

Regulation of Estrogen Receptor Nuclear Export by Ligand-Induced and p38-Mediated Receptor Phosphorylation

Heehyoung Lee and Wenlong Bai*

Department of Pathology, University of South Florida College of Medicine, and Program of Molecular Oncology and Drug Discovery, H. Lee Moffitt Cancer Center, Tampa, Florida 33612-4799

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Estrogen receptors are phosphoproteins which can be activated by ligands, kinase activators, or phosphatase inhibitors. Our previous study showed that p38 mitogen-activated protein kinase was involved in estrogen receptor activation by estrogens and MEKK1. Here, we report estrogen receptor-dependent p38 activation by estrogens in endometrial adenocarcinoma cells and in vitro and in vivo phosphorylation of the estrogen receptor α mediated through p38. The phosphorylation site was identified as threonine-311 (Thr³¹¹), located in helix 1 of the hormone-binding domain. The mutation of threonine-311 to alanine did not affect estrogen binding of the receptor but compromised its interaction with coactivators. Suppression of p38 activity or mutation of the site inhibited the estrogen-induced receptor nuclear localization as well as its transcriptional activation by estrogens and MEKK1. The inhibition of the p38 signal pathway by a specific chemical inhibitor blocked the biological activities of estrogens in regulating endogenous gene expression as well as endometrial cancer cell growth. Our studies demonstrate the role of estrogen receptor phosphorylation induced by the natural ligand in estrogen receptor's cellular distribution and its significant contribution to the growth-stimulating activity of estrogens in endometrial cancer cells.

Estrogens are female sex steroid hormones that control development, maintenance, and regulation of the female reproductive phenotype and behavior. They also stimulate the growth of normal and transformed epithelial cells of the female reproductive systems. The effect of estrogens is mediated through both estrogen receptors α and β (ER α and ER β), which belong to the nuclear hormone receptor superfamily, a group of ligand-regulated, zinc finger-containing transcription factors (11, 40). The superfamily includes not only receptors for classical steroids such as estrogens, androgens, progesterones, and glucocorticoids, but also receptors for steroid analogues and nonsteroid ligands such as vitamin D, thyroid, and retinoic acids, as well as orphan receptors for which the ligand is unknown.

Unlike the thyroid and vitamin D receptors, which reside in the nucleus in the absence of ligands, receptors for classical steroids such as ER α are targeted to the nucleus after binding with estrogens or selective estrogen receptor modulators such as tamoxifen. In contrast, the pure ER α antagonist ICI 182,780 directs the ER α to the cytoplasm (6). Of importance in this respect are three clusters of basic amino acids, similar to the nuclear localization signals found in simian virus 40 large T antigen, which were identified in the DNA binding domain and the hinge region of ER α (44). The nuclear localization signals are constitutively active and do not seem to explain the estrogen effect on the ER α nuclear localization (44). It is therefore possible that estrogen-induced targeting of ER α to the nucleus is mediated through other mechanisms.

Studies in recent years have provided increasing evidence

that nuclear localization is also controlled through nuclear export signals (17, 38, 42). A number of studies on exported proteins have shown that typical nuclear export signals are hydrophobic, leucine-rich sequences that signal the nuclear export complex containing exportin/Crm1 and RanGTP to transfer nuclear export signal-carrying proteins to the cytoplasm (8, 12, 29, 37).

Besides ligands, nonsteroid stimuli such as kinase activators, phosphatase inhibitors, neurotransmitters, and growth factors were also shown to activate the ER α (35). They either activate the receptor ligand independently or enhance the ligand-induced activity. Consistent with the cross talk with kinase/phosphatase pathways, ER α has been found to be phosphorylated at different sites by various kinases, including the external signal-regulated kinase (4, 19), cyclin A-CDK2 (32), c-SRC (25), protein kinase A (5), and pp90^{RSK1} (16). Except for tyrosine-537, all known ER α phosphorylation sites are on serine residues (1). Single-site mutation or simultaneous mutation at multiple sites reduced the transcriptional activity of the receptor (13). With the exception of the serine-236 phosphorylation by protein kinase A (5), most studies indicated the general role of ER α phosphorylation to be the regulation of the transcriptional activity of the receptor by modulating the interaction between the ER α activation domains and transcriptional coactivators (9, 28, 39).

