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Estradiol receptor: phosphorylation on tyrosine in uterus and interaction with anti-phosphotyrosine antibody

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Estradiol receptor from rat uteri incubated with [^{32}P]orthophosphate has been purified by diethylstilbestrol–Sepharose followed by heparin–Sepharose chromatography. The purified receptor, analyzed by centrifugation through sucrose gradients after incubation with monoclonal antibodies against purified estradiol receptor, appears to be labeled with ^{32}P. The receptor preparation has been further purified by immunoaffinity chromatography and submitted to SDS–polyacrylamide gel electrophoresis. A heavily ^{32}P-labeled 68 kd protein and a very lightly ^{32}P-labeled 48 kd protein, probably a proteolytic product of the 68 kd protein, were detected. Phosphoamino acid analysis of the receptor eluted from the immunoaffinity column shows that its ^{32}P-labeling occurs exclusively on tyrosine. This is the first report on phosphorylation on tyrosine of a steroid receptor in tissue. It is consistent with our previous finding that a uterus estradiol receptor-kinase, which confers hormone binding ability to the estradiol receptor, *in vitro* phosphorylates this receptor exclusively on tyrosine. Calf uterus receptor binds with high specificity and affinity to monoclonal anti-phosphotyrosine antibodies covalently bound to Sepharose (K_d = 0.28 nM). Dephosphorylation of the receptor by nuclei containing the calf uterus nuclear phosphatase abolishes the interaction with antibodies. These results suggest that also in calf uterus, estradiol receptor is phosphorylated on tyrosine. Anti-phosphotyrosine antibodies bound to Sepharose have been used to partially purify the estradiol receptor from calf uterus.

Key words: hormone binding/receptor-kinase/receptor-phosphatase/anti-receptor antibody

Introduction

Reversible covalent modifications regulate the activities of several enzymic and non-enzymic proteins. Protein phosphorylation-dephosphorylation is a major regulatory process of cellular activity that is controlled both by protein kinases and by phosphoprotein phosphatase (Cohen, 1982).

An important advance in the field of receptor biology resulted from the observation that several peptide hormone receptors undergo phosphorylation on tyrosine and catalyze tyrosine phosphorylation (Czech, 1985). Several retroviral transforming proteins are endowed with the same properties (Bishop, 1985). These findings suggest that phosphorylation on tyrosine is an initial event of hormone-induced cell multiplication and retroviral cell transformation.

In vitro phosphorylation on tyrosine of the purified estradiol receptor by an endogenous tyrosine kinase confers hormone-binding ability to this receptor (Migliaccio *et al.*, 1984). Evidence is now presented that this receptor is labeled with ^{32}P on tyrosine in whole rat uterus incubated in the presence of [^{32}P]orthophosphate.

In addition we report that calf uterus estradiol receptor binds with high affinity and specificity to anti-phosphotyrosine antibodies and that this interaction requires receptor phosphotyrosine. These findings with anti-phosphotyrosine antibodies support the conclusion, drawn from the experiments with whole rat uterus, that *in vivo* the estradiol receptor is phosphorylated on tyrosine.

Results

Purification and analysis of the estradiol receptor from rat uteri incubated with [^{32}P]orthophosphate

Rat uteri were incubated with [^{32}P]orthophosphate for 1 h at 39°C in Hepes buffer. This buffer contained, besides other compounds, Na_2MoO_4 and Na_3VO_4 which inhibit the calf uterus nuclear estradiol receptor phosphatase (Auricchio *et al.*, 1981a; A.Rotondi and F.Auricchio, unpublished data). The labeled uteri were mixed with carrier uteri and homogenized to prepare high speed supernatant. This supernatant was cycled several times through a diethylstilbestrol (DES)–Sepharose column. Recycling improves yield and purification of estrogen receptor. The receptor was eluted from the affinity resin with 1 μM [^{3}H]estradiol.

Pre-binding of 20 nM estradiol to cytosol receptor prevented receptor adsorption to DES–Sepharose. This shows that the receptor binds specifically to the affinity resin.

