Receptor Interconversion Model of Hormone Action. 2. Requirement of both Kinase and Phosphatase Activities for Conferring Estrogen Binding Activity to the Estrogen Receptor†

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ABSTRACT: Three interconvertible forms of the estrogen receptor have been identified in the oviduct of estrogen-stimulated chicks. The non-estradiol binding form (R_{nb}) can be converted to the lower affinity binding form (R_y, K_d = 0.8 nM) by a process requiring the γ-phosphoryl moiety of ATP. The enzymatic activity (F_y) essential for this "receptor potentiation" has been isolated from oviduct cytosol using ammonium sulfate fractionation, DEAE chromatography, and HPLC size-exclusion chromatography. The potentiation appears to require both kinase and phosphatase activities. The F_y kinase characteristically phosphorylates casein, histones, and glycogen synthase. Comparison of the kinase with casein kinase II, which also phosphorylates casein and glycogen synthase, indicates that F_y represents a distinct protein kinase since its activity is not stimulated by spermine or inhibited by heparin. F_y-mediated conversion of R_{nb} to R_y is blocked by the phosphatase inhibitors vanadate, fluoride, and pyrophosphate. The substrate specificity of the F_y phosphatase activity is distinct from that of the two well-characterized protein phosphatases 1 and 2A. Moreover, the requirement for F_y phosphatase activity in converting R_{nb} to R_y could not be mimicked by its substitution with purified protein phosphatases 1 or 2A. The unique substrate specificity of the oviduct protein phosphatase and protein kinase, which are apparently necessary to confer estradiol binding characteristics to the receptor, implies that these enzymes play a key role in the control of the estrogen receptor in its function as a transcription factor.

Steroid hormone receptors are perhaps the best characterized class of transcription factors. Posttranslational modifications of these receptors resulting in alterations in function have profound importance to our understanding of the regulation of gene expression. This laboratory has focused upon chick oviduct estrogen receptors and their interconversion between three different states as a model for posttranslational modification of transcription factors (Raymoure et al., 1985, 1986; McNaught & Smith, 1986; McNaught et al., 1986).

Regulation of the estrogen receptor concentration in target cells is postulated to occur by many mechanisms. Estrogen induces the formation of estrogen receptors in the chick oviduct and *Xenopus* liver (Taylor et al., 1980; Perlman et al., 1984). Estrogen receptor concentration is decreased by the antagonistic action of progesterone in the hamster and rat uterus (Evans et al., 1975; Hsueh et al., 1975) and chick oviduct (Sutherland et al., 1977). It has been suggested that estrogen can affect its own receptor concentration by decreasing the half-life of its receptor (Eckert et al., 1984), an event associated with nuclear processing (Horwitz & McGuire, 1978). However, all of these reports are concerned only with steroid binding forms of the receptor and have not addressed the role of a non-estrogen binding form of the receptor, which would have escaped detection in these assays.

Relatively recently, receptors for growth factors, hormones, and neurotransmitters have been demonstrated to be phosphoproteins. It is an attractive possibility that receptor function may be altered by phosphorylation/dephosphorylation mechanisms. While the literature has become replete with examples of steroid receptor phosphorylation, this laboratory has focused upon those events which can be related directly to receptor function. Since the steroid ligand is essential for a biological response, only those phosphorylation/dephosphorylation reactions which modify estradiol binding have been addressed.

Although expression of estrogen receptor cDNA in mammalian and amphibian cells results in an estrogen binding form of the receptor (Greene et al., 1986; Theulaz et al., 1988), in yeast only about 20% of the receptor molecules bind estradiol (Metzger et al., 1988). It is also of particular significance that in contrast to progesterone and androgen receptor mRNA, translation of in vitro synthesized estrogen receptor mRNA in the rabbit reticulocyte system produces receptor molecules which predominantly lack the capacity to bind estradiol (Kumar et al., 1986). We have shown previously that the nonbinding form (R_{nb}) of the receptor isolated from chick oviduct cytosol can be converted preferentially to the lower affinity form of the receptor (R_y) by an ATP/Mg^{2+}-dependent process. This process can be defined as "potentiation" (Sherman et al., 1983). The use of nonhydrolyzable nucleotide analogues of ATP demonstrated that potentiation requires the loss of the γ-phosphoryl moiety from ATP (Raymoure et al., 1985). In the preceding paper, we describe the isolation and characterization of an endogenous heat-labile enzymatic activity, F_y, that catalyzes this reaction (McNaught et al., 1990). In this paper, we have attempted to define the biochemical

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mechanism of conversion of R_{nb} to R_y. These experiments suggest that estrogen receptor potentiation is regulated by both phosphorylation and dephosphorylation reactions. Furthermore, the enzyme(s) involved has (have) characteristics of a phosphoprotein phosphatase.

Experimental Procedures

Chemicals. All reagents used were of analytical grade and obtained from commercial sources. 17β-[2,4,6,7-^{3}H]Estradiol (86 Ci/mmol) and adenosine 5′-[^{32}P]triphosphate triethylammonium salt (>5000 Ci/mmol) were purchased from Amersham (Arlington Heights, IL). Sepharose 4B (Pharmacia, Piscataway, NJ) and histones were supplied by Worthington Biomedical (Freehold, NJ). Casein was from Sigma (St. Louis, MO). Unlabeled protein standards and reagents for sodium dodecyl sulfate–polyacrylamide (SDS–polyacrylamide)[1] gel electrophoresis were purchased from Bio-Rad (Richmond, CA). Adenosine 5′-*O*-(3-thiotriphosphate) was supplied by Boehringer Mannheim (Indianapolis, IN).

Hormone Treatment. One-week-old White Leghorn chicks (Texas Animal Specialties, Humble, TX) were treated with daily subcutaneous injections of 2 mg of DES in sesame seed oil for a 2-week period. The animals were then withdrawn from hormone treatment.

Preparation of Cytosol. Minced oviducts were homogenized in TESH buffer [10 mM Tris-HCl, pH 7.4 (24 °C), 1.5 mM EDTA, and 12 mM monothioglycerol] with a Polytron tissue homogenizer (Brinkmann, Westbury, NY) in a ratio of 1:5 (weight to volume) with a 3 × 5 s pulses. The resultant homogenate was first centrifuged at 5000*g* for 30 min. Cytosol was obtained from the supernatant by centrifugation at 105000*g* for 90 min. Floating fatty material was carefully aspirated off after all centrifugation steps.