In the present studies, we report that, in ER α -expressing endometrial cancer cells, 17 β -estradiol activates the p38 mitogen-activated protein kinase (MAPK) pathway, which in turn mediates the phosphorylation of the ER α on threonine-311 (Thr³¹¹), promoting the receptor's nuclear localization and interaction with steroid receptor coactivators. Additional studies show that Thr³¹¹ phosphorylation in ER α is a critical determinant for its transcriptional and biological activities in endometrial cancer cells.

* Corresponding author. Mailing address: Department of Pathology, University of South Florida College of Medicine, 12901 Bruce B. Downs Blvd., MDC 11, Tampa, FL 33612-4799 Phone: (813) 974-0563. Fax: (813) 974-5536. E-mail: wbai@hsc.usf.edu.

MATERIALS AND METHODS

Plasmids. pLEN-hER α (2, 36), pLEN β gal (36), Flag-p38 (43), SR α MEKK1 (CT) (26), and EREe1bLuc have been described (20, 36). pBind expression vectors for SRC1 and TIF2 have been described (24). T311A was generated by site-directed mutagenesis with the QuickChange mutagenesis kit (Stratagene, La Jolla, Calif.) and confirmed by direct sequencing. pLEN-hER α was used as the template for the mutagenesis. The primers for the PCR were synthetic oligonucleotides with the sequences 5'-CCTGTCCCTGGCAGCCGACCAGATG-3' and 5'-CATCTGGTTCGGCTGCCAGGACAAGG-3'.

Transfection and reporter assays. Cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Transfection was performed with Lipofectamine (Gibco-BRL Life Technologies, Rockville, Md.) as described previously (20). After transfection, cells were placed in Dulbecco's modified Eagle's medium containing 5% charcoal-stripped fetal bovine serum, treated with different reagents, and assayed for luciferase and β -galactosidase activity as described previously (21). ER α transcriptional activity was measured by normalizing the luciferase activity to the corresponding β -galactosidase activity.

Immunoprecipitation and immunoblotting analysis. Detection of Flag-p38 and ER α expression by immunoblotting has been described (20). To determine the level of Thr phosphorylation of ER α , cells were lysed in modified radioimmunoprecipitation assay (RIPA) buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 2 mM EDTA, 1% NP-40, 5 mM sodium fluoride, and protease inhibitor cocktail. The lysates were immunoprecipitated with F-10 anti-ER α monoclonal antibody (Santa Cruz Biotechnology Inc., Santa Cruz, Calif.). The ER α precipitates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 8% polyacrylamide), transferred to nitrocellulose, probed with the antiphosphothreonine antibody (Sigma, St. Louis, Mo.) overnight, and visualized with the ECL kit (Amersham Pharmacia Biotech Inc., Piscataway, N.J.).

To detect the binding of coactivators to ER α , Ishikawa cells were transfected with pBind-SRC1, pBind-TIF2, or pBind as a control vector together with either wild-type or mutant ER α . Following immunoprecipitation with anti-ER α F-10 antibody, coactivator in the precipitates was detected with anti-Gal4 DNA-binding domain antibody (Santa Cruz).

In vitro kinase assays. p38 in vitro immunocomplex kinase assays were described before (20). For in vitro kinase assays with purified p38 kinase, recombinant ER α protein was incubated with 25 ng of active recombinant human p38 α /SAPK2a (2.4 U/ μ g; Upstate Biotechnology, Lake Placid, N.Y.).

Phosphoamino acid analysis, phosphopeptide mapping, and Edman degradation. Phosphoamino acid analysis, phosphopeptide mapping, and manual Edman degradation were performed as described previously (5, 13, 33).

