Function of Estrogen Receptor Tyrosine 537 in Hormone Binding, DNA Binding, and Transactivation†

Matthew R. Yudt,‡,§,|| Daria Vorojeikina,‡,§ Li Zhong,⊥ Debra F. Skafar,⊥ Shlomo Sasson,@ Thomas A. Gasiewicz,*,‡ and Angelo C. Notides‡,$

Departments of Biochemistry and Biophysics and of Environmental Medicine, University of Rochester School of Medicine and Dentistry, Rochester, New York 14642, Department of Physiology and Karmanos Cancer Institute, Wayne State University School of Medicine, Detroit, Michigan 48201, and Department of Pharmacology, Hebrew University School of Medicine, P.O. Box 12272, Jerusalem 91120, Israel

Received May 14, 1999; Revised Manuscript Received August 27, 1999

ABSTRACT: The human estrogen receptor (hER) is a ligand-activated transcription factor which, like many other members of the nuclear receptor protein family, exhibits a dimerization-dependent transcriptional activation. Several previous reports have provided evidence of the phosphorylation of the hER at tyrosine 537 (Y537). However, the exact function of a putative phosphorylation at this site remains controversial. Using a yeast transactivation assay, and in vitro biochemical approaches, we show that phosphorylation of tyrosine 537 is not required for the hER to bind hormone, or to activate transcription. An hER tyrosine 537 to phenylalanine (Y537F) mutant retains 70−75% of the transactivation potential of wild type hER in a yeast reporter system. Furthermore, the mutated receptor exhibits wild type hormone and DNA binding affinities. However, this mutation results in a decrease in receptor stability as measured by a decrease in the extent of hormone binding over time. The most striking difference between the wild type and Y537F hER is in the estradiol binding kinetics. Whereas the off-rate for estradiol exhibits a two-state binding mechanism, the Y537F mutant hER exhibits a monophasic estradiol off-rate. On the basis of these data and other reports describing the structure and activity of Y537 mutations, as well as knowledge of the three-dimensional structure of the hER ligand binding domain, we propose an alternate model wherein Y537F mutation favors an "open" pocket conformation, affecting the estrogen binding kinetics and stability of the hormone-bound, transcriptionally active "closed" pocket conformation. Although its phosphorylation is not essential for function of the hER, Y537 is nevertheless a critical residue intricately involved with the conformational changes of the hER and its ability to activate transcription.

The human estrogen receptor (hER)[1,2] is an essential component of a variety of signal transduction pathways, culminating in the transcription of estrogen-responsive genes (*1*). Multiple signaling factors are involved in the regulation of hER-mediated transcription, including the type of ligand bound (*2*), the interaction of the receptor with other protein coregulators (*3*), and the phosphorylation status of the receptor (*4*, *5*).

The hER, like all other members of the nuclear hormone receptor superfamily, is composed of multiple structural and functional domains (*6*). The ligand binding domain (LBD) not only binds its target ligands but contains a large dimerization interface, and a hormone-dependent transactivation function, termed AF-2 (*7*). The transcriptionally active hER is a homodimer. The AF-2 can be defined as the surface generated by the conformation of the carboxy-terminal α-helix (helix 12, hER amino acids 538−545) formed upon ligand binding. Crystal structure analysis has shown that different classes of ligands confer distinct and opposite orientations of this helix, and hence generate different binding surfaces for the transcription intermediary coactivator proteins (*8*, *9*).

Peptide mapping and Western blotting studies using antiphosphotyrosine antibodies provide suggestive evidence for Y537 phosphorylation (*10*, *11*). Several hypotheses have been developed for the function of Y537 phosphorylation. In calf uterine cytosol and MCF-7 breast cancer cells, Y537 phosphorylation reportedly plays a role in the hormone

† This work was supported by NIH Grant HD06707 and Center Grant ES01247. M.R.Y. was supported by a U.S. Army MRMC breast cancer research predoctoral fellowship (DAMD 17-96-1-6219). D.F.S. was supported by grants from the NSF (IBN-940736), the Karmanos Cancer Institute, and the National Cell Culture Center.

* To whom correspondence should be addressed. Phone: (716) 275-7723. Fax: (716) 256-2591. E-mail: gasiewt@envmed.rochester.edu.

‡ University of Rochester School of Medicine and Dentistry.

§ These authors contributed equally to this work.

|| Present address: NIEHS, P.O. Box 12233, MD E202, Research Triangle Park, NC 27709.

⊥ Wayne State University.

@ Hebrew University School of Medicine.

$ This paper is dedicated to the memory of the late Professor Angelo C. Notides.

[1] Abbreviations: hER, human estrogen receptor α; LBD, ligand binding domain; AF-2, activation function 2; ERE, estrogen response element; wt, wild type; Y537F, tyrosine 537 to phenylalanine mutation; TBE, Tris-borate-EDTA; TDEG, Tris-DTT-EDTA-glycine; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; H12, helix 12; SEM, standard error of the mean.

[2] There are two forms of the estrogen receptor, α and *β*. Estrogen receptor α was used for the studies described here, and hER denotes the α form, unless otherwise stated.

10.1021/bi9911132 CCC: $18.00
Published on Web 10/08/1999

binding activation of hER (*12*). Other studies have proposed additional roles for Y537 phosphorylation in receptor homodimerization, a prerequisite for DNA binding and transactivation (*10*, *13*).

Recently, the physiological importance of hER Y537 was suggested by the detection of a naturally occurring tyrosine to asparagine mutation (Y537N) at this site in a metastatic breast carcinoma (*14*). When transfected into mammalian cell cultures, the resulting Y537N mutant hER exhibited a potent estradiol-independent activation. This result was corroborated by two independent reports characterizing constitutively active mouse and human estrogen receptors mutated at Y537 (*15*, *16*). This ligand-independent activation phenomenon has been directly correlated with the binding of coactivators to the unliganded hER (*17*, *18*). However, in those studies, a tyrosine to phenylalanine mutant (Y537F) did exhibit hormone-dependent transcriptional activation, similar to that of the wild type (wt) protein (*15*, *16*).

To resolve these apparently contradictory reports, we have continued our study of hER Y537. We report here that the Y537F mutation does not significantly alter the hER affinity for either the estrogen response element (ERE) or estrogenic ligands. The Y537F hER exhibits a modest 25−30% decrease in the extent of hER-mediated transcription in a yeast expression reporter assay. The recombinant Y537F hER appears to be less stable than the wt in the presence and in the absence of estradiol. These observations are partially explained by the demonstration of altered estradiol binding kinetics between the wt and Y537F hER. In summary, we demonstrate that Y537 and its phosphorylation are not essential for hER dimerization and function, but nevertheless contribute to critical conformational changes and protein stability.

EXPERIMENTAL PROCEDURES

Materials. [^{3}H]Estradiol (50 Ci/mmol) and [γ-^{32}P]ATP (3000 Ci/mmol) were purchased from Dupont-NEN (Boston, MA). The ICI 182,780 was kindly provided by A. E. Wakeling (Zeneca Pharmaceuticals, Mereside, U.K.). Tamoxifen citrate was obtained from Stuart Pharmaceuticals (Wilmington, DE). Glass beads, 0.5 mm in diameter, for breaking yeast cell walls, were purchased from Biospec (Bartesville, OK). Bio-Rad's Muta-Gene in vitro mutagenesis kit was used for site-directed mutagenesis, and the dideoxy Sequenase kit from US Biochemical Corp. was used for sequencing. Anti-hER polyclonal antibody 6, directed against amino acids 259−278 (*19*), was affinity purified for use in Western blotting, and reacted equivalently with all hER mutants described in the text. Secondary antibodies were purchased from Santa Cruz Biotech.

Site-Directed Mutagenesis. Oligo-directed mutagenesis of the hER was performed by the method of Kunkel (*20*). The hER cDNA (HEGO) was cloned into M13mp19 to produce single-stranded DNA. The following oligonucleotides were used for mutagenesis: oligo 1 (Y537F), 5′-CAG-CAG-CTC-GAA-GAG-GGG-CAC-CAC-3′; and oligo 2 (L540Q), 5′-CAT-CTC-CAG-CTG-CAG-CTC-ATA-GAG-GGG-5′. Y537F-mutated DNA was used for the second round of mutagenesis together with oligo 2 to synthesize the double mutant Y537F/L540Q (dm). All mutations were verified by DNA sequencing. Mutated hER cDNA was cloned into the *Eco*RI site of both the yeast expression vector pSCW231 (*21*) and the baculovirus transfer vector pVL1393 (Invitrogen, San Diego, CA). The orientation of cloned inserts was verified by restriction enzyme digests with *Bgl*II and *Sma*I.

