Estradiol-Binding Mechanism and Binding Capacity of the Human Estrogen Receptor Is Regulated by Tyrosine Phosphorylation

Steven F. Arnold*, Michal Melamed, Daria P. Vorojeikina, Angelo C. Notides, and Shlomo Sasson†

Departments of Environmental Medicine and Biophysics
(S.F.A., D.P.V., A.C.N.)
University of Rochester
School of Medicine and Dentistry
Rochester, New York 14642

Department of Pharmacology (M.M., S.S.)
Hebrew University of Jerusalem
Faculty of Medicine, School of Pharmacy
Jerusalem, 91120, Israel

We have investigated the effects of tyrosine phosphorylation on the estradiol-binding mechanism and binding capacity of the human estrogen receptor (hER). The wild type hER and a point mutant form of the hER, in which tyrosine 537 was mutated to phenylalanine (Y537F hER), were expressed in Sf9 insect cells. The wild type hER, but not the Y537F hER, reacted with a anti-phosphotyrosine monoclonal antibody, indicating that tyrosine 537 was the only tyrosine phosphorylated on the hER. Scatchard and Hill analyses of the the binding interaction of [^{3}H]estradiol with the wild type hER indicated that the addition of millimolar phosphotyrosine, but not tyrosine, phosphate, or phosphoserine, abolished the cooperative binding mechanism of the hER. These observations are consistent with the idea that phosphotyrosine blocks dimerization and site-site interactions between the hER monomers. The wild type hER bound 10-fold more [^{3}H]estradiol than the Y537F hER. Treatment of the purified wild type hER with a tyrosine phosphatase decreased the binding capacity of the hER by approximately 90%, whereas, a serine/threonine phosphatase had no effect. The estrogen-binding capacity of the tyrosine-dephosphorylated hER was completely restored by rephosphorylation of tyrosine 537 with p60$^{c\text{-}src}$, a tyrosine kinase. These results indicate that p60$^{c\text{-}src}$ can restore estrogen binding to the tyrosine-dephosphorylated hER and that dimerization and cooperative site-site interaction of the hER occur via a phosphotyrosine-binding interaction. (Molecular Endocrinology 11: 48–53, 1997)

INTRODUCTION

The estrogen receptor (ER) is a member of the steroid/thyroid hormone receptor superfamily of ligand-activated transcription factors (1). The steroid hormone receptors bind hormone response elements and direct steroid hormone-specific gene transcription (2). Therefore, factors that affect the hormone binding of the steroid hormone receptors will also modulate hormone-directed transcription. For example, the hormone-binding capacity of the glucocorticoid receptor appears to be regulated by posttranslational phosphorylation (3). Munck and Brinck-Johnsen (4) made the initial observation that the hormone-binding capacity of the glucocorticoid receptor is modulated by intracellular ATP levels. These investigators have proposed a cyclic model in which the phosphorylation state of the glucocorticoid receptor, perhaps in a cell cycle-dependent manner, regulates hormone binding (5). In addition, the glucocorticoid and progesterone receptors, complexed with the heat shock proteins, facilitate hormone binding (6).

Auricchio and co-workers (7, 8) have proposed that the estradiol binding of the ER is regulated by tyrosine phosphorylation. They reported that the human ER (hER) is phosphorylated on tyrosine 537, and that a purified tyrosine kinase from calf uterus phosphorylates the hER, but only in an estradiol-receptor complex and Ca^{2+}-calmodulin dependent manner (9–11). They have also isolated a tyrosine phosphatase that dephosphorylated the ER and eliminated estradiol

† The authors dedicate this paper to their beloved friend, Angelo C. Notides, who passed away November 10, 1996.

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binding (12). However, they have not identified the specific protein kinase(s) or phosphatase(s) involved. Fawell and co-workers (13) have reported that a carboxy-terminal deletion of the mouse ER to amino acid 538 (or 534 of the hER) retained only 25% of its estradiol binding, while deletion to amino acid of 508 (or 504 of the hER) abrogated estradiol binding.

Recently, we identified tyrosine 537 as one of the phosphorylation sites on the native hER from MCF-7 cells and on the recombinant hER from Sf9 insect cells (14). In contrast to the reports of Auricchio and coworkers (7–12), the phosphorylation of tyrosine 537 on the native hER from MCF-7 cells is not regulated by estradiol (14).

