ESTROGEN RECEPTOR: LOSS OF DNA BINDING ABILITY FOLLOWING TRYPSIN OR Ca^{2+} TREATMENT

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1. Introduction

In a target cell, estradiol (E_2) binds to its cytosol receptor (Rc) and the only data available indicate that the resulting RcE_2 complex, modified slightly or not, is rapidly translocated to the nucleus where it interacts with some component(s) of the chromatin which have been called "acceptor"(s). DNA could be at least one part of this part of this nuclear acceptor(s) since an interaction between RcE_2 and DNA has been demonstrated *in vitro* using sucrose gradient ultracentrifugation [1], Sepharose [2] or DNA cellulose chromatography [3, 4] and paper disc filtration [5]. In addition, estradiol and other estrogens were shown to specifically increase the formation of the Rc–DNA complex [6], suggesting that the occupation of the estradiol binding site of the receptor increases the interaction of the RcE_2 complex with DNA. The 4 S-E_2 complexes, obtained after the treatment of the 8 S-E_2 receptor by trypsin [7] of calcium [8, 9] are known to be as efficient as the 8 S receptor in their ability to bind estradiol but unable to be converted into the 8 S complex. They seem to proceed through a limited proteolysis catalysed by trypsin or a calcium activated "transforming factor" [10]; however these proteins are different as far as their pI is concerned [11].

We report in the present paper that these 4 S entities are also inefficient in binding DNA. It is therefore suggested that the inter-subunit association site and/or the DNA acceptor site of Rc have been destroyed by trypsin or calcium treatment, whereas the estradiol binding sites were preserved. Consequently, we propose, as a working hypothesis, that these 4 S Ca^{2+} or trypsin binding proteins are inefficient in translating the hormonal message to the genome.

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2. Methods

The calf uterine cytosol prepared as described previously was incubated at 0° for 90 min with [6,7-^{3}H]-estradiol (CEA; specific activity = 48 Ci/mmole). It was then mixed with calf thymus DNA type I Sigma (180 μg/ml) at 2° for 2 hr. The samples were separately centrifuged through a 5–20% sucrose gradient at 105,000 *g* for 14 hr. The fractions were counted for radioactivity in a toluene scintillation mixture (50% efficiency) and the DNA concentration evaluated by absorption at 260 nm. The degree of the DNA–RcE_2 interaction was measured not only by the increase of radioactivity in the DNA region but also by the decrease of radioactivity in the unbound RcE_2 region [6]. The number of specific estradiol binding sites was measured by differential adsorption on dextran-coated charcoal at 0–4° [9]. Protein concentration was assayed according to its absorption at 280 and 260 nm and by the Lowry technique [12].

Before incubation with DNA, the cytosol containing the RcE_2 complex was treated as follows:

i) Partial purification of the 8 S Rc was performed either by ultracentrifugation through a 5–20% sucrose gradient and collection of the top of 8 S peak or by a 20% ammonium sulfate precipitation in the presence of KCl 0.4 M at 0–2° according to Puca [10]. The dissolved precipitate was subsequently desalted through a Sephadex G-25 column.

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ii) Trypsin (TPCK Worthington) was incubated with the cytosol (3–20 μg per mg protein) for 30 min at 0–2° according to Erdos [7]. Its activity could be inhibited by diisopropyl fluoro phosphate (1 mM) or soybean trypsin inhibitor (Worthington). The residual proteolytic activity of the mixture was checked using Hide powder azure, B grade (Calbiochem).

iii) Calcium treatment (4 mM) of the cytosol was applied either at 25° for 20 min without purification [9] or at 0–2° in the presence of 0.4 M KCl followed by 20% ammonium sulfate precipitation [10].

3. Results

3.1. *Interaction of the DNA with the partially purified 8 S-E_2 complex*

After a 20% ammonium sulfate precipitation of calf uterine cytosol, a 30-fold purified receptor was obtained, which migrated mainly as an 8 S peak, although some aggregates were also formed. The fact that this purified material was able to bind DNA (fig. 1) with a rather higher efficiency than the crude cytosol, supports the idea that the Rc interacts directly with DNA. Conversely, when the 8 S E_2 complex was purified 5-fold by sucrose gradient sedimentation, its ability to bind DNA was decreased. The addition of the 4 S region to this isolated 8 S peak could not restore the initial binding to DNA.

