Phosphorylation of Tyrosine 537 on the Human Estrogen Receptor Is Required for Binding to an Estrogen Response Element*

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We report here that the phosphorylation of tyrosine 537 on the human estrogen receptor (hER) controls the receptor’s dimerization and DNA binding ability. The DNA-binding form of both the hER from human MCF-7 mammary carcinoma cells and the hER overexpressed in SF9 insect cells was isolated using estrogen response element (ERE) affinity chromatography. Western blot analyses demonstrated that the DNA-binding form of the hER from MCF-7 or SF9 cells was (i) phosphorylated at tyrosine 537, (ii) localized in the nucleus of estradiol-treated MCF-7 cells with an apparent molecular mass of 67 kDa, and (iii) hyperphosphorylated at serine residue(s). The non-DNA-binding form of the hER was (i) devoid of phosphorylation at tyrosine 537, (ii) cytosolic with an apparent molecular mass of 66 kDa, and (iii) hypophosphorylated at serine residue(s). The dephosphorylation of the purified hER at phosphorylase 537 with a tyrosine phosphatase eliminated binding to an ERE in a gel mobility shift assay. The binding of the tyrosine-dephosphorylated hER to an ERE was restored by the rephosphorylation of tyrosine 537 with Src family tyrosine kinases, p60src or p56lck. Mutation of tyrosine 537 to phenylalanine confirmed that the phosphorylation of tyrosine 537 is necessary for the hER to bind an ERE. An anti-hER antibody restored the binding of the tyrosine-dephosphorylated hER to an ERE, indicating that the bivalent anti-hER antibody brought together the two inactive hER monomers. A far-Western blot confirmed that phosphorylase 537 is required for hER homodimerization.

These experiments establish that dimerization of the hER and DNA binding are regulated by phosphorylation at tyrosine 537. This is the first demonstration of the regulation of dimerization of a steroid hormone receptor by phosphorylation. These results are significant since p60src is overexpressed in estrogen-dependent breast cancers and may act to enhance the activity of the hER.

The steroid/thyroid hormone receptors are ligand-dependent transcription factors that function by binding to hormone response elements on target genes and regulating transcription (1). The processes controlling steroid-specific gene transcription are poorly understood, although receptor-associated coactivators have been reported to be involved (2). Nonetheless, it has been demonstrated that most steroid/thyroid hormone receptors, including the human estrogen receptor (hER), bind to their hormone response elements as hetero- or homodimers (3, 4). It has been proposed that the dimerization of the steroid/thyroid hormone receptors is mediated through a leucine zipper motif, a series of hydrophobic heptad repeats in the carboxyl termini of the receptors (5, 6). Recently, Bourguet et al. (7) showed that the ligand-binding domain of human retinoid X receptor α has a dyad symmetry composed of packed helices at the receptor’s interface, but not a leucine zipper structure. While the dimerization of most steroid hormone receptors is required for binding to DNA, accessory proteins and post-translational modifications have also been suggested to contribute to DNA binding (8–10). For example, purification of the progesterone receptor decreases its DNA binding ability, suggesting that an accessory protein (e.g. high mobility group protein-1) is required for high affinity DNA binding (8). Additionally, the phosphorylation of steroid/thyroid hormone receptors has been shown to modulate their DNA binding affinity (11). The phosphorylation of the retinoic acid and progesterone receptors increases, while the phosphorylation of thyroid hormone receptor-α and nerve growth factor-I-B decreases their affinity for their respective response elements (12–15).

The hER, like other members of the steroid/thyroid hormone receptor superfamily, undergoes a hyperphosphorylation at serine residues following hormone binding (16). The dephosphorylation of the hER with potato acid phosphatase reduces but does not eliminate the receptor’s affinity for an ERE (16). The enhanced affinity of the hER for its ERE was found to be mediated by the estradiol-induced phosphorylation of serine 167, the major phosphorylation site on the hER (11). Casein kinase I specifically phosphorylates serine 167 of the hER (17). Serine 118 of the hER was identified as a growth factor-regulated phosphorylation site, mediated by mitogen-activated protein (MAP) kinase; however, its role in transcriptional activation of the hER remains to be defined (11).

