Estrogen Receptor-α Phosphorylated at Ser^{118} Is Present at the Promoters of Estrogen-Regulated Genes and Is Not Altered Due to HER-2 Overexpression

Gregory E. Weitsman,[1] Lin Li,[1] George P. Skliris,[1] James R. Davie,[1] Kanyarat Ung,[1] Yulian Niu,[2] Linda Curtis-Snell,[2] Ladislav Tomes,[2] Peter H. Watson,[2] and Leigh C. Murphy[1]

[1]Department of Biochemistry and Medical Genetics and [2]Department of Pathology, Manitoba Institute of Cell Biology, University of Manitoba, Winnipeg, Manitoba, Canada

Abstract

Detection of estrogen receptor (ER)-α phosphorylated at Ser^{118} (P-Ser^{118}-ER-α) may be an indicator of an intact ligand-dependent ER-α in breast tumors *in vivo* and may predict responsiveness to endocrine therapy. The current study addresses whether P-Ser^{118}-ER-α is functionally involved in ER target gene transcription and if this is modulated by HER-2 overexpression. Using chromatin immunoprecipitation analysis, P-Ser^{118}-ER-α was found associated with the promoters of several estrogen-regulated genes in MCF-7 breast cancer cells 30 minutes following estrogen treatment. Coactivators AIB1 and p300 were coimmunoprecipitated with P-Ser^{118}-ER-α following estrogen treatment. The overexpression of HER-2 protein in MCF-7 cells did not affect estrogen induction of phosphorylation of Ser^{118} or its presence at the promoters of several estrogen-regulated genes. U0126, an inhibitor of mitogen-activated protein kinase (MAPK) pathway, had no effect on P-Ser^{118}-ER-α. The lack of effect of HER-2 overexpression on P-Ser^{118}-ER-α expression in cell models is supported by similar levels of expression of P-Ser^{118}-ER-α in ER$^+$/HER-2-overexpressing and ER$^+$/HER-2$^-$ breast tumors *in vivo*. Using inhibitors of cyclin-dependent kinase 7 (Cdk7), [(5,6-dichloro-1-β-D-ribofuranosylbenzimidazole and 2-(R)-1-ethyl-2-hydroxyethylamino)-6-benzylamino-9-isopropylpurine], and IκB kinase-α (IKK-α; BAY-11-7082), we show that IKK-α, but not Cdk7, is at least in part involved in estrogen-mediated phosphorylation at Ser^{118} in MCF-7 cells. Our data provide direct evidence for a functional role of P-Ser^{118}-ER-α in estrogen-regulated signaling and do not support the hypothesis that resistance of breast tumors to tamoxifen therapy involves ligand independent activation of ER-α due to constitutive phosphorylation of Ser^{118} by constitutive activation of MAPK pathway. (Cancer Res 2006; 66(20): 10162-70)

Introduction

Estrogen receptor (ER)-α is a phosphoprotein (1). There are several different sites on ER-α that can be phosphorylated and may modulate ER-α action and stability, although the exact functional roles *in vivo* of phosphorylation at the various sites are unclear (1). Phosphorylation of ER-α can be enhanced by estradiol binding and/or via activation of other signal transduction pathways (1). A well-studied phosphorylation site on ER-α is Ser^{118}, located in the NH_2-terminal domain of ER-α where the ligand independent activation function of the receptor is also located. Both estrogens and growth factors, such as epidermal growth factor (EGF) and insulin-like growth factor (IGF)-I, can result in phosphorylation of ER-α at Ser^{118} (P-Ser^{118}-ER-α) and direct P-Ser^{118}-ER-α by p44/42 mitogen-activated protein kinase [MAPK; extracellular signal-regulated kinase 1/2 (ERK1/2)] occurs *in vitro* (2). In addition, Ser^{118} in ER-α is a major phosphorylation site resulting from activation of the ras-raf-MAPK pathway *in vivo* (1) and this process is ligand independent. In contrast, it is unclear which kinase is responsible for the 17β-estradiol (E_2)–dependent P-Ser^{118}-ER-α, although cyclin-dependent kinase 7 (Cdk7) and IκB kinase-α (IKK-α) are candidates (3, 4). It has been suggested that ligand-independent ER-α activation may be involved in the mechanisms of altered estrogen action and antiestrogen resistance in breast cancer. P-Ser^{118}-ER-α can result from activation of several growth factor receptor signal transduction pathways [e.g., EGF receptor/HER-2 and the IGF receptor (1) and up-regulation of the former is associated with resistance to endocrine therapy in both cell line models and *in vivo* (5–8)]. Therefore, the role and mechanism of P-Ser^{118}-ER-α has not yet been resolved satisfactorily and is being investigated extensively.

Our previous studies of the role of P-Ser^{118}-ER-α *in vivo* have suggested that detection of P-Ser^{118}-ER-α in primary breast tumors is associated with a more differentiated phenotype, other markers of good prognosis, and a better response to tamoxifen (9, 10). We concluded that, in contrast to the situation in cell line models, P-Ser^{118}-ER-α is a marker of a functional, intact ligand-dependent ER-α signaling pathway in primary breast tumors *in vivo* and that P-Ser^{118}-ER-α was likely to be involved intimately in the mechanism by which the E_2/ER-α complex regulates gene transcription and possibly other mechanisms of E_2/ER-α signaling. However, the data *in vivo* are correlative studies and, although suggestive, do not provide evidence of a direct involvement of P-Ser^{118}-ER-α in E_2/ER-α-mediated activities. In this current study, we provide evidence for the direct involvement of P-Ser^{118}-ER-α in E_2/ER-α-mediated transcription of some classic as well as nonclassic target genes and provide evidence that expression of P-Ser^{118}-ER-α is unaffected by overexpression of HER-2/*neu* both in cell line models and *in vivo*.

Materials and Methods

Materials. Tissue culture DMEM, E_2, 4-hydroxytamoxifen (4-OHT), Cdk7 inhibitors, (5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) and 2-(R)-1-ethyl-2-hydroxyethylamino)-6-benzylamino-9-isopropylpurine [(R)-roscovitine], were purchased from Sigma-Aldrich Co. (St. Louis, MO). ICI

Note: J.R. Davie holds a Canada Research Chair.

Requests for reprints: Leigh Murphy, Manitoba Institute of Cell Biology, University of Manitoba, 675 McDermot Avenue, Winnipeg, Manitoba, Canada, R3E 0V9. Phone: 204-787-4071; Fax: 204-787-2190; E-mail: lcmurph@cc.umanitoba.ca.

doi:10.1158/0008-5472.CAN-05-4111

182780 was a gift from AstraZeneca (Macclesfield, United Kingdom). IKK-α inhibitor BAY-11-7082 was from Alexis Biochemicals (Lausen, Switzerland). Tissue culture plasticware was from Corning Glass Works (Corning, NY). Human recombinant EGF was obtained from Upstate Biotechnology (Lake Placid, NY). The antibodies against P-Ser^{118}-ER-α (16JR), phosphorylated ERK1/2, and phosphorylated RNA polymerase II subunit Rpb1-COOH-terminal domain (CTD; Ser^2/Ser^5) were from Cell Signaling Technology, Inc. (Beverly, MA); the antibody against ER-α (HC20, rabbit polyclonal) was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); the antibody against AIB1 was from BD Biosciences (Mountain View, CA); the antibody against ERK1/2 was from Invitrogen Corp. (Carlsbad, CA); the antibody against p300 was from Upstate (Charlottesville, VA); the antibodies against HER-2 and ER-α (6F11, mouse monoclonal) were from Novocastra Ltd. (Newcastle upon Tyne, United Kingdom); and the antibody against β-actin was from Sigma-Aldrich. Peroxidase-conjugated goat anti-mouse IgG and goat anti-rabbit IgG were from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). All other reagents were of analytic grade.

Cell lines. MCF-7 (T5) breast cancer cells, the derivative MCF-7-Neo and MCF-7/HER-2 cell lines (stably transfected with the control vector alone and a HER-2-overexpressing vector, respectively, a generous gift from Dr. M. Alaoui-Jamali, McGill University, Montreal, Quebec, Canada; ref. 11), and ZR-75-1 and T47D cells were maintained in DMEM containing 1 g/L glucose supplemented with 5% fetal bovine serum (FBS) and antibiotics. For experiments, the medium was changed to medium containing 5% (v/v) 2× charcoal/dextran-treated FBS [5% charcoal stripped (CS)] 24 hours after seeding and, 72 hours later, cultures were treated with E_2 (10 nmol/L), EGF (100 ng/mL), ICI 182780 (100 nmol/L), and 4-OHT (100 nmol/L). The vehicle alone (ethanol) was added to control cultures, and its concentration was kept below 0.01% (v/v).

