Estradiol receptor has proteolytic activity that is responsible for its own transformation

(serine protease/aprotinin/steroid hormone action)

G. A. Puca, C. Abbondanza, V. Nigro, I. Armetta, N. Medici, and A. M. Molinari

Istituto di Patologia Generale ed Oncologia, III Cattedra, I Facolta di Medicina e Chirurgia, Sant Andrea delle Dame 2, 80138 Naples, Italy

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ABSTRACT We have investigated the effect of various protease inhibitors and substrates on the hormone- and temperature-dependent binding of partially purified estradiol-receptor complex to isolated nuclei. Only serine protease substrates and inhibitors significantly depressed estradiol receptor transformation. At 20°C, we observed 50% inhibition with about 3 μM aprotinin or with 1.4 mM disopropyl fluorophosphate. Aprotinin also blocked those size and charge modifications of receptor that are characteristic of the transformation process. The estradiol receptor was able to bind to aprotininagarose only under transforming conditions; i.e., the interaction was hormone- and temperature-dependent and inhibited by molybdate. Disopropyl fluorophosphate, a covalent reagent for serine esterases, competitively inhibited the binding and specifically eluted the estradiol-receptor complex that had been bound to aprotinin-agarose. These results indicate that estradiol receptor transformation is due to the effect of a serine protease and that the receptor itself is endowed with this catalytic activity, which is triggered by the steroid.

Transformation of steroid receptors has been widely studied in cell-free systems and has been defined as that process which confers affinity to the steroid-receptor complex for nuclei and DNA. In all systems so far investigated, transformation is accompanied by a decrease in the molecular size and the net electric charge of the receptor. Receptor transformation is an irreversible first-order reaction, inhibited by millimolar concentration of molybdate, and is triggered by the specific steroid (for review, see refs. 1–3). Evidence for the physiological role of this reaction (4–7) supports the theory that transformation is the critical step in steroid hormone action. Numerous mechanisms have been proposed to explain the molecular basis of this event, but none has been definitively accepted.

We report here that the receptor-transforming activity of exogenous alkaline phosphatase is due to a contaminating proteolytic activity of the enzyme preparation, that serine protease inhibitors and substrates impair transformation of the estradiol receptor, and that estradiol receptor acquires upon transformation a serine binding site for aprotinin. Therefore, we suggest that estradiol receptor transformation is due to the action of a serine protease activated by the hormone and that the catalytic protein is the hormone receptor itself.

MATERIALS AND METHODS

[2,4,6,7-3H]Estradiol-17β (84–111 Ci/mmoll; 1 Ci = 37 GBq) was from New England Nuclear. Nonradioactive estradiol-17β was from Calbiochem. Antipain dihydrochloride, soybean trypsin inhibitor (SBTI), pepstatin A, trypsin inhibitor type II-0 (ovomucoid) from chicken egg white, leupeptin hemisulfate, a1-antitrypsin, tryptophan methyl ester (Trp-OMe), phosphoramidone, phenylmethylsulfonil fluoride, aprotinin, calf intestinal alkaline phosphatase types I-S and VII-S, and p-nitrophenyl phosphate were from Sigma. N-benzoyl-l-arginine methyl ester (Bz-Arg-OMe), disopropyl fluorophosphate (iPrP-F), and N-benzoyl-l-tyrosinamide were from Serva (Heidelberg). N-benzoyl-l-arginine ethyl ester (Bz-Arg-OEt) was from Becton Dickinson. Sodium molybdate was from Baker. DEAE-cellulose (DE-52) was from Whatman. Superoxide 6B and Sepharose 4B-CL came from Pharmacia. Values of molecular weight (Mf) and Stokes radius of standard proteins were from the literature (8).