Hormone binding assays. Cells were incubated for 2 h with 3 H-labeled 17 β -estradiol (New England Nuclear, Boston, Mass.) at the indicated concentrations. To determine nonspecific binding, a 200-fold excess of unlabeled diethylstilbestrol (DES; Sigma, St. Louis, Mo.) was added in addition to 3 H-labeled 17 β -estradiol in a parallel set of samples. Cells were washed five times with ice-cold PBS and extracted in ethanol, and radioactivity was counted in a scintillation counter. Specific binding was calculated by subtracting nonspecific binding in cells incubated with excess DES from the total binding in cells incubated with [3 H]17 β -estradiol alone. Each data point was analyzed in duplicate.

Preparation of nuclear and cytoplasmic extracts. For preparation of nuclear and cytosolic extracts, cells were scraped into hypotonic buffer containing 20 mM HEPES (pH 7.9), 1 mM EDTA, 1 mM EGTA, 20 mM NaF, 1 mM Na $_3$ VO $_4$, 1 mM Na $_2$ P $_2$ O $_7$, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1% NP-40, and protease cocktail. Nuclei were separated from the cytosol by centrifugation at 11,750 \times g for 20 min at 4°C. After centrifugation, pellets containing nuclei were resuspended in the same hypotonic buffer but containing 420 mM KCl and 20% glucose. To be representative of the ER α distribution in a cell, the same volume of buffer was used to prepare the nuclear and cytoplasmic fractions for each preparation, and equal volumes of cytosolic and nuclear extracts were subjected to immunoblotting analyses.

Immunofluorescence staining. Ishikawa cells were seeded onto chamber slides and transfected with 0.1 μ g of either wild-type or T311A mutant ER α expression constructs. Forty-eight hours later, the cells were fixed in 2% paraformaldehyde, permeabilized with phosphate-buffered saline (PBS) containing 1% Triton X-100 and 1% bovine serum albumin, and blocked in blocking buffer (PBS containing 1% bovine serum albumin and 0.1% NP-40). Cells were then incubated with anti-ER α antibody F-10. After extensive washing with PBS, anti-mouse immunoglobulin-fluorescein isothiocyanate (FITC) conjugate (Sigma, St. Louis, Mo.) was applied in blocking buffer as the secondary antibody. Nuclei were stained with 2',6'-diamidino-2-phenylindole (DAPI) in antifade mounting medium (Vec-

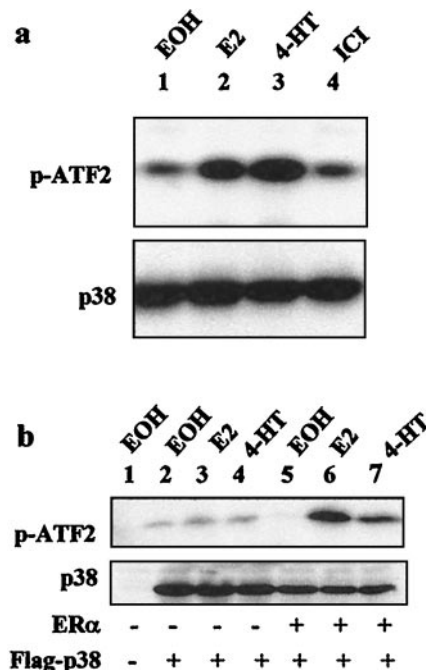


FIG. 1. 17 β -Estradiol and 4-hydroxytamoxifen activate p38 MAPK in endometrial cancer cells. (a) Activation of p38 by ER α agonists but not pure antagonist. ER α -negative Ishikawa cells were transfected with 0.5 μ g of Flag-p38 and 0.5 μ g of pLEN-hER α and treated with 10 $^{-8}$ M 17 β -estradiol (E2), 10 $^{-6}$ M 4-hydroxytamoxifen (4-HT), 10 $^{-7}$ M ICI 182,780 (ICI), or ethanol (EOH) as a vehicle control for 30 min. The kinase activity was determined by immunocomplex kinase assays with GST-ATF2 as a substrate (upper panel). The level of Flag-p38 expression was determined by Western blotting with the M2 antibody (lower panel). p-ATF2, phosphorylated GST-ATF2. (b) ER α dependency of p38 activation by ER α agonists. ER α -negative Ishikawa cells were transfected with Flag-p38 and pLEN-hER α or control vectors. Cells were treated and the p38 activity was assayed as explained for panel a.

tor laboratories, Burlingame, Calif.) before analysis under a fluorescence microscope.