Preparation of Recombinant hERs. Spodptera frugipedra (*Sf9*) cells were infected with baculovirus containing wt or mutant hER cDNA, grown for 4 days at 27 °C, and lysed by repeated freeze−thaw cycles in a hypotonic buffer [20 mM Tris, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 1 mM Na_3VO_4, 0.5 mM PefaBlock (Boehringer Manheim), and 80 μM leupeptin (pH 7.4)]. KCl was added to a final concentration of 500 mM, and extracts were incubated on ice for 30 min before centrifugation at 28000*g*. Supernatants were used as soluble cell extracts, or the hER was partially purified by 40% ammonium sulfate precipitation. Protein extracts from *Saccharomyces cerevisiae* were prepared by the glass bead method according to Kaiser et al. (*22*).

Hormone Binding Assays. Ammonium sulfate-precipitated hERs were used with final hER concentrations of 2 or 10 nM in a TDEEK buffer [40 mM Tris-HCl (pH 7.4), 1 mM DTT, 1 mM EDTA, 1 mM EGTA, 0.2 mM PefaBlock, 0.5 mM leupeptin, 15% glycerol, and 150 mM KCl]. Ovalbumin was added to achieve a final protein concentration of 5 mg/mL. For saturation binding experiments, the receptor preparation was incubated with various concentrations of [^{3}H]-estradiol (0.3−100 nM). The extent of nonspecific binding was measured by a parallel incubation with a 200-fold molar excess of unlabeled estradiol. Following incubation for 16 h on ice, an aliquot of each mixture was removed to determine the total [^{3}H]estradiol concentration. The unbound hormone was removed by incubation with a dextran-coated charcoal solution (0.03% and 0.3% final concentrations). Following liquid scintillation counting, the extent of specific binding was obtained by subtracting the extent of nonspecific binding from that of total binding. The K_d was determined by Scatchard analysis.

Electrophoretic Mobility Shift Assays. Whole cell extracts or ammonium sulfate fractions of *Sf9*-produced hER were prepared as described above. The gel mobility experiments were performed by incubation of equivalent amounts (approximately 6 nM) of wt or mutant receptor extracts in binding buffer [20 mM HEPES (pH 7.4), 1 mM EDTA, and 0.2 mM Na_3VO_4] with protease inhibitors (1 mM leupeptin and 0.5 mM PefaBlock). Final concentrations of salt and protein were maintained at 100 mM and 0.2 mg/mL, respectively. A double-stranded 27 bp probe was end labeled with [γ-^{32}P]ATP and gel purified, and the specific activity was determined using thin-layer chromatography and liquid scintillation counting (1−10 fmol = 20 000 cpm). One microgram of poly(dI-dC) (Pharmacia Biotech) was used to block nonspecific DNA binding. Following incubation for 1 h on ice, the samples were electrophoresed on a 5% nondenaturing polyacrylamide gel for 2.5 h at 175 V and 4 °C in a 0.5× TBE running buffer. The gels were dried and exposed to PhosphorImager plates. Band intensities were quantified using ImageQuant software (Molecular Dynamics) and saturation plots created and analyzed using Sigma Plot. For Scatchard analysis, bands were excised and analyzed by Cherenkov counting.

Yeast Strains, Growth Conditions, and Transfections. The *S. cerevisiae* yeast strain 939 was used for all experiments (*23*). Yeast cells were grown in minimal yeast medium

[0.67% (w/v) yeast nitrogen base without amino acids and 2% (w/v) glucose] supplemented with the required amino acids. Yeast cells were transformed by the lithium acetate procedure (*24*). The cells were then plated on minimal yeast medium supplemented with leucine (100 mg/mL). In these transformations, plasmid DNA consisted of a 1:2 mixture of the yeast expression vector pSCW231-hER or appropriate mutant hER and the reporter plasmid YRPE2 (*25*). This plasmid contains two copies of a consensus estrogen-responsive element upstream of the cyc promoter linked to a *lacZ* reporter gene. Successfully transformed yeast cells were able to restore auxotrophy and grow on synthetic glucose minimal plates without uracil and tryptophan (SD/-ura-trp).

Quantitative β-Galactosidase Assays. Yeast cells from a single colony were grown overnight at 30 °C to an OD_{600} of 1.0−1.5. Following dilution to an OD_{600} of 0.1, 2 mL aliquots were transferred to 18 mm diameter glass tubes where 20 μL of the appropriate ligand solution was added. Incubation was continued in an orbital shaker at 250 rpm for 16 h. The ligand-treated cells were collected by centrifugation, and β-galactosidase activity was assayed using whole, permeabilized cells. The values were expressed in Miller units as previously described (*21*).

Western Blots. Western blots were created as described previously (*19*), using a polyclonal antibody raised in our laboratory for hER detection. Quantification of band intensities was carried out by densitometry using a range of protein concentrations and multiple film exposures. For antiphosphotyrosine blotting, the manufacturer's recommended protocol was followed. Several antiphosphotyrsoine antibodies were used, including the PY20 presented here, and PY66 antibodies from Santa Cruz Biotechnology (Santa Cruz, CA), as well the 4G10 antibody from Upstate Biotechnology (Lake Placid, NY).

Stability of the Hormone-Free Receptor. The wt and mutant Y537F hER expressed as histidine-tagged fusions in *Sf9* insect cells were used for these experiments in addition to the non-histidine-tagged receptor. Cell pellets were lysed in TDEG buffer with 20 mg/mL ovalbumin and 0.4 mM PMSF, 0.1 mM leupeptin, 1.5 μM aprotinin, and 2 μM E64 in a Dounce tissue homogenizer and centrifuged at 220000*g* for 30 min. Soluble extracts were then incubated in the absence of hormone at 25 °C for up to 4 h with aliquots being removed at the indicated times. Hormone binding activity was measured after incubation for 1 h at 0 °C with labeled estradiol as described above.

Dissociation of [^{3}H]Estradiol from the wt and Y537F Mutant hER. Extracts from *Sf9* insect cells expressing either the wt or Y537F mutant hER were prepared and incubated with 20 nM [^{3}H]estradiol for 60 min at 25 °C. The unbound steroid was removed by incubation with 0.5 volume of dextran-coated charcoal suspension in TDE buffer for 10 min on ice. The charcoal was pelleted by centrifugation (5 min at 1000*g*). The supernatant was removed, and 10 μM unlabeled estradiol was added and the incubation continued at 25 °C. At the indicated times, 0.2 mL aliquots were removed and incubated with 0.1 mL of the dextran-coated charcoal suspension for 10 min on ice. The charcoal was pelleted with centrifugation, and 0.1 mL of the supernatant was removed for measurement of bound [^{3}H]estradiol using liquid scintillation counting. The extent of inactivation of the receptor was measured in parallel incubations in which the first dextran-coated charcoal treatment was eliminated.

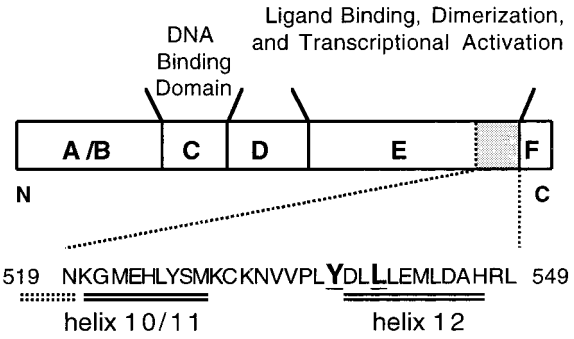

FIGURE 1: Sequence surrounding helix 12 of the hER ligand binding domain. The boundaries of the structural and functional domains of the hER are illustrated in relation to the helix 12−AF-2 core region in the ligand binding domain. Amino acids mutated in this study are shown as tall bold letters (Y537 and L540). The location of helices 11 and 12 is also indicated by the underlined sequences (*8*).

The data were analyzed using GraphPad PRISM, and were fit to either a two-phase or a one-phase exponential decay as indicated. The values of the fast dissociation component were corrected by subtraction of the contribution of the slow phase component. All values were plotted semilogarithmically, with the *Y*-axis being the percentage of bound [^{3}H]-estradiol.

RESULTS

Estrogen Receptor Mutations in the AF-2 Core Region of the Hormone Binding Domain. The mutation of tyrosine 537 to phenylalanine was initially introduced so that the putative role of phosphorylation on this residue in receptor dimerization and activation could be investigated (*13*). A second mutation, L540Q, was also prepared, which has been documented to disrupt transcriptional coactivator interactions with the hER and exhibits a dominant negative phenotype (*26*, *27*). A third hER mutant, Y537F/L540Q, containing the mutations at both residues was also prepared. Via study of the Y537F mutation alone and in the context of a second mutation not only close by but also with an established phenotype, the role of this residue in receptor function can be further dissected. Figure 1 shows the location of these residues in relation to the known secondary structure associated with the region.