Western blotting. Cell extracts were prepared as described previously (12). Briefly, cells were seeded in 6-cm Petri dishes. After treatment, cells were washed with ice-cold PBS and harvested in 200 μL SDS buffer [100 mmol/L Tris-HCl (pH 6.8), 20% glycerol, 4.0% SDS], boiled for 15 minutes, and placed on ice for 10 minutes. Following protein concentration determination (bicinchoninic acid protein detection kit, Pierce Biotechnology, Inc. Rockford, IL) 15 μL of 0.2% bromophenol blue (BPB) in β-mercaptoethanol (β-ME) were added to each sample. To extract proteins from the beads after immunoprecipitation, 60 μL of the SDS buffer containing BPB and β-ME were added and samples were boiled for 15 minutes. The extracts were subjected to SDS-PAGE (30 μg protein/lane or 30 μL/lane for immunoprecipitated samples); Western blot analysis and quantification of enhanced chemiluminescence were carried out by Bio-Rad Gel Doc 2000 Chemi Doc gel documentation systems (Bio-Rad Laboratories, Inc., Hercules, CA).

Chromatin immunoprecipitation. Chromatin immunoprecipitation (ChIP) was done as described previously (13) with modifications. Briefly, cells were cultured for 3 days in 5% CS. After treatment with estrogen (10 nmol/L, 30 minutes), cells were washed twice with ice-cold PBS and cross-linked with 1% formaldehyde at room temperature for 10 minutes. Then, cells were incubated with 125 mmol/L glycine solution for 10 minutes at room temperature (14) and harvested in 1 mL ice-cold PBS. Cells were rinsed twice with ice-cold PBS and resuspended in lysis buffer [5 mmol/L PIPES (pH 8.0), 85 mmol/L KCl, 0.5% NP40], incubated on ice for 10 minutes, and centrifuged for 30 seconds at 16,000 × *g* at 4°C. The pellet was resuspended in 1 mL nuclei lysis buffer [50 mmol/L Tris-HCl (pH 8.1), 10 mmol/L EDTA, 1% SDS] supplemented with leupeptin (1 μg/mL), aprotenin (1 μg/mL), phenylmethylsulfonyl fluoride (10 mmol/L), and NaF (100 mmol/L) followed by incubation at room temperature for 10 minutes. Samples were sonicated thrice at 40% output setting (Braun-Sonic 1510 sonicator) followed by centrifugation for 15 minutes at 16,000 × *g* at 4°C. Supernatant (50 μL) was taken as an input control and the rest of the sample was diluted 10 times in ChIP dilution buffer [0.01% SDS, 1.1% Triton X-100, 1.2 mmol/L EDTA, 16.7 mmol/L Tris (pH 8.1), 167 mmol/L NaCl] and divided into two portions. Anti-P-Ser^{118}-ER-α antibody (2 μg) and nonrelevant isotype-matched antibody (2 μg) were added to each portion. After overnight incubation at 4°C, immunocomplexes were collected by addition of 50 μL G-Sepharose (50% slurry) for 3 hours at 4°C and centrifugation for 30 seconds at 16,000 × *g* at 4°C. Precipitates were washed thrice with ChIP dilution buffer and treated for 30 minutes with DNase-free RNase (20 μg per sample) at 37°C followed by proteinase K (0.5 mg/mL) for 60 minutes at 55°C. To reverse formaldehyde cross-linking, the samples were incubated overnight at 65°C. DNA fragments were purified with a QIAquick Spin kit (Qiagen, Valencia, CA). The DNA concentration of samples was determined using PicoGreen reagent (Invitrogen) according to the manufacturer's instructions. Equal amounts of DNA were subjected to PCR for 30 to 35 cycles of amplification (15). The presence of the promoter regions of *pS2/TFF1, progesterone receptor* (*PR*), *cyclin D1*, and *c-myc* genes were detected by PCR using pairs of primers as follows. *pS2*: ER-interacting promoter region, 5′-GACGGAATGGGCTTCATGAGC-3′ and 5′-GATAACATTTGCCTAAGGAGG-3′ (amplifying the −486 to −100 region; refs. 15, 16) and the irrelevant promoter region, 5′-CAGTCTGGCAAATCATTCCCAAAC-3′ and 5′-CACATCTGAGAGGTAAGAGGAGGTG-3′ (amplifying the +5,114 to +5,323 region; ref. 17); *PR*: ER-interacting activator protein-1 (*AP-1*) site, 5′-GGCTTTGGGCGGGGCTCCCTA-3′ and 5′-TCTGCTGGCTCCGTACTGCGG-3′ (amplifying the −54 to +186 region; ref. 18), ER-interacting *Sp1* site, 5′-GGACAAACGACAGCCACAGTT-3′ and 5′-GGGAAGGAGGAGGGGGTTT-3′ (amplifying the +515 to +692 region; ref. 19), and irrelevant region, 5′-GGATCCATTTTATAAGCTCA-3′ and 5′-GTATCCTCAGCGTGACAAA-3′ (amplifying the −711 to −436 region; ref. 18, 19); c-*myc*: ER-responsive region, 5′-GAAAAAAGATCCTCTCTCGCTAATCTC-3′ and 5′-CTGCCTCTCGCTGGAATTACTACA-3′ (amplifying the −46 to +215 region relative to the P1 promoter; ref. 20) and irrelevant region, 5′-GAGGGTCAAGTTGGACAGTG-3′ and 5′-AGCTCCGTTTTAGCTCGTT-3′ (amplifying the +4,560 to +4,713 region with reference to Genbank accession no. AY214166); and *cyclin D1*: ER-interacting region, 5′-AACAAAACCAATTAGGAACCTT-3′ and 5′-ATTTCCTTCATCTTGTCCTTCT-3′ (amplifying the −1,039 to −770 region; ref. 21) and irrelevant region, 5′-GCTGAAACTAATTGATCTGGAG-3′ and 5′-CCATTGTTAAGCCCTTAAGTC-3′ (amplifying the −3,247 to −2,931 region; ref. 21).

Human breast tumors. All invasive breast cancers used in the current study were obtained from the Manitoba Breast Tumor Bank (MBTB; Department of Pathology, University of Manitoba, Winnipeg, Manitoba, Canada; ref. 22), which operates with the approval from the Faculty of Medicine, University of Manitoba, Research Ethics Board. The histopathology of all MBTB cases has been assessed and entered into a computerized database to enable selection based on composition of the tissue as well as clinicopathologic variables. After selection, cases were rereviewed on H&E sections by a breast histopathologist (P.H.W.). A tissue microarray (TMA) composed of a consecutive series of 191 primary breast tumor biopsies selected from a database of ~5,000 cases was constructed. The case series was representative of the entire database in that it comprised ~70% ER$^+$ tumors as determined by ligand-binding analysis. This TMA was screened for HER-2-overexpressing breast tumors (immunohistochemistry score of 3+, on a 4-point scale; 0-3+, as used in routine clinical assessment). Those tumors that were ER-α$^+$ [>3 fmol/mg protein by ligand-binding assay (LBA)] and HER-2 overexpressing were selected for analysis of their P-Ser^{118}-ER-α status (n = 16) using sections from the original biopsy block. A control group (n = 16) was selected from the tumor bank as HER-2$^-$ but matched to the ER$^+$/HER-2-overexpressing tumors for level of expression of ER-α and PR (determined by LBA), as well as grade and histologic type (Table 1).

Immunohistochemical assay (immunohistochemistry). Serial sections (5-μmol/L thick) of the TMA or biopsy block were cut, mounted on Superfrost/plus slides (Fisher Scientific, Pittsburgh, PA), and stained using immunohistochemistry with commercially available specific antibodies: HER-2 (mouse monoclonal, clone CB11, Novocastra) at 1:50 and P-Ser^{118}-ER-α (Cell Signaling Technology) at 1:600 as described previously (10). Slides were viewed and scored under a standard light microscope.