The receptor from DES–Sepharose was cycled through hep-

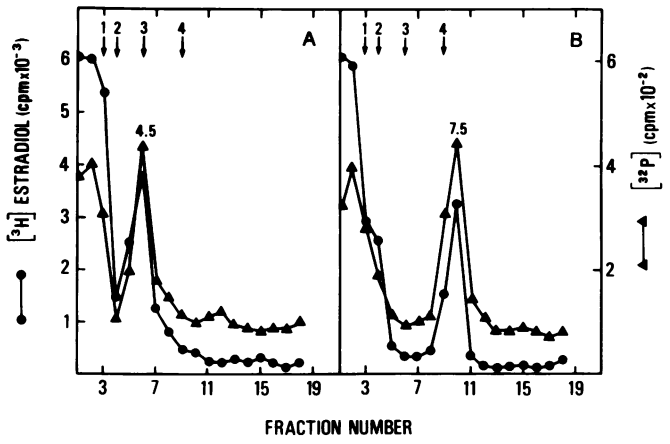

Fig. 1. Sucrose gradient centrifugation of receptor–antibody complex. Two aliquots of 50 μl each of estradiol receptor purified by DES–Sepharose and heparin–Sepharose from rat uteri incubated with [^{32}P]orthophosphate were exhaustively dialyzed against TD buffer supplemented with 0.4 M KCl after separate overnight incubation in the presence of 0.4 M KCl with control immunoglobulins **(panel A)** or with antibodies against the receptor (JS 34/32) **(panel B)** at a molar ratio of receptor:antibody of 1:150 (final volume 250 μl). Samples were subjected to 10–30% sucrose gradients in 0.4 M KCl. Arrows indicate the reference proteins: 1, cytochrome C, 1.9S; 2, ovalbumin, 3.2S; 3, BSA, 4.5S; 4, IgG, 7S.

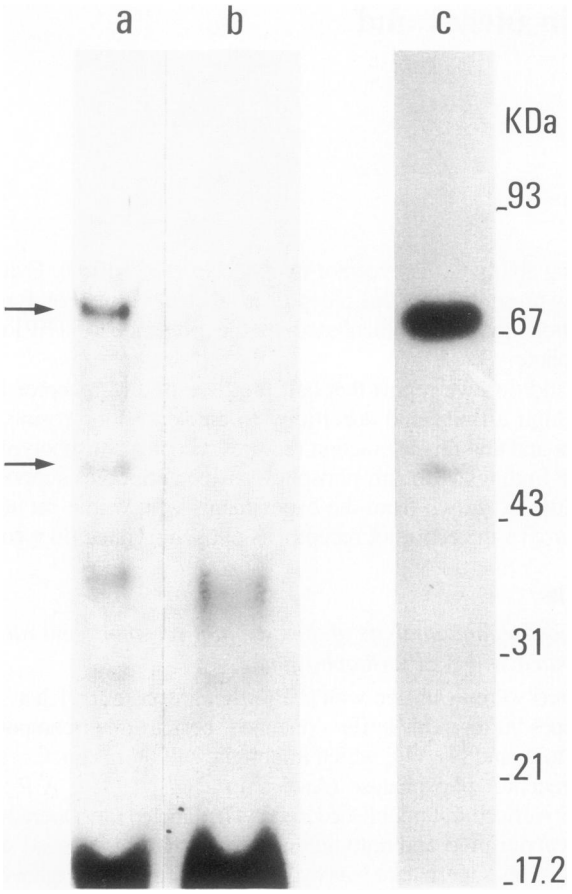

Fig. 2. SDS–polyacrylamide gel electrophoresis of the estradiol receptor purified from rat uteri incubated with [^{32}P]orthophosphate. Receptor was purified by DES–Sepharose, heparin–Sepharose and immunoaffinity chromatography. Receptor preparation (0.6 ml) was added with 10% TCA using myoglobin as a carrier. The pellet was washed twice with ether:ethanol (1:1) and dissolved with 60 μl of SDS–PAGE sample buffer (0.05 M phosphate buffer pH 7.2, containing 1% SDS, 4% 2-mercaptoethanol, 30% glycerol, 0.02% bromophenol blue) then heated at 100°C for 3 min. A 50-μl aliquot was submitted to SDS–PAGE. After the run, the gel was stained with silver stain (Bio-Rad), dried and exposed to autoradiography. **Lane A:** silver staining of the receptor sample added with myoglobin; **lane B:** silver staining of myoglobin control sample; **lane C:** autoradiography of **lane A**. The arrows show the two protein bands of the receptors sample.

arin–Sepharose and finally eluted from this resin by a buffer containing heparin.

Two samples of the estradiol receptor eluted from heparin–Sepharose were equilibrated with 12 nM [^{3}H]estradiol. These samples were incubated with an excess of immunoglobulin purified either from a control hybridoma derived from the fusion of myeloma cells with spleen cells from non-immunized mice or from the JS 34/32 clone produced by fusing myeloma cells with spleen cells from mice immunized with purified estrogen receptor (Moncharmont *et al.*, 1982). They were analyzed by centrifugation on sucrose gradients. The results of this experiment are presented in Figure 1. The [^{3}H]estradiol peak bound to the receptor incubated wtih control immunoglobulins co-sediments at 4.5S with a peak of ^{32}P. Pre-incubatin of the receptor preparation with JS 34/32 antibodies against purified receptor causes both peaks to shift to 7.5S. Since this shift is due to the formation of an antiody–receptor complex in a 1:1 molar ratio (Moncharmont *et al.*, 1982) it is clear that the ^{32}P peak shifted to 7.5S by the antibodies belongs to the receptor.