Alkaline phosphatase and MTT assays. Alkaline phosphatase activity was determined in ER α -positive Ishikawa cells as described previously (22). Cell growth was measured in methylthiazole tetrazolium (MTT) assays as described previously (21).

RESULTS

Activation of p38 by estrogens in endometrial adenocarcinoma cells. Our previous studies (20) showed that estrogens require p38 to activate ER α in endometrial cancer cells. To determine whether estrogens activate p38 in these cells, Ishikawa cells lacking ER α expression (20) were transfected with ER α and Flag-p38 expression vectors. Following treatment with 17 β -estradiol, 4-hydroxytamoxifen, or ICI 182,780, the p38 protein was immunoprecipitated with the M2 anti-Flag antibody, and its activity was determined by immunocomplex kinase assays with glutathione *S*-transferase (GST)-ATF2 as a substrate. As shown in Fig. 1a, treatment with 17 β -estradiol induced p38 activity (Fig. 1a, lane 2 of top panel) compared to the vehicle control (Fig. 1a, lane 1 of top panel). Interestingly, 4-hydroxytamoxifen also increased the p38 activity (Fig. 1a, lane 3 of top panel), supporting our previous data showing the

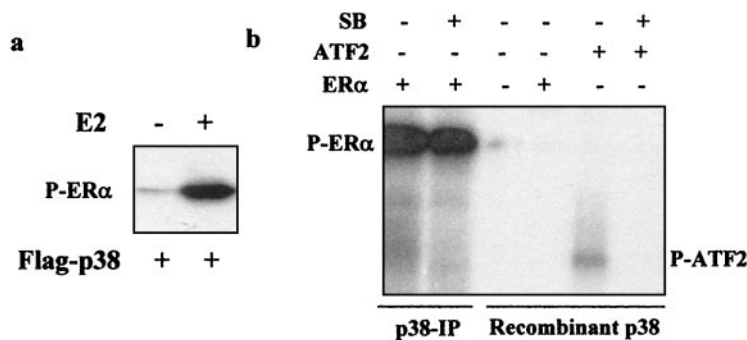


FIG. 2. Phosphorylation of purified ER α protein by p38 immunocomplex isolated from estrogen-treated cells. (a) ER α -negative Ishikawa cells were transfected with 0.5 μ g of Flag-p38 and 0.5 μ g of pLEN-hER α and treated with 17 β -estradiol (E2) or not for 30 min. The phosphorylation of recombinant ER α protein by p38 immunoprecipitates was determined in *in vitro* immunocomplex kinase assays. P-ER α , phosphorylated ER α . (b) Lack of ER α phosphorylation by purified recombinant p38. Phosphorylation of recombinant ER α by p38 immunocomplex (p38-IP) or recombinant human p38 α purified from bacteria in the presence or absence of 50 μ M SB 203580 (SB) was determined. As controls, recombinant ATF2 was phosphorylated by purified p38, and the phosphorylation was inhibited by SB 203580.

involvement of the MEKK1-p38 MAPK pathway in the uterine-specific agonistic activity of tamoxifen (20). On the other hand, the pure ER α antagonist ICI 182,780 did not induce p38 activity (Fig. 1a, lane 4 of top panel). The level of p38 expression was not affected by the hormonal treatment (Fig. 1a, bottom panel), demonstrating that 17 β -estradiol and 4-hydroxytamoxifen increased its specific activity.