Quantification technique and statistical analysis. The expression of P-Ser^{118}-ER-α was assessed using semiquantitative scoring (immunohistochemistry scores). Immunohistochemistry scores include an assessment of both staining intensity (scale 0-3) and the percentage of positive cells (0-100%), which when multiplied range from 0 to 300. Cases were evaluated by three authors (G.P.S., L.C.M., and P.H.W.) independently and where discordance was found, cases were reevaluated together to reach

Table 1. Relationship between the expression of P-Ser^{118}-ER-α and HER-2 in breast tumors

HER-2$^+$						HER-2$^-$					
Tumor no.	ER*	PR*	Type	Grade †	P-Ser^{118}-ER-α score	Tumor no.	ER*	PR*	Type	Grade †	P-Ser^{118}-ER-α score
1	3.5	8.2	D	6	+	1	3.6	2.6	D	9	+
2	5	4.9	D	8	−	2	4.3	1.3	D	7	+
3	5.5	12.7	D	7	−	3	4.8	2.9	D	9	+
4	6.9	5	D	8	−	4	4.8	11.8	D	5	+
5	8.4	16.6	D	8	−	5	5.5	6.2	D	7	−
6	9.7	12.8	D	7	−	6	6.2	10.5	D	8	−
7	11.7	6.8	D	9	−	7	9.4	15.1	D+L	7	+
8	13	11.7	D	6	−	8	12	12.4	D	7	−
9	20	5.3	L	6	−	9	23	6.6	D	6	+
10	27	8.5	D	9	+	10	31	96	D	7	+
11	27	12.7	L	6	+	11	32	3.4	D	7	+
12	27	30	D	8	+	12	32	5.3	D	8	+
13	28	35	D	8	+	13	32	15.3	D	6	+
14	31	57	D	8	−	14	37	23	D	6	+
15	52	28	D	4	+	15	48	24	D	9	+
16	59	68	D	6	+	16	48	24	D	6	+
Average	20.9	20.2		7.1		Average	20.9	16.3		7.1	
SD	16.5	19		1.4		SD	16.3	22.6		1.2	

NOTE: D, infiltrating ductal carcinoma; L, infiltrating lobular carcinoma.
*fmol/mg protein determined by LBA; ER$^+$ >3 fmol/mg.
†Nottingham system.
‡Positive P-Ser^{118}-ER-α, immunohistochemistry score of >0.

agreement. Tumors with membrane staining at intensities of 3+ were considered HER-2-overexpressing tumors, and the cut point for positivity for P-Ser^{118}-ER-α was immunohistochemistry score of >0. Differences between tumor subgroups (HER$^+$ versus HER$^-$) were tested using the Mann-Whitney rank-sum test, two-sided. Associations between HER-2 and P-Ser^{118}-ER-α were tested using contingency methods (Fisher's exact test) and the assigned cutoff values for positivity and negativity. All tests were done using Prism 4.02 (GraphPad, Inc., San Diego, CA) statistical analysis software. The calculation of the percentage inhibitory effect of BAY-11-7082 on estrogen-induced phosphorylation of Ser^{118} used the ratio of the signal obtained with anti-P-Ser^{118}-ER-α to total ER-α antibodies in samples treated with estradiol in the absence (R_E) or presence (R_{EI}) of inhibitor according to the equation $(1 - R_{EI} / R_E) \times 100$.

Results

Effect of ligand on expression of P-Ser^{118}-ER-α in MCF-7 human breast cancer cells. The time course and ligand dependence of P-Ser^{118}-ER-α in MCF-7 (T5) cells was determined (Fig. 1). The level of P-Ser^{118}-ER-α increased markedly between 15 and 60 minutes following E_2 treatment (10 nmol/L E_2). During this time, no marked alteration of total ER-α levels occurs (Fig. 1*A* and *B*). A 536 ± 218% (mean ± SD; n = 12) increase in the level of P-Ser^{118}-ER-α was observed 30 minutes after E_2 treatment. Thereafter, the level of P-Ser^{118}-ER-α decreased and so also do the levels of total ER-α, such that the relative levels of P-Ser^{118}-ER-α to total ER-α remained elevated above the control-untreated levels.

In contrast, treatment with 100 ng/mL human EGF increased P-Ser^{118}-ER-α transiently with a maximum induction at 15 minutes of 194 ± 59% (mean ± SD; n = 3) over control levels and decreased to background untreated levels by 30 minutes (Fig. 1*B*). Combining E_2 and EGF treatment had an additive effect, in terms of maximum levels of induction, but in contrast to the effect of E_2 alone, the levels of P-Ser^{118}-ER-α were not sustained and began to decrease 30 minutes after treatment (data not shown).

Treatment with ICI 182780 (1-500 nmol/L) for 30 minutes also increased the level of P-Ser^{118}-ER-α in MCF-7 (T5) cells, reaching a maximum at 100 nmol/L (data not shown). The maximum effect of ICI 182780 (100 nmol/L) was similar to the effect of E_2 [509 ± 41% (mean ± SD; n = 3) compared with 536 ± 218%; Fig. 1*B*]. However, in contrast to E_2, there was no maintenance of this level after 30 minutes of treatment and its decline occurred faster than after treatment with E_2 (Fig. 1*B*). Treatment with 4-OHT (10-500 nmol/L) for 30 minutes also resulted in elevated levels of P-Ser^{118}-ER-α, again reaching a maximum at 100 nmol/L (data not shown). The level of P-Ser^{118}-ER-α induced by 4-OHT was significantly lower than that caused by E_2 or ICI 182780 [257 ± 170% (n = 5) compared with 536 ± 218% and 509 ± 41%, respectively]. At longer time points and in contrast to the effect of ICI 182780, 4-OHT increased the level of total ER-α (Fig. 1*B*).

P-Ser^{118}-ER-α is present at the promoters of estrogen-regulated genes in MCF-7 human breast cancer cells. If P-Ser^{118}-ER-α is functionally involved in mediating estrogen-regulated transcription, then P-Ser^{118}-ER-α should be located at the promoters of genes whose transcription is directly regulated by the E_2/ER-α complex. ChIP analysis was used to determine this.

Initially, the antibody to P-Ser^{118}-ER-α was validated for immunoprecipitation of P-Ser^{118}-ER-α under ChIP analysis conditions. Figure 2*A* shows that, under these conditions, the antibody to P-Ser^{118}-ER-α is able to specifically immunoprecipitate ER-α

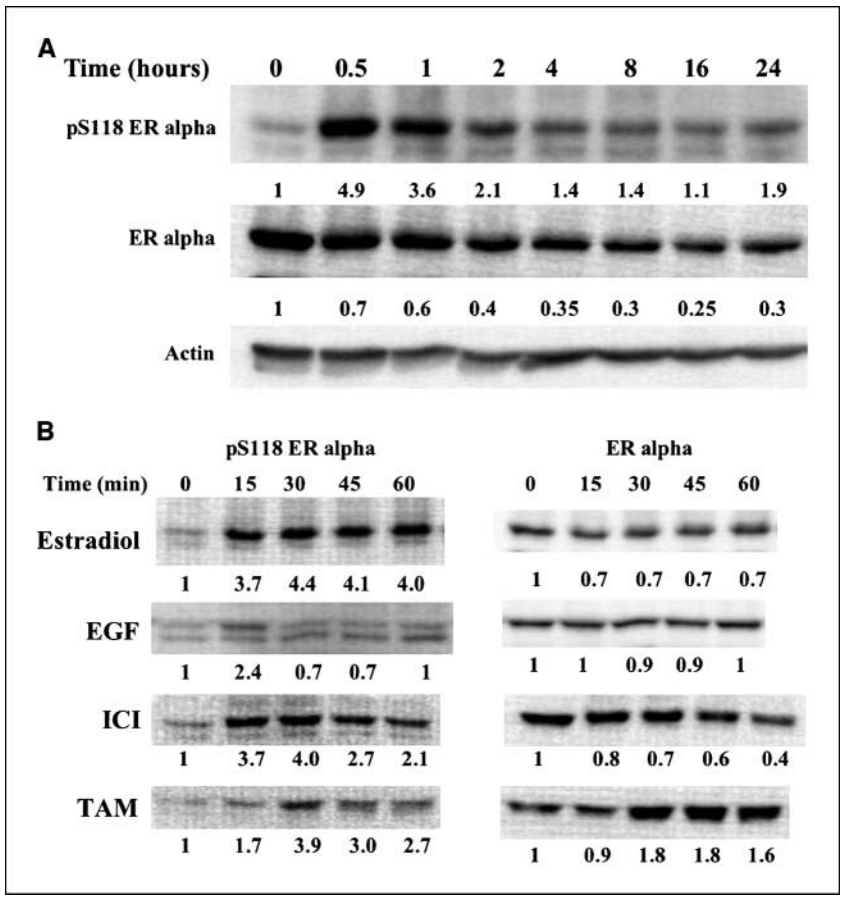

Figure 1. Expression of P-Ser^{118}-ER-α in MCF-7 cells. MCF-7 (T5) cells were seeded in 6-cm dishes (2×10^5 per dish). Twenty-four hours later, medium was changed to medium containing 2× charcoal-treated serum. Ninety-six hours after seeding, cells were treated with estradiol (10 nmol/L; *A* and *B*), EGF (100 ng/mL; *B*), ICI 182780 (*ICI*; 100 nmol/L; *B*), and 4-OHT (*TAM*; 100 nmol/L; *B*) for indicated times. Cell extracts were prepared and analyzed as described in Materials and Methods. Western blotting used the rabbit polyclonal anti-ER-α (HC20). The level of protein expression in control cultures was assigned the value of one. Results are representative of one of three independent experiments.

after 30 minutes of E_2 treatment, but little, if any, ER-α is immunoprecipitated after 30 minutes of vehicle alone treatment (Fig. 2*A*). No ER-α is detected after immunoprecipitation using a irrelevant isotype-matched antibody (Fig. 2*B*). As well following E_2 treatment, increased AIB1 and p300 were coimmunoprecipitated with P-Ser^{118}-ER-α (Fig. 2*D*). As well when AIB1 is specifically immunoprecipitated, P-Ser^{118}-ER-α is coimmunoprecipitated after E2 treatment only (data not shown).