Transcriptional Activation of wt and Mutant Estrogen Receptors in Yeast. A yeast expression reporter system was constructed so the transcriptional activation properties of the wt and mutant hERs could be investigated (*21*). The hERs expressed in yeast were transcriptionally active and hormone-responsive (Figure 2). The relative transcriptional activities of wt and mutant hERs were measured after treatment with 10 nM estradiol (Figure 2A). The Y537F, L540Q, and Y537F/L540Q mutants (dm) exhibited approximately 25, 50, and 75% decreases, respectively, in the level of transactivation relative to that of wt. Yeast cells containing an expression vector lacking the hER cDNA or those containing a cDNA inserted in the opposite orientation (inverted) showed no estradiol response.

A Western blot with a purified anti-hER polyclonal antibody indicated a consistent and equivalent level of the

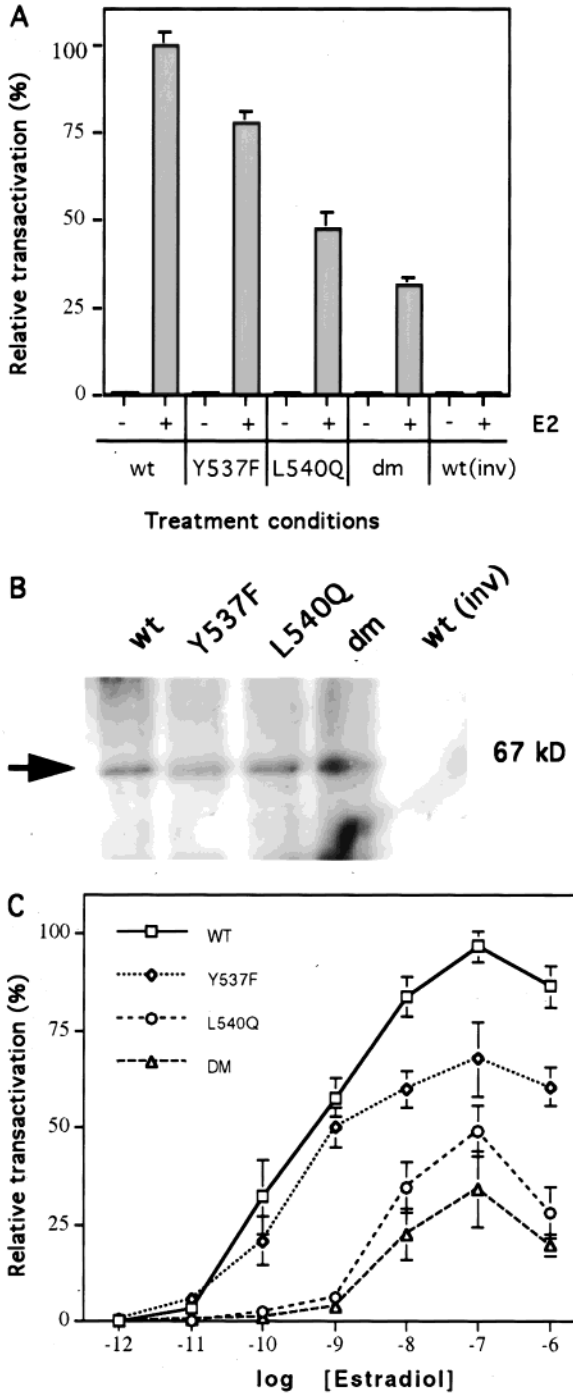

FIGURE 2: Transcriptional activation of wt and mutant hER in yeast. (A) The yeast strains containing either the wt or indicated mutant hER and the ERE reporter plasmid were grown at 30 °C for 18 h in the presence or absence of 10 nM estradiol. Transcriptional activities of hER were measured by the β-galactosidase assay and are shown as a percentage of the maximal response, measured in Miller units. The data shown are the means ± SEM for 8−20 individual samples from three to five different experiments. (B) Western blot of yeast-expressed hERs. Yeast cells, prepared as described above, were lysed in SDS sample buffer, boiled for 5 min, and subjected to Western blot analysis with anti-hER antibody. A representative result from three experiments is shown. The wt(inv) contains yeast extract in which the yeast expression vector has an inverted orientation of the hER cDNA sequence, resulting in no observable protein expression. (C) The ability of wt and mutant hERs to activate transcription over a range of estradiol concentrations was measured as described for panel A. Each curve is the mean ± SEM of three separate experiments, carried out in triplicate.

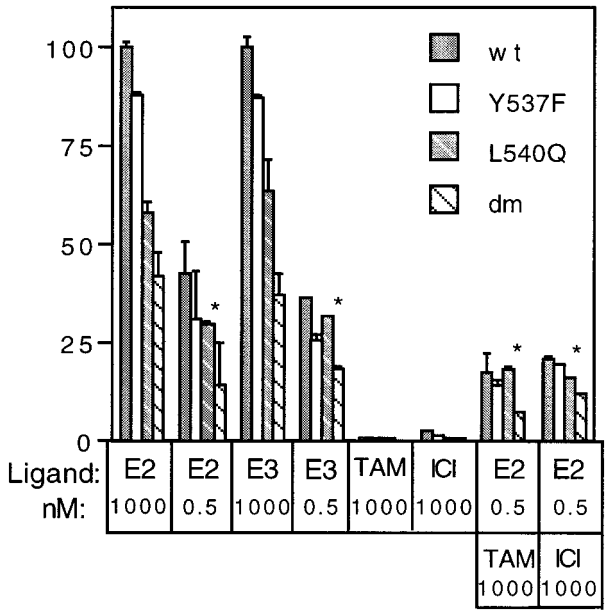

FIGURE 3: Steroid specificity of the mutant and wt hERs. The yeast cells expressing the wt and mutant hERs along with the reporter plasmid YRPE2 were grown in medium containing the indicated ligand at 30 °C for 16 h. E2 is 17-β-estradiol and E3 estriol. The ability of the antiestrogens tamoxifen citrate (TAM) and ICI 182,780 (ICI) to block estradiol-mediated transactivation of the hERs was assessed at the concentration of E2 corresponding to an approximately half-maximal response: 5 nM for wt and Y537F or 50 nM (*) for L540Q and dm hERs.

hER protein in all strains (Figure 2B). Although random deviations observed in yeast-expressed wt and mutant hERs can be quantified by densitometry of Western blots (within 10−20%), the observed differences in the level of transactivation remained consistent. No hER protein was detected in yeast cells lacking the hER cDNA expression vector or in the cDNA inverted strain. Thus, differences in transcriptional activity were not due to differences in the levels of expressed protein.

Maximal activation of transcription occurred near 10 nM estradiol for each mutant (Figure 2C). Higher concentrations of ligand had little effect, indicating that the mutant proteins, even when stimulated maximally by estradiol, do not attain the same levels of activation as wt.

Transcriptional Activation of hERs by Selected Agonists and Antagonists. We compared the ligand specificity and effectiveness of various hER agonists and antagonists in intact yeast cells. As shown in Figure 3, the activation of *lacZ* by both wt and mutants was strictly estrogen specific. No induction of β-galactosidase activity was detected in the presence of other steroids, such as progesterone, dexamethasone, or testosterone (data not shown). Estriol was able to stimulate reporter expression at concentrations that were 10-fold higher than those required for maximal induction with estradiol. The antiestrogens ICI 182,780 and tamoxifen citrate had no effect on wt or mutant hER transactivation when added alone but were able to antagonize the estradiol-induced transactivation. The responses to these compounds in yeast are in good agreement with published mammalian cell culture and in vivo data (*28*, *29*). These data suggest that none of the mutations significantly alters the ligand binding properties of the hER.

