Estradiol-induced Phosphorylation of Serine 118 in the Estrogen Receptor Is Independent of p42/p44 Mitogen-activated Protein Kinase*

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Phosphorylation of Ser\textsuperscript{118} of human estrogen receptor α (ER) enhances ER-mediated transcription and is induced by hormone binding and by activation of the mitogen-activated protein kinase (MAPK) pathway. We discovered that phosphorylation of Ser\textsuperscript{118} reduces the electrophoretic mobility of the ER. Using this mobility shift as an assay, we determined the in vivo stoichiometry and kinetics of Ser\textsuperscript{118} phosphorylation in response to estradiol, ICI 182,780, epidermal growth factor (EGF), and phorbol 12-myristate 13-acetate (PMA). In human breast cancer MCF-7 cells, estradiol induced a steady state phosphorylation of Ser\textsuperscript{118} within 20 min with a stoichiometry of 0.67 mol of phosphate/mol of ER. Estradiol did not activate p42/p44 MAPK, and basal p42/p44 MAPK activity was not sufficient to account for phosphorylation of Ser\textsuperscript{118} in response to estradiol. In contrast, both EGF and PMA induced a rapid, transient phosphorylation of Ser\textsuperscript{118} with a stoichiometry of ~0.25, and the onset of Ser\textsuperscript{118} phosphorylation correlated with the onset of p42/p44 MAPK activation by these agents. Either the EGF- or PMA-induced Ser\textsuperscript{118} phosphorylation could be inhibited without influencing estradiol-induced Ser\textsuperscript{118} phosphorylation. The data suggest that a kinase other than p42/p44 MAPK is involved in the estradiol-induced Ser\textsuperscript{118} phosphorylation. We propose that the hormone-induced change in ER conformation exposes Ser\textsuperscript{118} for phosphorylation by a constitutively active kinase.

Estrogen receptor α (ER)\textsuperscript{1} is a member of a superfamily of nuclear receptors that act as transcription factors when bound to specific lipophilic hormones (1). In common with other steroid hormone receptors, the ER has an N-terminal domain with a hormone-independent transcriptional activation function (AF-1), a central DNA-binding domain, and a C-terminal ligand-binding domain with a hormone-dependent transcriptional activation function (AF-2) (1, 2). In addition to being activated by binding to its ligand, estradiol, the ER can also be activated as a transcription factor by several nonsteroidal agents (2).

Phosphorylation of the ER increases in response to estradiol (2). A major site of estradiol-induced phosphorylation is serine 118 (Ser\textsuperscript{118}), which is located in AF-1 (3–5). In a number of cell types, mutation of Ser\textsuperscript{118} to Ala causes a reduction in estradiol-dependent transcriptional activation (4, 6–8). Ser\textsuperscript{118} is located within the sequence PPQLSPFLQL which has a high degree of homology with the optimum peptide substrate identified for p44 MAPK (9). Ser\textsuperscript{118} is also phosphorylated in response to EGF and PMA, possibly via p42/p44 MAPK (6, 7). Transcriptional activation of the ER by EGF is diminished when Ser\textsuperscript{118} is mutated to Ala (6–8). Thus both estradiol binding and activation of the MAPK pathway lead to phosphorylation of Ser\textsuperscript{118}, and this phosphorylation is involved in regulating ER-mediated transcription.

The evidence that p42/p44 MAPK directly phosphorylates Ser\textsuperscript{118} is based on in vitro observations that activated p42/p44 MAPK phosphorylated the N-terminal domain of the ER but not a mutant of this construct in which Ser\textsuperscript{118} was replaced with Ala (6). Additional supporting evidence is provided by the observation that in vivo a constitutively activated MAPK kinase (MAPKK) stimulated phosphorylation of the N-terminal domain of the ER but did not stimulate phosphorylation of a mutant of this construct in which Ser\textsuperscript{118} was replaced with Ala (6). However, it has still not been established whether p42/p44 MAPK directly phosphorylates Ser\textsuperscript{118} in the context of the full-length ER in vivo. We demonstrate that the onset of Ser\textsuperscript{118} phosphorylation correlates with the onset of p42/p44 MAPK activation in response to EGF and PMA. These data are consistent with the hypothesis that p42/p44 MAPK phosphorylates Ser\textsuperscript{118}. However, our data demonstrate that p42/p44 MAPK is not responsible for the observed Ser\textsuperscript{118} phosphorylation in response to estradiol binding by the ER. Our results raise the possibility that Ser\textsuperscript{118} may be the site of phosphorylation by either two or more kinases in response to various stimuli.