Occupancy of a classically E_2-regulated promoter, *pS2/TFF1*, by P-Ser^{118}-ER-α was investigated first by ChIP analysis using the specific P-Ser^{118}-ER-α antibody. As shown in Fig. 2*C*, primers flanking the −486 to −100 region of the *pS2/TFF1* promoter known to bind ER-α and required for E_2 regulation of transcription amplified specifically the appropriate fragment (387 bp) 30 minutes after E_2 treatment. Little, if any, amplification was found in the control-untreated cells or when an irrelevant antibody [β-galactosidase (β-gal)] was used for immunoprecipitation or when an irrelevant region was amplified (Fig. 2*C*). Next, the occupancy of a nonclassically E_2-regulated promoter, *PR*, by P-Ser^{118}-ER-α was tested using ChIP analysis. Both the half *ERE/AP-1* (−54 to +186) and the half *ERE/Sp1* (+515 to +692) have been identified as regions involved with ER-α binding and E_2-regulation of transcription of the human PR (18, 23). Figure 2*C* also shows that P-Ser^{118}-ER-α is specifically associated with the half *ERE/AP-1* as well as with the half *ERE/Sp1* region of the *PR* gene and at −1039 to −770 promoter region of the *cyclin D1* gene after 30 minutes of E_2 treatment (21). In addition, location of P-Ser^{118}-ER-α after 30 minutes of E_2 treatment at the −46 to +215 promoter region of the *c-myc* gene was identified (Fig. 2*B*). This region has been shown previously to be involved in E_2 regulation of c-*myc* transcription in breast cancer cells (20).

These data are consistent with the occupancy of estrogen-regulated promoters by P-Ser^{118}-ER-α after E_2 treatment and the ability of P-Ser^{118}-ER-α to recruit known coactivators to E_2-regulated promoters after E_2 treatment and support a role of P-Ser^{118}-ER-α in the mechanism by which ER-α directly regulates transcription of target genes.

Effect of overexpression of HER-2 on P-Ser^{118}-ER-α expression in MCF-7 human breast cancer cells. To determine if HER-2 overexpression in an ER-α$^+$ cell line (Fig. 3*A*) affected the expression of P-Ser^{118}-ER-α, the time course and level of P-Ser^{118}-ER-α after E_2 treatment was compared in MCF-7/HER-2 and MCF-7-vector alone (MCF-7-Neo) controls. As shown in Fig. 3*B*, neither basal levels of P-Ser^{118}-ER-α following estrogen depletion nor the E_2 induction of P-Ser^{118}-ER-α was different between the HER-2-overexpressing cells versus the vector alone control. The maximum level of P-Ser^{118}-ER-α achieved after 30 minutes of E_2 treatment was 634 ± 296% (mean ± SD; $n = 6$) in the HER-2-overexpressing cells and 640 ± 195% ($n = 6$) in the vector alone-expressing cells. It is interesting that high levels of activated ERK1/2 do not contribute to the P-Ser^{118}-ER-α because inhibition of the upstream kinase of ERK1/2 by the selective inhibitor U0126 did not change the expression of P-Ser^{118}-ER-α in HER-2-overexpressing cells (Fig. 3*C*). We also found that occupancy of the promoters of *pS2/TFF1* [HER-2 versus Neo, 5.7 ± 2.2–fold versus 6.2 ± 3.1–fold induction due to E_2, not significant (NS); mean + SD; $n = 6$], *PR* (AP-1 site

HER-2 versus Neo, 2.9 ± 0.3–fold versus 2.2 ± 0.6–fold induction, NS; n = 4; Sp1 site HER-2 versus Neo, 2.1 ± 0.04–fold versus 1.8 ± 0.4–fold induction, NS; mean ± SD; n = 3), *c-myc* (HER-2 versus Neo, 2.1 ± 0.2–fold versus 1.6 ± 0.7–fold induction, NS; mean ± SD; n = 4), and *cyclin D1* (HER-2 versus Neo, 5.7 ± 5.4–fold versus 4.7 ± 3.9–fold induction, NS; mean ± SD; n = 3) genes, as examples of E_2-regulated genes, by P-Ser^{118}-ER-α is similar in both HER-2 and Neo cells (Fig. 3*D*).

These data suggest that HER-2 overexpression has little, if any, effect on P-Ser^{118}-ER-α expression specifically.

Relationship of P-Ser^{118}-ER-α expression to HER-2 overexpression in human breast tumors *in vivo*. The lack of an effect of HER-2 overexpression on P-Ser^{118}-ER-α expression in these breast cancer cell line models was surprising and suggested that altered P-Ser^{118}-ER-α expression was unlikely to be involved in tamoxifen resistance often observed in patients whose tumors are ER$^+$ and HER-2 overexpressing. To investigate this further and to determine the relevance of these breast cancer cell line models to human breast tumors *in vivo*, P-Ser^{118}-ER-α expression was evaluated in relation to HER-2$^+$ status within a TMA of 191 primary breast tumors. Within this cohort, 16 (n = 16) ER$^+$- and HER-2-overexpressing breast tumors were identified and P-Ser^{118}-ER-α levels were then compared with control tumors within the cohort that were matched for similar ER-α and PR expression, tumor type, and grade but did not express HER-2 (n = 16; Fig. 4*A* and *C*; Table 1). Although there was a trend toward decreased