An estradiol-independent phosphorylation site at tyrosine 537 in the carboxyl terminus of the hER has been identified by amino acid sequencing of 32P-labeled tryptic peptides of the hER (18). Furthermore, the Src family tyrosine kinases, p60src and p56lck, were shown to specifically phosphorylate tyrosine 537 on the hER, while protein-tyrosine phosphatase 1B (PTP1B) and Src homology 2 protein-tyrosine phosphatase 1 (SHPPT1) dephosphorylated phosphorylase 537 (18). Inter-

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† The abbreviations used are: hER, human estrogen receptor; ERE, estrogen response element; MAP, mitogen-activated protein; PTP1B, protein-tyrosine phosphatase 1B; SHPTP1, Src homology 2 protein-tyrosine phosphatase 1; PVDF, polyvinylidene difluoride; MOPS, 4-morpholinopropanesulfonic acid; Ab, antibody; 32P-Y537 hER, purified recombinant hER 32P-labeled at tyrosine 537 with p60src; 32P-S167 hER, purified recombinant hER 32P-labeled at serine 167 with casein kinase II.
estingly, the tyrosine kinase activity of p60<sup>c-src</sup> in human breast cancers has been shown to be elevated as compared with other cancers (19). The human MCF-7 mammary carcinoma cell line overexpresses p60<sup>c-src</sup> and has been a useful paradigm for investigating estrogen-dependent processes associated with human breast cancers (20). Thus, we sought to gain insight into the consequences of the phosphorylation of tyrosine 537 on the hER by p60<sup>c-src</sup>.

We show here that the phosphorylation of tyrosine 537 is a regulatory mechanism that controls the capacity of the hER to undergo the monomer to dimer transition. The phosphorylation of tyrosine 537 is also a prerequisite for the estrogen-dependent hyperphosphorylation of the serine residue(s), nuclear retention, and DNA binding of the hER.

**EXPERIMENTAL PROCEDURES**

Materials—17β[6,7-<sup>3</sup>H]estradiol (45.6 mCi/mmol) was purchased from DuPont NEN. Leupeptin, pepstatin, and chymostatin were obtained from Peninsula Laboratories, Inc. (Belmont, CA). The 4G10 anti-phosphotyrosine monoclonal antibody, p60<sup>c-src</sup> (1 pmol of PO<sub>4</sub>/min/mg), p56<sup>lck</sup> (1 pmol of PO<sub>4</sub>/min/mg), MAP kinase (1 pmol of PO<sub>4</sub>/min/mg), MAP kinase phosphatase, (40 pmol of PO<sub>4</sub>/mg), and STO-3G (3 pmol of PO<sub>4</sub>/min/mg) were from Upstate Biotechnology, Inc. (Lake Placid, NY). Casein kinase II (3 pmol of PO<sub>4</sub>/min/mg) was a generous gift of Drs. E. G. Krebs and D. W. Litchfield (University of Washington, Seattle, WA).

Site-directed Mutagenesis of the hER—Oligonucleotide site-directed mutagenesis of the hER was performed according to the method of Kunkel (13). The oligonucleotide template was prepared from M13mp19 containing the hER DNA grown in Escherichia coli strain CL236. A 28-base pair oligonucleotide primer containing a mutation of tyrosine 537 to phenylalanine and introducing a novel restriction site, XhoI, was used. All mutants were verified by restriction enzyme digestion with XhoI and DNA sequencing. The mutated cDNA of the hER was cloned into the EcoRI site of the pVL1393 baculovirus transfer vector (Invitrogen, San Diego, CA). The orientation of the cloned fragment was confirmed by digestion with BgII.

Cell Culture, Baculovirus Expression, and hER Preparation—The growth conditions of the human MCF-7 cells and the S9 insect cells and the expression of the wild-type hER in S9 cells have been described (16, 22). Expression of the mutant Y537F hER was accomplished by transfecting the baculovirus vector AcNPV-Y537F hER into S9 cells using BaculoGold linearized baculovirus DNA (Pharmingen, San Diego, CA.) and Lipofectin reagent (Life Technologies, Inc.). Whole cell extracts from MCF-7 or S9 cells were prepared as described previously (16, 22).