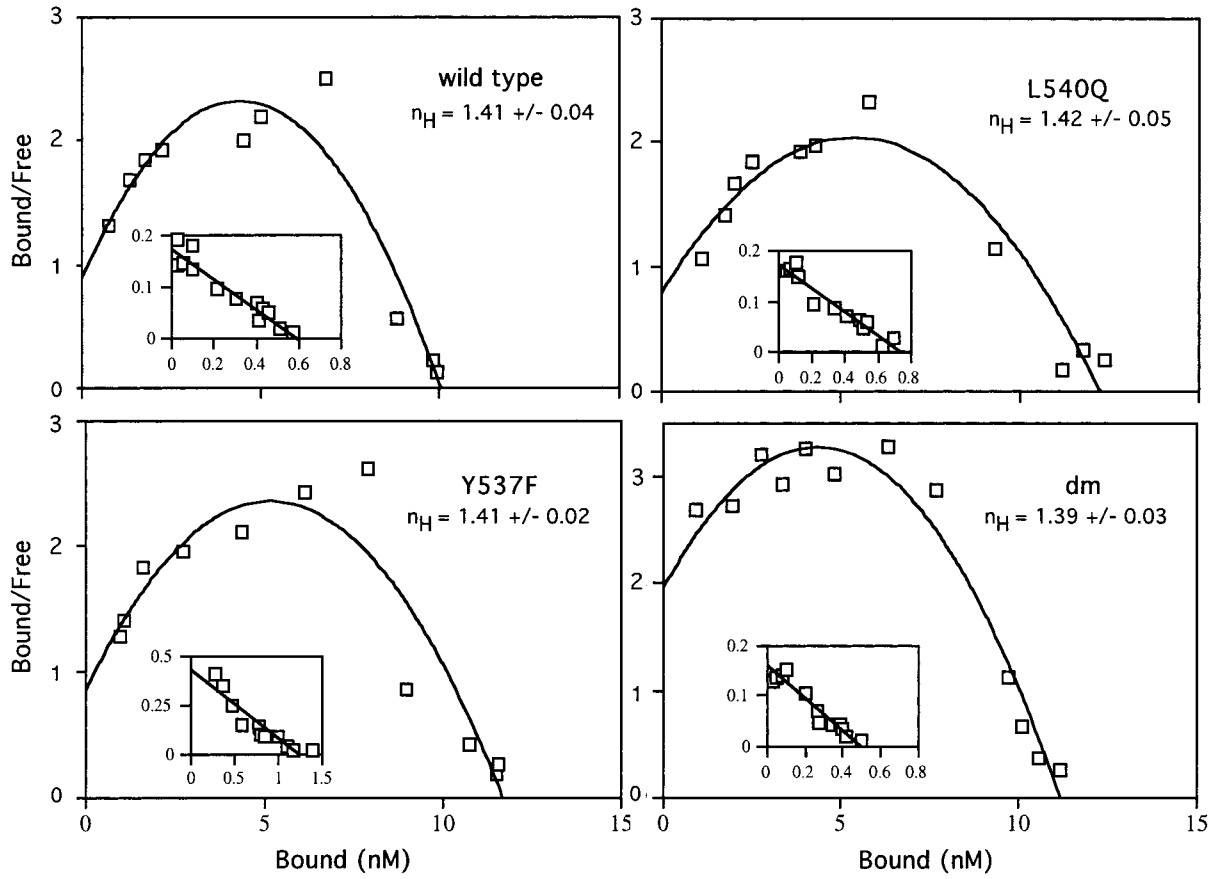

FIGURE 4: Saturation analyses of the [^{3}H]estradiol binding of the mutant and wt hERs. Aliquots of extracts containing 0.8−12 nM receptors were incubated for 16 h on ice with increasing concentrations of [^{3}H]estradiol. The extent of specific ligand binding was determined by subtraction of the extent of nonspecific binding in the presence of a 200-fold excess of radio inert estradiol from the total extent of binding observed. The data were analyzed by the methods of Scatchard and Hill. The relative affinities were estimated from fitted saturation plots and did not vary between wt and mutants more than 50% for any given measurement. The Hill coefficients (n_H) are indicated for each receptor with the SEM for three independent measurements. A representative Scatchard plot is shown for each mutant at both high and low (inset) concentrations.

Estradiol Binding Analysis of wt and Mutant hERs. The hER mutants studied here were also produced in baculovirus-infected *Sf9* cells to obtain sufficient quantities of receptor for binding analyses. Receptors produced in *Sf9* and yeast cells exhibit characteristics similar to those of the native hER (*19*). The wt and mutant baculovirus hERs exhibited similar [^{3}H]estradiol binding affinities (K_d = 0.8−2.0 nM) at both low and high hER concentrations (Figure 4). We compared the cooperativity of the mutants with that of wt hER to address the question of either mutation affecting the necessary site−site interactions attributed to the cooperative nature of estradiol binding (*30*). At concentrations between 10 and 12 nM, all hERs exhibited maximum cooperativity with a Hill coefficient of 1.4 (±0.02−0.05). At intermediate concentrations (2−4 nM hER), the affinity and observed partial cooperativity (Hill coefficient of 1.1−1.3) were also similar. These experiments were repeated two or three times with different receptor preparations under identical conditions, with no significant deviations. Low concentrations of hERs (<1 nM) yielded no cooperative estradiol binding and did not vary significantly in relative affinities (Figure 4 insets).

The Y537F Mutation Alters the Capacity but Not the Affinity of the hER for an Estrogen Response Element (ERE). The *Sf9*-produced hERs were analyzed by electrophoretic mobility shift assays to determine if the altered transcriptional responses of the mutant hERs were a result of variations in their DNA binding properties. Cell extracts were first standardized for hER content by Western blotting and densitometry to determine relative concentrations, to within 10% error, of total hER among the receptor preparations (data not shown). Using equivalent amounts of total hER, the apparent ERE affinity was estimated from saturation curves with ^{32}P-labeled ERE in both the presence and absence of estradiol. Saturation and Scatchard plots for the wt and mutants were generated by maintaining equivalent receptor and protein concentrations and increasing the amount of ERE added to the reaction mixture (Figure 5). The wt and mutant hERs exhibited similar affinities for the ERE in either the absence or presence of estradiol. It should be emphasized that the amount of hER used in these experiments was calibrated on the basis of the total number of receptors as quantified by Western blotting, which will not necessarily represent equivalent hormone binding sites. The exact relationship between binding sites and receptor stability is unclear, but it is likely that a mutation-induced loss of protein stability would decrease the number of hormone binding sites, and result in an apparent decrease in the concentration of active (hormone-binding) receptor.

A distinct difference in DNA binding capacity was observed between the wt and Y537F mutant proteins upon treatment with estradiol. After preincubation of wt hER with

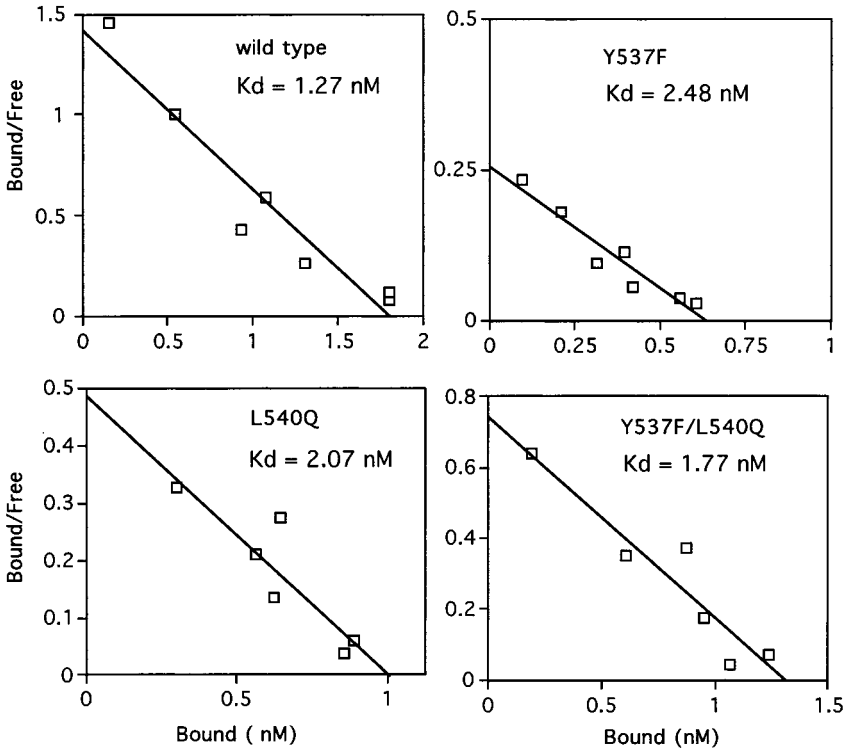

FIGURE 5: DNA binding analysis of wt and mutant hERs Relative expression levels of the wt and mutants were calibrated by titrating cell extracts over a range of hER concentrations on a Western blot. Equivalent amounts of the indicated hERs were then subjected to electrophoretic mobility shifts assays (EMSAs). To obtain affinity measurements, constant amounts of cell extract were incubated with increasing concentrations of ^{32}P-labeled ERE probe. Following electrophoresis, hER−ERE and free ERE bands were excised and analyzed by Cherenkov counting. Affinity constants were derived as described by the method of Scatchard. A representative analysis is shown with the calculated affinity constants for that experiment. The mean K_d values obtained from three different experiments ranged from 1 to 5 nM and were not significantly different.

estradiol, the maximum level of binding to the ERE increased more than 2-fold compared to that of non-estradiol-treated samples, while the apparent K_d remained essentially unchanged. An identical estradiol incubation with an equivalent amount of Y537F receptor had only a small effect on ERE binding (Figure 6). This result suggests the stabilizing effect of estradiol on the hER is absent in the Y537F mutant.