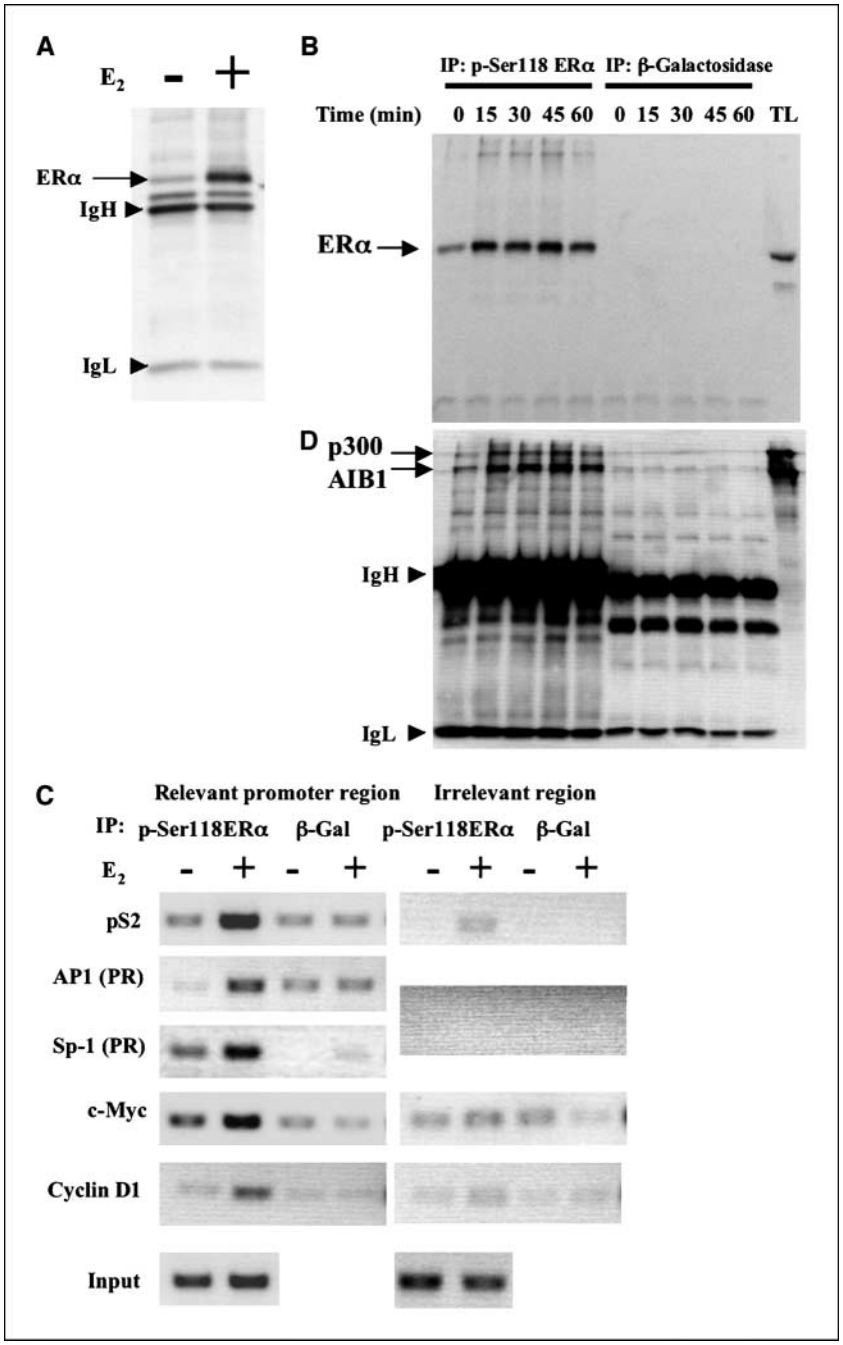

Figure 2. P-Ser^{118}-ER-α is present at the promoter of *pS2, c-myc, cyclin D1*, and *PR* genes and interacts with coactivators. MCF-7 cells were seeded in 15-cm dishes (2×10^6 per dish). Twenty-four hours later, medium was changed to medium containing 2× charcoal-treated serum. Ninety-six hours after seeding, cells were treated with estradiol (10 nmol/L; E_2) for 30 minutes (*A* and *C*) or indicated times (*B* and *D*). Cell extracts and detection of DNA related to promoter regions of *pS2, c-myc, cyclin D1*, and *PR* genes were carried out as described in Materials and Methods. *A, arrow,* ER-α is immunoprecipitated by anti-phosphorylated Ser^{118} antibody. Western blotting used the mouse monoclonal anti-ER-α (6F11). *Arrowheads,* IgG (heavy, IgH chain; light, IgL chain). *B, arrow,* ER-α is immunoprecipitated (*IP*) by anti-phosphorylated Ser^{118} antibody (P-Ser^{118}-ER-α) but not by an irrelevant isotype-matched anti-β-gal antibody. Western blotting used rabbit polyclonal anti-ER-α (HC20). *C,* detection of the occupancy of the promoter regions of *pS2/TFF1, c-myc, cyclin D1*, and *PR* genes by P-Ser^{118}-ER-α. PCR results for the ER response regions (*left*) and an irrelevant region (*right*) of the promoters. Results are representative of one of four independent experiments. The input represents amplified signals before immunoprecipitation. This was done for all primer sets but only the input results obtained using primers flanking *pS2/TFF1* promoter are shown. *D,* P-Ser^{118}-ER-α interacts with coactivator AIB1 and p300. After treatment with estradiol (10 nmol/L), cells were cross-linked with DSP and ER-α was immunoprecipitated by antibodies against phosphorylated Ser^{118} (mouse). Proteins were detected by Western blot analysis using specific antibodies against p300 (mouse) and AIB1 (mouse) as described in Materials and Methods. Results are representative of one of four independent experiments. *TL,* total lysate of MCF-7 cells.

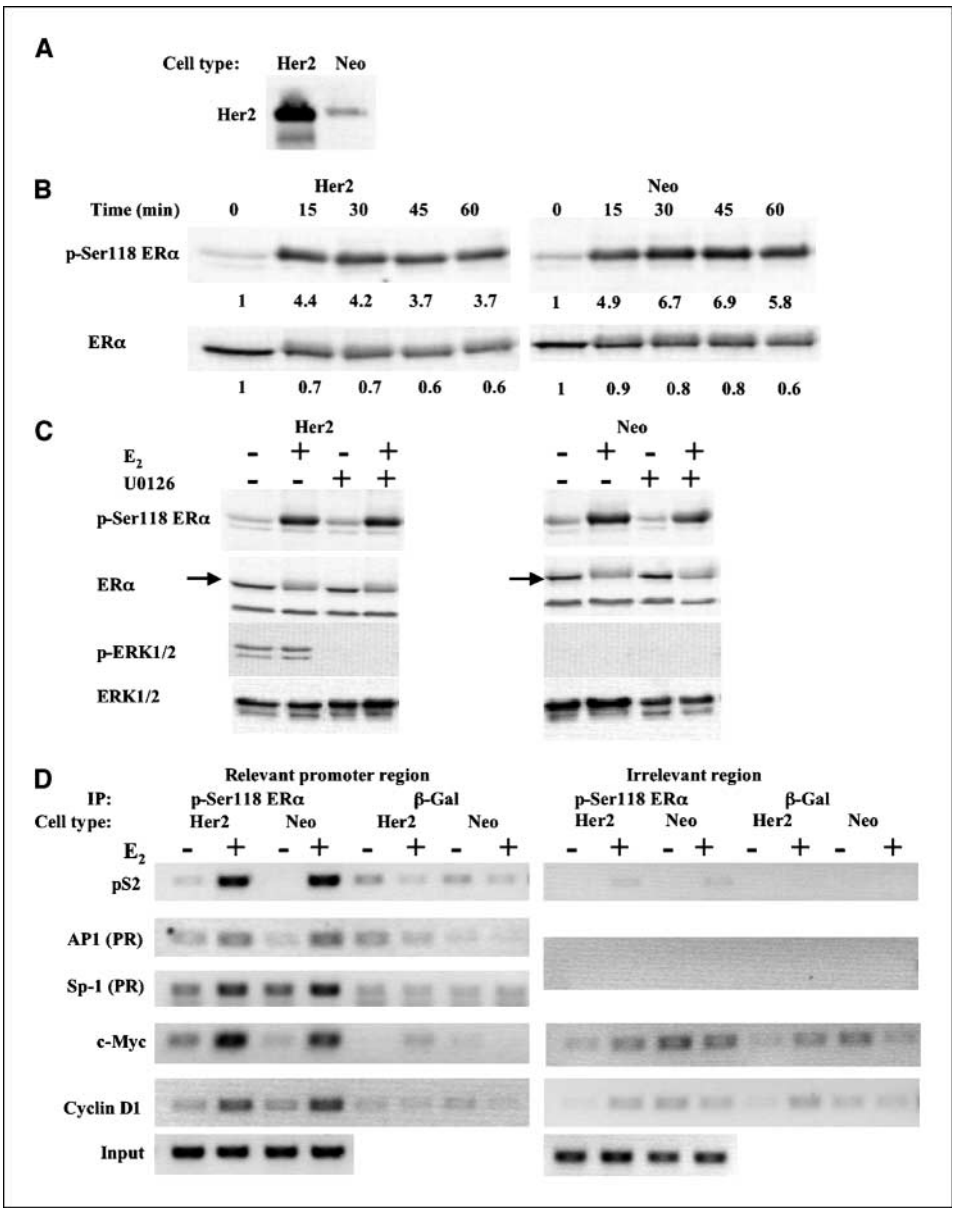

Figure 3. Effect of overexpression of HER-2 on P-Ser^{118}-ER-α and occupancy of the promoter regions of estrogen-regulated genes in MCF-7 human breast cancer cells. MCF-7 cells overexpressing HER-2 or empty vector (Neo) were seeded in 6-cm dishes (2×10^5 per dish; *A-C*) or in 15-cm dish (2×10^6 per dish; *D*). Twenty-four hours later, medium was changed to medium containing 2× charcoal-treated serum. Ninety-six hours after seeding, cells were treated with estradiol (10 nmol/L) for indicated times (*B*) or for 30 minutes (*C* and *D*). Cells were pretreated for 30 minutes with U0126 (10 μmol/L) before the addition of estrogen (*C*). Cell extracts and detection were carried out as described in Materials and Methods. *A,* expression of HER-2 in untreated cells. *B* and *C,* results are representative of one of four independent experiments. *D,* detection of DNA related to promoter regions of *pS2/TFF1, PR,* c-*myc*, and *cyclin D1* was carried out as described in Materials and Methods. Immunoprecipitation with antibodies to P-Ser^{118}-ER-α and irrelevant antibody β-gal. PCR results for the ER-α response regions (*left*) and an irrelevant region (*right*) of the promoters. The input represents amplified signals before immunoprecipitation. This was done for all primer sets but only the input results obtained using primers flanking the *pS2/TFF1* promoter are shown. Results are representative of one of three to six independent experiments.