**EXPERIMENTAL PROCEDURES**

**Chemicals—**ICI 182,780 was a gift from Zeneca Pharmaceuticals, Cheshire, United Kingdom.

**Expression Vectors and Receptor Mutants—**Pierre Chambon (Unité 154 of Biologie Moléculaire et de Génétique de l’INSERM, Strasbourg Cedex, France) kindly provided pSG5-ER (the human estrogen receptor cDNA (10)). The mutants S118A-ER, S104/106/118A-ER, S154A-ER, and S167A-ER were created by site-directed mutagenesis of pSG5-ER using the megaprimer method (11). The luciferase reporter plasmid, 2ERE-MpGL2, has been described (8). pSI-β-galactosidase contains the β-galactosidase gene in the pSI vector from Promega.
Ser118 Phosphorylation of the Human Estrogen Receptor

Cell Culture, Transfection, Cell Harvest, and Immunoblotting—COS-1, MCF-7, T-47D, and NMuMG cell lines were obtained from the American Type Culture Collection, Manassas, VA. All media used in this study were phenol-red free, and all fetal bovine serum used was charcoal-dextran treated (CFBS). COS-1, T-47D, and NMuMG cells were maintained in DMEM/Ham’s Nutrient Mix F-12 (Life Technologies, Inc.) supplemented with 10% CFBS. COS-1 media contained 50 units/ml penicillin and 50 μg/ml streptomycin. T-47D and NMuMG cell media contained antibiotics-antimycotic (Life Technologies, Inc.). MCF-7 cells were maintained in DMEM supplemented with 5% CFBS, 50 units/ml penicillin, and 50 μg/ml streptomycin. Cells were incubated 24 h in medium without serum prior to treatment with steroids or other agents unless indicated otherwise.

COS-1 cells were transfected by the diethylaminoethyl-dextran method as described previously (12). Each 6-cm plate was transfected with 0.5 μg of pS5G-ER or pS5G-ER mutant plus 2 μg of pLG2-Vector (Promega) as carrier DNA. Following dimethyl sulfoxide treatment, the cells were incubated for 24 h in serum-free medium before treatment with estradiol or PMA and harvesting.

Following treatment with steroids or agents as indicated in the figure legends, the cells (on 6-cm plates) were washed with warm PBS and 0.5 ml of 2× SDS-PAGE sample buffer (0.12 M Tris-Cl, pH 6.8, 4% SDS, 20% glycerol, 0.2 mM DTT, 0.008% bromphenol blue) near 100 °C was added to the plate. The cells were scraped into a 1.5-ml centrifuge tube at a heat block at 100 °C. The tubes were heated for 10 min, cooled on ice, and stored at -20 °C until samples were analyzed on SDS-PAGE.

At the time of electrophoresis, samples were harvested for 5 min at 100 °C, and 30-μ aliquots were layered on 7% SDS-PAGE and electrophoresed until the 43-kDa marker reached the bottom of the gel. Proteins on the gels were transferred to nitrocellulose membranes in 25 mM Tris-HCl, pH 8.3, 192 mM glycine, and 20% methanol. For detection of the ER, immunoblotting was performed with monoclonal antibody a78–1-C6 (mAb 78) (13) for COS-1 extracts or monoclonal antibody EVG F9 (mAb F9) (14) for MCF-7, T-47D, or NMuMG extracts.

For detection of activated p42/p44 MAPK, immunoblotting was performed with anti-ACTIVE MAPK (Promega). For detection of total p42/p44 MAPK, immunoblotting was performed with an antibody-pan ERK (Transduction Laboratories). All immunoblots used either anti-mouse or anti-rabbit IgG (for normalizing luciferase data) and 1.5 μg of 2ERE-MpGL2 (luciferase reporter plasmid) (for detection of total p42/p44 MAPK).