In this report, we propose that the phosphotyrosine and the residues surrounding phosphotyrosine 537 mediate dimerization by a phosphotyrosine of one hER monomer coupling to a phosphotyrosine-binding domain of the complementary hER monomer. We show that phosphorylation of tyrosine residue 537 of the hER induces conformational changes that regulate estradiol binding, hER dimerization, and the positive site-site cooperative [^{3}H]estradiol binding mechanism of the hER.

RESULTS

Expression of the Y537F hER Mutant in Sf9 Cells

Tyrosine 537 of the hER was mutated to phenylalanine, and the resulting mutant receptor, Y537F hER, was expressed in Sf9 insect cells using the baculovirus expression system. An ammonium sulfate precipitate of the wild type hER and the Y537F hER were separated by SDS-gel electrophoresis and electrotransferred to a poly(vinylidene) difluoride (PVDF) membrane for a Western blot. The anti-hER antibody recognized the wild-type hER and the Y537F hER at 66 kDa (Fig. 1). The PVDF membrane was stripped and reprobed for tyrosine phosphorylation of the hER with the 4G10 monoclonal anti-phosphotyrosine antibody. The anti-phosphotyrosine antibody reacted with the wild type hER, but not with the Y537F hER, indicating that tyrosine 537 was the only phosphorylated tyrosine residue on the hER (Fig. 1). Furthermore, the Y537F hER immunopurified with the anti-hER antibody revealed that it reacted with the anti-hER but not the anti-phosphotyrosine antibody. A 40% ammonium sulfate fraction of mock-infected Sf9 cells revealed no reactivity with the anti-hER or anti-phosphotyrosine antibody at 66 kDa (data not shown).

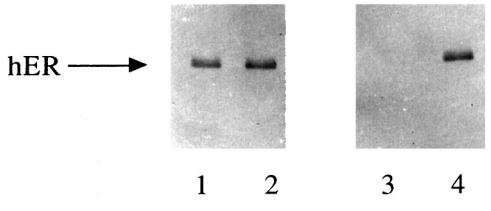

Fig. 1. A Western Blot of the Wild Type hER and the Y537F hER from Sf9 Cells

Y537F hER (lanes 1 and 3) and wild type hER (lanes 2 and 4) were separated by SDS-gel electrophoresis and electrotransferred to a PVDF membrane. The membrane was probed with an anti-hER antibody (*left panel*) then stripped and reprobed with an anti-phosphotyrosine antibody (*right panel*).

Estradiol Binding of the Wild Type hER as Compared with the Y537F hER

The effect of tyrosine phosphorylation on the estradiol-binding capacity of the hER was investigated by the comparing the ability of the wild type hER and the Y537F hER to bind [^{3}H]estradiol. Increasing amounts (25–100 μg protein of ammonium sulfate precipitate) of the wild type hER or the Y537F hER were incubated with [^{3}H]estradiol for 12 h at 4 C and the [^{3}H]estradiol-hER complex was measured by the hydroxyapatite (HAP) assay. The wild type hER bound approximately 10-fold more [^{3}H]estradiol than the Y537F hER (Fig. 2). The binding of [^{3}H]estradiol to the Y537F hER was not enhanced after incubation at 25 C for 1 h and remained approximately 10-fold less than the wild type hER (data not shown).

Estradiol Binding of the Purified Wild Type hER

To eliminate the possibility that the tyrosine to phenylalanine mutation produced a deleterious conformational change in the hER that was responsible for the