P-Ser^{118}-ER-α expression in HER-2-overexpressing tumors compared with nonexpressing tumors (median immunohistochemistry score HER-2 overexpressors = 0 versus median for nonexpressors = 10; P = 0.089, Mann-Whitney, two-tailed) and a trend toward an inverse association of HER-2 overexpression and P-Ser^{118}-ER-α positivity (P = 0.073, Fisher's exact test), these were not statistically significant (Fig. 4*B* and *D*). Therefore, the data are consistent with the conclusion that increased level or frequency of P-Ser^{118}-ER-α expression is not associated with HER-2 overexpression in primary breast tumors that remain ER-α$^+$ *in vivo*.

Which kinase is potentially involved in the E_2-induced P-Ser^{118}-ER-α? Presently, two main kinases have been suggested to be responsible for E_2-induced P-Ser^{118}-ER-α, Cdk7 (3), and IKK-α (4). To determine the involvement of these in our cell line models DRB and BAY-11-7082, inhibitors of Cdk7 (24) and IKK-α (25), respectively, were used. Figure 5*A* shows that DRB (50 μmol/L) was unable to inhibit E_2-induced P-Ser^{118}-ER-α in MCF-7 cells, although as expected, DRB was able to inhibit Cdk7 as determined by reduced levels of the phosphorylated Rpb1-CTD subunit of RNA polymerase II (Fig. 5*A*; ref. 26). In addition, when another Cdk7 inhibitor, roscovitine (50 μmol/L), was used, it similarly had no effect on E_2-induced P-Ser^{118}-ER-α despite being able to reduce phosphorylation of the Rpb1-CTD subunit of RNA polymerase II (Fig. 5*B*). In contrast, BAY-11-7082 (at saturating concentrations of 20 μmol/L; ref. 25) inhibited E_2-induced P-Ser^{118}-ER-α in MCF-7 cells either not expressing or overexpressing the HER-2 protein, as well as in ZR-75-1 and T47D cells (Fig. 5*C*). These data are consistent with IKK-α being involved, at least in part, in E_2-induced P-Ser^{118}-ER-α in these cell lines, with no detectable contribution from Cdk7 under these conditions.

Discussion

The above data are the first to our knowledge to directly show the presence of P-Ser^{118}-ER-α at the promoters of several estrogen-regulated genes in MCF-7 breast cancer cells, following

estrogen treatment. The results are consistent with the hypothesis that P-Ser^{118}-ER-α has a central role in the mechanism by which estrogen mediates signal transduction, at the level of regulation of gene expression. In particular, following estrogen treatment, we have identified P-Ser^{118}-ER-α present at both sites in the promoter of the *PR* gene that have been implicated previously in E_2 regulation of PR expression in MCF-7 cells (18, 19). These data are consistent with the association of P-Ser^{118}-ER-α with PR expression (measured by LBA) in breast tumors *in vivo* (9, 10) and support the hypothesis that P-Ser^{118}-ER-α expression in human breast tumors reflects an intact estrogen signaling pathway. Consequently, P-Ser^{118}-ER-α may be a more precise predictor of endocrine therapy responsiveness than ER-α alone. Indeed, we have shown previously that, in node-negative patients whose tumors are ER$^+$, those patients whose tumors have detectable P-Ser^{118}-ER-α had a better disease outcome when treated with tamoxifen than those who do not (10). Therefore, P-Ser^{118}-ER-α adds more precise information to current endocrine response biomarkers in breast tumors *in vivo*. The data presented in this study provide a molecular basis underlying the associations we have identified previously.

Previous data in the literature suggest that antiestrogens may have differential effects on P-Ser^{118}-ER-α expression. In MCF-7 cells, treatment with ICI 182780 led to increased P-Ser^{118}-ER-α (27), whereas no effect was seen in Cos-1 cells transiently expressing ER-α (3) and treatment with 4-OHT increased P-Ser^{118}-ER-α levels in both experimental models (ref. 3; see results above). However, no quantitative assessment of the stoichiometry of phosphorylation due to 4-OHT compared with E_2 was done (28). Our data show that all three ligands can induce P-Ser^{118}-ER-α; however, whereas E_2 and ICI 182780 can induce similar levels, induction due to 4-OHT was significantly less. Therefore, the differential effect of ICI 182780 seen previously is likely due to the different cell lines used in the experiments (MCF-7 versus Cos-1). These data suggest that P-Ser^{118}-ER-α, although important for ER-α action, does not discriminate between agonist versus antagonist activity, and its alteration per se may not be directly related to endocrine resistance.

With respect to the possible role of P-Ser^{118}-ER-α in estrogen action, several activities have been suggested. Mutations of Ser^{118} to an alanine in several studies, but not all (29), suggest that lack of phosphorylation at Ser^{118} results in reduced transcriptional activity in response to E_2 in multiple cell lines (1), whereas addition of a negative charge (mimicking phosphorylation) by replacement of Ser^{118} with a glutamic acid increased transcriptional activity (1). Consistent with a role in transcriptional activity is the observation that P-Ser^{118}-ER-α is important in the recruitment of some coactivators (e.g, p68 helicase; ref. 30). However, all these studies were done using transient transfection analyses and reporter gene read-outs, which are variable with respect to level of ER-α expression, and of course, the promoters are artificial and do not

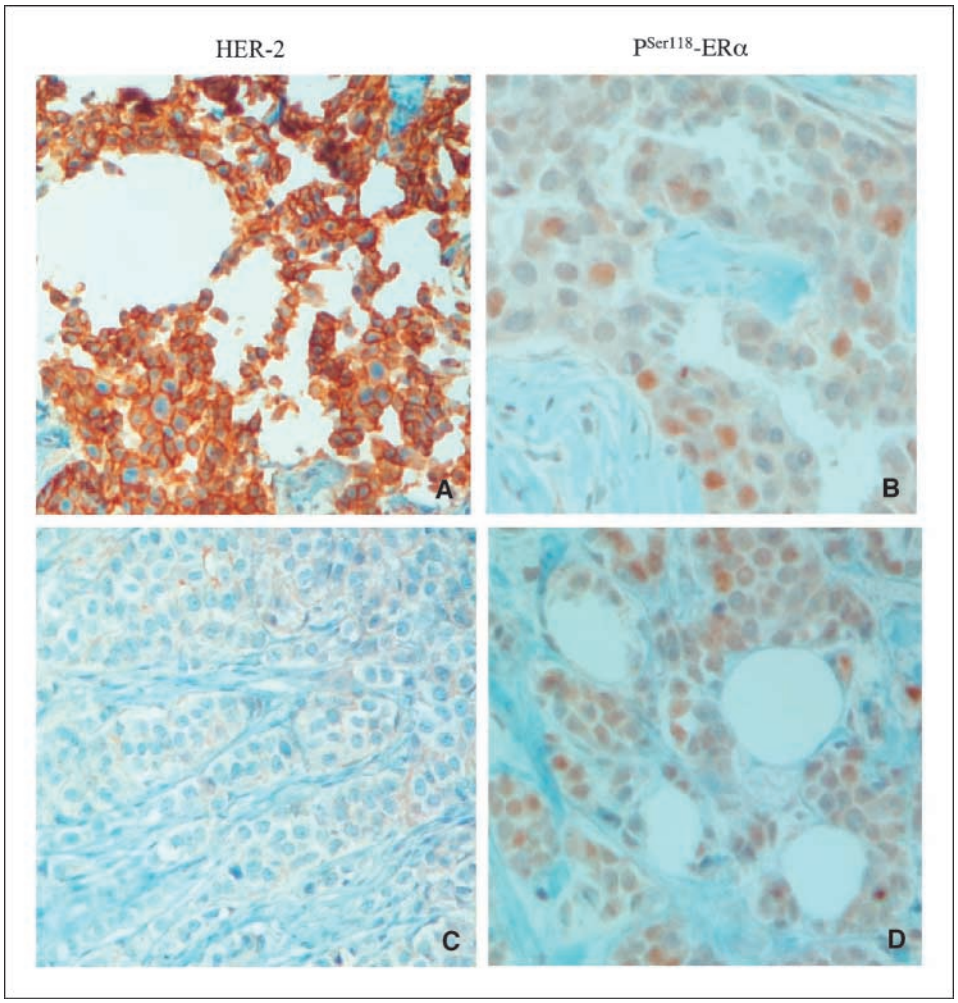

Figure 4. Expression of P-Ser^{118}-ER-α in HER-2-overexpressing breast tumors. Representative sections of two tumors stained by immunohistochemistry for HER-2 (*A* and *C*, + and −, respectively) and semiserial sections stained for P-Ser^{118}-ER-α (*B* and *D*).