Ammonium Sulfate Fractionation. Saturated ammonium sulfate, neutralized (pH 7.4) with NH_4OH, was prepared in TESH buffer and added dropwise until the final solution was at the specific ammonium sulfate composition. The suspension was then stirred at 4 °C for 30 min. The precipitate was collected by centrifugation at 10000*g* for 20 min. The supernatant was discarded, and the pellets were redissolved in an appropriate volume of TESH buffer. The resulting solutions, containing the partially purified estrogen receptor from the 30% pellet, and the enzymatic activity F_y from the 40–60% pellet, were centrifuged at 105000*g* for 30 min to sediment insoluble material.

Steroid Binding Assay. Saturation analyses were performed with [^{3}H]estradiol at final concentrations ranging from 0.08 to 10 nM. Nonspecific binding was quantitated with the introduction of a 100-fold molar excess of radioinert diethylstilbestrol (DES). Assays were incubated at 30 °C for 3 h followed by an additional period for 18 h at 4 °C. Dextran-coated charcoal was used to separate bound from free steroid. Saturation assays were analyzed according to the method of Scatchard (1949).

Preparation of Affinity Resin. CNBr-activated Sepharose 4B (1 g) was coupled with the monoclonal antibody against the estrogen receptor (100 μg) dissolved in 0.1 M $NaHCO_3$ (pH 8.3) containing 0.5 M NaCl at 4 °C overnight. The excess ligand was removed by washing with coupling buffer. Finally the resin was incubated with 1 M ethanolamine, pH 9.0, for 2 h at 25 °C. The freshly prepared affinity resin was washed alternately with 500 mL of 0.1 M acetate (pH 4.0) and 0.1 M Tris buffer (pH 8.0) containing 0.5 M NaCl. The resin was stored at 4 °C in 0.1 M Tris, pH 8.0, buffer.

Immunoprecipitation of the Receptor. The ammonium sulfate fraction was immunoadsorbed to monoclonal antibody H-222 (Abbott Laboratories, Chicago, IL) coupled to Sepharose 4B. Unbound material was washed copiously with TESH buffer containing 0.4 M NaCl and once with TESH buffer alone. Resultant pellets were used for assaying the phosphorylation and estrogen binding capacity of the receptor.

Phosphorylation Incubation System. The immunoadsorbed estrogen receptor was phosphorylated in the presence of [γ-^{32}P]ATP (5000 Ci/mmol), in TESH buffer containing 100 μM cold ATP (resulting in a final specific activity of 10 Ci/mmol), 5 mM $MgCl_2$, 10 mM sodium molybdate, 10 nM unlabeled estradiol, and F_y for 3 h at 30 °C. The total incubation volume was 200 μL. The phosphorylated samples were centrifuged at 4 °C and the resulting pellets washed twice with iced TESH buffer containing 0.4 M NaCl and 4 times with TESH containing 10 mM molybdate. The phosphorylated receptor was eluted from the pellets by boiling for 5 min at 100 °C in gel loading buffer containing SDS and then electrophoresed on a 10% Porzio and Pearson (1977) one-dimensional slab gel (1.5 mm). The gels were stained with Coomassie Blue R250 and dried, and subjected to autoradiography at room temperature using XAR-5 X-ray (Kodak) film.

Myosin (M_r 200000), β-galactosidase (M_r 116000), phosphorylase B (M_r 92500), bovine serum albumin (M_r 66200), ovalbumin (M_r 45000), carbonic anhydrase (M_r 31000), soybean trypsin inhibitor (M_r 21500), and lysozyme (M_r 14400) were used as molecular weight standards.

Steroid Binding Assay of Immunopurified Receptor. The immunoadsorbed estrogen receptor was labeled in the presence of 5 nM [^{3}H]estradiol, 100 μM ATP, 5 mM $MgCl_2$, 10 mM molybdate, and F_y in a final volume of 200 μL for 3 h at 30 °C. In parallel, a control assay was carried out in the absence of F_y. Nonspecific binding was determined by inclusion of a 100-fold molar excess of DES. After being extensively washed with TESH buffer containing 0.4 M NaCl, the labeled immunoadsorbed receptor was extracted with 1 mL of 100% ethanol and the extracted [^{3}H]estradiol counted.

Phosphoamino Acid Analysis. After electrophoresis, protein bands were excised from polyacrylamide gels. Gel bands were cut into small pieces and allowed to swell in a small volume of 0.05 M NH_4HCO_3. Peptides were extracted by incubating the gel slices overnight at 37 °C in the presence of trypsin (50 μg/mL). The supernatant containing ^{32}P-phosphopeptides was collected, and the gel fragments were redigested with trypsin. The second supernatant was pooled with the first and lyophilized. The dried protein mixture was mixed with 200 μL of 6 N HCl. The samples were hydrolyzed in sealed tubes at 110 °C for 2 h, evaporated to dryness, and reconstituted in 1.2 mL of HPLC buffer (10 mM KP/12.5% methanol, final pH 3.0) containing the phosphoamino acid markers (phosphothreonine, phosphoserine, phosphotyrosine). Phosphoamino acids were separated (Yang et al., 1982) on a Partisil-10 SAX exchange column (Whatman). The column was run at room temperature (flow rate 1 mL/min). One-milliliter fractions were collected; 0.7-mL aliquots were assayed for ^{32}P; 0.3-mL aliquots were treated with ninhydrin to detect amino acids.

Purification of Activating Factor (F_y). Purification and characterization of F_y are described in more detail in the preceding paper (McNaught et al., 1990). The 40–60% ammonium sulfate fractionated cytosol was dialyzed overnight and then chromatographed on a DEAE minicolumn. The 250

[1] Abbreviations: DES, diethylstilbestrol; SDS, sodium dodecyl sulfate.