The following buffers were used: (A) 7.5 mM phosphate buffer, pH 7.4 at 20°C, containing 1 mM dithioerythritol, 2 mM EGTA, and additional 10 mM molybdate, as specified; (B) 50 mM phosphate buffer, pH 7.4 at 20°C, containing 1 mM dithioerythritol, 2 mM EGTA, 10 mM molybdate, and additional salts as specified; (C) 7.5 mM phosphate buffer, pH 7.4 at 20°C, containing 250 mM succrose, 25 mM KCl, 1 mM dithioerythritol, and 0.2 mM phenylmethylsulfonil fluoride.

Frozen (−70°C) calf uteri were used for cytosol preparation. Tissue was homogenized by an Ultraturrax homogenizer (Janke and Kunkel, TP 18/10) in 3 volumes of buffer B. The homogenate was centrifuged at 105,000 × g for 60 min, and the supernatant was collected. Estradiol-binding activity of cytosol and of partially purified receptor was measured by the dextran-coated charcoal (DCC) method (9). Nonspecific binding was determined by parallel incubation in the presence of a 300-fold excess of unlabeled estradiol.

Estradiol receptor was partially purified by DEAE-cellulose chromatography. Cytosol (200 ml), prepared in buffer B plus 50 mM KCl, was incubated with 30 g of DEAE-cellulose preequilibrated in the same buffer. After 60 min of agitation at 4°C, the slurry was packed in a column (internal diameter, 2 cm) and washed with 150 ml of the equilibration buffer, and bound material was eluted with a 100-ml linear gradient of 50–400 mM KCl in the same buffer. Fractions of 3 ml were collected. Specific estradiol-binding activity in aliquots of each fraction was measured, and the peak fractions of estradiol-binding activity were combined and stored at 4°C after dialysis in buffer A. Protein concentrations of such pools varied from 2 to 3 mg/ml, and estradiol-binding activity from 80,000 to 120,000 cpm/0.1 ml. Recovery of the receptor varied between 40% and 60% of the original binding activity of the cytosol. The continuous presence of 10 mM molybdate and 50 mM phosphate, together with the absence of estradiol, was necessary to decrease spontaneous modifications at 0°C.

Estradiol receptor partially purified by DEAE-cellulose chromatography, which in a sucrose gradient settled at 7.8 S, was eluted as a sharp peak (identical to the cytosolic receptor) from a calibrated Superose 6B column (see Results). From a calibration plot of Mf of standard proteins vs.

Abbreviations: SBTI, soybean trypsin inhibitor; iPrP-F, disopropyl fluorophosphate; DCC, dextran-coated charcoal.
elution volumes, the molecular weight of the 7.8S receptor was found to vary from 215,000 to 250,000 (Fig. 4 Inset), very similar to the value that can be calculated from the Stokes radius (66.83 ± 1.83 Å; n = 6) and sedimentation coefficient (8). This partially purified estradiol receptor is called 8S receptor in the following text. Incubation of 8S receptor at 20°C in buffer A in the presence of estradiol, but not in its absence, induced a decrease in the high Mr complex and the appearance of smaller species, with the predominance of a molecule of apparent Mr 75,000 (Stokes radius = 39.8 ± 0.84 Å; n = 4). A shoulder between the two peaks, corresponding to an apparent Mr of 120,000 (Stokes radius = 49 ± 2 Å; n = 4) was consistently present. When the nuclei were incubated at 0°C with the preheated estradiol-receptor complex and then centrifuged, the amount of Mr 240,000 component in the supernatant remained unchanged, while the Mr 120,000 and smaller components decreased and could be extracted quantitatively from nuclei with buffer B containing 400 mM KCl. Incubation of 8S receptor with nuclei and hormone in the buffer used for the nuclear extraction did not modify the elution of the estradiol–receptor complex. Analogously, we have reported (10) the estradiol-dependent appearance at 20°C of modified hormone–receptor complexes that were eluted from DEAE-cellulose at lower salt concentration and that were the only forms able to bind to nuclei at 0°C. These more basic forms were also extracted from the uterine nuclei of rats injected with physiological doses of estradiol (11).