To show that p38 activation by estrogens requires ER α , the induction of p38 activity by 17 β -estradiol or 4-hydroxytamoxifen was assayed in the ER α -negative Ishikawa cells transfected or not with ER α . As shown in Fig. 1b, 17 β -estradiol and 4-hydroxytamoxifen did not affect p38 activity in the absence of ER α (Fig. 1b, lanes 3 and 4), whereas in ER α -transfected cells, treatment with either 17 β -estradiol (Fig. 1b, lane 6) or 4-hydroxytamoxifen (Fig. 1b, lane 7) again increased p38 activity compared to the vehicle control (Fig. 1b, lane 5). No phosphorylation of ATF2 was detected in the absence of Flag-p38 (Fig. 1b, lane 1), showing the specificity of ATF2 phosphorylation to p38. These data demonstrate that ER α agonists activate p38 via ER α in endometrial cancer cells.

Phosphorylation of ER α by p38 immunocomplex but not recombinant p38 purified from bacteria. Since MEKK1-activated p38 immunoprecipitated from cells phosphorylated the recombinant ER α protein in *in vitro* immunocomplex kinase assays (20), we next tested whether p38 immunoprecipitates from estrogen-treated cells would also phosphorylate the ER α protein. ER α -negative Ishikawa cells were transfected with p38 and ER α expression vectors, and p38 immunocomplex kinase assays were performed as described for Fig. 1 with the exception that recombinant human ER α protein was used as a substrate. As shown in Fig. 2a, the ER α protein was weakly phosphorylated by the basal p38 immunoprecipitated from cells treated with vehicle (Fig. 2a, lane 1). Treatment with 17 β -estradiol induced ER α phosphorylation considerably (Fig. 2a, lane 2), suggesting that the ER α protein is a substrate for the p38 immunocomplex isolated from estrogen-treated cells.

To determine whether it is the p38 or an associated kinase that phosphorylates ER α , the phosphorylation of ER α protein was tested with active p38 α purified from bacteria. As shown in Fig. 2b, while recombinant p38 phosphorylated ATF2, which was blocked by SB 203580, it did not phosphorylate ER α in

parallel reactions. In parallel analysis, p38 immunocomplexes phosphorylated ER α protein, as expected, but p38 inhibitor added to the kinase reactions did not block this phosphorylation. These analyses suggest that it is a p38-associated kinase that phosphorylated ER α in the immunocomplex.

p38-mediated phosphorylation of ER α on Thr residues *in vitro* and *in vivo*. In previous studies (20), none of the presently known ER α phosphorylation sites were found to be required for ER α activation by MEKK1, suggesting that p38 MAPK may induce the phosphorylation of the ER α at novel sites. To determine which type of amino acid is phosphorylated, ER α protein phosphorylated *in vitro* by p38 immunocomplex (Fig. 2a, lane 2) was excised from the SDS-PAGE gel and subjected to phosphoamino acid analysis. The result re-