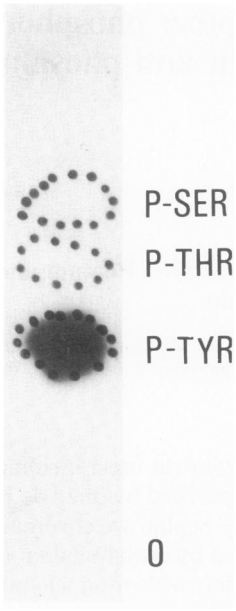

Fig. 3. Phosphoamino acid analysis of the estradiol receptor purified from rat uteri incubated with [^{32}P]orthophosphate. Receptor was purified by DES–Sepharose, heparin–Sepharose and immunoaffinity chromatography. Receptor preparation (0.3 ml) was subjected to acid hydrolysis by incubation under vacuum of 6 N HCl at 110°C for 3 h. The sample was diluted with water to 4 ml, then lyophilized and solubilized with 30 μl of water containing 30 μg of phosphoserine (P-SER), phosphothreonine (P-THR) and phosphotyrosine (P-TYR). 20 μl of this sample were subjected to electrophoresis at pH 3.5. The plate was stained with ninhydrin and then exposed to autoradiography. The dotted lines represent the standard superimposed on autoradiography.

Since the experiment in Figure 1 shows that the ^{32}P-labeled estradiol receptor specifically interacts with JS 34/32 antibody, this antibody was coupled to Sepharose (Cuatrecasas, 1970) and used to further purify the receptor eluted from heparin–Sepharose. The receptor was eluted from the antibody–Sepharose column at alkaline pH and neutralized immediately after the elution (G.A.Puca, A.M.Molinari, N.Medici and C.Abbondanza, in preparation). A fraction of the sample was concentrated by acid precipitation using myoglobin as a carrier. The pellet was dissolved and submitted to SDS–polyacrylamide gel electrophoresis (Figure 2). Silver nitrate stained several protein bands, but only two of them (those indicated by arrows in Figure 2) belonged to the receptor preparation since they were not detectable in the control sample of myoglobin. The mol. wts of these two proteins were 68 and 48 kd respectively. Autoradiography showed a more phosphorylated band coincident with the 68 kd protein and a barely phosphorylated band coincident with the lighter protein.

A sample of the immunopurified receptor was subjected to acid hydrolysis, concentrated by lyophilization, solubilized in a small volume of water and analyzed by one-dimensional electrophoresis at pH 3.5. The electrophoresis plate was exposed for autoradiography. The only phosphorylated amino acid detectable was phosphotyrosine (Figure 3).

High affinity interaction of calf uterus estradiol receptor with anti-phosphotyrosine antibodies coupled to Sepharose

This experiment, as well as the following experiments performed with anti-phosphotyrosine antibodies, suggests that the calf uterus estradiol receptor is also phosphorylated on tyrosine.

Three 0.5-ml samples, each containing 2850 fmol of the [^{3}H]-estradiol–receptor complex extensively purified by heparin–

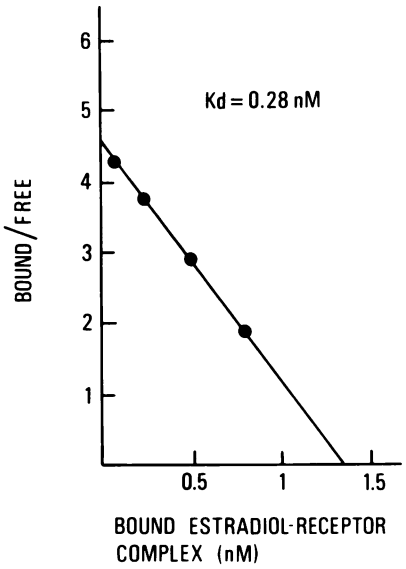

Fig. 4. Measurement of the affinity of the estradiol–receptor complex for anti-phosphotyrosine antibodies coupled to Sepharose. A sample of 1.5 ml calf uterus cytosol [^{3}H]estradiol–receptor complex preparation extensively purified was incubated with 0.1 volumes of packed BSA–Sepharose beads for 2 h at 0°C to remove molecules non-specifically bound to protein–Sepharose. The suspension was centrifuged and different aliquots of the supernatants containing from 25 to 500 fmol [^{3}H]estradiol-specific binding sites were diluted with TGD buffer to 0.35 ml. Each aliquot was incubated under gentle shaking overnight at 0°C with 35 μl of packed anti-P-Tyr–Sepharose beads, then centrifuged. The specific hormone-binding sites found in the supernatant after charcoal treatment were used as a measure of free [^{3}H]hormone–receptor complex. Bound [^{3}H]hormone–receptor complex was calculated from the difference between specific binding sites present in samples before and after incubation with anti-P-Tyr–Sepharose. B/F, bound/free hormone–receptor complex.