Y537 Stabilizes the Hormone-Binding Activity of the hER. The difference in protein stability between the wt and Y537F was tested directly in two ways. First, cell extracts containing the wt and Y537F mutant hER were prepared in the absence and in the presence of carrier protein (ovalbumin, 20 mg/mL). The presence or absence of carrier protein had little effect on the binding of the wt hER to [^{3}H]estradiol (Figure 7A). However, in the absence of the carrier protein, the Y537F mutant hER exhibited little [^{3}H]estradiol binding activity; the activity was increased approximately 10-fold by addition of the carrier (Figure 7A). Western blotting revealed no differences in the recovery of hER protein, or sensitivity to proteolysis (not shown).

Second, cell extracts expressing either wt or Y537F receptor and containing the carrier protein were incubated for up to 4 h at 25 °C in the absence of hormone, followed by measurement of the remaining hormone binding activity. The wt hER retained its hormone binding activity essentially unchanged for up to 4 h, whereas the Y537F hER lost 30% of its binding activity during that time (Figure 7B). This effect was most striking in extracts prepared using low-ionic strength buffer. In contrast, when cells were prepared in the presence of 300 mM KCl, the hormone binding activity of both receptors increased approximately 3-fold during the first hour of incubation (Figure 7C). The activity of the wt receptor remained stable for up to 4 h. The hormone binding activity of the Y537F hER remained near maximal for up to 2 h, but a small (approximately 15%) decrease from maximal levels was observed at 4 h (Figure 7C). Western blotting revealed no detectable differences in immunoreactivity between aliquots taken at the beginning or the end of the incubation (not shown).

Dissociation of [^{3}H]Estradiol from the wt and Y537F Mutant hER. The dissociation of [^{3}H]estradiol from the wt hER exhibited a biphasic or two-component dissocation curve (Figure 8). The half-life of the first, fast component was 13.0 ± 0.6 min, while the half-life of the second, slow component was 118 ± 3 min. By contrast, the dissocation of [^{3}H]-estradiol from the Y537F mutant hER appeared to be linear, with a half-life of 65 ± 9.8 min (Figure 8). Moreover, when the data for dissocation from the Y537F mutant were fit to equations for two-phase dissocation and one-phase dissociation, the equation for one-phase dissocation gave a better fit (not shown). No effect of receptor concentration on the dissocation kinetics of either the wt or the mutant was observed under these conditions.

DISCUSSION

Tyrosine 537 of the hER is located in a flexible loop region at the base of the amphipathic α-helix (H12) of the ligand binding domain which undergoes extreme conformational changes in response to hormone binding (*8*, *9*). We have studied the function of Y537 by biochemical analysis of

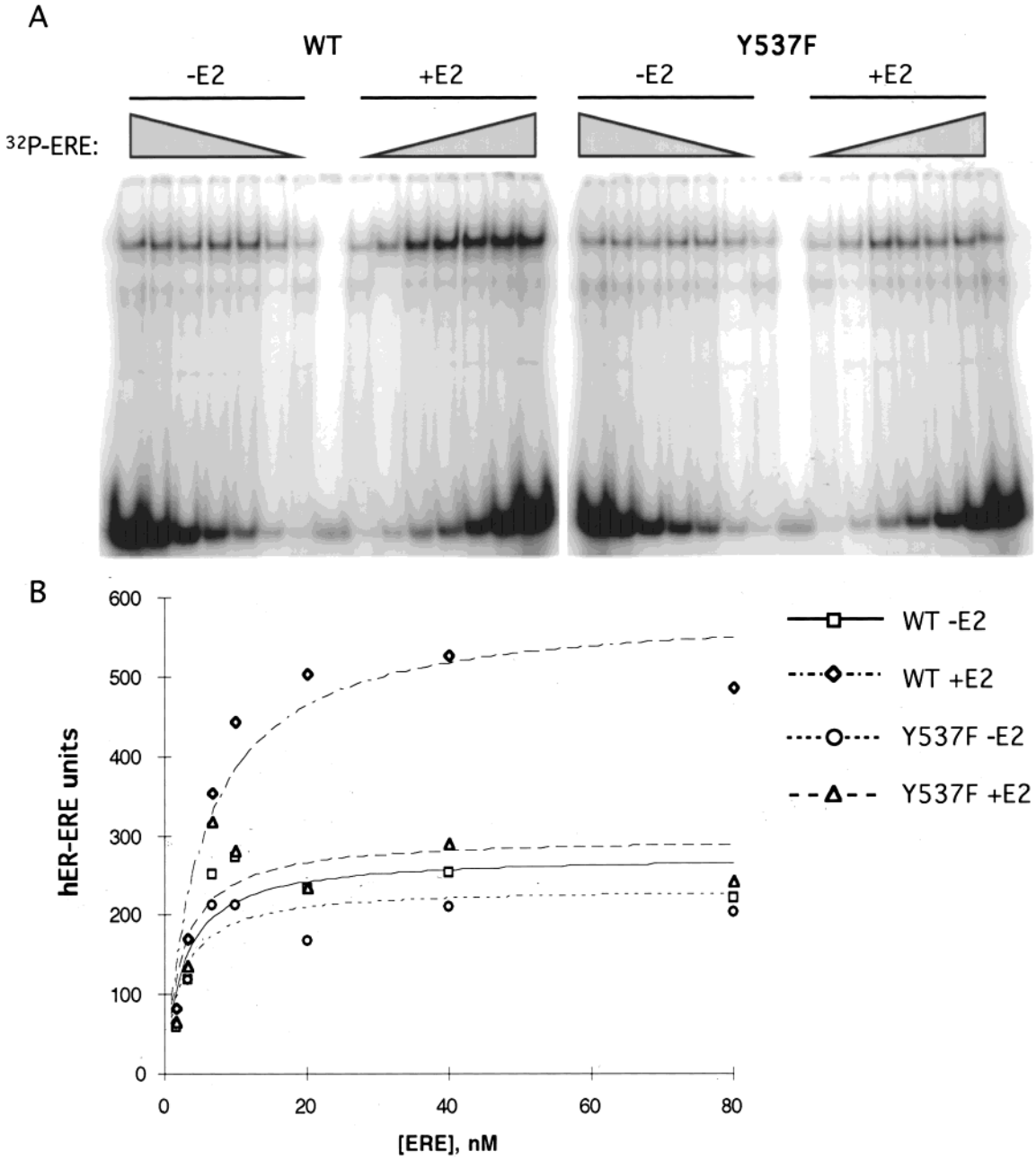

FIGURE 6: DNA binding capacity of the hER is compromised in Y537F. **(**A) Equivalent amounts of wt and Y537F hER cell extracts were incubated overnight on ice in both the absence and presence of 10 nM estradiol before incubation with increasing concentrations of ^{32}P-labeled ERE probe. The overnight incubation of wt hER in the absence of hormone did not decrease the level of ERE binding as compared with the same receptor used immediately in a gel shift (data not shown). (B) The data presented in panel A were plotted as a function of ERE concentration. The ordinate is relative units as measured on the PhosphorImager from an equal exposure time for each gel. The relative affinities of wt and Y537F hER do not vary more than 2-fold from experiment to experiment. A representative graph from three separate experiments is shown, with the smooth line indicating the data fit to a hyperbolic equation.

specific mutations, and provide a comparison with studies of other relevant mutations within this region (Table 1). We find that a tyrosine to phenylalanine mutation reduces hER stability, alters the hormone binding kinetics, and attenuates receptor-mediated transcriptional response in yeast by approximately 25%. Furthermore, in combination with the dominant negative L540Q hER mutant, the effect on transcription is additive. These data support a hypothesis in which hER Y537 is an important component of the intricate protein structure−function mechanism of hER activation.

Yeast Transactivation Assays and hER Mutants. Yeast-based transcription assays are common approaches to studying mammalian hormone receptors (*31*). Yeast-expressed hER has been shown to exhibit DNA and hormone binding affinities similar to those of the mammalian expressed receptor (*32*−*34*). We have established a transactivation assay for the hER in *S. cerevisae* which is completely hormone-dependent and responds to steroids in a manner comparable to those of other reported yeast and mammalian expression systems (*21*, *35*). Yeast expressing the receptor exhibit no measurable basal transcriptional activation in the absence of estrogen treatment. Another distinction of the yeast system is that unlike transient transfection assays, a relatively low and consistent amount of hER protein is expressed, estimated to be around 5−10 nM (A. Notides, unpublished data).