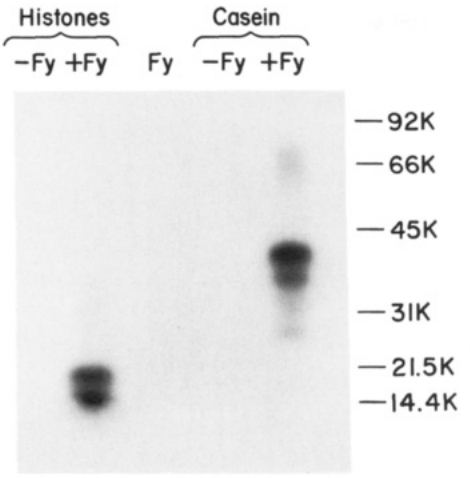


FIGURE 1: Protein kinase activity of pure F_y. One hundred micrograms of casein or histones was phosphorylated in the presence of HPLC-purified F_y under conditions described under Experimental Procedures. The samples were TCA precipitated, and the pellets were reconstituted in 130 μL of loading buffer (Porzio & Pearson, 1977). One hundred thirty microliters of each sample was loaded on a 10% SDS slab gel. From left to right, the autoradiograph shows histones and casein treated with [^{32}P]ATP in the absence or presence of F_y.

mM eluate was then further purified on HPLC using a TSK G3000 gel permeation column.

Determination of Protein Kinase Activity of F_y. Aliquots of DEAE-purified F_y or HPLC-purified F_y were incubated in the presence of 5 mM $MgCl_2$, 10 mM molybdate, 100 μg of casein or histones, 10 μCi [^{32}P]ATP (7500 Ci/mmol), and 100 μM cold ATP in a final volume of 130 μL at 30 °C for 30 min. The reaction was terminated by the addition of 130 μL of 20% trichloroacetic acid at 0 °C, and the mixture was centrifuged at 10000 rpm for 30 min. The resultant pellet was rinsed twice with 100% ethanol and air-dried before adding the electrophoresis sample buffer. After electrophoresis, the bands were excised and analyzed for phosphoamino acids. The same procedure was used to analyze the phosphorylated histones. To demonstrate that the histone or casein preparations were devoid of any endogenous kinase activity, the above reactions were performed in the absence of F_y.

To compare the kinase activity of F_y with casein kinase II, we have used purified casein kinase II and incubated with [^{32}P]ATP, cold ATP, and Mg^{2+}. Casein kinase II was purified from liver (Hara et al., 1981). Spermine and heparin were used to enhance or to inhibit casein kinase II activity, respectively. Parallel incubation assays were carried out in the presence of F_y at different levels of purification. The samples were precipitated with trichloroacetic acid and ^{32}P counted.

Determination of Phosphoprotein Phosphatase Activity of F_y. [^{32}P]Phosphorylase *a* was prepared by incubating phosphorylase *b* with phosphorylase kinase, [^{32}P]ATP, and Mg^{2+}. One mole of phosphate per mole of protein was incorporated into a specific serine residue (Cohen, 1973). Glycogen synthase (Cohen, 1986) was phosphorylated with cAMP-dependent protein kinase (Beavo et al., 1974) up to 0.8 mol/mol of substrate. Histone H_1 was also phosphorylated with cAMP-dependent protein kinase. The phosphatase activity of F_y was determined by the release of ^{32}P from phosphorylase *a*, histone H_1, or glycogen synthase (Shenolikar & Ingebritsen, 1984).

RESULTS

Kinase Activity of F_y. DEAE Bio-Gel purified F_y was incubated with histones and casein in the presence of [^{32}P]-ATP. The products were electrophoresed on a 10% polyacrylamide–SDS gel. Examination of resulting autoradiographs revealed that casein and histones were phosphorylated in the presence of F_y (data not shown). Phosphorylation did not occur in the absence of F_y. To determine whether HPLC-purified F_y had kinase activity, casein and histones were again used as substrates in the presence of [^{32}P]ATP/Mg^{2+}. Figure 1 demonstrates that the highly purified enzyme was capable of phosphorylating both casein and histones. Phosphoaminoacid analyses indicated that F_y primarily phosphorylates serine residues (Figure 2). No autophosphorylation of F_y was evident (Figure 1). Table I further illustrates the kinase activity of F_y using glycogen synthase as substrate and demonstrates that kinase activity copurifies with F_y.

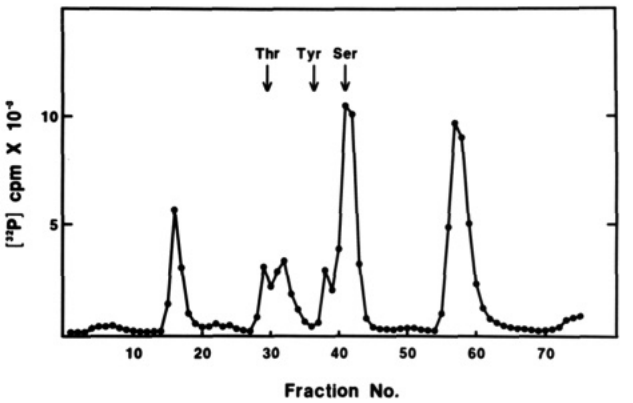


FIGURE 2: Phosphoamino acid analysis of casein phosphorylated with HPLC-purified F_y. Casein was phosphorylated by ATP/Mg^{2+} in the presence of F_y and isolated by SDS–polyacrylamide gel electrophoresis. The phosphorylated protein was eluted from the gel, hydrolyzed, and analyzed as described under Experimental Procedures.

Table I: Effect of Purification on Kinase Activity of F_y Using Glycogen Synthase as Substrate[a]

	^{32}P incorpn (cpm)	
	expt 1	expt 2
glycogen synthase blank	1506 ± 138	2138 ± 312
F_y $(NH_4)_2SO_4$ fractionated and dialyzed	3682 ± 199	3852 ± 301
DEAE purified	4882 ± 642	3228 ± 94
HPLC purified	3248 ± 115	4638 ± 398

[a] Glycogen synthase (5 μM) purified from rabbit skeletal muscle was phosphorylated by F_y fractions (20 μL) having equivalent activity in converting R_{nb} to R_y in a total assay volume of 100 μL containing 100 μM ATP (10^5 cpm/nmol) and 1.0 mM $MgCl_2$ for 10 min at 30 °C. The reaction was stopped by cooling to 4 °C and adding 1 mL of 20% trichloroacetic acid and 100 μL of bovine serum albumin. ^{32}P incorporated into trichloroacetic acid precipitated fractions was monitored by Cerenkov counting. The results represent the average of triplicate values.