Sepharose and chromatography on 17β-estradiol-17-hemisuccinyl-hexane-agarose (Puca *et al.*, 1980) were incubated in TGD buffer and gently shaken at 0°C for 2 h with 0.1 volumes of packed Sepharose 4B beads to which BSA (BSA–Sepharose), bovine immunoglobulins (IgG–Sepharose) or anti-phosphotyrosine antibodies (anti-P-Tyr–Sepharose) had been linked. The suspensions were incubated at 0°C for 2 h, then centrifuged at 4000 r.p.m. for 10 min at 4°C. The amount of estradiol–receptor complex bound to Sepharose was calculated from the difference between the receptor present in the samples before and after incubation with Sepharose. Only 5% of the receptor was bound to BSA–Sepharose and IgG–Sepharose, whereas 80% of the receptor was bound to anti-P-Tyr–Sepharose.

The affinity of the calf uterus [^{3}H]estradiol–receptor complex preparation for anti-phosphotyrosine antibodies coupled to Sepharose was measured. Increasing amounts of the hormone–receptor complex were added to a fixed amount of anti-P-Tyr–Sepharose and the preparation was gently shaken at 0°C overnight. Suspensions were centrifuged and the hormone–receptor complex that remained in the supernatants was assayed. The data were plotted according to Scatchard and are shown in Figure 4. The K_d value was 0.28 nM. The high affinity of the estradiol receptor for the anti-phosphotyrosine antibody was exploited to purify the receptor.

Microbatch immunopurification of calf uterus estradiol receptor

A 3-ml sample of crude cytosol [^{3}H]estradiol–receptor complex preparation was incubated with 0.25 ml of packed anti-P-Tyr–Sepharose beads at 0°C overnight and then centrifuged. The

Table I. Purification of calf uterus estradiol receptor with anti-P-tyrosine antibody

Step	Volume (ml)	Protein (mg)	Specific [^{3}H]17β-estradiol binding sites (fmol)	Purification factor	Yield (%)
Cytosol	3	30.9	5484	1	100
Anti-P-Tyr–Sepharose	0.3	0.017	1400	464	26

Table II. Lack of interaction of dephosphorylated estradiol receptor with anti-phosphotyrosine antibodies coupled to Sepharose

	Phosphorylated receptor		Dephosphorylated receptor	
	fmol	%	fmol	%
Cytosol after incubation with nuclei	2794	100	1164	100
Anti-P-Tyr–Sepharose supernatant	147	5	1081	93

Calf uterus cytosol (2 ml) [^{3}H]estradiol–receptor complex preparation containing 3958 fmol of the complex were incubated at 25°C for 20 min with homologous nuclei in TGD buffer. During the incubation, nuclear receptor phosphatase inactivated (and dephosphorylated) 1164 fmol of the [^{3}H]estradiol-specific binding sites of the receptor. This cytosol, containing phosphorylated hormone-binding receptor (2794 fmol) and dephosphorylated receptor was incubated overnight at 0°C with 0.2 ml of anti-P-Tyr–Sepharose under gentle shaking. The suspension was centrifuged and the supernatant assayed for estradiol-specific binding sites under standard conditions (phosphorylated receptor) and after incubation at 15°C in the absence and presence of ATP (to assay the dephosphorylated receptor).

Sepharose pellet was washed twice with 2 volumes of 0.2 M KCl in TGD buffer to remove molecules non-specifically bound to anti-P-Tyr–Sepharose. Finally, the receptor was eluted from the Sepharose pellet with 0.2 ml of TGD buffer containing 0.5 M NaSCN and 40 mM phenyl phosphate hapten (hapten buffer). The results of this procedure are shown in Table I. The estradiol receptor was significantly purified (at least 464-fold over cytosol) by the anti-P-Tyr–Sepharose. We have used this step in combination with heparin–Sepharose and obtained highly purified receptor (A.Migliaccio, A.Rotondi and F.Auricchio, in preparation).