All immunoblots and Western blots were developed with SuperSignal West Pico (Pierce) and exposed for 1 h to Kodak X-AR5 film. Autoradiograms were viewed with a UV transilluminator. Eradicated films were scanned (Umax PowerLook 25) and analyzed with ImageQuant software (Molecular Dynamics, Inc.). The level of radioactivity was determined using the ImageQuant software and plotted versus the amount of ER on the densitometric reading was performed. The relative intensities of the ER bands were quantitated from various exposures of the film.

Immunoprecipitation and Protein Phosphatase 1 Treatment—MCF-7 cells were treated for 38 min with 1 nM estradiol and then rinsed with PBS. Cells were lysed in buffer A (50 mM NaCl, 1% Nonidet P-40 (Nonidet P-40), 20 mM Na3MoO4, 20 mM NaF, 2 mM Na3VO4, 20 μM β-glycerophosphate, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml each of pepstatin, leupeptin, and aprotinin, 200 μM o-phenylenediamine, 10 mM MgCl2, and 100 units/ml DNase 1 (Life Technologies, Inc.)). The ER was immunoprecipitated with mAb a78 as described (5). The immunoprecipitates were washed with protein phosphatase 1 (PP1) buffer (New England Biolabs), and two aliquots were suspended in a final volume of 30 μl of PP1 buffer with 1 μl Mcl2. One tube received 1 unit of PP1, and both tubes were incubated for 5 min at 30 °C. Reactions were stopped by addition of SDS-PAGE sample buffer and heating for 5 min at 100 °C. Sample proteins were analyzed on SDS-PAGE, electrotransferred, and immunoblotted as described above.

Transcriptional Analysis—For transcriptional analysis, MCF-7 cells were seeded at a density of 5 × 105 cells per 6-cm dish in medium containing 5% CBFS and antibiotic-antimycotic the day before transfection. Cells were transfected by 15 μl of lipofectAMINE in 2 ml of DMEM according to the instructions provided by Life Technologies, Inc. with 0.5 μg of 2ERE-MpGL2 (luciferase reporter plasmid), 1.9 μg of pS104/106A-ER (for normalizing luciferase data), and 1.5 μg of pLG2-Vector (Promega) as carrier DNA. The cells were left overnight in the incubator and washed once with PBS before the addition of DMEM with the appropriate concentration of estradiol, modulators, or vehicle. After treatment for 5.5 h, the cells were lysed with Reporter Lysis Buffer (Promega). Luciferase and β-galactosidase activity were measured as described previously (5).

In-gel Kinase Assay—For the in-gel kinase assay, MCF-7 cells were washed with PBS and lysed with ice-cold extraction buffer (20 mM Tris-Cl, pH 7.5, 5 mM EGTA, 0.5% Triton X-100, 6 mM DTT, 50 mM β-glycerophosphate, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na3VO4, 10 μg/ml aprotinin and leupeptin, and 2 μg/ml pepstatin). The supernatant was cleared by centrifugation, and SDS-PAGE sample buffer was added. The supernatant was electrotransferred on 7% SDS-PAGE with 0.05 mg/ml myelin basic protein polymerized in the gel. Following electrophoresis, the proteins were denatured and renatured as described (15). The gels were incubated for 30 min at 22 °C in 40 mM Hepes-NaOH, pH 8.0, 0.2 mM DTT, 0.1 mM EGTA, 5 mM MgCl2, 25 μM ATP (40 μCi/ml [γ-32P]ATP)). The gels were washed as described (15), dried, and autoradiographed.

RESULTS

Development of an Assay for the Detection of Ser118 Phosphorylation—Many proteins migrate more slowly on SDS-PAGE due to phosphorylation of particular sites. Estradiol addition to cells causes the ER to migrate as a doublet on SDS-PAGE (5, 8, 16). We showed that in human ER expressed in COS-1 cells the major difference in the phosphorylation of the upper and lower bands was phosphorylation of Ser118 in the upper band. Furthermore, a mutant ER in which Ser118 was replaced with Ala was phosphorylated at a level only 20% that of the wild type (5) and did not upshift when cells were treated with estradiol (5).

Thus, phosphorylation of Ser118 appeared to be required for the electrophoretic upshift. This electrophoretic upshift could be used to monitor the phosphorylation of Ser118, greatly facilitating the characterization of kinases that phosphorylate Ser118. Therefore, we wanted to confirm that only the phosphorylation of Ser118 was required for the electrophoretic upshift.