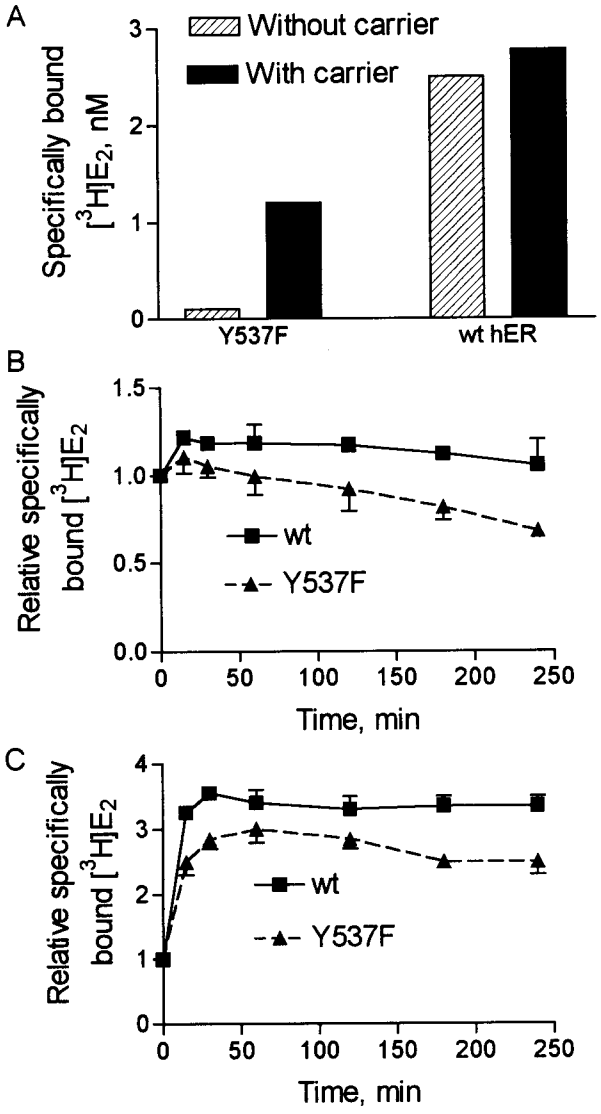

FIGURE 7: Stability of the wt and Y537F mutant ERs. **(**A) Cell extracts were prepared with and without 20 mg/mL ovalbumin carrier protein. The extracts were incubated with 20 nM [^{3}H]-estradiol for 1.5 h in an ice/water bath. A parallel incubation containing 20 nM [^{3}H]estradiol and a 200-fold molar excess of unlabeled estradiol was carried out to determine the level of specific binding. Free and bound steroids were separated with the dextran-coated charcoal assay, and the amounts were measured using liquid scintillation counting. These data are typical of four independent experiments. (B) Cell extracts were prepared from *Sf9* cells expressing the wt or Y537F hER, in a low-ionic strength TDEG buffer containing 20 mg/mL ovalbumin. The extracts were incubated without hormone at 25 °C for up to 4 h, and the remaining hormone binding activity was measured as described in Experimental Procedures. The data are expressed relative to the initial concentration of specifically bound [^{3}H]estradiol. The initial concentrations of bound [^{3}H]estradiol were 3.7 and 3.8 nM (wt), and 6.7 and 7.3 nM (Y537F). The data are the average of two independent experiments. (C) Cell extracts were prepared as described for panel B, with the addition of 0.3 M KCl. The initial concentrations of bound [^{3}H]estradiol were 1.6 and 3.2 nM (wt), and 1.1 and 2.6 nM (Y537F). The data are the average of two independent experiments.

The yeast expressing Y537F hER exhibited a small but measurable decrease in the maximum level of transactivation which was further diminished in combination with the L540Q AF-2 mutant. In our system, the mutant and wt receptors were expressed at equivalent levels (within 10%) independent

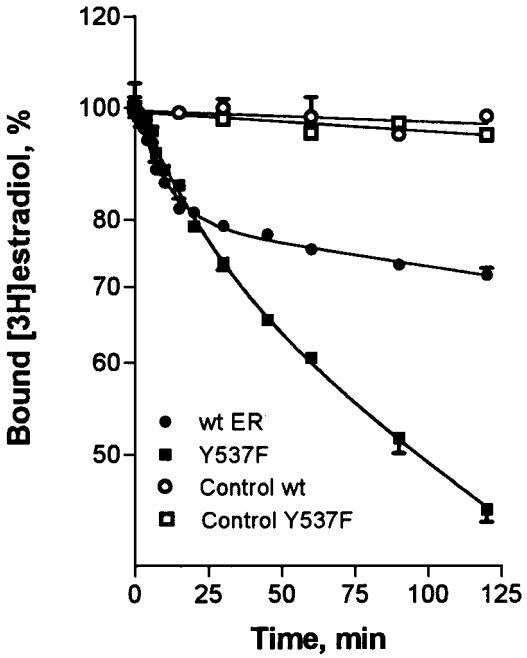

FIGURE 8: Dissociation of [^{3}H]estradiol from the wt and Y537F mutant hER. Extracts from *Sf9* insect cells expressing either wt (●) or Y537F hER (■) were prepared, and the dissociation of [^{3}H]-estradiol from the receptors was assessed as described. Receptor concentrations were between 1.05 and 2.67 nM for wt and 0.86 and 5.1 nM for the Y537F hER. The values that are shown are the mean and SEM of four independent experiments. The lines represent the best fit to two-phase exponential decay (wt) or one-phase exponential decay (Y537F). Inactivation of the wt (○) and Y537F hERs (□) was assessed in parallel incubations and was less than 5%.

Table 1: Summary of hER Y537 and Helix 12 Mutations and Their Effects on Hormone-Induced Transcription and the Observed Phenotype[a]

hER	transcriptional response	phenotype	ref
wt	hormone-dependent	wt protein	
Y537F	hormone-dependent	decreased stability	*10*−*16*
L540Q	hormone-dependent	dominant negative	*26*, *27*
Y537S Y537A Y537E Y537D Y537N L536P	hormone-independent	constitutively active	*14*−*18*
L539A/L540A M543A/L544A	none	transactivation (AF-2) deficient	*47*−*49*
D538V	hormone-dependent	reduced response	*51*

[a] Receptors were studied in various systems. Refer to the indicated references for each mutant. The Y537 mutants exhibit various degrees of constitutive activity. Where appropriate, mouse ER residues are indicated as the corresponding human sequences.

of added estradiol, and all responded to agonists and antagonists in a manner consistent with mammalian cell cultures or in vivo systems (*36*). Although several yeast studies show a lack of estradiol antagonsim using tamoxifen and ICI 182,780, and in fact report of a partial agonist response with these compounds, we and others have observed estradiol antagonism with these compounds in yeast (refs *21* and *52* and references therein). Several explanations have been proposed to account for these differences, including yeast strain and promoter specificity, different uptake ratios

and metabolism of antiestrogen compounds in yeast, and the presence or absence of specific factors associated with transcriptional activation of the hER.

Despite the known tyrosine kinase and phosphatase activity in *S. cerevisiae* (*37*, *38*), we have not determined whether in fact the Y537 site of the hER expressed in yeast was phosphorylated. On the other hand, the differences between wt and Y537F obtained with the yeast transcription assay (Figure 2A) were quantitatively similar to those obtained in mammalian cell transcription assays performed by us and others (D. Vorojeikina, unpublished data, and refs *15* and *16*). This suggests that the role of Y537 phosphorylation in transcriptional activation is probably insignificant.

Tyrosine 537 Phosphorylation. Tyrosine phosphorylation of the hER was first reported 14 years ago where it was found that ^{32}P-labeling of the rat uterine estrogen receptors occurred on tyrosine residues (*39*). This receptor also reacted with antiphosphotyrosine antibodies (*40*). It was shown by deletion and mutational analysis that tyrosine 537 was required for maximal hormone binding activity of in vitro-synthesized ER (*41*). These authors proposed that tyrosine 537 phosphorylation was required for estradiol activation of the hER.

An alternative role for Y537 phosphorylation was proposed in which tyrosine phosphorylation not only regulates hormone binding activity (*42*) but also is required for the DNA binding and dimerization of the hER (*13*). In this model, Y537 phosphorylation is essential for the interaction of one monomer with another tyrosine-phosphorylated monomer to constitute an active hER dimer. The data presented here, which were verified with multiple preparations of sequence-verified recombinant hER mutants, as well as similar data presented by others (*15*, *16*), do not support this hypothesis. Several explanations for the differences from earlier observations exist. The observed decrease in the stability of the Y537F hER described here may have been interpreted as an effect of phosphorylation state. Additionally, differences in expression levels during the original preparation of the Y537F mutant could have resulted in an apparent loss of activity when compared to that of the wt. Since we do observe a decrease in receptor stability with the Y537F mutant, small changes in sample handling and preparation could magnify differences with the wt hER.