Dephosphorylated receptor does not interact with anti-phosphotyrosine antibodies coupled to Sepharose

Incubation of cytosol estradiol receptor with nuclei or purified nuclear receptor-phosphatase inactivates a portion (30–60%) of its hormone-binding sites (Auricchio *et al.*, 1981a; Migliaccio *et al.*, 1982). Dephosphorylation is responsible for this inactivation. In fact, incubation with ATP in the presence of the endogenous receptor-kinase restores the lost hormone-binding sites (Auricchio *et al.*, 1981b; Migliaccio *et al.*, 1982). *In vitro* experiments have shown that the nuclear phosphatase removes ^{32}P incorporated into the receptor that has been phosphorylated in the presence of [γ-^{32}P]ATP by the Ca^{2+}-calmodulin stimulated receptor-kinase (Auricchio *et al.*, 1984). Since this kinase phosphorylates the receptor exclusively on tyrosine (Migliaccio *et al.*, 1984) it is clear that the nuclear phosphatase dephosphorylates the phosphotyrosine of the receptor.

To verify if receptor-phosphotyrosine rather than other receptor phosphoamino acids is responsible for the high affinity interaction of the receptor with anti-phosphotyrosine antibody we have

studied the interaction of the receptor dephosphorylated by the nuclear phosphatase.

Calf uterus cytosol was incubated with homologous nuclei to yield a mixture of phosphorylated, hormone-binding and dephosphorylated, non-hormone binding receptor. As expected from the results presented above, after incubation of such a mixture with anti-P-Tyr−Sepharose, the hormone-binding (phosphorylated) receptor bound to anti-P-Tyr−Sepharose. In contrast, the inactive (dephosphorylated) receptor did not bind and appeared in the resin supernatant (Table II). That the dephosphorylation is responsible for the inactivation of the receptor present in the supernatant is demonstrated by the reactivation of the inactivated receptor by incubation of the anti-P-Tyr−Sepharose supernatant with ATP.

These results strongly suggest that dephosphorylation on phosphotyrosine of the receptor prevents the inactivated estradiol receptor from interacting with anti-P-Tyr−Sepharose.

Discussion

It has been shown recently that the hormone binding of purified estradiol receptor is inactivated by a nuclear phosphatase (Auricchio *et al.*, 1981a) that removes phosphate from the receptor (Auricchio *et al.*, 1984), and that hormone binding is reactivated by a member of a new class of protein kinases, namely a calmodulin-stimulated kinase that phosphorylates the receptor exclusively on tyrosine (Migliaccio *et al.*, 1984). These enzymes possess properties that support our hypothesis that they regulate hormone binding to the receptor *in vivo*: the extraordinary affinity of both enzymes for the estradiol receptor ($K_m \sim 1$ or < 1 nM) (Auricchio *et al.*, 1981a,b); the presence of the nuclear phosphatase only in estradiol target tissues (Auricchio and Migliaccio, 1980); and stimulation of the kinase by physiological concentrations of estradiol (F.Auricchio and A.Migliaccio, in preparation). The fact that the kinase confers hormone-binding ability to the estradiol receptor through phosphorylation on tyrosine (Migliaccio *et al.*, 1984) is an additional point of interest since phosphorylation of proteins on tyrosine is a rare event that is implicated in hormone-induced cell growth and cell transformation.

The findings presented in this paper show that ^{32}P-labeled estradiol receptor can be purified from whole rat uterus incubated in the presence of [^{32}P]orthophosphate.

The experiment presented in Figure 1 proves that the receptor purified by DES−Sepharose and heparin−Sepharose from rat uterus is ^{32}P-labeled since it shows that the ^{32}P peak coincident with the receptor at 4.5S is shifted to 7.5S together with the receptor by antibodies against the receptor. The same thing occurs with purified calf uterus estradiol receptor phosphorylated on tyrosine by incubation with [γ-^{32}P]ATP and calmodulin-stimulated kinase (Migliaccio *et al.*, 1982). As in that case, we now conclude that the estradiol receptor is a phosphoprotein.

Immunoaffinity was used to further purify the ^{32}P-labeled receptor. For this step the JS 34/32 antibodies were used because the sucrose gradient centrifugation showed that these antibodies specifically reacted with the ^{32}P-labeled receptor. To our knowledge this is the first time that immunoaffinity chromatography has been used in addition to the affinity steps usually used to obtain highly purified estradiol receptor. This combination should produce the highest possible purification of the receptor, although it drastically decreases the final yield of the protein (see Materials and methods).

On the basis of SDS−polyacrylamide gel electrophoresis the final receptor preparation contained two ^{32}P-phosphorylated proteins with mol. wts of 68 and 48 kd. It is reasonable to assume that the barely ^{32}P-phosphorylated 48 kd protein is a proteolytic product of the 68 kd receptor. The estradiol receptor with a mol. wt between 45 and 50 kd has been reported only in the presence of the heavier estradiol receptor, which suggests that it corresponds to an *in vitro* degradation product of the larger protein (Van Osbree *et al.*, 1984; Katzenellenbogen *et al.*, 1983; Walter *et al.*, 1985).