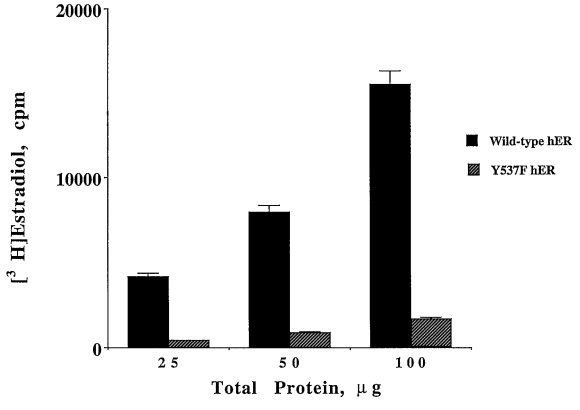

Fig. 2. Binding of [^{3}H]Estradiol by the Wild Type hER or the Y537F hER

Twenty five to 100 μg protein of the 40% ammonium sulfate fraction of the Sf9 cell expressing the wild type hER or the Y537F hER (each containing 30 fmol/μg protein) were incubated with 100 nM [^{3}H]estradiol or [^{3}H]estradiol plus a 200-fold excess of unlabeled estradiol for 12 h at 4 C. The [^{3}H]estradiol-hER complex was adsorbed to HAP and washed, and the radioactivity was measured. Each value represents the specifically bound [^{3}H]estradiol and is representative of four determinations in two separate experiments.

loss of estradiol binding, the estradiol binding and tyrosine phosphorylation of the wild type hER, purified to apparent homogeneity by ERE-affinity chromatography (15, 16), was investigated. The purified wild type hER reacted with anti-phosphotyrosine antibody, indicating it was phosphorylated on tyrosine 537 (14, 16). Dephosphorylation of the wild type hER with PTP1B, a tyrosine phosphatase, reduced the estrogen-binding capacity of the receptor by 90% (Fig. 3). The loss of estradiol binding by the wild type hER was blocked by the addition of 1 mM sodium vanadate, an inhibitor of PTP1B activity, indicating that the loss of estradiol binding was dependent on the phosphatase activity. Furthermore, after treatment with PTP1B, the purified hER did not react with the anti-tyrosine antibody, and proteolysis products were not detected by Western blot analysis (data not shown). Treatment of the purified wild type hER with potato acid phosphatase, a serine/threonine phosphatase, did not alter the estradiol-binding capacity of the hER (Fig. 3).

Conditions for the stoichiometric *in vitro* phosphorylation of the hER by the src family tyrosine kinase, $p60^{c\text{-}src}$, on tyrosine 537 have been reported (14). The estradiol binding of the tyrosine-dephosphorylated hER was increased 80% by rephosphorylation with $p60^{c\text{-}src}$ and ATP (Fig. 4). Estradiol binding was not restored to the tyrosine-dephosphorylated hER with the $p60^{c\text{-}src}$ in the absence of ATP (data not shown). This is consistent with our previous finding that the src family tyrosine kinases incorporate approximately 0.8 mol of [^{32}P]phosphate/mol of hER (14). The rephosphorylation of the tyrosine-dephosphorylated hER was specific for phosphotyrosine 537 because casein kinase II, which specifically phosphorylates serine 167

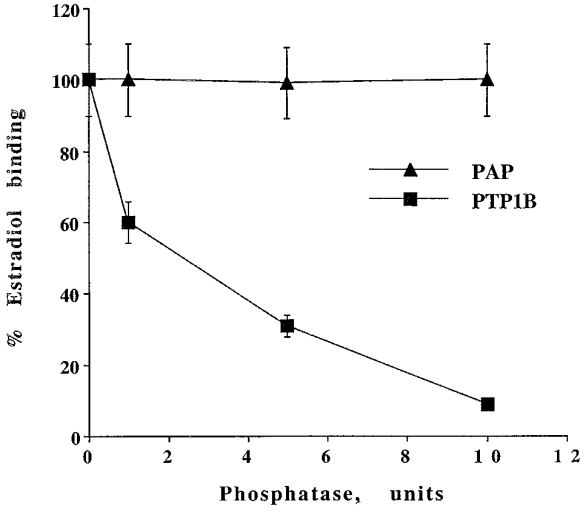

Fig. 3. Elimination of [^{3}H]Estradiol Binding by the Purified Wild Type hER

The purified wild type hER was dephosphorylated with potato acid phosphatase (PAP) or PTP1B, a phosphotyrosine phosphatase, then assayed for [^{3}H]estradiol binding. Each value is an average of three determinations in two separate experiments. The 100% value was 1250 ± 125 dpm.