Casein kinase II is a protein serine/threonine kinase which in common with F_y phosphorylates glycogen synthase and its activity is independent of cyclic nucleotides and calcium (Hara et al., 1981). To further characterize F_y, we compared its kinase activity with casein kinase II. In contrast to casein kinase II, HPLC-purified F_y kinase activity is not enhanced by spermine (1 mM) or inhibited by heparin (100 nM). Casein kinase II was also unable to substitute for F_y in converting R_{nb} to R_y, suggesting that casein kinase II is not the major protein kinase activity associated with F_y.

Effect of F_y/ATP/Mg^{2+} on Immunopurified R_{nb}. Having established that HPLC-purified F_y possessed kinase activity, it was important to determine whether this activity was involved in the conversion of R_{nb} to R_y. For these experiments, immunopurified R_{nb} was utilized as substrate. Oviduct estrogen receptors consisting of mainly the R_{nb} form were

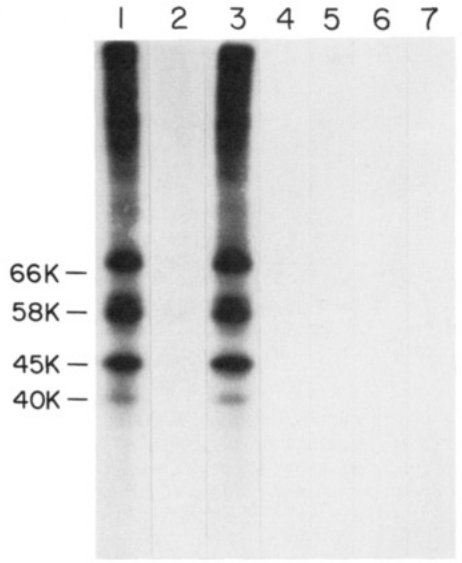


FIGURE 3: Phosphorylation of immunoprecipitated estradiol receptor. Ammonium sulfate fractionated receptor (30%) was further purified by using the matrix-bound monoclonal antibody immunopurification procedure (Experimental Procedures). The immunocomplex was washed extensively with TESH buffer containing 0.4 M NaCl before incubation of bound receptor with 1 mCi of [γ-^{32}P]ATP, 100 μM cold ATP, 5 mM $MgCl_2$, 10 mM MoO_4, and 10 nM unlabeled estradiol for 3 h at 30 °C. The total incubation volume was 200 μL. ^{32}P-Polypeptides were eluted from the affinity matrix by boiling for 5 min at 100 °C in gel loading buffer and were then electrophoresed on a 10% Porzio and Pearson (1977) one-dimensional slab gel. Autoradiography was performed for 15 min at room temperature. Lane 1, control incubation; lane 2, the same experimental procedure as in lane 1 with the inclusion of F_y; lane 3, control incubation in the presence of heat-inactivated F_y; lane 4, incubation in the absence of Mg^{2+}; lane 5, incubation in the absence of Mg^{2+} and in the presence of Ca^{2+}; lane 6, immunobeads in the absence of ammonium sulfate fractionated cytosol; lane 7, control CNBr-activated Sepharose 4B treated in the absence of H222 in the presence of ammonium sulfate precipitated R_{nb}.

partially purified by ammonium sulfate fractionation and then immunopurified by using an estrogen receptor monoclonal antibody affinity resin (H222). We had previously demonstrated that this particular antibody has a high affinity for all three forms (R_x, R_y, R_{nb}) of the chick estrogen receptor (Raymoure et al., 1986).

Two series of experiments were conducted, the first using DEAE-purified F_y and the second using HPLC-purified F_y. Immunopurified receptor was incubated with 100 μM [^{32}P]-ATP, 5 mM $MgCl_2$, 10 mM molybdate, and 10 nM estradiol at 30 °C for 3 h. In parallel, F_y and heat-inactivated F_y were added to the incubation. Although 100 μM is a suboptimal concentration of ATP for the conversion of R_{nb} to R_y (McNaught et al., 1990), this concentration was selected to increase the sensitivity of the phosphorylation reactions by maximizing the specific activity of [^{32}P]ATP. The estrogen receptor was analyzed for phosphorylation by elution from the affinity resin followed by SDS–polyacrylamide gel electrophoresis and autoradiography. Parallel experiments were conducted except that unlabeled ATP and [^{3}H]estradiol ± 200 nM DES were used. In these experiments, rather than analyzing the receptor on the immunobeads by electrophoresis and autoradiography, the immobilized receptor in its steroid binding form was quantitated by measurement of the amounts of [^{3}H]estradiol specifically bound.

The results of treating the immunopurified R_{nb} with DEAE-purified F_y are shown in Figure 3. Surprisingly, in the absence of F_y, receptor phosphorylation occurred. Four major radioactive polypeptides of 68, 58, 45, and 40 kDa were

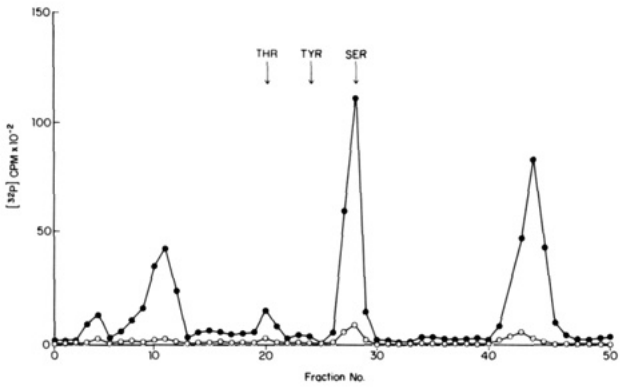


FIGURE 4: Phosphoamino acid analysis of immunopurified estrogen receptor. The phosphorylated receptor was eluted from immunobeads containing the estrogen receptor monoclonal antibody and analyzed by SDS–polyacrylamide gel electrophoresis. The phosphorylated receptor bands were eluted from the gel, hydrolyzed, and analyzed by HPLC as described under Experimental Procedures. Treatment with heat-inactivated F_y (●); treatment with active F_y (○). Parallel incubations in the presence of [^{3}H]estradiol confirmed that active F_y converted R_{nb} to R_y.