Western Blot Analysis—Proteins were resolved on a SDS-10% polyacrylamide gel for 10 h at 30 mA and electrotransferred to a PVDF membrane. The native or recombinant hER (10 nM) was dephosphorylated by the addition of 100 pmol of PTP1B or 5 pmol of SHPTP1 conjugated to agarose beads. The phosphatase-agarose beads were removed by centrifugation at 70°C.

In Vitro Phosphorylation/Dephosphorylation of the hER—The Native or recombinant wild-type hER was phosphorylated by the addition of the reaction buffer for p60<sup>c-src</sup> (40 mM Tris-HCl, pH 7.4, and 10 mM MgCl<sub>2</sub>), p56<sup>lck</sup> (125 mM Tris-HCl, pH 7.0, 62.5 mM MgCl<sub>2</sub>, 12.5 mM MnCl<sub>2</sub>, and 0.125 mM Na<sub>2</sub>VO<sub>4</sub>), casein kinase II (50 mM Tris-HCl, pH 7.6, and 10 mM MgCl<sub>2</sub>), or MAP kinase (60 mM MOPS, pH 7.2, 50 mM β-glycerophosphate, 2 mM EGTA, 30 mM MgCl<sub>2</sub>, 0.2 mM NaF, and 8 mM dithiothreitol). The reaction was initiated by the addition of 1 mM ATP and 5 units of p60<sup>c-src</sup>, 50 units of p56<sup>lck</sup>, 50 ng of casein kinase II, or 50 ng of MAP kinase. The reactions were incubated at 30°C for 15 min and then stopped by placing the tube at 4°C and adding phosphatase inhibitors. The native or recombinant hER (10 nM) was dephosphorylated at 37°C for 30 min in 50 mM HEPES, pH 7.4, 150 mM NaCl, and 5% glycerol with 1 μg of PTP1B or 5 μg of SHPTP1 conjugated to agarose beads. The phosphatase-agarose beads were removed by centrifugation at 4°C and washed with buffer. The supernatant and the washes were pooled and used to detect phosphorylated and unphosphorylated hER.

Far-Western Blot Analysis—The purified recombinant hER was dephosphorylated/epiphosphorylated as described above, resolved by SDS gel electrophoresis, and electrotransferred to a PVDF membrane. For the probe, the purified recombinant hER was labeled with [<sup>32</sup>P]ATP and p60<sup>c-src</sup> or casein kinase II (25).

**RESULTS**

Western Blot Analysis of the hER with an Anti-hER and an Anti-Phosphorysotyne Antibody—The native hER from untreated MCF-7 cells detected with an anti-hER antibody migrated at 66 kDa (Fig. 1, lane 1), whereas estradiol treatment of the MCF-7 cells produced two forms of the hER, a 66-kDa and a 67-kDa form (Fig. 1, lane 3). The 67-kDa form of the hER results from hyperphosphorylation of serine residues as previously shown by [<sup>32</sup>P]phosphate incorporation and phosphoamino acid analysis (16, 26). In addition, a serine phosphatase, but not a tyrosine phosphatase, reverts the 67-kDa form to the 66-kDa form of the hER (data not shown).

The 66-kDa form of the native hER from untreated MCF-7 cells was recognized by the 4G10 anti-phosphotyrosine monoclonal antibody (Fig. 1, lane 2). However, after estradiol treatment of the MCF-7 cells, the anti-phosphotyrosine antibody recognized only the 67-kDa form and not the 66-kDa form of the hER (Fig. 1, lane 4). These data indicate that only the fraction of the hER in the 66-kDa form, which was phosphorylated at...
tyrosine, was converted to the 67-kDa form after estradiol treatment of the MCF-7 cells. To further aid in deciphering the function of tyrosine phosphorylation, the wild-type hER and the mutant Y537F hER were expressed in Sf9 cells.