Several attempts were made in our laboratories to distinguish the Y537F mutation from wt by Western blotting with antiphosphotyrosine antibodies. Although the phosphotyrosine antibodies do react with the hER (Figure 9), no significant differences were observed between wt or Y537F receptors expressed in *Sf9* insect cells, suggesting either a lack of tyrosine 537 phosphorylation in the recombinant hER or the presence of alternative tyrosine-phosphorylated sites. Interestingly, the possibility of alternative tyrosine phosphorylation sites on the hER has recently been suggested (*43*). To date, the only direct evidence of Y537 phosphorylation that has been reported was a result of radiolabel sequencing of the hER tryptic peptides from *Sf9* and MCF-7 cells (*10*). However, the amount of phosphorylation on Y537 was only 5−6% of the total amount of hER phosphorylation. Thus, the extent and the functional role of tyrosine 537 phosphorylation therefore remain unclear.

The decreased stability of the Y537F hER can be explained by several lines of reasoning which do not involve phosphorylation. As seen in Figure 10, the hydroxyl group of Y537 forms a hydrogen bond with asparagine 348, within helix 3 on the estradiol-bound hER (*8*). The potentially stabilizing effect of this interaction in the hormone-bound hER is obviously absent in the phenylalanine mutant. Furthermore, phenylalanine itself is known to have a helix destabilization effect when studied in relation to other hydrophobic amino acids (*44*).

The wt hER, expressed in *Sf9* insect cells, exhibits biphasic dissocation kinetics, as has previously been reported for the calf uterine hER (*45*). The values of the half-lives of the

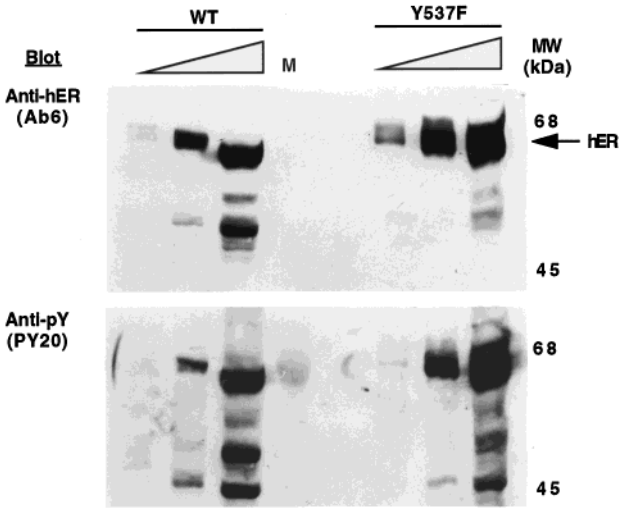

FIGURE 9: Reactivity of hER to antiphosphotyrosine antibodies. To analyze the wt and Y537F hER for reactivity to antiphosphotyrosine antibodies, increasing amounts of total cell extracts containing approximately equivalent amounts of both wt and Y537F hER were run on SDS−PAGE and transferred to PVDF membranes. The blots were first probed with the PY20 antiphosphotyrosine antibody from Santa Cruz Biotech (Anti-PY) according to the manufacturer's protocol. The membrane was then stripped and reprobed with the anti-hER antibody (Ab 6). Similar results were obtained when the order of probing was reversed. Other antiphosphotyrosine antibodies also reacted with the hER (see Experimental Procedures). The raised triangle corresponds to increasing amounts of total protein in each lane corresponding to approximately 2.5, 10, and 25 μg of total protein, respectively. The hER band at 66 kDa is denoted to the right along with the positions of the molecular mass markers (MW, kilodaltons).

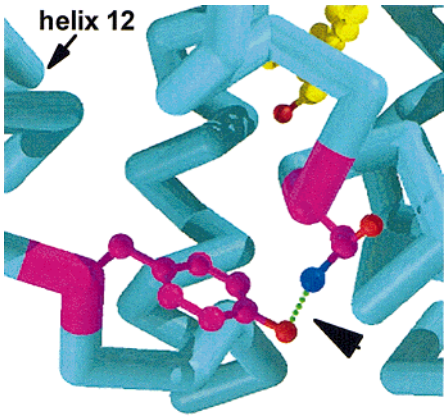

FIGURE 10: Potential hydrogen bond between Y537 and N348. Residues Y537 and N348 are shown in magenta; estradiol is shown in yellow. The potential hydrogen bond is represented by a dotted green line and is highlighted by an arrow. This figure was generated using the coordinates of the hER ligand-binding domain deposited in the PDB (1ere) (*8*) using Molecular Images software.

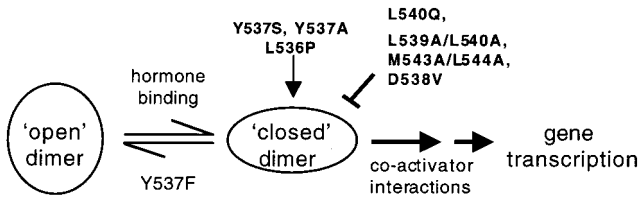

FIGURE 11: Model illustrating the effects of various helix 12 region mutations on a hypothetical pathway of hER activation. The activation of the dimeric wt hER proceeds through a hormone binding event which results in a conformational change of the helix 12 region and the conversion from an open to a closed hormone binding pocket. The indicated mutations of Y537 and the neighboring helix 12 amino acids are proposed to affect either the formation of the closed complex (left half-reaction) or the interaction with coactivators (right half-reaction). The constitutively active Y537S, Y537A, and L536P mutants are shown in a closed pocket conformation, independent of hormone binding. The Y537F mutation is shown to affect the open−closed conformational equilibrium or form a third distinct conformation. The mutations that are shown are discussed in the text. In this model, specific amino acids can easily be understood as having a role in either or both activation steps.

two phases are somewhat different for the two receptors, 5.8 and 173 min for the calf ER versus 14.9 and 118 min for the recombinant hER, respectively. We do not know whether this variance is due to differences in the solution conditions between experiments, or reflects actual differences between the bovine and human receptors. The biphasic dissocation kinetics for the wt hER and positive cooperative equilibrium binding reported here for both the wt and Y537F mutant are consistent with the ERs being in the dimeric state.

In contrast with the wt hER, the Y537F mutant exhibited monophasic dissocation kinetics, with a half-life of approximately 70 min. These results were surprising, since the equilibrium studies demonstrated that the wt and Y537F mutant hER bound [^{3}H]estradiol with the same affinity. The results suggest that mutation of Y537 affected the association rate, as well as the dissocation rate, of the interaction between estradiol and the ER. This observation is consistent with the conclusions of Carlson et al. (*46*), who studied deletion mutants of the hER and the Y537S mutant of the hER. However, in their report, neither cooperative binding of estradiol to the receptor nor biphasic dissocation kinetics for dissociation of estradiol from the receptors were observed. Overall, the data are consistent with the idea that the loss of the predicted hydrogen bond between Y537 and N348 dramatically alters the kinetics of the receptor−hormone interaction. The ability of the Y537 to form a hydrogen bond is also important in the stability of the hormone-free receptor.

Analysis of Y537 and H12 Mutations. Several constitutively active Y537 mutant hERs have been identified in which serine, alanine, glutamic acid, and asparagine replace the tyrosine (*14*−*16*). The degree of constitutive activity has been correlated with the formation of a stabilized interface for receptor coactivators or transcription factors (*16*). These receptors apparently gain hormone-independent function by folding into a "closed" position where H12 covers the ligand binding pocket (*46*). Our data indicate that the phenylalanine substitution has a small, but opposite, destabilizing effect. We propose a model in which the tyrosine to phenylalanine change favors a more "open", or "loose", conformation in both the presence and absence of hormone (Figure 11). The change in hydrogen bonding and the increased hydrophobicity of the phenylalanine could account for the difference between phenylalanine and tyrosine at this position by preventing a stable conformation of the hER. Similarly, the increased size of phenlylalanine, relative to serine and alanine, could explain the lack of Y537F constitutive activity as observed for Y537S and Y537A hER. This model assumes a two-state mechanism for hormone binding for both wt and mutant hERs. Alternatively, the Y537F hER could adopt a distinct conformation in the hormone-free state. While the Y537 mutants are able to bind coactivators (*16*), the L540Q and other helix 12 AF-2 AD mutations, such as the double mutants L539A/L540A and M543A/L544A, do not (*47*−*49*). Mutations of other neighboring amino acids have been found to affect hER function as well. Constitutive activity has been demonstrated for an L536P hER mutant (*50*), and another mutation, D538V, exhibits a muted transcriptional response to estradiol in yeast, similar to that of the L540Q hER (*51*). These mutations are summarized in Table 1 and suggest a dual role for the helix 12 region in providing both a stable and a specific surface for coactivators, since these mutations can apparently affect either function (Figure 11).