apparent (Figure 3, lane 1), indicating that a kinase copurifies with R_{nb}. The intensity of phosphorylation of the first three bands was identical; the fourth band contains less ^{32}P. The inclusion of α_2-macroglobulin as a protease inhibitor had no impact on the appearance of the 58-, 45-, and 40-kDa phosphorylated peptides. Figure 3 (lane 4) also shows that in the absence of Mg^{2+}, or in the presence of Ca^{2+} (lane 5), no endogeneous kinase activity is observed. To confirm that the phosphorylation products were indeed associated with receptor and not other proteins nonspecifically absorbed to the immunoaffinity resin, control reactions in the absence of receptor (lane 6) or in the absence of antibody on the affinity resin (lane 7) were run. In these reactions, no phosphorylation was evident. Figure 3 (lane 3) also shows the 68-, 58-, 45-, and 40-kDa phosphorylation products when heat-inactivated F_y was included in the incubation. However, lane 2 of Figure 3 shows that in the presence of active F_y, no phosphorylation is discernible. Parallel experiments designed to monitor acquisition of specific [^{3}H]estradiol binding indicated an increase from 0.56 to 1.7 pmol in the presence of active F_y. This degree of ATP/Mg^{2+}-dependent augmentation was similar to that observed when Scatchard analysis was performed prior to immunopurification of R_{nb}. When control resin (Sepharose 4B without H222 monoclonal antibody) was incubated with the same amount of receptor and F_y, and labeled under the same conditions, no specific [^{3}H]estradiol binding was observed. These data show that R_{nb} does not nonspecifically bind to the resin in the absence of monoclonal antibody. F_y alone was also incubated with the affinity resin to examine whether F_y artifactually had the capacity to confer estradiol binding properties to the affinity resin. Once again, no estradiol-specific binding was observed, demonstrating that potentiation of [^{3}H]estradiol binding was indeed through the conversion of R_{nb} to R_y by the activating enzyme(s). In summary, increased [^{3}H]estradiol binding to the immunoadsorbed receptor occurs only in the presence of active F_y and ATP/Mg^{2+}; the increase in binding is accompanied by an overall decrease in ^{32}P incorporation.

The 68-, 58-, 45-, and 40-kDa phosphorylated proteins were each excised from the SDS gels, trypsin treated, and hydrolyzed with HCl. Phosphoamino acid analysis showed that each peptide was phosphorylated on a serine residue and that serine phosphorylation was reduced 10-fold in the presence of F_y (Figure 4). It is important to note that although phospho-

Table II: Effect of Purification on Phosphorylase *a* Phosphatase Activity of F_y[a]

F_y	^{32}P released (nmol min^{-1} mL^{-1}) expt 1	expt 2
$(NH_4)_2SO_4$ fractionated and dialyzed	0.99	1.02
DEAE purified	0.45	0.51
HPLC purified	0.009	0.062

[a] Phosphorylase phosphatase activity was assayed with 10 μM phosphorylase *a* according to Shenolikar and Ingebritsen (1984) using amounts of F_y which were equivalent in their degree of potentiation of ammonium sulfate purified R_{nb}.

rylation of the receptor was inhibited, the same F_y preparation also had kinase activity (Table I). To determine whether phosphorylation/dephosphorylation is controlled by estradiol, experiments identical with those described above were performed in the absence of estradiol. Exclusion of the hormone had no effect on the phosphorylated state of the receptor (data not shown).

In the second series of experiments, HPLC-purified F_y was used. In the absence of F_y consistent with the above experiment, R_{nb} was again phosphorylated. However, in the presence of HPLC-purified F_y, in direct contrast to the experiments in which partially purified F_y was used, no observable increase or decrease in the phosphorylation state of immunopurified R_{nb} was evident. Moreover, in parallel incubations where [^{3}H]estradiol binding was monitored, there was no augmentation of binding in the presence of HPLC-purified F_y. In control experiments using partially purified rather than immunopurified R_{nb}, augmentation of [^{3}H]estradiol binding was observed.

Collectively, these two series of experiments indicate that F_y kinase activity alone is insufficient to convert R_{nb} to R_y. It is apparent that additional activity present either in partially purified R_{nb} or in partially purified F_y is required. A reduction in the phosphorylated state of the receptor always accompanied the R_{nb} to R_y conversion.

Phosphatase Activity Plays a Role in the Conversion of R_{nb} to R_y. When receptor, phosphorylated according to Figure 3, lane 1, was washed to remove excess ATP/Mg^{2+} and then treated with F_y, although some dephosphorylation occurred, complete dephosphorylation of the 67-kDa band was only observed in the presence of ATP/Mg^{2+}; this was accompanied by increased high-affinity [^{3}H]estradiol binding. Neither dephosphorylation nor increased estradiol binding occurred in the presence of ATP/Mg^{2+} and F_y at 4 °C. These results suggest that the conversion of R_{nb} to R_y requires phosphatase activity.

By use of ^{32}P-labeled phosphorylase *a* as substrate, phosphatase activity of F_y was compared at three levels of purification (Table II). Treatment with partially purified F_y resulted in substantial release of ^{32}P from phosphorylase *a*, and parallel experiments showed conversion of immunopurified R_{nb} to R_y. In contrast, HPLC-purified F_y showed no significant protein phosphatase activity and was unable to convert immunopurified R_{nb} to R_y. Interestingly, the dialyzed 40–60% ammonium sulfate fraction and the DEAE-purified and HPLC-purified F_y were all capable of converting partially purified R_{nb} to R_y. Upon assaying partially purified R_{nb} or the nonimmunoabsorbed fraction of R_{nb}, both preparations were shown to contain serine phosphatase activity. Furthermore, assaying of fractions from the HPLC purification showed that a broad peak of weak phosphatase activity eluted 10 fractions earlier than F_y kinase activity. The weak phosphatase activity present in these fractions was insufficient in the absence of other factors to cause the conversion of R_{nb} to R_y.