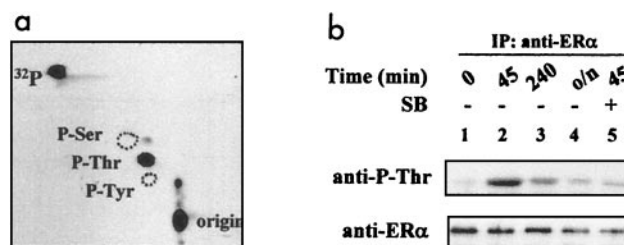


FIG. 3. ER α is phosphorylated by the p38 immunocomplex on Thr residues. (a) Phosphoamino acid analysis. ER α phosphorylated by the p38 immunocomplex was treated with HCl for hydrolysis, mixed with phosphoamino acid markers, and separated by two-dimensional electrophoresis on a TLC plate. The positions of phosphoamino acids from the markers (indicated by dashed circles) were revealed by ninhydrin spray, and 32 P-radiolabeled phosphoamino acids from the sample were located by autoradiography. 32 P, free 32 P from the hydrolysis; P-Ser, phosphoserine; P-Thr, phosphothreonine; P-Tyr, phosphotyrosine. (b) p38-mediated ER α phosphorylation on Thr residues *in vivo* induced by 17 β -estradiol. ER α -positive Ishikawa cells were treated with 10 $^{-8}$ M 17 β -estradiol for the indicated times with 50 μ M SB 203580 (SB) or vehicle. o/n, overnight. The Thr phosphorylation of endogenous ER α protein was detected by Western blotting with antiphosphothreonine antibody (anti-P-Thr) (upper panel) following immunoprecipitation with the F-10 anti-ER α antibody. The level of ER α in the immunoprecipitates was determined by immunoblotting with the same F-10 antibody (lower panel).

vealed that ER α phosphorylation by p38 occurs exclusively on Thr residues (Fig. 3a).

Since the p38 immunocomplex phosphorylated ER α in vitro exclusively on Thr residues, in contrast to all known ER α phosphorylation sites, we examined whether estrogens induce Thr phosphorylation of ER α in vivo with an antibody that specifically recognizes proteins containing phosphorylated Thr residues. ER α -positive Ishikawa cells (30) were treated with 17 β -estradiol for various time periods. The endogenous ER α protein was then immunoprecipitated with an anti-ER α antibody and subjected to immunoblotting with either the anti-ER α or the antiphosphothreonine antibody. As shown in Fig. 3b, treatment with 17 β -estradiol for 45 min resulted in a considerable increase in Thr phosphorylation of the endogenous ER α protein (Fig. 3b, lane 2) compared to the control (Fig. 3b, lane 1). The Thr phosphorylation returned to the basal level after the cells were treated for 4 h (Fig. 3b, lane 3) or overnight (Fig. 3b, lane 4), indicating that it is a transient event. Treatment of cells with SB 203580 blocked in vivo ER α phosphorylation on Thr residues (Fig. 3b, lane 5), demonstrating that the endogenous p38 MAPK pathway in endometrial cancer cells is required for the phosphorylation. Compared with the decreased level of the ER α protein (Fig. 3b, lower panel), the increase in the phosphothreonine signal at 45 min cannot be attributed to a change of ER α level and thus must represent an increase in specific Thr phosphorylation.

Identification of Thr³¹¹ as the p38-mediated phosphorylation site on ER α . To determine which of the ER α Thr residues is phosphorylated, in vitro-phosphorylated ER α protein was eluted from the gel, digested with trypsin, and subjected to two-dimensional separation on thin-layer chromatography (TLC) plates. The phosphopeptide mapping analysis showed the presence of two major phosphopeptides (Fig. 4a), suggesting that p38 may phosphorylate the ER α at two separate sites. Alternatively, the two spots may represent isoforms or products of partial tryptic digestion of the same phosphopeptide. The minor spots in the chromatogram are likely due to phosphorylation of the ER α protein by background kinases contaminating the p38 immunoprecipitates.

Following the tryptic mapping, each of the two major spots was recovered from the TLC plates and subjected to manual Edman degradation. As shown in Fig. 4b and c, both peptides released their ³²P at cycle 8, indicating that the phosphorylated amino acids are at the eighth position counting from the amino terminus of the tryptic peptides.

Next, phosphorylated ER α protein was digested with both trypsin and a secondary endoproteinase, Glu-C or Asp-N, which cuts peptides at the carboxyl terminus of glutamate and the amino terminus of aspartate, respectively. As indicated by the altered migration of the peptides after double enzyme digestion, both phosphotryptic peptides were cleaved by Glu-C (Fig. 4d) and Asp-N (data not shown). This suggests that both peptides contain Glu and Asp residues in their sequences. Manual Edman degradation of the two peptides obtained by secondary digestion with either Glu-C (Fig. 4e and 4f) or Asp-N (data not shown) released the ³²P at cycle 8, suggesting that the tryptic peptides contain no Asp or Glu residues at positions amino terminal to the phosphothreonine.