The wild-type hER expressed in Sf9 cells reacted with an anti-hER antibody, confirming the presence of the wild-type hER at 66 and 67 kDa (22). The 67-kDa form, but not the 66-kDa form, of the wild-type hER from whole cell extracts of Sf9 cells (or following purification of the hER by immunoprecipitation) showed strong reactivity with the anti-phosphotyrosine antibody (Fig. 1, lanes 6 and 8). Antibody 6 immunoprecipitates, before or after estrogen treatment of MCF-7 cells, the 66- or 67-kDa form of the hER with equal efficiency (data not shown). The presence of the 67-kDa form of the wild-type hER was independent of estradiol treatment of the Sf9 cells (Fig. 1, lanes 5 and 7). The lack of estrogen-dependent phosphorylation of the hER in Sf9 insect cells may be due to the 10,000-fold overexpression of the hER in insect as compared with mammalian cells (22), cell-specific differences in protein kinase regulatory pathways, or loss of regulatory factors during baculoviral infection. An extract from Sf9 cells infected with mock baculovirus showed no reactivity with the anti-hER or the anti-phosphotyrosine antibody at 66 or 67 kDa (data not shown). The mutant Y537F hER expressed in Sf9 cells migrated only at 66 kDa in the absence or presence of estradiol treatment of the Sf9 cells (Fig. 1, lanes 9 and 11) and did not show reactivity with the anti-phosphotyrosine antibody (lanes 10 and 12). These results confirm our earlier findings that the hER is phosphorylated at tyrosine 537 (18) and that the 4G10 anti-phosphotyrosine monoclonal antibody specifically recognizes the phosphorylation at tyrosine 537 on the hER. These data also indicate that the phosphorylation of tyrosine 537 is a necessary prerequisite for the serine hyperphosphorylation of the hER and consequently the formation of the 67-kDa form of the hER.

Intracellular localization of the 67-kDa form of the hER in MCF-7 cells was exclusively nuclear after estradiol treatment. Following a 20-min exposure to [3H]estradiol, the nuclear fraction of the MCF-7 cells contained 90% of the specifically bound [3H]estradiol, while only 10% was present in the cytoplasmic fraction (data not shown). Equivalent amounts of cytosolic and nuclear proteins were separated by SDS gel electrophoresis and electrotransferred to a PVDF membrane. Western blot analysis with an anti-hER antibody revealed that the nuclear fraction contained the 67-kDa form of the hER, while the cytosolic fraction contained the 66-kDa form (Fig. 2, lanes 1 and 3). Consistent with the observations described above, only the 67-kDa form of the hER reacted with the anti-phosphotyrosine antibody in a Western blot (Fig. 2, lanes 2 and 4). In the absence of estradiol treatment of MCF-7 cells, the hER was recovered only in the cytosolic fraction, demonstrating that estrogen binding promotes nuclear retention of the hER (Ref. 16 and data not shown). These results suggest that the biologically active hER is the 67-kDa form since it is hyperphosphorylated and retained in the nucleus after estradiol treatment of MCF-7 cells.

ERE Affinity Chromatography of the hER—The hyperphosphorylated 67-kDa form, whether from estradiol-treated MCF-7 cells or untreated Sf9 cells expressing the wild-type hER, had a higher affinity than the 66-kDa form for an ERE affinity column. Ammonium sulfate fractions of the MCF-7 or Sf9 cell extracts were subjected to ERE affinity chromatography. Western blot analysis of the flow-through and salt-eluted fractions from the ERE affinity column revealed that the salt-eluted fractions from the MCF-7 or Sf9 cell preparations contained only the 67-kDa form of the hER (Fig. 3, A and B, lane 5). In contrast, the flow-through fraction contained only the 66-kDa form of the native hER and the recombinant hER (Fig. 3, A and B, lane 3). The anti-phosphotyrosine antibody data indicated that the native hER and the recombinant hER in the eluate fraction, but not in the flow-through fraction, were phosphorylated at tyrosine 537 (Fig. 3, A and B, lanes 4 and 6). Rechromatography of the flow-through fraction of the recombinant hER on the ERE affinity column revealed that the hER still eluted in the flow-through fraction, indicating that selective ERE binding was not due to a limited number of ERE-binding sites on the column (data not shown). Furthermore, the presence of estradiol did not alter the binding of the hER to the ERE affinity column since the 67-kDa form of both the unliganded recombinant hER and the liganded native hER bound to the ERE affinity column (Fig. 3, A and B). The mutant Y537F hER did not bind to the ERE affinity column and was present only in the flow-through fraction (Fig. 3C).

The 67-kDa form of the recombinant hER was purified to near homogeneity by ERE affinity chromatography. Coomassie Blue staining indicated that the 67-kDa form of the hER was the only protein present (Fig. 4, lane 1). Western blot analysis with the anti-phosphotyrosine antibody detected only the hER (Fig. 4, lane 2).