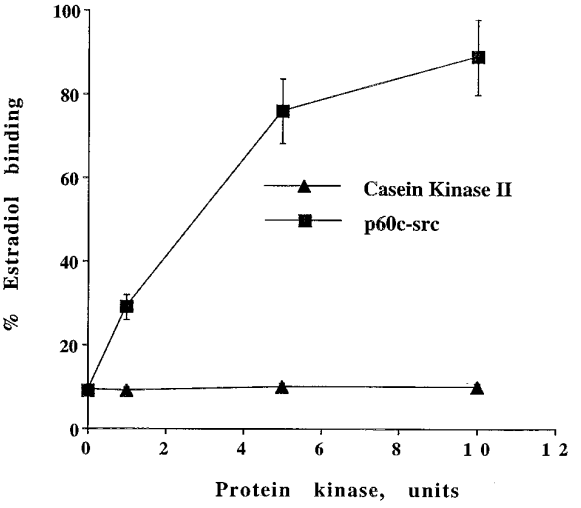

Fig. 4. Restoration of [^{3}H]Estradiol Binding to the Purified Wild Type hER

The tyrosine-dephosphorylated hER was rephosphorylated with casein kinase II or $p60^{c\text{-}src}$ and then assayed for [^{3}H]estradiol binding. Each value is an average of three determinations in two separate experiments. The 100% value was 1250 ± 120 dpm.

on the hER (17, 18), had no effect on the estradiol-binding capacity. Phosphorylation of the purified phosphorylated hER with $p60^{c\text{-}src}$ further increased its estradiol-binding capacity by 20–30%, indicating that purified wild type hER was not completely phosphorylated on tyrosine 537 (18).

Effect of Phosphotyrosine on the Equilibrium-Binding Mechanism of [^{3}H]Estradiol with of the Wild-Type hER

ERs from various species including hER bind estradiol in a positive cooperative manner (20). [^{3}H]Estradiol binding with 10 nM wild type hER shows a convex Scatchard plot and a Hill coefficient (nH) of 1.39 (Fig. 5), indicative of positive cooperative binding interaction and receptor homodimerization (20, 21). When the binding assay was performed in the presence of 0.4 mM phosphotyrosine, the Scatchard plot appeared linear, and the nH was reduced to 1.03, indicating absence of positive cooperative binding interaction and conformational changes associated with receptor homodimerization. This inhibitory effect was specific to phosphotyrosine, because 0.4 mM phosphoserine in the binding assay did not affect the positive cooperative binding interaction of [^{3}H]estradiol with the hER, which remained highly cooperative (nH = 1.41).

DISCUSSION

This study demonstrates that the tyrosine 537 to phenylalanine mutation of the hER severely reduced the

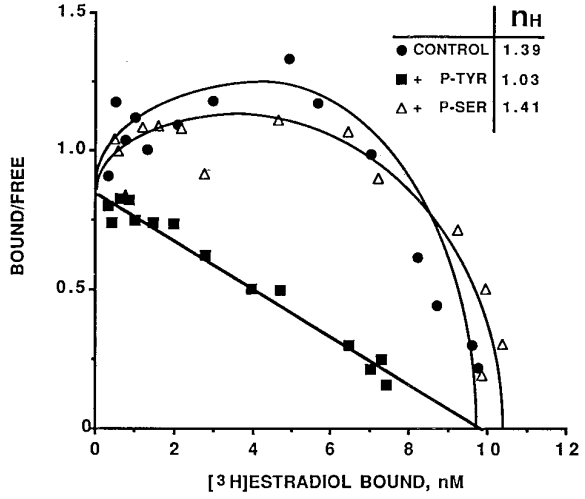

Fig. 5. Equilibrium Binding Analysis of [^{3}H]Estradiol Binding with hER.

The receptor (10 nM) was incubated without or with 0.4 mM phosphotyrosine or phosphoserine and [^{3}H]estradiol (0.5–60 nM) as described in *Materials and Methods.* The binding data were analyzed and presented according to Scatchard and Hill.

receptor's ability to bind estradiol. Purified wild type hER dephosphorylated at tyrosine 537 also displayed reduced estradiol-binding capacity. The estradiol binding of the wild type hER was restored by site-specific phosphorylation on tyrosine 537 with the tyrosine protein kinase, p60$^{c\text{-}src}$.