Phosphorylation of the ER occurs only on Ser residues (3, 17, 18) with the exception of a low level of phosphorylation on Tyr in the human ER (19). Le Goff et al. (3) reported that a mutant of the ER lacking the A/B region (amino acids 1–175) showed no estradiol-induced phosphorylation, indicating that the hormone-induced phosphorylation sites are predominantly in the A/B region. To determine whether phosphorylation of another Ser in the A/B region in addition to Ser118 is required for the electrophoretic upshift, we prepared mutants in which other Ser residues in the A/B region known or suspected to be phosphorylated were replaced by Ala residues (2). Ser-104 and Ser-106 were replaced with Ala residues to create the mutant Ser-118A, Ser-154A, and Ser-167A. The wild type and mutant ERs were transfected into COS-1 cells. Cell lysates were immunoblotted with an anti-ER antibody. Data displayed in Fig. 1, lanes 1–4 show that after subtraction of the control, estradiol converted 30% of the wild type ER to the upshifted form, PMA converted about 65% of the receptor, and estradiol plus PMA converted 83% of the receptor. In contrast, Fig. 1, lanes 5–8 show that neither estradiol nor PMA caused a change in mobility with the mutant S185A-ER. All of the other mutants, S167A-ER (lanes 9–12), S154A-ER (lanes 13–16), and S104/106A-ER (lanes 17–20) responded to estradiol and PMA similarly to the wild type. Thus, the change in mobility of the ER did not require phosphorylation of Ser residues 104, 106, 154, or 167.

Ser118 Phosphorylation of the ER in Mammary Cell Lines—To determine whether estradiol causes phosphorylation of Ser118 in endogenous ER in mammary cell lines, MCF-7 (human breast carcinoma), NMuMG (mouse mammary gland normal epithelium), and T-47D (human breast duct carcinoma, paracell effusion) cells, were incubated for 30 min with estradiol or vehicle. Estradiol induced an electrophoretic upshift of the ER in all three mammary cell lines (Fig. 2A, immunoblot).

To confirm that the upshift was due to phosphorylation, MCF-7 cells were treated with estradiol, and the ER was immunoprecipitated. Aliquots of the immunoprecipitate were incubated with and without PP1. ER incubated without PP1 (Fig. 2A, immunoblot).
2B, lane 1) had 35% of the ER upshifted, whereas only 3% of the ER was upshifted (lane 2) in ER incubated with PP1. The amount of ER in the lower band in lane 2 is equivalent to the amount of ER in the two bands in lane 1 combined, indicating that dephosphorylation of the ER in the upper band converted it into the lower band. The fraction of ER recovered in the upper band following immunoprecipitation was less than when the cells were treated directly with hot SDS sample buffer (Fig. 2A), presumably due to the action of phosphatases during immunoprecipitation. These results together with the data with mutants confirm that Ser118 was phosphorylated in endogenous ER in human breast cells and most likely on the analogous Ser122 in mouse mammary cells.

**Phosphorylation of Ser**\(^{118}\)** in Response to Estradiol and ICI 182,780 of the ER**—To determine the dose response of Ser\(^{118}\) phosphorylation to estradiol in vivo, MCF-7 cells were treated with increasing concentrations of estradiol. Estradiol-induced phosphorylation of Ser\(^{118}\) was maximal at approximately 1 nM estradiol, with an average of 67% of the receptor phosphorylated on Ser118 (Fig. 3). Thus the stoichiometry of Ser\(^{118}\) phosphorylation is higher in cells that normally express ER as compared with heterologously expressed ER in COS-1 cells.

To determine whether ICI 182,780 induces Ser\(^{118}\) phosphorylation, MCF-7 cells were treated with various concentrations of ICI 182,780. Interestingly, although ICI 182,780 is a pure ER antagonist (20), 30 nM of this compound induced phosphorylation of Ser\(^{118}\) to the same extent as estradiol (Fig. 3).

We found that ICI 182,780 caused a rapid decrease in total ER protein, an observation in agreement with reports for ICI 164,384 (20, 21). Treatment of MCF-7 cells with 10 nM estradiol for 1 h did not significantly alter the total amount of ER protein, whereas treatment with 10 nM ICI 182,780 for 1 h reduced the total amount of ER protein by 70% compared with the control level (data not shown).