Table III: Substrate Specificity of the Protein Phosphatase (F_y) from Chick Oviduct[a]

substrate	PrP-1	PrP-2A	F_y AS	DEAE	HPLC
phosphorylase *a* (1 μM)	100	100	100	100	ND
glycogen synthase site 1a/1b (2 μM)	160	320	40	30	ND
histone H_1 (15 μM)	340	690	20	10	ND

[a] Protein phosphatase 1 (PrP-1) and protein phosphatase 2A (PrP-2A) catalytic subunits (specific activity 3000 and 2100 nmol min^{-1} mg^{-1}, respectively) were purified from rabbit skeletal muscle (Shenolikar & Ingebritsen, 1984). Approximately equivalent amounts of phosphorylase *a* phosphatase (1 nmol/min) were used. The activity, using phosphorylase *a*, was arbitrarily set as 100%. Protein phosphatase activity using glycogen synthase and histone H_1 was compared relative to phosphorylase *a*. Protein phosphatase activity in the HPLC fraction was too low to be estimated with accuracy (ND = non detectable). Equivalent amounts of F_y were used based on their activity in converting ammonium sulfate (AS) purified R_{nb} to R_y.

Comparison of the substrate specificity of the oviduct–protein phosphatase with purified protein phosphatases 1 and 2A from rabbit skeletal muscle indicated differences in their ability to dephosphorylate three phosphoprotein substrates, phosphorylase *a*, glycogen synthase, and histone H_1 (Table III). Neither of the two well-characterized phosphatases was able to convert R_{nb} to R_y, suggesting that the oviduct phosphatase is highly substrate specific.

The impact of several phosphatase inhibitors on F_y-dependent R_{nb} to R_y was investigated. As shown in Figure 5, in the absence of F_y (open bars), only low levels of specific estradiol binding are observed. Incubation with F_y/ATP/Mg^{2+} produced a 9-fold increase in specific binding (closed bars). The inclusion of 0.5 mM (panel A) sodium fluoride in the buffer inhibited R_{nb} to R_y conversion by 11% (lane 3). The conversion was inhibited 50% by 1 mM sodium fluoride (lane 4) and totally inhibited by 5, 10, or 50 mM (panel A, lanes 5, 6, and 7, respectively). Sodium fluoride was also shown to inhibit ATP/Mg^{2+}-dependent serine phosphatase activity but not F_y kinase activity. Sodium pyrophosphate at 0.5 mM inhibited the reaction by 75% and was completely inhibitory at 1, 5, and 10 mM (panel B). Sodium vanadate was completely inhibitory at 0.5 or 1 mM (panel C).

Conversion of R_{nb} to R_y in the Presence of Thio-ATP. The results presented above demonstrated that both partially purified R_{nb} and F_y contain a phosphatase. However, in addition to the phosphatase, F_y kinase activity is essential for the R_{nb} to R_y conversion. To determine which of these enzymes is primarily responsible for conferring steroid binding to the estrogen receptor, thio-ATP was used and compared to ATP for its efficiency in converting R_{nb} to R_y. As shown in Figure 6, R_{nb} is converted to R_y using 5 mM ATP/Mg in the presence of F_y (closed circles). Only low levels of specific estradiol binding are observed in the absence of F_y. Figure 6 also shows that substitution of 5 mM thio-ATP for ATP does not result in R_{nb} to R_y conversion. Parallel studies using a 10-fold reduction in ATP concentration confirmed that although there was a small difference in the affinity of estradiol binding when 0.5 mM ATP was used, the degree of conversion to R_y was equivalent to that obtained with 5 mM ATP.

Discussion

In our laboratory, we have indirectly detected a nonbinding form of estrogen receptor in the estrogen-stimulated chick