Gel Mobility Shift Assay of the hER—The phosphorylation of tyrosine 537 on the hER was necessary for its binding to an ERE in a gel mobility shift assay. A whole cell extract of Sf9 cells expressing the wild-type hER was incubated with a 32P-labeled ERE, and the complexes were resolved by nondenaturing gel electrophoresis. The recombinant hER formed a specific

**Fig. 2.** Intracellular localization and tyrosine phosphorylation of the hER from estradiol-treated MCF-7 cells. Cytosolic and nuclear fractions were prepared from [3H]estradiol-treated MCF-7 cells. The proteins (100 µg) were separated by SDS gel electrophoresis and then transferred to a PVDF membrane. The membrane was immuno-blotted with the anti-phosphotyrosine antibody (pY Ab) (lanes 2 and 4) and then stripped and reblotted with the anti-hER antibody (hER Ab) (lanes 1 and 3).
hER complex that was supershifted with an anti-hER antibody (i.e. the mobility of the hER Ab–hER–ERE complex was further retarded, confirming the presence of the hER in the complex) (Fig. 5, lanes 1 and 2). The DNA specificity was shown by the addition of a 50-fold excess of unlabeled ERE, which eliminated the appearance of the hER–ERE complex (Fig. 5, lane 3). The recombinant or native hER in the salt-eluted fraction from the ERE affinity column formed a hER–ERE complex (Fig. 5, lanes 4 and 6). In contrast, the recombinant or native hER in the flow-through fraction did not form a hER–ERE complex (Fig. 5, lanes 5 and 7).

The 67-kDa form of the native hER treated with the tyrosine phosphatase PTP1B resulted in a dose-dependent reduction of the hER–ERE complex (Fig. 6A, lanes 2–4) and reactivity with the anti-phosphotyrosine antibody (Fig. 6B, lanes 2–4). With 1 μg of PTP1B, the hER–ERE complex and the tyrosine phosphorylation of the hER were completely eliminated (Fig. 6, A and B, lane 4). The tyrosine dephosphorylation by PTP1B was specific since 1 mM Na3VO4, a tyrosine phosphatase inhibitor, prevented the loss of the hER–ERE complex (Fig. 6A, lane 7).
and the tyrosine dephosphorylation of the hER (Fig. 6B, lane 7). The addition of Sf9 mock cytosol (without hER) did not restore the binding of the tyrosine-dephosphorylated hER to an ERE (data not shown). The tyrosine-dephosphorylated hER nevertheless migrated as a 67-kDa protein, indicating that PTP1B did not result in serine dephosphorylation, which is responsible for the 67-kDa form of the hER.

The rephosphorylation of the tyrosine-dephosphorylated hER by p60<sub>c-src</sub> resulted in a dose-dependent increase in the hER-ERE complex (Fig. 6A, lanes 5 and 6). The action of the p60<sub>c-src</sub> was not observed in the absence of ATP (data not shown). A Western blot confirmed that the tyrosine phosphorylation of the hER by the p60<sub>c-src</sub> restored reactivity with the anti-phosphotyrosine antibody (Fig. 6B, lanes 5 and 6). Western blot analysis with the anti-hER antibody confirmed the presence of the hER in all the samples (Fig. 6B). These results indicate that the binding of the native hER to an ERE is dependent on the phosphorylation of tyrosine 537.

The purified recombinant wild-type hER showed identical characteristics, with respect to tyrosine phosphorylation and binding to an ERE, as the native hER. The tyrosine dephosphorylation of the recombinant wild-type hER (Fig. 7A, lanes 1 and 2) eliminated ERE binding (Fig. 7A, lanes 1 and 2). The tyrosine dephosphorylation of the recombinant wild-type hER was inhibited with 1 mM Na<sub>3</sub>VO<sub>4</sub> (Fig. 7B, lanes 11 and 12) and prevented the loss of the hER-ERE complex by inhibiting the activity of the tyrosine phosphatases (Fig. 7A, lanes 11 and 12).

