucleosome. The comigration of RN-estradiol with chromatin was much more important for lower digestion of nuclei (not shown).

DNA-binding Ability of RN. The RN estradiol extracted by micrococcal nuclease was incubated either with or without calf thymus DNA, and its migration was analyzed in a sucrose gradient. With heavy DNA (>15S), the 6 and 4S peaks disappeared, while the majority of the radioactivity was pelleted. An aggregation of RN was unlikely, since the [^3H]estradiol RN was displaced but not pelleted by a 6S

sonically disrupted DNA. We concluded that the RN was able to interact *in vitro* with DNA. During the same experiments, we checked that the 8S RC, but not the 4S Ca^{2+} RC, was able to interact with DNA. However, the different DNA-binding abilities to RN and of the 4S Ca^{2+} RC could be due to other components than the receptors themselves. To eliminate these possibilities, we performed reconstitution experiments. The addition of nuclear extracts or cytosol to the Ca^{2+} RC did not restore any DNA-binding ability of the Ca^{2+} RC, thus confirming that this property

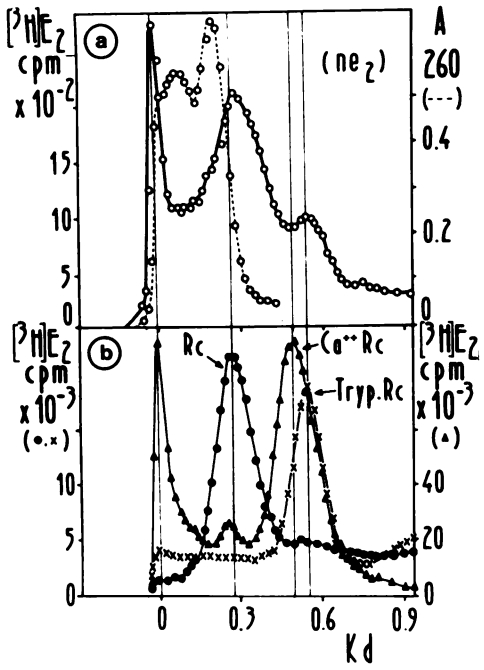

Chart 2. Ultrogel ACA 34 chromatography. The micrococcal nuclear extracts (*a*) and the cytosol (*b*) containing the "native" RC, the Ca^{2+} RC, and the RC partially proteolyzed by trypsin were prepared as described in "Materials and Methods." Absorbance at 260 nm (- - -) and radioactivity (——) were measured in fraction aliquots. The distribution coefficient (K_d) was calculated from the relationship

$$K_d = \frac{V_e - V_0}{V_t - V_0}$$

V_e is the elution volume of each fraction, V_0 is the void volume evaluated by dextran blue, and V_t is the total volume accessible to the solvent measured by β-mercaptoethanol.

was irreversibly lost during proteolysis (2). Conversely, the interaction of the 6S RN-estradiol complex with heavy DNA was not prevented by adding the partially purified Ca^{2+} RC which had been occupied by nonradioactive estradiol excluding the possibility of an inhibitor in this Ca^{2+}-treated cytosol. These results indicated that the 6 to 4S RN-estradiol extracted by micrococcal nuclease, actually interacted *in vitro* with DNA like the 8S RC and contrary to the Ca^{2+} RC.

Gel Filtration Analysis. Using Ultrogel ACA 34 chromatography, we compared in a low-salt medium the elution pattern of the RN-estradiol with those of the native and partially proteolyzed RC (Chart 2). The major peak entering into the gel was eluted just like the native RC and did not comigrate with the bulk chromatin. The minor peak migrated like the proteolyzed RC. The Stokes radius of the major peak of RN was similar to that of the 8S RC but was much higher than those of the Ca^{2+} RC (Table 1) and the RC partially proteolyzed by trypsin (MW ≃37,000). Reconstitution experiments ruled out the possibility that the high Stokes radius of RN was due to interactions of a Ca^{2+} transformed RC with other molecules contained in the nuclear extract. The "micrococcal RN" appeared to be reversibly dissociated by 0.5 M KCl as was the 8S RC since its Stokes radius and sedimentation constant were markedly decreased after salt treatment.[4] The anhydrous molecular weight of the micrococcal RN was found to be slightly smaller than that of the 8S RC but much higher than that of the partially proteolyzed RC (Table 1). The interest of the molecular weight values reported in Table 1 is mainly a comparative one since they might vary with the separation procedure and the degree of purity of the receptor (9).