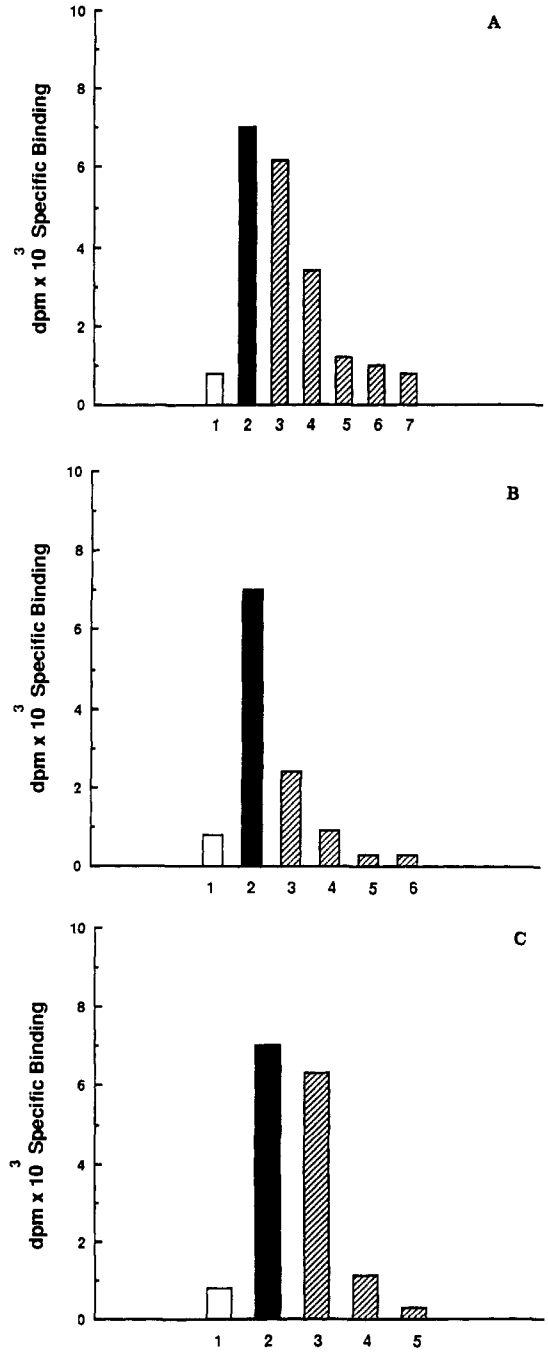


FIGURE 5: Influence of phosphatase inhibitors on F_y-mediated augmentation of chick estrogen receptor. Ammonium sulfate fractionated cytosol was prepared from the oviducts of 2-week-old chicks stimulated for 14 days with DES and withdrawn from estrogen treatment (Raymoure et al., 1986). The binding assay was performed in the presence of 2 nM [^{3}H]estradiol, 5 mM ATP/Mg^{2+}, and 10 nM molybdate. Nonspecific binding was assessed by addition of 100-fold excess DES to duplicate tubes. Assays were incubated for 3 h at 30 °C and then for 18 h at 4 °C. Unbound steroid was separated by addition of dextran-coated charcoal. The specific binding was determined by subtraction of nonspecific from total binding. (Panel A) Control (lane 1); treated with F_y (lane 2); F_y in the presence of 0.5 (lane 3), 1 (lane 4), 5 (lane 5), 10 (lane 6), and 50 mM (lane 7) sodium fluoride. (Panel B) Control (lane 1); treated with F_y (lane 2); F_y in the presence of 0.5 (lane 3), 1 (lane 4), 5 (lane 5), and 10 mM (lane 6) sodium pyrophosphate. (Panel C) Control (lane 1); treated with F_y (lane 2); F_y in the presence of 0.1 (lane 3), 0.5 (lane 4), and 1 mM (lane 5) sodium vanadate.

oviduct after estrogen withdrawal. In this report, we have described characteristics of a unique enzyme system (F_y) having the intrinsic property of potentiating the chick oviduct estrogen receptor.

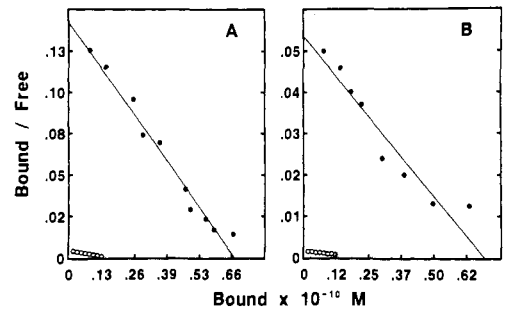


FIGURE 6: Conversion of the nonbinding form of chick estrogen receptor to R_y in the presence of F_y: comparison of ATP with thio-ATP. Ammonium sulfate fractionated cytosol (30%) was prepared from DES-stimulated chicks following withdrawal of DES treatment (Raymoure et al., 1986). Binding assays were carried out with 60 pM–10 nM [^{3}H]estradiol. Nonspecific binding was quantitated with the inclusion of a 100-fold molar excess of cold DES. The data were analyzed according to Scatchard (1949). (Panel A) Assays were performed with 5 mM ATP/Mg^{2+} and 10 mM molybdate in the absence (O) or presence (●) of F_y for 3 h at 30 °C. (Panel B) With 5 mM $MgCl_2$, 10 mM molybdate, and F_y in the presence of 5 mM thio-ATP (O) or 0.5 mM ATP (●), for 3 h at 30 °C.

The demonstration of non-steroid binding forms of estrogen receptor has been reported by two other groups. Auricchio's group have described the augmentation of estrogen binding by nucleotides in mouse, calf, and rat uterine cytosol, and their data suggest it is associated with phosphorylation of the receptor on a tyrosine residue (Aurricchio et al., 1981, 1982; Migliaccio et al., 1984, 1986). We have been unable to confirm this finding in the chick oviduct system; however, the chick oviduct contains two distinct forms of the estrogen receptor, R_x and R_y (McNaught & Smith, 1986; Raymoure et al., 1986). Since we have only characterized the R_{nb} to R_y conversion, it is possible that conversion to R_x involves tyrosine phosphorylation. In agreement with Auricchio's findings, direct phosphorylation of the estrogen receptor on a serine or threonine residue is not necessary for potentiation of the estradiol receptor. Fleming et al. (1983) reported that cyclic GMP mediated an increase in estradiol binding in both rat uterine cytosol and MCF7 cell cytosol.

Partial purification and characterization of F_y result in separation of kinase and phosphatase activities, each of which appear to be required for estrogen receptor potentiation. In vitro this conversion is not dependent upon the presence of estradiol (McNaught et al., 1990). Similarly, we have shown herein that estradiol is not required in vitro for stimulation of kinase and phosphatase activity. In the preceding paper, partially purified R_{nb} was used as substrate to monitor purification of F_y (McNaught et al., 1990). When immunopurified R_{nb} was used as substrate, partially purified F_y converted immunopurified R_{nb} to its estradiol binding form. However, in parallel experiments, HPLC-purified F_y was incapable of conferring steroid binding characteristics to immunopurified R_{nb}. The loss of this property was associated with separation of phosphatase activity from kinase activity in F_y during HPLC purification. The apparent anomaly that HPLC-purified F_y kinase activity was capable of potentiating partially purified R_{nb} is probably explained by the presence of contaminating phosphatase activity in R_{nb} which was shown to be removed during immunopurification.

Experiments were designed to characterize the ATP/Mg^{2+}-dependent enzymatic activities required for receptor potentiation. The ammonium sulfate, DEAE, and HPLC fractions containing F_y activity all had the ability to phosphorylate casein, mixed histones, histone H_1, and rabbit skeletal muscle glycogen synthase. Glycogen synthase is