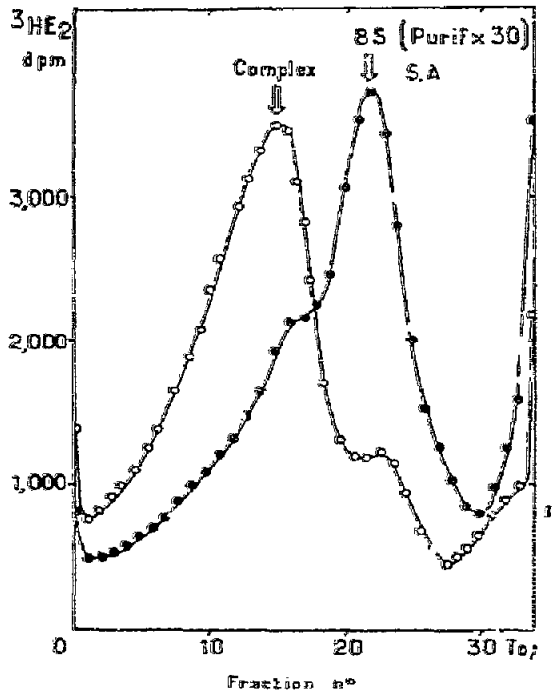

Fig. 1. Interaction between the partially purified estradiol receptor complex and DNA. Calf uterine cytosol containing [^{3}H]E_2 (10 mM) was purified by 20% ammonium sulfate precipitation according to Puca [10] and desalted through a Sephadex G-25 column. The exclusion volume was incubated in the presence (o—o—o) or absence (•—•—•) of DNA, for 2 hr at 0.2°, and subsequently centrifuged through a sucrose gradient.

These results suggest that the irreversible modification of the DNA binding site of the 8 S protein varied according to the purification procedure.

3.2 *Effect of trypsin on the DNA–RcE_2 interaction*

The 4 S binding protein obtained after trypsin treatment of the crude cytosol, was found to be inefficient in interacting with DNA, contrary to the 8 S cytosol receptor (fig. 2a). This effect of trypsin was actually irreversible and probably due to its catalytic activity, since diisopropyl fluorophosphate or soybean trypsin inhibitor prevented but did not reverse the formation of the 4 S trypsin binding protein. The number of estradiol binding sites and the apparent dissociation of the complex were not modified by the enzymatic treatment as tested by charcoal adsorption and sucrose gradient centrifugation, thus confirming the conclusion of Erdos [7].

Similar results were obtained with the partially (5-fold) purified 8 S receptor (fig. 2b) which had been treated by trypsin, and incubated with DNA. As when using the crude cytosol, [^{3}H]estradiol peaked exclusively in the 4 S region, whereas the 8 S control which had not been treated by trypsin, was still able to interact with DNA. This experiment indicates that the 4 S-E_2 complex is due to a transformation of the 8 S receptor and that the disappearance of the binding activity for DNA is not due to the purification procedure but to an actual effect of trypsin. In order to know whether the enzyme had altered the receptor itself or some other cytosol protein implicated in the DNA–Rc interaction, untreated cytosol containing the 4 S trypsin binding protein and a trypsin inhibitor. Following this treatment, the 4 S trypsin entity was unable to be reactivated in order to bind DNA and/or to give the 8 S receptor, suggesting that the lack of interaction with DNA is actually due to a modification of the cytosol receptor itself.