DEAE-Cellulose Chromatography. Most often, the RN was eluted as 2 broad peaks with 0.23 M and 0.30 M KCl concentrations, suggesting an heterogeneity of the receptor. The elution pattern was very similar to that obtained with the 8S RC. The proportion of the 2 peaks varied according to the experiments both for the 8S RC and for the micrococcal RN. In any case the RN elution pattern was very different from that of the 4S Ca^{2+} RC, which was eluted to 0.1 M KCl.

Discussion

The digestion of endometrial nuclei with micrococcal

Table 1

Comparison between the "micrococcal" RN with "native" RC and Ca^{2+} RC

These results were obtained with nonpurified RN from lamb endometrium. The molecular weights were calculated from the S value, and the Stokes radius was calculated according to the method of Siegel and Monty (24). Similar molecular weights have been found for RC (25) and for Ca^{2+} RC (15). Similar elution from DEAE-cellulose has been reported for RC and Ca^{2+} RC (16).

	Sedimentation coefficient (S)	Stokes radius (nM)	M.W.	DEAE-cellulose KCl elution (M)	DNA binding
"Micrococcal" RN	6–4	5.2	130,000, 90,000	0.23, 0.30	+
"Native" RC	8–6	5.3	170,000, 130,000	0.23, 0.30	+
"Ca^{2+}" RC	≃4.5	2.9	55,000	0.10	–

nuclease at 0–2° allowed us to extract the RN-estradiol complex under mild conditions. Up to 80% of the total DNA and of the saturable RN could be solubilized by this procedure and directly characterized in a low-salt medium. As shown in Table 1, the "micrococcal" RN appears to be clearly different from the partially proteolyzed RC obtained after Ca^{2+} treatment (16). We checked that the heavier apparent molecular weight of the "micrococcal" RN was not due to artifactual aggregation of a proteolyzed RN with other nuclear molecules. In addition, a functional difference was noticed, since the "micrococcal" RN was able to bind DNA, while this property is known to be irreversibly lost after partial proteolysis of RC (2). Conversely, the "micrococcal" RN was similar to the "native" 8S RC since it was also reversibly dissociated by KCl and displayed the same Stokes radius and DNA-binding ability (Table 1). However, the sedimentation constant and the apparent molecular weight of RN were slightly smaller than those of the "native" RC. Since RC and RN were analyzed in a distinct environment, it is not known whether their different rates of migration indicated a real difference between the 2 receptors or different interactions with other molecules of the cytosol or nuclear extracts (26).

These results support the hypothesis previously proposed (2, 27) according to which the proteolyzed receptors are not transferable to the nucleus and would be artifactual or exit forms of receptors. It is therefore very unlikely that the Ca^{2+}-activated proteolysis is biologically responsible for the nuclear translocation of RC. The mechanism by which RC is dimerized or associated with another protein during the translocation (9, 15) cannot be excluded here since it was based on studies performed in high-salt medium and on different animals. However, the lower molecular weight of RN does not favor this possibility. RN homogeneity is questionable, since after nuclear lysis at least 2 binding peaks were distinguished by sucrose gradient, gel filtration, and DEAE-cellulose analysis. The significance of these 2 entities, either 2 different receptor subunits, A and B, as proposed for the progesterone receptor (21), or a transformation of one native receptor into a secondary receptor formed either *in vivo* or artifactually *in vitro* were not specified here. Definitive conclusions will be obtained when the native receptor protein is purified to homogeneity from both the cytosol and nuclear compartments.

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