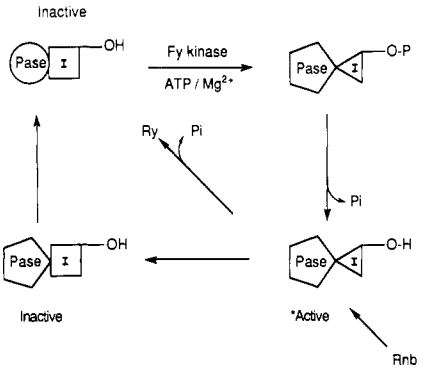
phosphorylated on seven different serines by five different protein kinases; the phosphorylation sites and the kinases have been well characterized (Cohen, 1986). Of these, cAMP-dependent protein kinase, Ca^{2+}/calmodulin-dependent multifunctional protein kinase II, casein kinase II, and casein kinase I (which also phosphorylates glycogen synthase to some extent) are the only protein kinases which also demonstrate significant phosphorylation of casein and histone H_1. HPLC-purified F_y has serine kinase activity and an apparent molecular weight of aproximately 40K (McNaught et al., 1990). The Ca^{2+}/calmodulin-dependent protein kinase II, which occurs as isoenzymes with subunit sizes between 50 and 60 kDa, is always seen with holoenzyme sizes ranging from 300 000 to 760 000 (Shenolikar et al., 1986). Ca^{2+}/calmodulin-dependent protein kinase II has been reported to phosphorylate the estrogen receptor in vitro (Schulman et al., 1985). However, an active monomeric form of this enzyme has not yet been demonstrated. Furthermore, in contrast to F_y, this enzyme shows an absolute requirement for calcium and calmodulin (which would be removed both by the ammonium sulfate precipitation step and by chromatography on DEAE-cellulose).

The purified catalytic subunit of cAMP-dependent protein kinase, isolated from rabbit skeletal muscle (Beavo et al., 1974), and casein kinase II partially purified from rat liver were examined for their ability to potentiate the receptor. Indeed, casein kinase II like enzyme activity has been reported to copurify with the progesterone receptor (Longeat et al., 1987). However, neither protein kinase was effective in augmenting estradiol binding. F_y kinase was also examined for its ability to be stimulated by 1 mM spermine or inhibited by 100 ng/mL heparin (characteristics of casein kinase II), or for inhibition by the specific protein inhibitor of cAMP-dependent protein kinase (Whitehouse & Walsh, 1983). The results of these experiments indicated that the kinase activity present in F_y was different to these kinases. We conclude that neither cAMP-dependent protein kinase nor casein kinase II is identical with F_y.

Protein phosphatase activity in the partially purified F_y fractions was measured by using phosphorylase *a* as substrate. This activity was separated from the kinase activity by HPLC gel filtration. The phosphatase reduced the phosphorylation of phosphoserine in the immunopurified receptor with equal efficacy. Cellular protein phosphatases with broad specificity are largely accounted for by two enzymes termed protein phosphatase 1 (PrP-1) and protein phosphatase 2A (PrP-2A). Indeed, phosphorylase *a* has been used to selectively assay these enzymes (Ingebritsen & Cohen, 1983). However, in spite of the similarities in properties of the phosphatases, highly purified catalytic subunits of PrP-1 (M_r 37 000) isolated from rabbit skeletal muscle (Resink et al., 1983) and PrP-2a (M_r 35 000) from bovine adrenals (Mumby et al., 1987) were ineffective in eliciting receptor potentiation.

The phosphatase inhibitors vanadate, fluoride, and pyrophosphate blocked potentiation of estradiol binding. The concentration of each compound which inhibited F_y-induced R_{nb} to R_y conversion precisely paralleled the inhibition of phosphorylase *a* phosphatase activity of PrP-1 and PrP-2A. However, the substrate specificity of the F_y phosphatase activity is different to both these enzymes (Table III). The specific nature of the oviduct phosphatase is also illustrated by the demonstration that its associated kinase activity is preferred when histones or histone H_1 is a substrate, but phosphatase activity is dominant when receptor is used as a substrate.

Scheme I: Model Depicting Activation of Phosphatase Activity by F_y Kinase Resulting in Conversion of R_{nb} to R_y[a]



[a] Pase, phosphatase; I, inhibitory regulatory subunit.

Finally, ATP-γ-S (thio-ATP) was used to assess the contribution of the kinase and phosphatase in the conversion of the receptor. Thio-ATP is used by many protein kinases, albeit less effectively (i.e., with slower rates and/or higher K_m) than ATP, to phosphorylate proteins. These resulting phosphoproteins are essentially resistant to dephosphorylation by phosphoprotein phosphatases (Ballou & Fischer, 1986; Gratecos & Fischer, 1974). Thus, the effects of a stable modification can be observed. In this respect, thio-ATP, even at 5 mM, failed to substitute for 0.5 mM ATP in the R_{nb} to R_y conversion process. These results strongly argue for a dephosphorylation process being involved in receptor potentiation.

Scheme I represents our speculation on the role of the kinase and phosphatase in estrogen receptor potentiation (conversion of $R_{nb} \rightarrow R_y$). Precedents for this scheme have been reviewed recently (Ballou & Fischer, 1986). The "ATP·Mg-dependent phosphatase" has been demonstrated in a variety of tissues. This enzyme exists essentially as a "latent or inactive" form and is activated in the presence of ATP/Mg^{2+} by a protein kinase. The kinase causes phosphorylation of a regulatory subunit of the phosphatase to result in a conformational change and activation of the phosphatase. Subsequent autodephosphorylation of the regulatory subunit still maintains the phosphatase in an active conformation. According to this scheme, it is postulated that F_y in an ATP/Mg^{2+}-dependent manner converts the chick oviduct phosphatase into the highly active form responsible for the dephosphorylation of R_{nb}, hence, its conversion to R_y, i.e., receptor potentiation. While in general terms our data support this speculation, more extensive studies involving the complete purification and characterization of the chick oviduct F_y phosphatase and kinase are ongoing.

In summary, two facts suggest a major role for protein phosphatase activity in the conversion process: (a) F_y purified via HPLC, which contains protein kinase but no phosphatase, was ineffective in the absence of chick oviduct phosphatase activity to convert R_{nb} to R_y; (b) several protein phosphatase inhibitors, at concentrations which inhibit phosphoserine phosphatase activity, effectively blocked the conversion process.

In conclusion, although we cannot absolutely exclude the involvement of other factors, chick oviduct phosphoprotein phosphatase activity appears to be essential in the conversion of the estrogen receptor from non-estradiol binding form to a potentiated receptor. This effect could not be mimicked by the addition of protein kinases or phosphatases isolated from other sources. The reason for this remains unknown but may be related to differences in the properties of the chick oviduct enzymes. Additional work is necessary to characterize these enzymes and further define their function on the regulation

of estrogen receptors in their role as transcription factors.

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