There are several mechanisms by which tyrosine phosphorylation may regulate the estradiol-binding capacity of the hER. First, the tyrosine phosphorylation of the hER may result in a conformational change in the ligand-binding domain. The tyrosine phosphorylation may promote the formation of an estradiol-binding pocket with a higher affinity for estradiol than the non-tyrosine-phosphorylated hER. Katzenellenbogen and co-workers (22) have suggested amino acids 515 to 535 at the carboxy terminus are important for the formation of the ligand-binding pocket.

A second, and more likely, possibility is that tyrosine 537 phosphorylation, receptor homodimerization, and estrogen binding of the hER are linked. We have demonstrated that the hER requires tyrosine phosphorylation for dimerization through a phosphotyrosyl-SH2 domain-binding mechanism (16, 18). The enhancement of hER dimerization by tyrosine phosphorylation may increase the receptor's estradiol-binding capacity and affinity by its cooperative estrogen-binding mechanism. The cooperative binding mechanism involves site-site interactions between monomers of the dimeric ER in which estradiol binding by one monomer induces conformational changes in the dimeric receptor that results in an increased affinity of the second monomer for estradiol (20). Therefore, phosphorylation of tyrosine 537 on the hER increases the capacity and affinity for estradiol by a change in the estradiol-binding mechanism, from a noncooperative to a cooperative hormone-binding mechanism, through an acquisition of the receptor to undergo dimerization.

The basal phosphorylation of tyrosine 537, which occurred independently of estrogen binding, is in the hormone-binding region of the receptor that regulates dimerization (16, 18). It has been shown that this phosphorylation is required for binding of hER to an estrogen response element (18). The dimerization of the hER is probably mediated by coupling between phosphotyrosine 537 of the hER and a phosphotyrosine-binding domain (*i.e.* SH2-like domain) on the hER (16). Further support for this mechanism comes from the ability of phosphotyrosine, but not phosphoserine, to eliminate the estradiol-induced cooperative binding interaction and receptor dimerization. This effect of phosphotyrosine is concentration-dependent (data not shown). It therefore seems that phosphotyrosine competes with the phosphorylated tyrosine 537 to an SH2-like domain in the receptor, thereby hindering the dimerization process.

We hypothesize that the regulation of estradiol binding by tyrosine phosphorylation occurs through the p60$^{c\text{-}src}$ family of tyrosine kinases that are coupled to cell-signaling pathways. Very relevant and analogous with these findings are the signal transducers and activators of transcription (STAT) proteins (23). Tyrosine phosphorylation of STATs promotes their homo- and heterodimerization, which allows their translocation to the nucleus and interaction with specific recognition elements to initiate transcription (23). The dimerization of the STAT proteins is mediated by reciprocal coupling between phosphotyrosine on one monomer and a SH2 domain on the opposing monomer (24).

In conclusion, we have demonstrated that phosphorylation of tyrosine 537 is responsible for the regulation of estradiol binding of the hER. We believe that the tyrosine phosphorylation of the hER is regulated by cell-signaling pathways, perhaps in a cell cycle-specific fashion, which controls the hER's ability to direct estradiol-dependent gene transcription.

MATERIALS AND METHODS

Materials

17β-[6,7-^{3}H(N)]estradiol (45.6 Ci/mmol) was purchased from DuPont/New England Nuclear (Boston, MA). Leupeptin was obtained from Peninsula Laboratories (Belmont, CA). The potato acid phosphatase was from Boehringer Mannheim (Indianapolis, IN). The 4G10 monoclonal anti-phosphotyrosine antibody, p60$^{c\text{-}src}$ (1 pmol PO_4 transferred/min/mg), and PTP1B (4 nmol PO_4 removed/min/mg) were from Upstate Biotechnology (Lake Placid, NY). The casein kinase II (3 μmol PO_4 transferred/min/mg) was a generous gift of Drs. D. W. Litchfield and E. G. Krebs. Pepstatin, phenylmethylsulfonyl fluoride (PMSF), phosphotyrosine, and phosphoserine were from Sigma (St. Louis, MO). All other chemicals were reagent grade.