**Induction of Ser**\(^{118}\)** Phosphorylation in Response to Estradiol, EGF, and PMA**—Ser\(^{118}\) is also phosphorylated in response to both EGF and PMA as compared with estradiol. Within 5 min of estradiol treatment, phosphorylation of Ser\(^{118}\) was already noticeable (Fig. 4A) and was maximal within 20 min, remaining at that maximal level at 60 min. In response to EGF, approximately 24% of the receptor was phosphorylated on Ser\(^{118}\), and this maximal level was reached within 5 min after addition of ligand (Fig. 4B). In response to PMA, approximately 26% of the receptor was phosphorylated on Ser\(^{118}\), and phosphorylation reached a maximum...
level within 10 min (Fig. 4C). Thus, the extent and time course of induction of Ser\textsuperscript{118} phosphorylation differed considerably between estradiol, EGF, and PMA. Estradiol causes a rapid but prolonged phosphorylation, whereas EGF and PMA cause rapid and transient phosphorylation of Ser\textsuperscript{118}.

Next, to ensure that we were studying Ser\textsuperscript{118} phosphorylation under conditions in which EGF and PMA influence ER-mediated transcription, we determined the effect of both EGF and PMA on ER-dependent transcription in MCF-7 cells. These cells were cotransfected with a reporter construct that contained a luciferase gene regulated by two palindromic estrogen response elements (ERE) and a plasmid that constitutively expresses a protein kinase of 62 kDa (Fig. 6B). These experiments were necessary to eliminate transcriptional responses that were independent of the ERE. Following transfection, the cells were treated for the minutes indicated with 10 nM estradiol (A), 100 ng/ml EGF (B), or 100 nM PMA (C). Vehicle was added for the 0 min value. Lysates were analyzed as described in Fig. 1. Quantitation of the time course of Ser\textsuperscript{118} phosphorylation in response to the various agents was determined from immunoblots by densitometry. The means ± S.D. are shown.

**Estradiol Does Not Activate p42/p44 MAPK, whereas EGF and PMA Both Activate p42/p44 MAPK**—It has been reported that estradiol can stimulate signal transduction pathways that lead to p42/p44 MAPK activation in MCF-7 cells (22). To investigate the possibility that p42/p44 MAPK was responsible for Ser\textsuperscript{118} phosphorylation in response to estradiol, serum-starved MCF-7 cells were treated with either estradiol, EGF, or PMA, and Ser\textsuperscript{118} phosphorylation and p42/p44 MAPK activation were determined. As Fig. 6A shows, the onset of Ser\textsuperscript{118} phosphorylation paralleled the onset of activation of p42/p44 MAPK by EGF. The activated form of p42/p44 MAPK was detected by using an antibody that specifically recognizes the p42/p44 MAPK activation by PMA. However, activation of p42/p44 MAPK was not observed in response to estradiol. The immunoblot with an antibody that recognizes both activated p42/p44 MAPK and unactivated forms of p42/p44 MAPK demonstrated that approximately equal levels of p42/p44 MAPK were present in each lane. In other experiments, extended exposure of the anti-active MAPK immunoblot demonstrated the presence of basal p42/p44 MAPK activity. However, this low level of activity was not enhanced by estradiol (Fig. 7A).

As an alternative approach to determine whether estradiol activates p42/p44 MAPK, an in-gel kinase assay was performed using extracts from MCF-7 cells that had been treated with estradiol, PMA, or vehicle (C) in the presence or absence of 1 nM estradiol. Cells were harvested, and luciferase and β-galactosidase activities were measured. To correct for differences in transfection efficiency, the luciferase activities were normalized to the β-galactosidase activities. To correct for transcriptional responses not due to the ER, the normalized luciferase activity of cells transfected with MpGL2 was subtracted from the normalized luciferase activity of cells transfected with 2ERE-MpGL2. The data are reported as the % of response obtained compared with the response obtained with estradiol. The means ± S.E. are shown. ** indicates a p value of <0.005, and * indicates a p value of <0.05. For p values, PMA and EGF were compared with control, and estradiol with either EGF or PMA were compared with estradiol.