Isolated nuclei were prepared (12) from the crude nuclear pellet of calf uterus in buffer C. Concentration of DNA was determined (13) and adjusted to 1.2 mg/ml with the buffer. Nuclei, which were stored in 2- to 3-ml aliquots at −70°C, were thawed to 4°C at the beginning of the experiment. Binding of the [3H]estradiol-8S receptor complex to nuclei (0.1 ml, 120 μg of DNA) was assayed in duplicate. The binding of the complex was time-, temperature-, and estradiol-dependent and was blocked by 10 mM molybdate. Nonspecific binding, obtained by incubating unheated [3H]-estradiol–8S receptor complex with nuclei, was subtracted from values obtained. At the end of the incubation, the nuclei were pelleted in a swinging bucket rotor at 3000 × g for 10 min and washed three times with 4 ml of buffer C. The pellet was resuspended in 1 ml of the same buffer for measurement of radioactivity by liquid scintillation counting.

Aprotinin, SBTI, ovomucoid, and protamine were coupled to CNBr-activated Sepharose CL-4B in 25 mM phosphate buffer (pH 7.6) according to Cuatrecasas (14). Under our conditions, 330 μg of aprotinin and 3 mg of SBTI, ovomucoid, or protamine were covalently bound per g of agarose.

RESULTS

Effect of Exogenous Alkaline Phosphatase on Transformation of Estradiol Receptor Is Not Due to Phosphatase Activity. Analogous to what was described for the glucocorticoid receptor system (15, 16), commercial calf intestinal alkaline phosphatase (type I-S) added to uterine cytosol or to partially purified 8S estradiol receptor induces changes of the binding molecule similar to those observed when cytosol or partially purified 8S receptor are incubated at 20°C in the presence of estradiol; i.e., it decreases the sedimentation of the 8H-containing peak from 8 to 4 S and the KCl concentration required to elute receptor from DEAE-cellulose from 200 mM to 50 mM. The effect of this enzyme occurs at 0°C, but not at higher temperature, and does not require estradiol (data not shown). We assayed in vitro nuclear binding of 8S receptor at 0°C in the presence of various concentrations of Sigma type I-S alkaline phosphatase (Fig. 1A). The greatest nuclear binding corresponded to 60% of the binding obtained by heating the 8S receptor in the presence of estradiol for 30 min at 20°C (17,393 cpm). This suggested that the transforming activity of exogenous alkaline phosphatase might not be due to the phosphatase activity of the enzyme. This conjecture became certainty when we used the Sigma type VII-S enzyme, which has a much higher specific activity than type I-S. Even though the specific activity of the type VII-S enzyme toward the synthetic substrate p-nitrophenyl phosphate was 163-fold greater, we had to use 20-fold more enzyme to obtain substantial transformation. A very high, nonphysiological concentration of exogenous calf intestinal alkaline phosphatase was required to enhance transformation of the glucocorticoid receptor (15). Superose 6B chromatography of the low-specific-activity, type I-S enzyme (Fig. 1B) showed that the transforming activity was separable utilizing the phosphatase activity. No RNase, DNase, phosphoprotein phosphatase, or phosphodiesterase activity corresponded to the transforming-activity peak. However, caseinolytic activity was coincident with transforming activity (data not shown). The transforming activity of calf intestinal alkaline phosphatase was stimulated by SBTI (100 μg/ml), partially inhibited by 5 mM Trp-OHMe, and unaffected by leupeptin,

![Figure 1](https://example.com/fig1.png)
pepstatin, or aprotinin (all at 100 μg/ml). This suggested that a chymotrypsin-like enzyme destroyed by a trypsin-like enzyme could be responsible for transformation. The coincidence of caseinolytic activity and the transforming activity of exogenous alkaline phosphatase was interesting, since the product of the exogenous phosphatase was structurally (for size and charge) and functionally (being able to bind to nuclei) similar to the product of the endogenous transforming system. We considered, therefore, that a specific protease might be involved in the hormone- and temperature-dependent transformation of the estriol receptor.