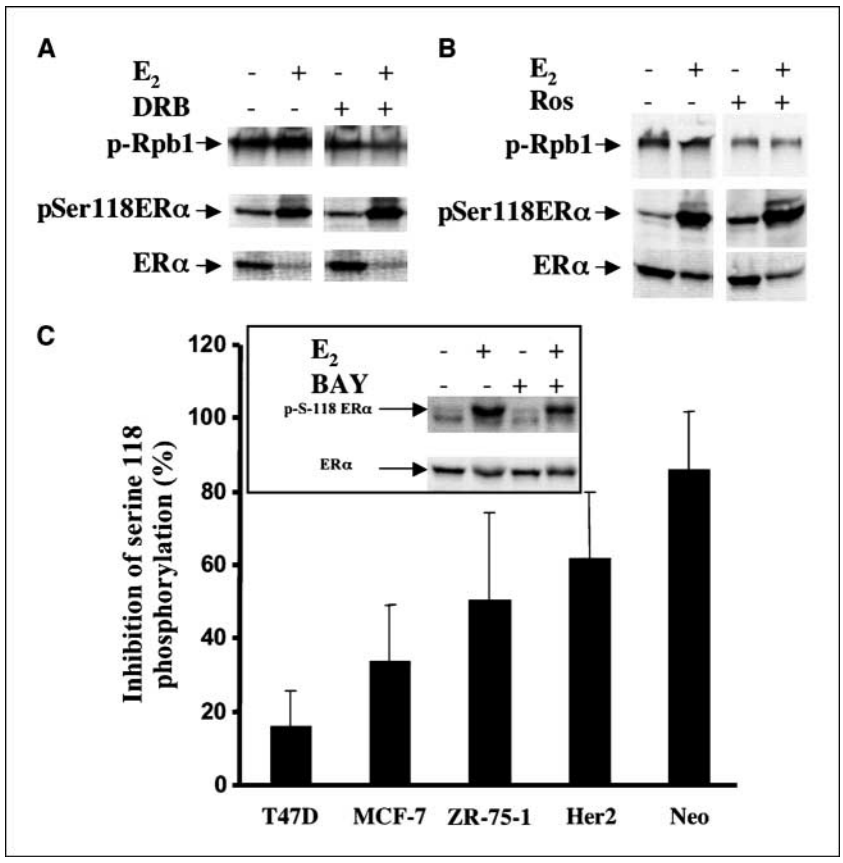

Figure 5. Involvement of Cdk7 and IKK-α in the P-Ser^{118}-ER-α. Cells were seeded in 6-cm dishes (2×10^5 per dish). Twenty-four hours later, medium was changed to medium containing 2× charcoal-treated serum. Ninety-six hours after seeding, cells were treated with DRB (50 μmol/L; *A*) and roscovitine (*Ros*; 50 μmol/L; *B*), or BAY-11-7082 (*BAY*; 20 μmol/L; *C*) for 60 minutes before exposure to estradiol (10 nmol/L) for an additional 30 minutes. Cell extracts, detection, and calculation of the inhibitory effect of BAY-11-7082 were carried out as described in Materials and Methods. *Columns,* mean (MCF-7 cells, $n = 6$; all other cells, $n = 3$) *bars,* SD. *Inset,* representative Western blot of MCF-7 cells treated with estrogen in the presence or absence of the inhibitor.

represent the complexity of natural promoters nor are they in an endogenous chromatin environment (31). Our current data complement and enhance such studies showing that P-Ser^{118}-ER-α is indeed present at the endogenous promoters of multiple estrogen-regulated target genes due to estrogen treatment. More recently, P-Ser^{118}-ER-α was suggested to also have roles in mediating proteasomal degradation of ER-α (32) and regulating splicing of RNA (33), both of which are important in overall estrogen regulation of gene expression. Our study does not address these functions.

MAPK/ERK1/2 (2), Cdk7 (3), and IKK-α (4) have been implicated in the P-Ser^{118}-ER-α. Previous data have excluded MAPK/ERK1/2 in the E_2-induced P-Ser^{118}-ER-α in MCF-7 but have suggested that Cdk7 and/or IKK-α are the most likely candidates (1, 4). Our data using inhibitors that target either Cdk7 or IKK-α support the conclusion that, in MCF-7 and other ER$^+$ human breast cancer cells, IKK-α is, at least in part, responsible for the E_2-induced P-Ser^{118}-ER-α. This would also be consistent with the time course of recruitment of ER-α to the promoter of the *pS2/TFF1* gene after E_2 treatment that has been described in the literature (34), where ER-α is recruited to the *pS2/TFF1* promoter earlier than Cdk7 (10 versus 40 minutes). Our data showing that P-Ser^{118}-ER-α is present at the promoter of *pS2/TFF1* as early as 30 minutes (Fig. 2) suggest that recruitment of P-Ser^{118}-ER-α to the promoters of E_2-regulated genes is also significantly earlier than Cdk7 (34). Our data and the previously documented presence of IKK-α (4) at the promoter of estrogen-regulated genes at a similar time to P-Ser^{118}-ER-α (4) make it tempting to speculate that IKK-α is likely a relevant kinase in estrogen-responsive human breast tumors *in vivo* as well. Interestingly, the contribution of IKK-α to P-Ser^{118}-ER-α as determined using a selective inhibitor seems to differ between the different ER$^+$ cell lines. The reasons are unknown but may be due to biological variability, clonal selection, and/or differential inhibitor uptake and metabolism.

The ligand-independent P-Ser^{118}-ER-α due to up-regulation of growth factor receptor kinase pathways with associated increased activation of MAPK/ERK1/2 has been suggested to be a possible mechanism of tamoxifen resistance in breast tumors *in vivo*. However, previously, we did not find any association of increased activated ERK1/2 with *de novo* tamoxifen resistance *in vivo* (35), although another study has shown increased activated ERK1/2 associated with a poorer response to endocrine therapy (36). We therefore investigated basal P-Ser^{118}-ER-α expression and E_2 induction of P-Ser^{118}-ER-α in HER-2-overexpressing cells and controls. Surprisingly, no differences in basal level or E_2-induced P-Ser^{118}-ER-α level were found between the HER-2-overexpressing MCF-7 cells and controls. Furthermore, although the level of activated ERK1/2 in the HER-2-overexpressing cells is significantly elevated, inhibition of ERK1/2 did not effect either the basal or E2-induced levels of P-Ser^{118}-ER-α. These data are consistent with our data *in vivo* showing that P-Ser^{118}-ER-α levels are not significantly different in ER$^+$, HER-2-overexpressing breast tumors compared with ER$^+$/HER-2$^-$ tumors. Interestingly, clinical data are emerging to suggest that, although ER$^+$/HER-2$^+$ breast tumors are often resistant to tamoxifen therapy, they are not necessarily resistant to aromatase inhibitors, suggesting that E_2-activated ER-α is intact in many of these tumors and the resistance seen to tamoxifen may be due to altered coactivator activity, at least in part, due to HER-2-mediated phosphorylation of overexpressed coactivators, such as AIB1 (28, 37). Our data would be consistent

with these data because MCF-7 cells are known to overexpress AIB1 (38).

In conclusion, our data provide evidence for a direct functional involvement of P-Ser^{118}-ER-α in an intact estrogen-regulated signal transduction pathway and support previous data that the detection of P-Ser^{118}-ER-α in breast tumors may be a better biomarker than ER-α for endocrine sensitivity. Furthermore, our data do not support the hypothesis that resistance of breast tumors to tamoxifen therapy involves ligand-independent activation of ER-α due to constitutive phosphorylation of Ser^{118} via persistent activation of ERK1/2.

Acknowledgments

Received 11/15/2005; revised 8/9/2006; accepted 8/15/2006.

Grant support: Canadian Institutes of Health Research (CIHR), CIHR Training Grant Postdoctoral Fellowship (G.E. Weitsman), Manitoba Health Research Council Postdoctoral Fellowship and CancerCare Manitoba Foundation (CCMF; G.P. Skliris), and U.S. Army Medical Research and Materiel Command Predoctoral Fellowship (L. Li).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank the strong support of the CCMF for our facilities (Manitoba Institute of Cell Biology, Winnipeg, Manitoba, Canada) and Michelle Parisien for doing the MBTB database searches.