Casein kinase II or MAP kinase activity did not restore the ERE binding of the tyrosine-dephosphorylated hER (Fig. 7A, lanes 5, 6, 9, and 10). However, ERE binding was restored after the 67-kDa form of the purified recombinant hER (that was dephosphorylated with PTP1B or SHPTP1) was rephosphorylated with p60<sub>c-src</sub> or p56<sub>c-src</sub> (Fig. 7A, lanes 3, 4, 7, and 8). The restoration of ERE binding was specific since p60<sub>c-src</sub> or p56<sub>c-src</sub> in the absence of ATP did not restore the hER-ERE complex (data not shown). Western blot analysis with the anti-phosphotyrosine antibody confirmed the phosphorylation of tyrosine 537 by p60<sub>c-src</sub> or p56<sub>c-src</sub>, but not by casein kinase II or MAP kinase (Fig. 7B, lanes 3–10).

To confirm that the phosphorylation of tyrosine 537 is required for the hER to bind an ERE, the ERE binding properties of the mutant Y537F hER were assayed. As expected, a whole cell extract of Sf9 cells expressing the wild-type hER or the purified 67-kDa form of the recombinant wild-type hER produced a hER-ERE complex (Fig. 8, lanes 1 and 2). However, at 10 or 40 nM mutant Y537F hER, a hER-ERE complex was not formed (Fig. 8, lanes 3 and 4).

An “antibody rescue experiment” (27) suggested that the phosphorylation of the hER at tyrosine 537 regulates the monomer to dimer transition of the receptor (Fig. 9). The premise of the antibody rescue experiment is that the anti-hER antibody is bivalent and recognizes two molecules of hER. Therefore, the anti-hER antibody can bring together two inactive hER monomers and facilitate dimer formation and DNA binding. The purified 67-kDa form of the recombinant hER was incubated in the absence or presence of the anti-hER antibody or a nonspecific antibody. The anti-hER antibody, but not the nonspecific antibody, supershifted the hER-ERE complex (Fig. 9, lanes 1, 2, and 4). The 67-kDa form of the recombinant hER after dephosphorylation with PTP1B did not form a hER-ERE complex (Fig. 9, lane 5). Interestingly, we observed the appearance of a supershifted complex (hER Ab<hER-ERE) when the dephosphorylated hER was incubated with the anti-hER antibody (Fig. 9, lane 6). The hER Ab<hER-ERE complex was eliminated with an excess of unlabeled ERE (Fig. 9, lanes 3 and 9), but not unlabeled glucocorticoid response element (lane 8), indicating that the complex was ERE-specific. A supershifted hER Ab<hER-ERE complex was not formed with the anti-hER antibody when hER peptide 6, which was used to generate the anti-hER antibody, was added to the gel shift reaction (22). In addition, a nonspecific antibody did not produce a complex with
the dephosphorylated hER (data not shown). Thus, the anti-hER antibody facilitated the formation of the hER dimer and allowed ERE binding. Predictably, the mutant Y537F hER did not form a hER complement, whereas the addition of the anti-hER antibody to the mutant Y537F hER gel shift reaction produced a hER Ab-hER-ERE complex (Fig. 9, lanes 10 and 11).

These results suggest that the phosphorylation of tyrosine 537 on the hER is required for dimerization of the hER.

Far-Western blot analysis established that the tyrosine phosphorylation of the hER was necessary for dimerization. Dimer formation was maximal when both monomers of the hER were phosphorylated at tyrosine 537. Dimer formation was also observed when one of the monomers of the hER dimer was tyrosine-phosphorylated; however, the extent of dimerization was reduced to more than one-half. The far-Western blot analysis utilized the purified recombinant hER that was either 32P-labeled at tyrosine 537 with p60c-src (32P-Y537 hER) (Fig. 10A) or 32P-labeled at serine 167 with casein kinase II as a probe (32P-S167 hER) (Fig. 10B). The 32P-Y537 hER showed strong protein-protein interaction with the purified recombinant wild-type hER immobilized on a PVDF membrane (Fig. 10A, lane 1). However, far-Western analysis of the hER that was tyrosine-dephosphorylated prior to SDS gel electrophoresis showed a reduced interaction with 32P-Y537 hER (Fig. 10A, lanes 2 and 3). An immunoblot with the anti-tyrosine antibody confirmed that the PVDF-immobilized hER was tyrosine-dephosphorylated (Fig. 10C, lanes 2, 3, 6, and 7). One mM Na3VO4 inhibited the activity of PTP1B and SHPTP1 (Fig. 10C, lanes 4 and 5) and prevented the reduction of interaction with 32P-Y537 hER (Fig. 10A, lanes 4 and 5). The hER was tyrosine-dephosphorylated with PTP1B or SHPTP1 and then rephosphorylated with casein kinase II, MAP kinase, p60c-src, or p56lk. The tyrosine phosphorylation, but not the serine phosphorylation, of the hER restored the full dimerization potential to the dephosphorylated hER (Fig. 10A, lanes 8 and 9 versus lanes 6 and 7). Far-Western blot analysis with the 32P-S167 hER confirmed that hER dimerization was dependent on tyrosine and not serine phosphorylation (Fig. 10B). An immunoblot of the PVDF membrane with the anti-hER antibody