The present phosphoamino acid analysis of the extensively purified receptor shows that phosphorylation of estradiol receptor on tyrosine occurs also in whole uterus. Unfortunately the present data do not provide direct information on the role of this phosphorylation in the uterus. Nevertheless, the consistency of this finding with the results of our previous experiments performed on purified receptor with purified endogenous enzymes (Migliaccio *et al.*, 1982, 1984), suggests that in whole uterus this phosphorylation also confers hormone-binding ability to the receptor.

Monoclonal antibodies have been raised against the hapten azobenzylphosphonate, a close phosphotyrosine analog (Frackelton *et al.*, 1983). These antibodies have been covalently coupled to Sepharose beads and used to purify proteins phosphorylated on tyrosine, e.g. the phosphotyrosyl proteins from cells transformed by the Abelson murine leukemia virus (Frackelton *et al.*, 1983; Foulkes *et al.*, 1985) and the platelet-derived growth factor receptor (Daniel *et al.*, 1985).

The present findings show that estradiol receptor from calf uterus cytosol interacts with a very high affinity and specificity with the anti-P-Tyr-antibody covalently bound to Sepharose. This interaction seems to be due to the phosphotyrosine of the receptor because it disappears after inactivation of the receptor using a receptor phosphotyrosine phosphatase. This observation extends our findings of phosphorylation of the receptor in whole rat uterus as it suggests that in calf uterus the estradiol receptor is also phosphorylated on tyrosine. Furthermore, the data presented in Table II show that the use of anti-P-Tyr−Sepharose provides an almost total separation of the dephosphorylated receptor from the phosphorylated receptor, a prerequisite for the study of the molecular and biological properties of the dephosphorylated receptor.

The recent, basic achievement of estradiol and glucocorticoid receptor synthesis from complementary DNA (Walter *et al.*, 1985; Hollenberg *et al.*, 1985) will probably make an important contribution to the understanding of the role of post-translational modifications on the functions of steroid receptors. In this regard it is interesting that the complete complementary DNA sequence of the estrogen receptors from both breast cancer mice cells (MCF-7; Green *et al.*, 1986) and chicken oviduct (Krust *et al.*, 1986) contains information for four tyrosine residues that are potential sites of phosphorylation. Two of them are in the region required for tight binding of the hormone−receptor complex to the nucleus and one is in the hydrophobic domain required for hormone-binding. Our previous observation that *in vivo* nuclear 'translocation' of estradiol−receptor complex in mouse uterus is followed by release into cytosol of dephosphorylated, non-hormone binding receptor (Auricchio *et al.*, 1981c) might be attributed to dephosphorylation of these three tyrosine residues by the nuclear receptor-phosphatase. The sequence of estradiol receptor also contains information for two potential cAMP-dependent phosphorylation sites. This does not contradict the results presented here as these results do not exclude the possibility that, in addition to phosphotyrosine, other phosphoamino acids are present in the estradiol receptor which might be detected under different experimental conditions.

The avian erythroblastosis virus (AEV) causes erythroleukemia

in chickens. This transformation is due to the product of v-*erbB*. The function of this gene is stimulated by v-*erbA*. V-*erbB* contains the sequence of the tyrosine kinase associated with the EGF-receptor (Downward *et al.*, 1984) and v-*erbA* shows homology with the hormone-binding and DNA-binding domains of the estradiol receptor (Green *et al.*, 1986; Krust *et al.*, 1986). These homologies suggest that interaction similar to that observed between estradiol receptor and its tyrosine kinase (Migliaccio *et al.*, 1984) might occur between *erbA* and *erbB*. On the basis of this hypothesis, homology between estradiol receptor kinase and *erbB* would be expected.

In conclusion our present and previous findings show that, like the peptide hormone receptors (Czech, 1985), the estradiol receptor also interacts with a protein tyrosine kinase. The fact that peptide hormone receptors are covalently linked with tyrosine kinase, whereas this linkage does not exist in the case of estradiol receptor, does not, in our opinion, undermine the functional similarities between these receptors.

Materials and methods

Reagents

[^{3}H]Estradiol (99 Ci/mmol) and [^{32}P]orthophosphate (carrier-free) were from Amersham, UK. Estradiol, DES, DTT, DTA, EGTA, 2-mercaptoethanol, heparin grade 1, BSA, ATP disodium salt from equine muscle and phosphoamino acids were from Sigma, St. Louis, MO, USA. Bovine immunoglobulins were from Armour, Chicago, IL, USA.