Serine Proteinase Inhibitors and Substrates Inhibit Transformation of Estradiol Receptor, and Aprotinin Blocks Modifications Correlated with Transformation. None of the biologically active peptides we tried (antipain, pepstatin A, phosphoramidone, and leupeptin) had any effect either on the transformation of the 8S estriol receptor, or on the binding of estriol to the receptor, or on the nuclear binding of the transformed estriol–receptor complex. Among the protease substrates, Trp-O-Me, but not N-benzoyl-L-tyrosinamide, effectively reduced the nuclear binding of the estriol–receptor complex if it was present during the 20°C preincubation; it did not work if added after the preincubation (Fig. 2A). Trp-O-Me also affected the estriol binding capacity of the receptor, but less than it did nuclear binding (Fig. 2B). Bz-Arg-O-Me and Bz-Arg-OEt behaved similarly, although they were less effective. SBTI (60–600 μg/ml), ovomucoid (60 μg/ml), and α1-antitrypsin (60 μg/ml) were without effect. When present during the 20°C preincubation, aprotinin (50 μg/ml) almost completely inhibited the nuclear binding of the estriol–receptor complex. The inhibition was concentration-dependent and did not occur when aprotinin was added after the preincubation (Fig. 3). Fifty percent inhibition of transformation at 20°C occurred at about 3 μM aprotinin (Mₚ = 6500). Temperature and salt concentration had a striking effect on inhibition by aprotinin. Aprotinin was not effective under conditions where the transformation reaction was faster (i.e., at higher temperature or higher salt molarity). iPr₂P-F also inhibited transformation when present during the 20°C preincubation, but not when added after, giving 50% inhibition at 1.4 mM. Aprotinin blocked the hormone- and temperature-induced size changes (Fig. 4, closed circles) when incubation was carried out in the absence of salt, but not when transformation was stimulated by the presence of 0.15 M KCl (Fig. 4, open circles). At 3 mM, iPr₂P-F impeded the conversion of the Mₚ 240,000 native receptor into the lower Mₚ forms (data not shown). DEAE-cellulose chromatography showed that in the presence of aprotinin or iPr₂P-F, the temperature- and estriol-dependent appearance of more basic estriol–receptor complexes did not occur (data not shown).

Under Transforming Conditions, Estradiol Receptor Acquires a Serine Binding Site for Aprotinin. The estriol–receptor complex was able to bind to aprotinin-agarose at 20°C but not at 0°C (Fig. 5A), unless it was preincubated at 20°C. The binding did not occur in the presence of 10 mM molybdate (Fig. 5A) but was accelerated by the presence of estradiol (Fig. 5B). Addition of molybdate after the 20°C preincubation had no effect (data not shown). Thus, the receptor recognizes aprotinin only after transformation. No binding of estradiol receptor to SBTI-agarose or to ovomucoid-agarose was detected, even though aprotinin substitution was 10-fold greater. Although protamine-agarose did bind estradiol receptor, this interaction was neither dependent on estradiol and temperature nor inhibited by 10 mM molybdate. iPr₂P-F inhibited the interaction between the estradiol receptor and aprotinin-agarose in a concentration-dependent (Fig. 6A) and competitive manner (Fig. 6B). Further, 10 mM iPr₂P-F was able to elute specifically the transformed estriol–receptor complex that had been bound to aprotinin-agarose (Fig. 6 Inset). This indicates that the binding of transformed estriol–receptor complex to aprotinin-agarose occurs through a site containing an active serine residue.
**FIG. 4.** Superose 6B chromatography of [3H]estradiol–8S receptor complex incubated at 20°C in the presence of aprotinin plus or minus KCl. Aliquots of 8S receptor (binding capacity 977,760 cpm) were incubated at 20°C with 5 nM [3H]estradiol in the presence of aprotinin (50 μg/ml) with (●) or without (○) 150 mM KCl in a final volume of 3 ml in buffer A. After 30 min, the samples were cooled to 0°C and incubation continued for an additional 30 min. Free hormone was eliminated by the DCC method (9). Samples were applied consecutively to a calibrated column (17.5 × 2.6 cm) of Superose 6B equilibrated in buffer B containing 200 mM KCl and were eluted with the same buffer. Fractions (1.45 ml) were collected and analyzed for radioactivity. (Inset) M, vs. elution volumes: 1. human gamma globulin; 2. bovine plasma albumin; 3. ovalbumin; 4. half-mere hemoglobin; 5. myoglobin. Stippled areas represent untransformed receptor (A), intermediate shoulder (B), and transformed receptor (C) (see Materials and Methods). Void volume of the column was 24.9 ml; internal volume was 87.6 ml. Upward flow rate of 33 ml/hr was obtained with a peristaltic pump.