![Fig. 8. Gel mobility shift assay of the mutant Y537F hER.](image)

Whole cell extracts (WCE) from Sf9 cells expressing the wild-type hER (10 nM) (lane 1), the purified 67-kDa wild-type hER (10 nM) (lane 2), 10 nM mutant Y537F hER (lane 3), or 40 nM mutant Y537F hER (lane 4) were incubated with 500 ng of poly(dI-dC) for 15 min at 4 °C followed by the addition of the 32P-labeled ERE for 15 min at 4 °C. The protein-DNA complexes were resolved by nondenaturing gel electrophoresis.

![Fig. 9. Anti-hER antibody-induced dimerization and ERE binding of the hER.](image)

Ten nM purified 67-kDa wild-type hER from Sf9 cells was incubated alone (lane 1) or with the anti-hER antibody (hER Ab) (lane 2), the anti-hER antibody and a 200-fold excess of unlabeled ERE (lane 3), or a nonspecific antibody (lane 4). Ten nM purified 67-kDa wild-type hER from Sf9 cells dephosphorylated with PTP1B was incubated alone (lane 5) or with the anti-hER antibody (lane 6), the anti-hER antibody and 5 µg of hER peptide 6 (22) used to generate the anti-hER antibody (lane 7), the anti-hER antibody and a 200-fold excess of a glucocorticoid response element (GRE) (lane 8), or the anti-hER antibody and a 200-fold excess of unlabeled ERE (lane 9). A whole cell extract of Sf9 cells expressing the mutant Y537F hER (10 nM) was incubated alone (lane 10) or with the anti-hER antibody (lane 11), the anti-hER antibody and 5 µg of hER peptide 6 (lane 12), the anti-hER antibody and a 200-fold excess of a glucocorticoid response element (lane 13), or the anti-hER antibody and a 200-fold excess of unlabeled ERE (lane 14). The treated hER samples were incubated with 500 ng of poly(dI-dC) for 15 min at 4 °C followed by the addition of the 32P-labeled ERE for 15 min at 4 °C. The protein-DNA complexes were resolved by nondenaturing gel electrophoresis and visualized by autoradiography. hER Ab-hER-ERE denotes the supershifted anti-hER antibody complexed to two molecules of hER, which in turn is bound to the 32P-labeled ERE.
Fig. 10. Dimerization of the hER is maximal when both monomers are tyrosine-phosphorylated. The recombinant wild-type hER, purified in the presence of the phosphatase inhibitors and therefore fully phosphorylated (lane 1), was then dephosphorylated with the tyrosine phosphatase PTP1B or SHPTP1 in the absence (lanes 2 and 3) or presence (lanes 4 and 5) of Na₂VO₄, an inhibitor of tyrosine phosphatases. The dephosphorylated hER was then rephosphorylated with casein kinase II (CKII) (lane 6), MAP kinase (MAPK) (lane 7), p60\(^{c-src}\) (lane 8), or p56\(^{lck}\) (lane 9). The hER samples (1.5 pmol each) were subjected to SDS gel electrophoresis and then electrotransferred to a PVDF membrane. The hER, purified in the absence of phosphatase inhibitors and therefore partially dephosphorylated, was \(^{32}P\)-phosphorylated with p60\(^{c-src}\) (lane 6) or casein kinase II (B) and used as a probe for assaying monomer-monomer interaction of the hER. The PVDF membranes were also immunoblotted with the anti-tyrosine antibody (PY Ab) (C) or the anti-hER antibody (hER Ab) (D).

showed that the amount of hER in each lane was equivalent (Fig. 10D).