The analyses presented here are intended to clarify the role of tyrosine 537 in hER function. Although the possible phosphorylation of Y537 and the dimerization of the hER are apparently not directly related, mutations at this site do have significant consequences on receptor function. These effects, as well as those seen in mutations nearby, can be partially explained with an understanding of the structure of the LBD and the role of helix 12 in activation.

ACKNOWLEDGMENT

We thank Drs. Patricia Hinkle and Shohei Koide for their critical review of the manuscript. We also acknowledge Drs. Pierre Chambon and Dr. Hinrich Gronemeyer for the generous gift of plasmid pSG5 containing the HEGO cDNA.

REFERENCES

1. Mangelsdorf, D. J., Thummel, C., Beato, M., Herrlich, P., Schutz, G., Umesono, K., Blumberg, B., Kastner, P., Mark, M., Chambon, P., et al. (1995) *Cell 83*, 835−9.
2. Parker, M. G., Fawell, S. E., Danielian, P., and Arbuckle, N. (1991) *Biochem. Soc. Trans. 19*, 897−9.
3. Horwitz, K. B., Jackson, T. A., Bain, D. L., Richer, J. K., Takimoto, G. S., and Tung, L. (1996) *Mol. Endocrinol. 10*, 1167−77.
4. Le Goff, P., Montano, M. M., Schodin, D. J., and Katzenellenbogen, B. S. (1994) *J. Biol. Chem. 269*, 4458−66.
5. Denton, R. R., Koszewski, N. J., and Notides, A. C. (1992) *J. Biol. Chem. 267*, 7263−8.
6. Parker, M. G. (1995) *Vitam. and Horm. (San Diego) 51*, 267−87.
7. Tora, L., White, J., Brou, C., Tasset, D., Webster, N., Scheer, E., and Chambon, P. (1989) *Cell 59*, 477−87.
8. Brozowski, A. M., Pike, A. C., Dauter, Z., Hubbard, R. E., Bonn, T., Engstrom, O., Ohman, L., Greene, G. L., Gustafsson, J. A., and Carlquist, M. (1997) *Nature 389*, 753−8.
9. Shiau, A. K., Barstad, D., Loria, P. M., Cheng, L., Kushner, P. J., Agard, D. A., and Greene, G. L. (1998) *Cell 95*, 927−37.
10. Arnold, S. F., Obourn, J. D., Jaffe, H., and Notides, A. C. (1995) *Mol. Endocrinol. 9*, 24−33.
11. Migliaccio, A., Rotondi, A., and Auricchio, F. (1986) *EMBO J. 5*, 2867−72.
12. Castoria, G., Migliaccio, A., Green, S., DiDomenico, M., Chambon, P., and Auricchio, F. (1993) *Biochemistry 32*, 1740−50.

13. Arnold, S. F., Vorojeikina, D. P., and Notides, A. C. (1995) *J. Biol. Chem. 270*, 30205−12.
14. Zhang, Q. X., Borg, A., Wolf, D. M., Oesterreich, S., and Fuqua, S. A. (1997) *Cancer Res. 57*, 1244−9.
15. Weis, K. E., Ekena, K., Thomas, J. A., Lazennec, G., and Katzenellenbogen, B. S. (1996) *Mol. Endocrinol. 10*, 1388−98.
16. White, R., Sjoberg, M., Kalkhoven, E., and Parker, M. G. (1997) *EMBO J. 16*, 1427−35.
17. Lazennec, G., Ediger, T. R., Petz, L. N., Nardulli, A. M., and Katzenellenbogen, B. S. (1997) *Mol. Endocrinol. 11*, 1375−86.
18. Kalkhoven, E., Valentine, J. E., Heery, D. M., and Parker, M. G. (1998) *EMBO J. 17*, 232−43.
19. Obourn, J. D., Koszewski, N. J., and Notides, A. C. (1993) *Biochemistry 32*, 6229−36.
20. Kunkel, T. A. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 488−92.
21. Chen, C. W., Hurd, C., Vorojeikina, D. P., Arnold, S. F., and Notides, A. C. (1997) *Biochem. Pharmacol. 53*, 1161−72.
22. Kaiser, C., Michaelis, S., and Mitchell, A. (1994) *Methods in Yeast Genetics*, Cold Spring Harbor Laboratory Press, Plainview, NY.
23. Jones, E. W. (1991) *J. Biol. Chem. 266*, 7963−6.
24. Chen, D. C., Yang, B. C., and Kuo, T. T. (1992) *Curr. Genet. 21*, 83−4.
25. Pham, T. A., Hwung, Y. P., Santiso-Mere, D., McDonnell, D. P., and O'Malley, B. W. (1992) *Mol. Endocrinol. 6*, 1043−50.
26. Ince, B. A., Zhuang, Y., Wrenn, C. K., Shapiro, D. J., and Katzenellenbogen, B. S. (1993) *J. Biol. Chem. 268*, 14026−32.
27. Ince, B. A., Schodin, D. J., Shapiro, D. J., and Katzenellenbogen, B. S. (1995) *Endocrinology 136*, 3194−9.
28. Sasson, S., and Notides, A. C. (1983) *J. Biol. Chem. 258*, 8113−7.
29. Wakeling, A. E., and Bowler, J. (1988) *J. Steroid Biochem. 31*, 645−53.
30. Notides, A. C., Lerner, N., and Hamilton, D. E. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 4926−30.
31. White, J. H., Metzger, D., and Chambon, P. (1988) *Cold Spring Harbor Symp. Quant. Biol. 53*, 819−28.
32. Metzger, D., White, J. H., and Chambon, P. (1988) *Nature 334*, 31−6.
33. McDonnell, D. P., Nawaz, Z., Densmore, C., Weigel, N. L., Pham, T. A., Clark, J. H., and O'Malley, B. W. (1991) *J. Steroid Biochem. Mol. Biol. 39*, 291−7.
34. Wooge, C. H., Nilsson, G. M., Heierson, A., McDonnell, D. P., and Katzenellenbogen, B. S. (1992) *Mol. Endocrinol. 6*, 861−9.
35. Lyttle, C. R., Damian-Matsumura, P., Juul, H., and Butt, T. R. (1992) *J. Steroid Biochem. Mol. Biol. 42*, 677−85.
36. Tsai, M.-J., and O'Malley, B. W. (1994) *Annu. Rev. Biochem. 63*, 451−86.
37. Wilson, L. K., Benton, B. M., Zhou, S., Thorner, J., and Martin, G. S. (1995) *J. Biol. Chem. 270*, 25185−93.
38. Schieven, G., Thorner, J., and Martin, G. S. (1986) *Science 231*, 390−3.
39. Migliaccio, A., Rotondi, A., and Auricchio, F. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 5921−5.
40. Auricchio, F., Migliaccio, A., Castoria, G., Rotondi, A., and Di Domenico, M. (1987) *Methods Enzymol. 139*, 731−44.
41. Migliaccio, A., Di Domenico, M., Green, S., de Falco, A., Kajtaniak, E. L., Blasi, F., Chambon, P., and Auricchio, F. (1989) *Mol. Endocrinol. 3*, 1061−9.
42. Arnold, S. F., Melamed, M., Vorojeikina, D. P., Notides, A. C., and Sasson, S. (1997) *Mol. Endocrinol. 11*, 48−53.
43. Patrone, C., Gianazza, E., Santagati, S., Agrati, P., and Maggi, A. (1998) *Mol. Endocrinol. 12*, 835−41.
44. Padmanabhan, S., Marqusee, S., Ridgeway, T., Laue, T. M., and Baldwin, R. L. (1990) *Nature 344*, 268−70.
45. Weichman, B. M., and Notides, A. C. (1977) *J. Biol. Chem. 252*, 8856−62.
46. Carlson, K. E., Choi, I., Gee, A., Katzenellenbogen, B. S., and Katzenellenbogen, J. A. (1997) *Biochemistry 36*, 14897−905.
47. Danielian, P. S., White, R., Lees, J. A., and Parker, M. G. (1992) *EMBO J. 11*, 1025−33.
48. Cavailles, V., Dauvois, S., L'Horset, F., Lopez, G., Hoare, S., Kushner, P. J., and Parker, M. G. (1995) *EMBO J. 14*, 3741−51.
49. Mahfoudi, A., Roulet, E., Dauvois, S., Parker, M. G., and Wahli, W. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 4206−10.
50. Eng, F. C., Lee, H. S., Ferrara, J., Willson, T. M., and White, J. H. (1997) *Mol. Cell. Biol. 17*, 4644−53.
51. Wrenn, C. K., and Katzenellenbogen, B. S. (1993) *J. Biol. Chem. 268*, 24089−98.
52. Tsai-Pflugfelder, M., Gasser, S. M., and Walhi, S. (1998) *Mol. Endocrinol. 12*, 1525−41.

BI9911132