**DISCUSSION**

The molecular mechanism of transformation of the steroid hormone receptors that confers to the receptor the capacity to bind to the nuclei is not understood. Several reports (17–19) have suggested that a dephosphorylation reaction is involved, since some inhibitors of phosphatase block transformation in cell-free systems. Steroid receptors have been shown to be phosphorylated proteins (20–22), and exogenous calf intestinal phosphatase was able to induce transformation (15, 16). Our results are not consistent with a dephosphorylation hypothesis for receptor transformation. The receptor transformation induced by the exogenous phosphatases appears to be due to a contaminating proteolytic enzyme rather than due to a dephosphorylation reaction.

It has been reported (23) that a serine protease(s) might be involved in the mechanism of activation of glucocorticoid-receptor complex into a nuclear form. The great sensitivity of steroid receptors to proteolytic attack is well-known (24). Even though limited proteolysis of receptor, due to endogenous or exogenous enzymes, may lead to functionally active forms capable of binding to nuclei or DNA (25–27), receptor proteolysis has been generally implicated in the degradation rather than in the transformation of receptor. One of the conceptual obstacles to accepting proteolysis as the activating step was the fact that no proteolytic enzyme so far studied (28–30) was able to distinguish between the occupied and the unoccupied receptor. Further, the role of nonreceptor molecules in the transformation process has been excluded repeatedly in recent publications. It has been reported that transformation can occur in highly purified receptor preparations (31, 32) and that the molecular weight of the steroid binding receptor subunit does not change upon transformation (31) or nuclear translocation (33, 34). The irreversibility of the transformation reaction may be explained by chemical changes that would render reassociation impossible.

Our results on the interaction of the transformed estradiol receptor with aprotinin and the effect of iPrP-F on this interaction indicate that a serine protease activity responsible for the transformation may be in the native receptor molecule itself. It is devoid of catalytic activity in the absence of the hormone but becomes activated as a consequence of the conformational change brought about by the hormone. The dissociation of the oligomeric native receptor would be a consequence and not the mechanism of this process, as it has been postulated (35). The initial, very specific cleavage of one or a few peptide bonds may also occur in a nonsteroid-binding component of the oligomeric native receptor (36) and thus render the process irreversible, without changing the molecular weight of the estradiol-binding subunit.

If the hormone is the allosteric effector of the serine protease responsible for the transformation process, it is necessary that the hormone binding site and the catalytic site be near each other (37). The effect of serine protease substrates and inhibitors on the hormone binding of all steroid receptors (38–41) and the analogy of the transforming...
process, both in terms of conditions (hormone requirement, effect of ionic strength, nucleotides, dilution, aging, molybdate, chelators, and sulfhydryl reagents) and in terms of molecular changes (alter mobility on DEAE-cellulose and decrease in size) (1–3), suggest a common mechanism for steroid receptor transformation. The fact that, until now, proteolysis has been proposed as a mechanism only for the glucocorticoid receptor (23) can be explained because the putative intrinsic proteolytic activity of the receptor may be highly specific and resistant to the common protease inhibitors. Aprotinin, which in our case is the best inhibitor, has no effect when used in crude cytosol or when transformation in partially purified 8S receptor preparations is accelerated by temperature and ionic strength.

Whether the proteolytic activity of the estrogen receptor is required only for the transformation process or whether it also participates in the modification of other functions and structures at the various cellular levels where the receptor acts remains to be established.

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