Based on the above analysis, the p38 phosphorylation site(s) should satisfy the following criteria. The site(s) is a Thr resi-

due. It should be located at the eighth position from an arginine or lysine. The tryptic peptides should contain both Glu and Asp residues. The Glu and Asp residues must be located at positions carboxyl terminal but not amino terminal to the phosphothreonine. Examination of the ER α protein sequence revealed that only Thr³¹¹, located in the amino terminus of the ER α ligand-binding domain, satisfies all these criteria and is therefore the p38 phosphorylation site (Fig. 4g). It becomes obvious that the two major spots generated by protease digestions both contain Thr³¹¹, suggesting that they are most likely two isoforms of the same peptide.

To determine whether Thr³¹¹ is the major threonine phosphorylated in vivo, we mutated the site to alanine and analyzed the threonine phosphorylation of the mutant ER α (T311A). As shown in Fig. 5, the mutation eliminated the 17 β -estradiol-induced ER α phosphorylation on Thr residues (Fig. 5, upper panel) but did not affect the level of ER α expression (Fig. 5, lower panel), suggesting that Thr³¹¹ is the major site phosphorylated in vivo. Together with the data in Fig. 3b showing that the p38 inhibitor blocks 17 β -estradiol-induced ER α phosphorylation on Thr residues, the analysis shows that the p38 pathway induced by estrogens mediates Thr³¹¹ phosphorylation in vivo.

Regulation of ER α transcriptional activity by Thr³¹¹ phosphorylation. Since our previous study showed that inhibition of p38 prevented estrogen- and MEKK1-induced ER α activation (20), it was expected that mutation of Thr³¹¹ would decrease the ER α activity and abolish the receptor's response to MEKK1 and p38. Therefore, the transcriptional activities of wild-type ER α and the T311A mutant were compared.

As shown in the upper panel of Fig. 6a, 17 β -estradiol activated the wild-type ER α about 15-fold but induced negligible activity in the mutant ER α . Since the level of ER α expression was not affected by the mutation (Fig. 6a, lower panel), this demonstrates that the mutation decreased the specific activity of the receptor. The activity of the mutant receptor was essentially undetectable unless a very high level of the expression plasmids was transfected into the cells, at which point the activity of the wild-type receptor started decreasing (Fig. 6b), presumably due to the squelching effect of excessively expressed receptors.

Further studies showed that the activity of the T311A mutant was not increased by MEKK1, whereas wild-type ER α was activated threefold by MEKK1 (Fig. 6c). In addition, SB 203580 did not decrease the activity of the T311A mutant (Fig. 6d), while the transcriptional activity of wild-type ER α induced by 17 β -estradiol was decreased by 60% by the p38 inhibitor (data not shown). These studies demonstrate that Thr³¹¹ phosphorylation is required for ER α activation by both ligands and kinases.

Lack of effect of Thr³¹¹ mutation on receptor's ability to bind ligand. Since Thr³¹¹ is located in helix 1 of the receptor's ligand-binding domain, we first determined whether the mutation of Thr³¹¹ to alanine affected hormone binding. Wild-type or mutant ER α was transfected into ER α -negative Ishikawa cells, and hormone binding was determined after the cells were incubated with ³H-labeled 17 β -estradiol at different concentrations. As shown in Fig. 7a, wild-type and mutant receptors exhibited no difference in specific estrogen binding at both saturating and nonsaturating doses. Consistent with the lack of