DISCUSSION

This study provides the first evidence that the tyrosine phosphorylation of the hER regulates the capacity of the receptor to homodimerize and bind an ERE. We have presented evidence, from several different experimental approaches, that the phosphorylation at tyrosine 537 of the hER is a prerequisite for ERE binding. We observed that the nuclear form, but not the cytosolic form, of the hER is tyrosine-phosphorylated. Only the nuclear hER bound an ERE in a gel mobility shift assay. The dephosphorylation of the purified hER with a tyrosine phosphatase resulted in the loss of ERE binding, while rephosphorylation with p60\(^{c-src}\) or p56\(^{lck}\), but not with serine-protein kinases, restores ERE binding. These observations were confirmed by the inability of the mutant Y537F hER to bind an ERE. The antibody rescue experiments show that ERE binding is restored to the tyrosine-dephosphorylated hER or the mutant Y537F hER by virtue of the ability of the anti-hER antibody to promote dimer formation. Western blot analysis demonstrated that the tyrosine phosphorylation of at least one of the hER monomers, but preferably both, is required for dimer formation. Overall, our results indicate that the phosphorylation of tyrosine 537 facilitates the transition of the hER from the monomer to dimer form.

The reactivity of the anti-phosphotyrosine antibody with the native hER was equivalent in the absence (i.e. the 66-kDa form of the hER) or presence (i.e. the 67-kDa form of the hER) of estradiol treatment of MCF-7 cells, confirming earlier observations that the phosphorylation of tyrosine 537 is not increased by estradiol treatment (18). In a previous report, the ERE-Teflon matrix containing ~500 nmol of double-stranded ERE/ml of Teflon fiber bound both the 66- and 67-kDa forms of the hER (22). In the experiments described here, the ERE content was only 1–5% of that previously used and consequently revealed selectivity for the 67-kDa form of the hER.

Recently, tyrosine phosphorylation has been reported to regulate the dimerization of the signal transducer and activators of transcription (STAT) family of DNA-binding proteins (9, 10). The STAT proteins dimerize through the association of a phosphorylated tyrosine residue on one monomer and with a SH2 domain on the opposing monomer (9, 10). This mechanism of dimerization allows for an additional level of control of the activity of a transcription factor. A computer search of the amino acid sequence of the hER has failed to reveal a region of significant homology to the SH2 domain of p60\(^{c-src}\).

We hypothesize that the 67-kDa form of the hER is the transcriptionally active form of the receptor. In the absence of estradiol treatment of MCF-7 cells, the 66-kDa form of the native hER exists as two populations of receptors: one that is phosphorylated at tyrosine 537 and one that is not. After estradiol treatment of MCF-7 cells, only the population of hER that is phosphorylated at tyrosine 537 will undergo serine hyperphosphorylation, as monitored by the production of the up-shifted 67-kDa form of the hER. We and others (28) have shown that the phosphorylation of tyrosine 537 is required for the hER to bind estradiol in vitro and in vivo. Numerous mechanisms can account for the nuclear retention of the 67-kDa form of the native hER; estradiol has been shown to increase the binding of the calf estrogen receptor to an ERE by isocratic elution chromatography (29), although the influence of hormone binding is not observed with the ERE affinity column or gel mobility shift analysis. We have shown that the phosphorylation of serine 167 on the hER increases the affinity of the hER for an ERE (11). In addition, the hER associates in an estradiol-dependent fashion with transcriptional proteins, such as transcriptional intermediary factor 1 (TIF1) (2). Nonetheless, the DNA binding ability of the hER is regulated at two levels of post-translational phosphorylation: first, the phosphorylation of tyrosine 537 is required for hER binding to an ERE, and second, the phosphorylation of serine 167 increases the hER (already phosphorylated at tyrosine 537) affinity for an ERE.

In conclusion, the post-translational phosphorylation of tyrosine 537 on the hER is required to produce the biologically active hER. Furthermore, the oncogenic potential of protein-tyrosine kinases, such as p60\(^{c-src}\), may alter the activity of the hER in estrogen-dependent breast cancer.

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