Sepharose 4B, dextran T70 and the calibration kit for the electrophoresis of low mol. wt proteins were from Pharmacia, Uppsala, Sweden. Acrylamide, BIS, TEMED, ammonium persulphate, gelatin and Norit A were from Serva, Heidelberg, FRG. All other reagents were analytical grade.

Buffers

The following buffers were used: 50 mM Tris−HCl, 1 mM DTT, pH 7.4 (TD buffer); TD buffer with 0.2 mM EGTA (TGD buffer); 20 mM Tris−HCl, 1 mM DTT, 5 mM $MgCl_2$, pH 7.4 (TDM buffer). The buffer used for rat uteri incubation was 25 mM Hepes, 1.28 mM NaCl, 6.3 mM KCl, 2.8 kM $CaCl_2$, 1.3 mM $MgCl_2$, 10 mM glucose, 0.5 mM succinate, 0.5 mM pyruvate, 0.5 mM glutamate, pH 7.4.

The hapten buffer was TGD buffer supplemented with 40 mM phenyl phosphate and 0.5 M NaSCN.

Rat uterus incubation

Uteri were removed from 13 intact adult rats (Sprague−Dawley strain), stripped of connective tissue, and opened by a single longitudinal cut of the anterior suface. Uteri (~320 mg each) were kept on ice for ~20 min, washed with the incubation buffer, equilibrated with the room temperature and finally added to a 100 ml Ehrlenmeyer flask containing 40 ml of incubation buffer supplemented with 10 mM sodium molybdate and 10 mCi [^{32}P]orthophosphate. The flask was shaken in a Dubnoff shaker set at 50 excursions/min at 39°C for 1 h. Na_3VO_4 (80 μM) was added 5 min after the beginning of the incubation at 39°C.

Purification of estradial receptor from rat uterus

Receptor was purified by a modification and an extension of a previously reported procedure (Van Osbree *et al.*, 1984). After incubation, labeled uteri were washed twice with homogenization buffer, mixed with 10 unlabeled uteri from 7-day ovariectomized rats, minced and homogenized in the cold in 4 volumes of TGD buffer supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF), 80 μM Na_3VO_4 and 120 mM Na_2MoO_4, using an Ultraturrax homogenizer (Janke and Kunkel Co). Four bursts at 90 V lasting 10 s each at 1 min intervals were used. Cytosol was prepared from homogenate by centrifugation at 150 000 *g* for 30 min at 2°C in an L8-55 Beckman ultracentrifuge equipped with a Ti 70.1 rotor. Cytosol was cycled at 4°C overnight through a 2.5-ml DES−Sepharose column (7 cycles) equilibrated with TGD buffer containing 1 mM PMSF. DES−Sepharose was prepared by coupling DES to epoxy-activated Sepharose 4B as reported by Van Osbree *et al.* (1984). The affinity resin column was washed with TGD buffer containing 5 mM $MgCl_2$, 10% dimethylformamide, 0.5 M NaSCN, 1.0 μM 17β-estradiol (diluted with radioinert estradiol to a sp. act. of 10 Ci/mmol).

Immediately after elution the preparation was diluted 10-fold with TD buffer containing 5 mM $MgCl_2$ to prevent the high concentration of NaSCN from inactivating the estradiol receptor, and cycled at 12 ml/h overnight through a 1.5-ml heparin−Sepharose column also equilibrated with TD buffer supplemented with 5 mM $MgCl_2$. Heparin−Sepharose was prepared as previously reported (Cuatrecasas, 1970). The column was extensively washed until neither ^{3}H nor ^{32}P radioactivity was detected. Then the receptor was eluted by 2.5 ml TD buffer containing 4 mg heparin/ml. Two 100-μl samples were immediately used for [^{3}H]estradiol (13 800 c.p.m.) and ^{32}P (4900 c.p.m.) counts. The rest of the sample was kept at 4°C in the presence of 12 nM [^{3}H]estradiol (diluted with cold hormone to a sp. act. of 10 Ci/mmol). 1.5 ml of this sample was dialyzed against cold 50 mM phosphate buffer, pH 7.2 in the absence of DTT, then slowly poured on a 0.5-ml column filled with JS 34/32 antibody covalently coupled to Sepharose 4B (Cuatrecasas, 1970). The column was washed with 8 volumes of phosphate buffer containing 0.2 M KCl and finally the receptor was eluted with 1 ml of diluted ammonium hydroxide, pH 12. The sample was added with acetic acid to reach pH 7 in a final volume of 1.1 ml. Two 50-μl samples were used for [^{3}H]estradiol (767 c.p.m.) and ^{32}P (126 c.p.m.) counts.