References

1. Lannigan D. Estrogen receptor phosphorylation. Steroids 2003;68:1–9.
2. Kato S, Endoh H, Masuhiro Y, et al. Activation of the estrogen receptor through phosphorylation by mitogen activated protein kinase. Science 1995;270:1491–4.
3. Chen D, Washbrook E, Sarwar N, et al. Phosphorylation of human estrogen receptor α at serine 118 by two distinct signal transduction pathways revealed by phosphorylation-specific antisera. Oncogene 2002;21:4921–31.
4. Park K, Krishnan V, O'Malley B, Yamamoto Y, Gaynor R. Formation of an IKKα-dependent transcription complex is required for estrogen receptor-mediated gene activation. Mol Cell 2005;18:71–82.
5. Schiff R, Massarweh S, Shou J, Bharwani L, Mohsin S, Osborne C. Cross-talk between estrogen receptor and growth factor pathways as a molecular target for overcoming endocrine resistance. Clin Cancer Res 2004;10:331–6S.
6. Dowsett M, Harper-Wynne C, Boeddinghaus I, et al. HER-2 amplification impedes the antiproliferative effects of hormone therapy in estrogen receptor-positive primary breast cancer. Cancer Res 2001;61:8452–8.
7. Pietras R, Arboleda J, Reese D, et al. HER-2 tyrosine kinase pathway targets estrogen receptor and promotes hormone independent growth in human breast cancer cells. Oncogene 1995;10:2435–46.
8. Knowlden J, Hutcheson I, Jones H, et al. Elevated levels of epidermal growth factor receptor/c-erbB2 heterodimers mediate an autocrine growth regulatory pathway in tamoxifen-resistant MCF-7 cells. Endocrinology 2003;144:1032–44.
9. Murphy LC, Cherlet T, Adeyinka A, Niu Y, Snell L, Watson P. Phospho-serine-118 estrogen receptor-α detection in human breast tumors *in vivo*. Clin Cancer Res 2004;10:1354–9.
10. Murphy L, Niu Y, Snell L, Watson P. Phospho-serine-118 estrogen receptor-α expression in primary human breast tumors *in vivo* is associated with better disease outcome in women treated with tamoxifen. Clin Cancer Res 2004;10:5902–6.
11. Yen L, You XL, Al Moustafa AE, et al. Heregulin selectively upregulates vascular endothelial growth factor secretion in cancer cells and stimulates angiogenesis. Oncogene 2000;19:3460–9.
12. Ravid A, Koren R, Rotem C, Garach-Jehoshua O, Glaser T, Liberman U. 1,25-Dihydroxyvitamin D3 increases the cellular content of the calcium-activated neutral protease mu-calpain in renal cell carcinoma. Endocrinology 1994;135:2822–5.
13. Spencer V, Sun J, Li L, Davie J. Chromatin immunoprecipitation: a tool for studying histone acetylation and transcription factor binding. Methods 2003;31:67–75.
14. Wells J, Boyd K, Fry C, Bartley S, Farnham P. Target gene specificity of E2F and pocket protein family members in living cells. Mol Cell Biol 2000;20:5797–807.
15. Espino P, Li L, He S, Yu J, Davie J. Chromatin modification of the trefoil factor 1 gene in human breast cancer cells by the Ras/mitogen-activated protein kinase pathway. Cancer Res 2006;66:4610–6.
16. Berry M, Nunez A, Chambon P. Estrogen-responsive element of the human pS2 gene is an imperfectly palindromic sequence. Proc Natl Acad Sci U S A 1989;86:1218–22.
17. Berry A, Scott H, Kudoh J, et al. Refined localization of autosomal recessive nonsyndromic deafness DFNB10 locus using 34 novel microsatellite markers, genomic structure, and exclusion of six known genes in the region. Genomics 2000;68:22–9.
18. Petz L, Ziegler Y, Loven M, Nardulli A. Estrogen receptor a and activating protein-1 mediate estrogen responsiveness of the progesterone receptor gene in MCF-7 breast cancer cells. Endocrinology 2002;143:4583–91.
19. Petz L, Ziegler Y, Schultz J, Kim H, Kemper J, Nardulli A. Differential regulation of the human progesterone receptor gene through an estrogen response element half site and Sp1 sites. J Steroid Biochem Mol Biol 2004;88:113–22.
20. Dubik D, Shiu RP. Mechanism of estrogen activation of c-*myc* oncogene expression. Oncogene 1992;7:1587–94.
21. Cicatiello L, Addeo R, Sasso A, et al. Estrogens and progesterone promote persistent CCND1 gene activation during G_1 by inducing transcriptional derepression via c-Jun/c-Fos/estrogen receptor (progesterone receptor) complex assembly to a distal regulatory element and recruitment of cyclin D1 to its own gene promoter. Mol Cell Biol 2004;24:7260–74.
22. Watson P, Snell L, Parisien M. The NCIC-Manitoba Breast Tumor Bank: a resource for applied cancer research. CMAJ 1996;155:281–3.
23. Petz L, Nardulli A. Sp1 binding sites and an estrogen response element half-site are involved in regulation of the human progesterone receptor A promoter. Mol Endocrinol 2000;14:972–85.
24. Chen D, Washbrook E, Sarwar N, et al. Activation of estrogen receptor α by S118 phosphorylation involves a ligand-dependent interaction with TFIIH and participation of CDK7. Mol Cell Biol 2000;6:127–37.
25. Pierce J, Schoenleber R, Jesmok G, et al. Novel inhibitors of cytokine-induced IκBα phosphorylation and endothelial cell adhesion molecule expression show anti-inflammatory effects *in vivo*. J Biol Chem 1997;272:21096–103.
26. Oelgeschlager T. Regulation of RNA polymerase II activity by CTD phosphorylation and cell cycle control. J Cell Physiol 2002;190:160–9.
27. Joel P, Traish A, Lannigan D. Estradiol-induced phosphorylation of serine 118 in the estrogen receptor is independent of p42/p44 mitogen-activated protein kinase. J Biol Chem 1998;273:13317–23.
28. Shou J, Massarweh S, Osborne C, et al. Mechanisms of tamoxifen resistance: increased estrogen receptor-HER2/*neu* cross-talk in ER/HER2-positive breast cancer. J Natl Cancer Inst 2004;96:926–35.
29. Le Goff P, Montano MM, Schodin DJ, Katzenellenbogen BS. Phosphorylation of the human estrogen receptor. Identification of hormone-regulated sites and examination of their influence on transcriptional activity. J Biol Chem 1994;269:4458–66.
30. Endoh H, Maruyama K, Masuhiro Y, et al. Purification and identification of p68 RNA helicase acting as a transcriptional coactivator specific for the activation function 1 of human estrogen receptor α. Mol Cell Biol 1999;19:5363–72.
31. Archer T, Cordingley M, Wolford R, Hager G. Transcription factor access is mediated by accurately positioned nucleosomes on the mouse mammary tumor virus promoter. Mol Cell Biol 1991;11:688–98.
32. Callige M, Kieffer I, Richard-Foy H. CSN5/Jab1 is involved in ligand-dependent degradation of estrogen receptor α by the proteasome. Mol Cell Biochem 2005;25:4349–58.
33. Masuhiro Y, Mezaki Y, Sakari M, et al. Splicing potentiation by growth factor signals via estrogen receptor phosphorylation. Proc Natl Acad Sci U S A 2005;102:8126–31.
34. Metivier R, Penot G, Hubner M, et al. Estrogen receptor-α directs ordered, cyclical, and combinatorial recruitment of cofactors on a natural target promoter. Cell 2003;115:751–63.
35. Adeyinka A, Niu Y, Cherlet T, Snell L, Watson P, Murphy L. Activated mitogen-activated protein kinase expression during human breast tumorigenesis and breast cancer progression. Clin Cancer Res 2002;8:1747–53.
36. Gee J, Robertson J, Ellis I, Nicholson R. Phosphorylation of ERK1/2 mitogen-activated protein kinase is associated with poor response to anti-hormonal therapy and decreased patient survival in clinical breast cancer. Int J Cancer 2001;95:247–54.
37. Mora JFd, Brown M. AIB1 is a conduit for kinase-mediated growth factor signaling to the estrogen receptor. Mol Cell Biol 2000;20:5041–7.
38. Anzick S, Kononen J, Walker R, et al. AIB1, a steroid receptor coactivator amplified in breast and ovarian cancer. Science 1997;277:965–8.

Estrogen Receptor-α Phosphorylated at Ser^{118} Is Present at the Promoters of Estrogen-Regulated Genes and Is Not Altered Due to HER-2 Overexpression

Gregory E. Weitsman, Lin Li, George P. Skliris, et al.

Cancer Res 2006;66:10162-10170.