**FIG. 4.** Time course of Ser\textsuperscript{118} phosphorylation induced by estradiol, PMA, EGF, and PMA in MCF-7 cells. Serum-starved MCF-7 cells were treated for the minutes indicated with 10 nM estradiol (A), 100 ng/ml EGF (B), or 100 nM PMA (C). Vehicle was added for the 0 min value. Lysates were analyzed as described in Fig. 1. Quantitation of the time course of Ser\textsuperscript{118} phosphorylation in response to the various agents was determined from immunoblots by densitometry. The means ± S.D. are shown.

**FIG. 5.** PMA and EGF cause ligand-independent transcriptional activation of the ER in MCF-7 cells. Serum-starved MCF-7 cells were transfected with either 2ERE-MpGL2 (luciferase reporter plasmid) or MpGL2 without the EREs. Additionally, the cells were cotransfected with a vector encoding β-galactosidase. After transfection, cells were treated for 5.5 h with 100 nM PMA, 100 ng/ml EGF, or vehicle (C) in the presence or absence of 1 nM estradiol. Cells were harvested, and luciferase and β-galactosidase activities were measured. To correct for differences in transfection efficiency, the luciferase activities were normalized to the β-galactosidase activities. To correct for transcriptional responses not due to the ER, the normalized luciferase activity of cells transfected with MpGL2 was subtracted from the normalized luciferase activity of cells transfected with 2ERE-MpGL2. The data are reported as the % of response obtained compared with the response obtained with estradiol. The means ± S.E. are shown. ** indicates a p value of <0.005, and * indicates a p value of <0.05. For p values, PMA and EGF were compared with control, and estradiol with either EGF or PMA were compared with estradiol.
Fig. 6. p42/p44 MAPK is not activated in MCF-7 cells in response to estradiol. A, serum-starved MCF-7 cells were treated for the min indicated with 100 nM estradiol, 100 nM PMA, 100 ng/ml EGF, or vehicle (for 0 min value). Lysates were resolved on either 7% SDS-PAGE (for ER) or 10% SDS-PAGE (for p42/p44 MAPK). After transfer to nitrocellulose, the ER was detected with an anti-ER antibody, and active p42/p44 MAPK was detected with an antibody that recognizes only the dually phosphorylated form of p42/p44 MAPK. This later blot was stripped and reprobed with anti-p42/p44 MAPK antibody that detects both activated and nonactivated p42/p44 MAPK. B, extracts were made from MCF-7 cells that were serum-starved for 24 h and then treated for 5 min with 10 nM estradiol (E), 100 ng/ml EGF, 100 nM PMA, or vehicle (C). These extracts were used in an in-gel kinase assay containing myelin basic protein in the gel. The relative positions of the molecular mass standards are shown. The arrow indicates the position of p42/p44 MAPK activated by PMA and EGF.

myelin basic protein (Fig. 7B, lanes 1, 3, and 5 versus 2). Thus two different approaches both indicated that estradiol did not activate p42/p44 MAPK.

**EGF- and PMA-induced Ser**<sup>118</sup> **Phosphorylation of Human Estrogen Receptor**

The above results suggest that activation of p42/p44 MAPK may be involved in the phosphorylation of Ser<sup>118</sup> in response to EGF and PMA but not in response to estradiol. To further test this hypothesis we compared the effects of various inhibitors on either the EGF- or PMA-induced Ser<sup>118</sup> phosphorylation versus the estradiol-induced Ser<sup>118</sup> phosphorylation. Fig. 7A shows that PD98059, an inhibitor of MAPK-activating enzyme (MEK1/2) (24), diminished the EGF-induced Ser<sup>118</sup> phosphorylation but did not affect the estradiol-induced Ser<sup>118</sup> phosphorylation. Extended exposure of the anti-active MAPK immunoblot showed faint bands corresponding to active p42 MAPK and p44 MAPK. This basal activity of p42/p44 MAPK diminishes in extracts taken from cells pretreated with PD98059 before addition of estradiol or EGF. Estradiol did not increase p42/p44 MAPK activity above the basal level. Importantly, even though PD98059 was able to diminish basal p42/p44 MAPK activity, PD98059 did not affect estradiol-induced Ser<sup>118</sup> phosphorylation.