Preparation of the hER from Sf9 Cells

The production and expression of the recombinant baculovirus, AcNPV-hER, carrying the cDNA of the wild type hER, has been described (15). Whole cell extracts of the Sf9 cells containing the wild type hER or Y537F hER were prepared as described (14, 15). The whole cell extracts were made 40% saturated with ammonium sulfate, and the precipitate was collected by centrifugation. An ammonium sulfate preparation of the wild type hER in the absence of estradiol was purified on a ERE-Teflon affinity matrix as previously described with the inclusion of the phosphatase inhibitors: 50 mM sodium fluoride, 10 mM sodium pyrophosphate, 1 mM sodium orthovanadate, and 50 nM okadaic acid (15).

SDS-Gel Electrophoresis and Western Blot Analysis

Ammonium sulfate preparations, containing approximately 25 μg of protein, of the wild-type hER and the Y537F hER were added to Laemmli sample buffer and separated on a 10% acrylamide SDS-gel at 30 mA for 5 h (19). The SDS-gel was electrotransferred to a PVDF membrane (Millipore, Bedford, MA). The membrane was blocked in 2% (wt/vol) BSA for 3 h at room temperature, then probed with anti-hER antibody 6 (15). The bands were visualized by chemiluminesence using the enhanced chemiluminescence (ECL) system (Amersham, Arlington Heights, IL). The PVDF membranes were stripped for reprobing in 62.5 mM Tris-HCl, pH 6.7, 100 mM 2-mercaptoethanol, and 2% SDS for 30 min at 50 C, then rinsed with PBS and reprobed with the horseradish peroxidase-conjugated monoclonal 4G10 anti-phosphotyrosine antibody (1:1000 dilution) for 3 h.

Estradiol Binding of the hER

Ammonium sulfate preparations of the wild type hER or the Y537F hER (30 fmol/μg protein, for the experiment shown in Fig. 2) or the purified wild type hER (15 fmol, for the experiment shown in Fig. 3) were added to the binding buffer consisting of: 20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM EGTA, 100 mM KCl, 50 mM sodium fluoride, 10 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 50 nM okadaic acid, 10% (vol/vol) glycerol, 0.5 mM leupeptin, and 0.2 mM PMSF. The concentration of the Y537F hER was estimated by comparing the Y537F hER to known quantities of the purified wild type hER on a Western blot. The purified wild type hER was determined by its specific [^{3}H]estradiol binding, SDS-gel electrophoresis, and quantitative protein determination (data not shown). Mock-infected Sf9 whole cell extract or bovine γ-globulin was added to the binding buffer to give a final protein concentration of 1.5 mg/ml. [^{3}H]Estradiol at a final concentration of 100 nM was added, while the nonspecific binding was measured by a parallel incubation with [^{3}H]estradiol plus a 200-fold excess of estradiol for 12 h at 4 C. After the incubation, 100 μl of a 50% slurry of HAP in the binding buffer was added and allowed to adsorb the hER for 40 min at 4 C. The HAP was washed three times with 0.5 ml of the binding buffer. The HAP pellets were suspended in 0.5 ml ethanol, scintillation fluid was added, and the radioactivity was measured.

The effect of phosphotyrosine and phosphoserine on the equilibrium-binding mechanism of [^{3}H]estradiol to the wild type hER was determined according to Melamed *et al.* (21). Ammonium sulfate precipitates of the hER were dissolved in TDEE buffer (40 mM Tris-HCl, pH 7.4, l mM dithiothreitol, 1 mM EDTA, 1 mM EGTA) containing 0.1 mM PMSF. 0.2 mM leupeptin, 1 μg/ml pepstatin, 10% (vol/vol) glycerol, 100 mM KCl, 1 mM orthovanadate, 1 mM sodium pyrophosphate, and 10 mg/ml bovine γ-globulin. Tubes containing 200 μl of hER preparation were preincubated in duplicates without or with 0.4 mM phosphotyrosine or phosphoserine and varying [^{3}H]estradiol concentrations (0.5 to 60 nM) and incubated for 1 h at 25 C. Nonspecific binding was measured by a parallel incubation of the receptor with radioactive estradiol in the presence of a 200-fold excess of unlabeled estradiol. Nonspecific binding was less then 5% of total [^{3}H]estradiol binding. During the incubation period, a 50-μl sample from each tube was removed to determine the total [^{3}H]estradiol concentration. At the end of the incubation, the tubes were cooled on ice for 5 min; 100 μl of 1% (wt/vol) charcoal and 0.01% (wt/vol) dextran 500 suspension in TDEE buffer were then added and incubated for 10 min at 4 C. The suspension was then centrifuged, and a 100-μl aliquot of the supernatant was removed for scintillation counting. Determination of specific [^{3}H]estradiol binding and Scatchard (25) and Hill (26) analyses were performed as previously described (21). Stability of the receptor was assayed as described previously (21).