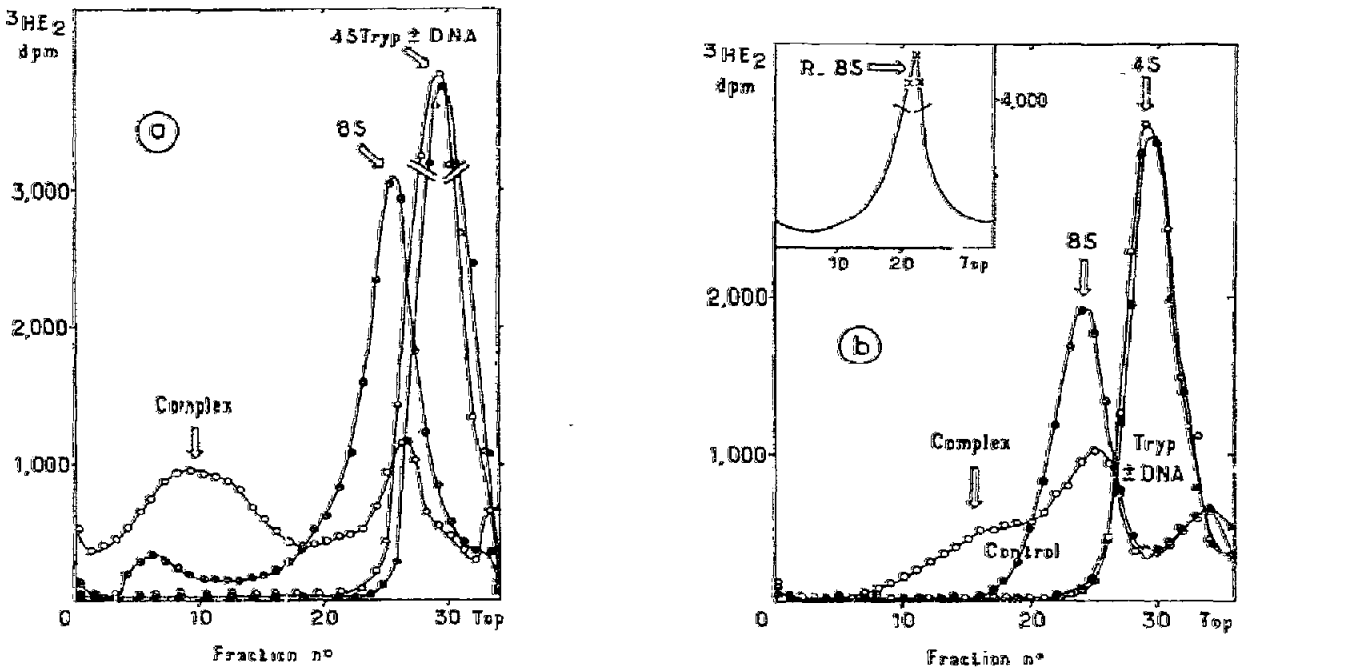

Fig. 2. Effect of trypsin on the Rc–DNA interaction. a) With the crude cytosol: Calf uterine cytosol previously incubated with [^{3}H]E$_2$ (1 nM) was treated by trypsin 30 min at 2° as indicated in Methods and agitated 2 hr with (△—△—△) or without (▲—▲—▲) DNA 180 μg/ml. The control cytosol was also incubated with (○—○—○) or without (●—●—●) the same amount of DNA. Each sample was centrifuged through a 5–20% sucrose gradient. b) With the 8 S region: Calf uterine cytosol containing [^{3}H]E$_2$ (2 nM) was first centrifuged through a sucrose gradient. The 8 S region was collected as indicated and dialysed under reduced pressure against 100 vol of Tris-EDTA buffer. One part was treated by trypsin 30 min at 2°, incubated at 2° with (△—△—△) or without (▲—▲—▲) DNA and recentrifuged through a 5–20% sucrose gradient. The cytosol control, incubated in the presence (○—○—○) or absence (●—●—●) of DNA was centrifuged under the same conditions.

3.3. *Effect of calcium on the DNA–RcE$_2$ interaction*

The crude cytosol which had been heated in the presence of calcium and [^{3}H]E$_2$ contained both an 8 S and 4 S E$_2$ binding peaks. When this cytosol was incubated with DNA, and then centrifuged through a sucrose gradient, two peaks of radioactivity, 12 S and 4 S, were found (fig. 3a). After addition of DNA, the 8 S radioactive peak was no longer visible, whereas the 4 S Ca^{2+} peak was almost unchanged. These results suggested that DNA was able to interact with the 8 S-E$_2$ complex but not with the 4 S Ca^{2+}-E$_2$ entity. However, the 4 S Ca^{2+} might have been the intermediate form under which the 8 S would interact with DNA. In order to eliminate this possibility the isolated 8 S and 4 S regions were incubated with DNA (fig. 3b) and then analysed by sucrose gradient centrifugation. The 4 S Ca^{2+} estradiol peak was not bound to DNA, contrary to the 8 S receptor. This result indicated that the molecular form of the receptor rather than the presence of calcium, was responsible for the loss of DNA binding activity. It had been previously verified that this 4 S Ca^{2+} peak was derived from the native Rc [9]. Additional control experiments have shown that the slow dissociation rate of the 4 S Ca^{2+}-E$_2$ complex as tested on dextran coated charcoal or dialysis, is compatible with the specificity of this binding protein. Moreover, the 4 S Ca^{2+} receptor, prepared according to Puca [10] was also unable to interact with DNA, in contrast to the 8 S receptor prepared in parallel in the presence of calcium confirming the previous observation of Toft [1].

4. Discussion

Trypsin or calcium treatment of the uterine cytosol are both known to transform the entire cytosol receptor (8 S) into smaller (≃ 4 S) E$_2$ binding units [7–9].