It has been suggested that there are two MEK1/2-independent pathways for activating p42/p44 MAPK; one pathway involves PKC, and the other pathway involves phosphatidylinositol 3'-kinase (25). Both staurosporine and GF109203X, inhibitors of PKC (26, 27), completely prevented phosphorylation of Ser<sup>118</sup> in response to PMA but had no effect on estradiol-induced phosphorylation induced by estradiol (Fig. 7, B and C). Pretreatment of MCF-7 cells for 30 min with 100 nM wortmannin, conditions shown to inhibit phosphatidylinositol 3'-kinase (28), did not reduce estradiol-induced Ser<sup>118</sup> phosphorylation (data not shown). An inhibitor combination of GF109203X, PD98059, and wortmannin also did not affect estradiol-induced Ser<sup>118</sup> phosphorylation (data not shown). Pretreatment of MCF-7 cells for 30 min with 20 μM SB203580, conditions shown to inhibit the activation of p38 MAPK (29), had no effect on the estradiol-induced Ser<sup>118</sup> phosphorylation (data not shown). These results provide additional evidence that estradiol does not activate a signal transduction pathway that leads to p42/p44 MAPK or p38 MAPK activation.

Of interest to note is that Trowbridge et al. (30) have suggested that the cyclin A/cyclin-dependent kinase 2 (cdk2) complex phosphorylates the ER. However, staurosporine, which inhibits a number of kinases including PKC and cdk2 at similar concentrations (26), had no effect on estradiol-induced phosphorylation (Fig. 7C). Moreover, pretreatment of MCF-7 cells for 30 min with 100 μM olomoucine, a condition that inhibits cdk1 and cdk2 (31), also did not affect estradiol-induced Ser<sup>118</sup> phosphorylation (data not shown). These data do not support a model in which cdk2 is the kinase responsible for Ser<sup>118</sup> phosphorylation in response to estradiol binding by the ER.

**Basal Activity of p42/p44 MAPK Is Not Responsible for Estradiol-induced Ser**<sup>118</sup> **Phosphorylation**—Our results demonstrate that p42/p44 MAPK is not activated in response to estradiol. It could be argued, however, that the estradiol-bound receptor is an extremely good substrate for p42/p44 MAPK and that even the activity of p42/p44 MAPK in the presence of PD98059 is sufficient for 67% of the ER molecules to be phosphorylated in response to estradiol. To test this hypothesis, we determined whether increasing p42/p44 MAPK activity would enhance estradiol-induced Ser<sup>118</sup> phosphorylation. Increasing amounts of EGF were added to MCF-7 cells that had been pretreated with a high concentration of estradiol for an extended period of time. This pretreatment was performed to ensure that ER binding to estradiol was not rate-limiting for Ser<sup>118</sup> phosphorylation. The lowest dose of EGF did not effect
estradiol-induced Ser\textsuperscript{118} phosphorylation even though p42/p44 MAPK activity was significantly increased over the basal level (Fig. 8). Higher levels of EGF which dramatically enhanced p42/p44 MAPK activity only increased Ser\textsuperscript{118} phosphorylation to the same extent as EGF alone and did not induce 100\% of the estradiol-bound ER molecules to be phosphorylated (Fig. 8).

**DISCUSSION**

Phosphorylation of Ser\textsuperscript{118} is involved in regulating the transcriptional activity of AF-1 in response to either estradiol or EGF (6–8). Although Ser\textsuperscript{118} had been identified as a putative substrate for p42/p44 MAPK, no information concerning the stoichiometry and kinetics of Ser\textsuperscript{118} phosphorylation in response to estrogen agonists or antagonists, EGF, or PMA was known. We developed a convenient and quantitative electrophoretic mobility assay to monitor specifically the \textit{in vivo} phosphorylation of Ser\textsuperscript{118}. Using this assay, we provide the first evidence that phosphorylation of Ser\textsuperscript{118} occurs in human breast cancer cells, a physiologically relevant target tissue. The rapidity of Ser\textsuperscript{118} phosphorylation in response to estradiol suggests that the phosphorylation is a direct response to hormone binding. Le Goff \textit{et al.} (3) have shown that ICI 164,384 increases phosphorylation of the ER but they did not determine the sites of phosphorylation. Interestingly, we have found that both estradiol and ICI 182,780 induce Ser\textsuperscript{118} phosphorylation to a similar extent. The receptor is known to have a different conformation after it binds agonist as opposed to antagonist (32). However, the conformational difference induced by estradiol and ICI 182,780 does not seem to affect the N-terminal region of the receptor.