Monoclonal antibodies against estrogen receptor

Production and characterization of the antibody against estrogen receptor purified from calf uterus (JS 34/32) has been described elsewhere (Moncharmont *et al.*, 1982). Control immunoglobulins were purified from hybridoma derived from the fusion of the myeloma cells with spleen cells from non-immunized mice.

Sucrose gradients centrifugation

250-μl samples were layered on 4.2 ml 10−30% sucrose gradients in TD buffer supplemented with 0.4 M KCl. After centrifugation for 14 h at 2°C with a SW 60 rotor in a Beckman L5-75 ultracentrifuge at 45 000 r.p.m. gradients were fractionated into 7-drop samples. Each fraction was added to 3 ml Beckman MP scintillation fluid and counted for radioactivity.

Polyacrylamide gel electrophoresis

SDS−slab gel electrophoresis was performed at 20°C using 7.5% acrylamide gel (w/v) (37.5:1 acrylamide:Bis ratio) 0.1% (w/v) SDS in 0.1 M phosphate buffer, pH 7.2. The 13-h run was performed at 40 mA at a constant current. The slab was stained with silver stain, treated with a Bio Rad gel dryer and exposed for autoradiography with a Lanex intensifying screen in a Dupont cassette for 21 days at −70°C to a Fuji film.

Phosphoamino acid analysis

The acid hydrolysis of the receptor purified from rat uterus was performed under vacuum in 6 N HCl at 110°C for 3 h. The sample was lyophilized, solubilized with water, supplemented with non-radioactive phosphoserine, phosphothreonine and phosphotyrosine, and subjected to thin-layer electrophoresis on cellulose thin-layer plates (250 μm) from Machery-Nagel (Duren, FRG) at pH 3.5 in acetic acid/pyridine/H_2O, 50:5:945, 1000 V for 45 min. The plates were stained with ninhydrin and finally exposed for autoradiography for 10 days at −70°C to X-AR Kodak ultrasensitive film.

Calf uterus homogenization and fractionation

Calf uterus 750-g pellet (nuclei) and cytosol were obtained by homogenizing (Ultra-turrax homogenizer) frozen calf uterus stored at −80°C in 4 volumes of TED or TGD sucrose buffer, as previously described (Auricchio *et al.*, 1981b).

Anti-phosphotyrosine antibody−Sepharose 4B preparation

Anti-phosphotyrosine antibody 2G8.D6 was prepared and coupled at a concentration of 15 mg/ml of packed beads as previously described (Frackleton *et al.*, 1983).

Cytosol [^{3}H]estradiol−receptor complex preparation

Cytosol was incubated with 4 nM [^{3}H]estradiol. Separate samples were incubated in the presence of a 100-fold excess of cold estradiol (low specific activity) for 12−18 h at 0°C. This separate incubation served to assay the specific ^{3}H-hormone-binding activity of cytosols and of receptor preparations purified from these cytosols.

Binding activity determination

Specific hormone binding activity of hormones was determined in duplicate as the difference between binding at 0°C for 12−18 h of high and low sp. act. ^{3}H-hormone calculated by subtracting the non-specific binding activity from the total. Bound hormone was separated from free hormone by DCC treatment. The DCC suspension consisted of 1% charcoal, 0.05% dextran T70, 0.1% gelatin in 50 mM Tris, pH 7.4. The DCC suspension was added to the same volume of the sample and incubated at 0°C for 10 min, then centrifuged at 4000 r.p.m. for 10 min at 4°C. The supernatant was measured for radioactivity.

Specific hormone-binding activity of receptor partially purified by anti-P-Tyr−Sepharose was determined as the difference between radioactivity eluted from Sepharose loaded with samples from cytosols preincubated with high and low sp. act. ^{3}H-hormone.

Assay of dephosphorylated receptor

This assay is based on the activation of specific hormone-binding activity by ATP in the presence of the receptor kinase (Auricchio *et al.*, 1981b). Each sample was added to 10 mM Na_2MoO_4 and 5 mM $MgCl_2$ and divided into two fractions. ATP (10 mM) was added to one fraction. Both fractions were incubated at 15°C for 10 min then assayed for the specific hormone-binding activity. Levels

of dephosphorylated receptor were calculated as the ATP-activated binding activity calculated from the difference in binding activity in the presence and absence of ATP.

Radioactivity measurement

Samples from 0.1 to 0.25 ml were added to 3 ml of scintillation liquid for radioactivity counting, and radioactivity was measured in a Beckman Spectrometer with a 45% efficiency.

Proteins

Proteins were assayed using a Bio-Rad protein assay kit.

BSA, bovine immunoglobulins and Sepharose preparations

BSA and bovine immunoglobulins were covalently coupled with CN-Br-activated Sepharose 4B at a concentration of 15 mg/ml of packed beads (Cuatrecasas, 1970).

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