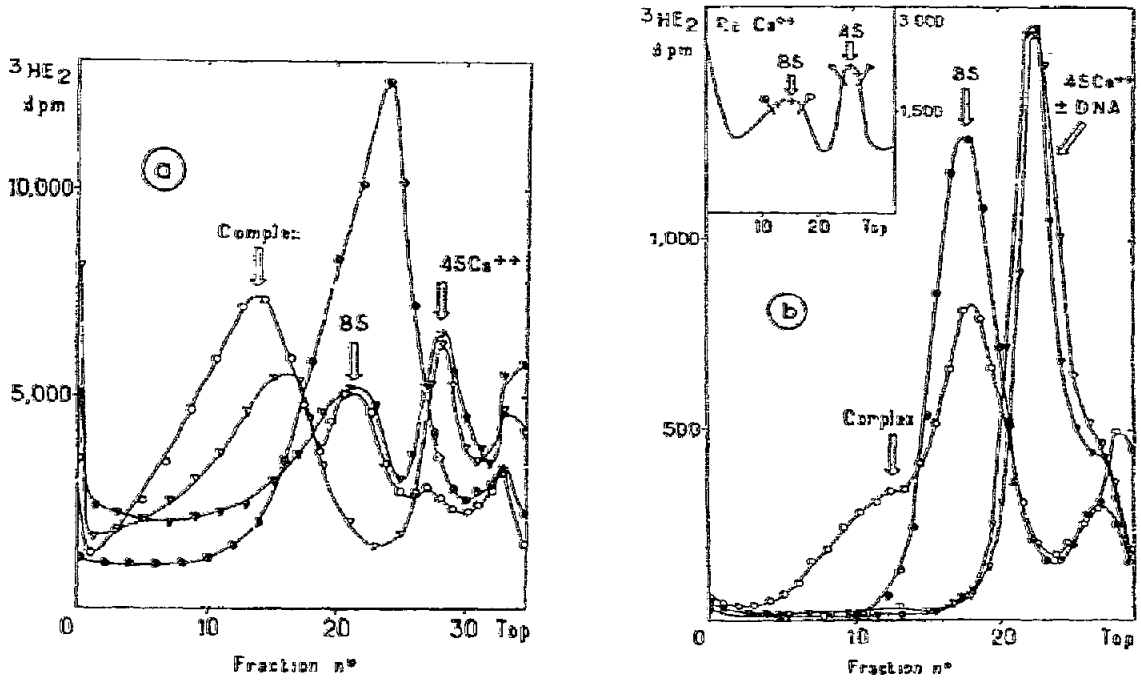

Fig. 3. Effect of calcium on the Rc–DNA interaction. a) With the crude cytosol: Calf uterine cytosol containing [^{3}H]E_2 (2 nM) was heated for 20 min at 25° with $CaCl_2$ (4 mM) then agitated with (▽—▽—▽) or without (▼—▼—▼) DNA, 180 μg/ml and finally centrifuged through a 5–20% sucrose gradient. The cytosol control was incubated for 20 min at 25° without $CaCl_2$ and incubated with (○—○—○) or without (●—●—●) DNA. b) With the isolated 8 S and 4 S Ca^{2+} regions: Calf uterine cytosol labelled with [^{3}H]E_2, 2 nM was heated for 20 min at 25° with $CaCl_2$ (4 mM) and then centrifuged through a sucrose gradient. The 4 S Ca^{2+} region was collected as indicated, dialysed under reduced pressure against 100 vol of Tris-EDTA and agitated with (▽—▽—▽) or without (▼—▼—▼) DNA, 180 μg/ml. The 8 S region was treated in parallel and incubated with (○—○—○) or without (●—●—●) DNA. All samples were analysed in a second 5–20% sucrose gradient.

These 4 S entities have lost the ability to reassociate into the initial molecular form of receptor, whereas the integrity of their estradiol binding sites was preserved. This paper reports another consequence of trypsin and calcium treatment of the receptor, which is to prevent its interaction with DNA. Since the crude cytosol was inefficient in reactivating the already formed 4 S trypsin protein for interacting with DNA, it is proposed that the lack of binding with DNA is the consequence of the partial proteolysis of Rc catalysed by trypsin. The reason for this inability to bind DNA could be due to some uncoupling between E_2 and DNA binding, through alteration of the receptor inter unit association site for instance, and/or, to a more or less direct modification of the receptor DNA acceptor site.

In any case, these observations indicate that E_2 and DNA binding sites have different locations on the receptor. Interaction of these 4 S entities with other possible nuclear "acceptor" components such as histones or non histone nuclear proteins [13] are not excluded and actually the 4 S Ca^{2+} protein has been shown to interact with crude uterine nuclei [14]. However, assuming that the interaction of the RcE_2 complex with DNA is of biological importance, we propose that, contrary to the entire 8 S receptor, these 4 S entities are not implicated in the triggering by estradiol of early metabolic response. They may either be interesting artifacts or as far as the 4 S Ca^{2+} protein is concerned some elimination form of the receptor, produced after the initiation of the hormonal effect.

Acknowledgements

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