Although EGF and PMA only transiently induce Ser\textsuperscript{118} phosphorylation, these agents are able to stimulate ER-mediated transcription. In agreement with published reports, we observed that EGF and PMA with or without estradiol enhanced ER-mediated transcription (7, 33–35). Phosphorylation of Ser\textsuperscript{118} appears to be important for ER-mediated transcription since a mutant ER in which Ser\textsuperscript{118} is replaced with Ala has a diminished transcriptional ability compared with the wild type ER (4, 6–8). One possible model to explain the mechanism by which EGF and PMA can enhance ER-mediated transcription is that transient Ser\textsuperscript{118} phosphorylation is sufficient to induce a cascade of events that eventually lead to transcriptional activation. Of interest to note is that a combination of EGF and estradiol additively enhance ER-mediated transcription, whereas a combination of PMA and estradiol synergistically...
effect transcription. It is possible that EGF only enhances Ser\textsuperscript{118} phosphorylation, whereas PMA can also influence another step in ER-mediated transcription. Thus the effects of EGF and estradiol are additive because both agents are influencing the same event, whereas the effects of PMA and estradiol are synergistic because these agents are influencing multiple steps that are involved in ER-mediated transcription.

Our results with EGF and PMA are consistent with Ser\textsuperscript{118} being a substrate for activated p42/p44 MAPK (6, 7). In contrast to our results with EGF and PMA, we obtained three different lines of evidence that p42/p44 MAPK is not the kinase responsible for the estradiol-induced Ser\textsuperscript{118} phosphorylation. First, activation of p42/p44 MAPK activity was not detectable in serum-starved MCF-7 cells treated with estradiol. These results are in disagreement with the work of Miaglia as et al. (22). Second, basal level of p42/p44 MAPK is not sufficient to account for the estradiol-induced Ser\textsuperscript{118} phosphorylation. This reasoning is based on the observation that the basal p42/p44 MAPK activity with PD98059 had no effect on estradiol-induced Ser\textsuperscript{118} phosphorylation. Furthermore, if basal p42/p44 MAPK activity was responsible for the estradiol-induced Ser\textsuperscript{118} phosphorylation, then elevation of p42/p44 MAPK activity should result in 100% of the estradiol-bound ER molecules being phosphorylated. This result was not observed. Third, inhibitors that diminished either the EGF or PMA enhance-ment of p42/p44 MAPK activity and induction of Ser\textsuperscript{118} phosphorylation did not affect the estradiol-induced phosphorylation. Taken together, our results strongly suggest that in vivo Ser\textsuperscript{118} of the human ER is a target for two or more kinases that are regulated differentially by second messengers.

Trowbridge et al. (30) reported that cdk2 enhances both ligand-dependent and independent transcription by ER. An in vitro kinase assay demonstrated that Ser\textsuperscript{104} and Ser\textsuperscript{106} of the human ER were the likely targets of cdk2 activity. However, we did not observe any effect of the inhibitors staurosporine and olomoucine which inhibit cdk2 on estradiol-induced Ser\textsuperscript{118} phosphorylation. Like Ser\textsuperscript{118}, Ser\textsuperscript{104} and Ser\textsuperscript{106} are adjacent to proline residues. In our \textsuperscript{32}P-labeling experiments, Ser\textsuperscript{104} and Ser\textsuperscript{106} were minor sites of phosphorylation compared with Ser\textsuperscript{118} (5, 8).

This paper provides the first evidence that more than one kinase regulates Ser\textsuperscript{118} phosphorylation of the human ER. One simple model to explain the estradiol-induced phosphorylation of Ser\textsuperscript{118} is that, upon hormone binding, the ER undergoes a conformational change that makes the ER a better substrate for a constitutively active kinase. Phosphorylation of Ser\textsuperscript{118} may allow the ER to integrate signals from hormone binding and activation of the MAPK pathway.

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