In Vitro Tyrosine Dephosphorylation/Phosphorylation of the Purified Recombinant hER

The *in vitro* dephosphorylation of the purified wild type hER (15 fmol in 20 μl) was carried out in phosphatase buffer [50 mM HEPES, pH 7.4, 150 mM NaCl, and 5% (vol/vol) glycerol]. Five microliters of PTP1B, conjugated to agarose beads, were added to the receptor and incubated for 30 min at 37 C. The products in the supernatant were recovered by centrifugation at 15,000 $\times$ *g* at 4 C and subsequent washing of the beads with phosphatase buffer. In other experiments, 10 μl of potato acid phosphatase were added for 1 h at 4 C, and the estradiol binding was performed as described above. The *in vitro* rephosphorylation reactions were done as follows: the purified dephosphorylated wild type hER (15 fmol) was suspended in 50 μl of p60$^{c\text{-}src}$ reaction buffer (20 mM Tris HCl, pH 7.4, and 50 mM $MgCl_2$) or 50 μl casein kinase II (CKII) reaction buffer (50 mM Tris HCl, pH 7.6, and 10 mM $MgCl_2$) and 1 mM ATP. Next, 3 U of p60$^{c\text{-}src}$ or 1 U of CKII were added to initiate the reaction. The reaction was carried out for 15 min at 30 C and was terminated by placing the tubes at 4 C, after which the estradiol binding was performed as described above.

Site-Directed Mutagenesis of the hER

Oligonucleotide site-directed mutagenesis of the hER was performed essentially according to the method of Kunkel (27). A single-stranded template was prepared from M13mp19 containing the hER cDNA grown in *Escherichia coli* strain CL236. A 28-bp primer that contained a mutation to change tyrosine 537 to phenylalanine and a novel restriction site, *Xho*I, was used. The oligonucleotide primer was phosphorylated for 45 min at 37 C by T4 polynucleotide kinase (Bio-Rad, Hercules, CA) in 50 mM Tris, pH 7.5, 10 mM $MgCl_2$, 5 mM dithiothreitol, and 1 mM ATP. The phosphorylated oligonucleotide and the UTP-containing DNA templates were mixed at a 5:1 molar ratio in 10 mM Tris, pH 7.5, and 5 mM $MgCl_2$ for the annealing reaction, heated to 70 C, and then slowly cooled to room temperature. A polymerase reaction was carried out in 20 mM Tris, pH 7.5, 10 mM $MgCl_2$, 5 mM dithiothreitol, 500 μM deoxynucleoside triphosphates, 1 mM ATP, 2.5 U of the Klenow fragment of DNA polymerase, 1 U of T4 DNA ligase, and 2 μg of gene 32 T4 protein and was carried out for 16 h at 16 C. The double-stranded DNA was used to transform *E. coli* strain NM 1193. All mutants were verified by the restriction enzyme digestion with *Xho*I and DNA sequencing. The mutated cDNA of the hER was cloned into the *Eco*RI site of the pVL1393 baculovirus transfer vector. The orientation of the cloned fragment was confirmed by a digestion with *Bgl*II. The resulting baculovirus vector, AcNPV-Y537F, was purified by CsCl centrifugation and transfected into Sf9 cells using the BaculoGold Linearized Baculovirus DNA (PharMingen, San Diego, CA.) and Lipofectin Reagent (GIBCO BRL, Grand Island, NY).

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Address requests for reprints to: Angelo C. Notides, Department of Environmental Medicine, Box EHSC, University of Rochester School of Medicine and Dentistry, Rochester, New York 14642.

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* Current address: Center for Bioenvironmental Research, Tulane University, New Orleans, Louisiana 70112.

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