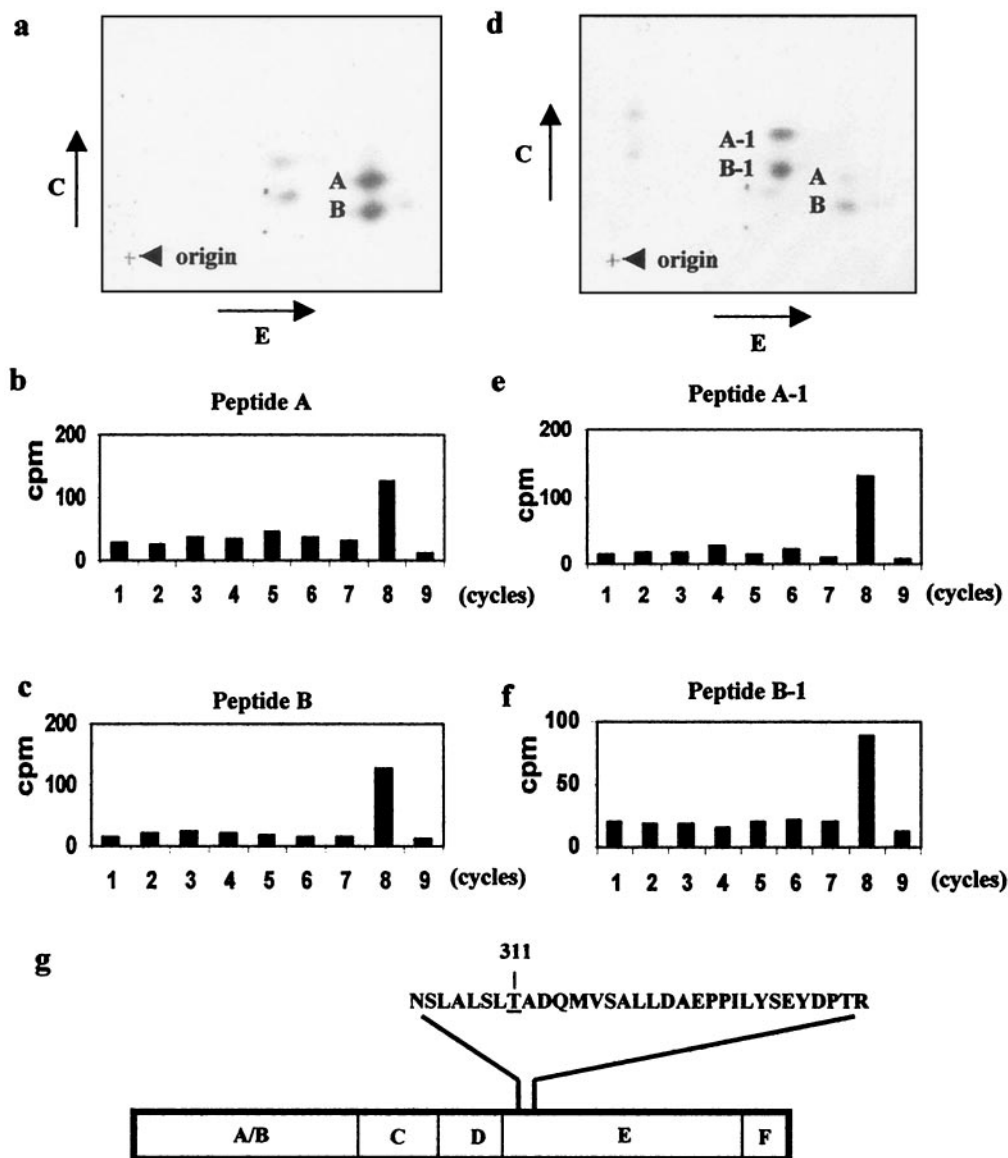


FIG. 4. Thr³¹¹ is the site phosphorylated by the p38 immunocomplex in vitro. (a) Phosphopeptide map of ER α after tryptic digestion. ER α phosphorylated in vitro by p38 was subjected to tryptic digestion and two-dimensional separation. The two major phosphopeptides were labeled A and B. E, electrophoresis; C, chromatography. (b and c) Edman degradation of tryptic peptides. Peptides A and B were recovered from the TLC plate and subjected to manual Edman degradation. Radioactivity released from each cycle was plotted. (d) Phosphopeptide map of ER α after trypsin/Glu-C double digestion. Tryptic digests of phosphorylated ER α were cut with Glu-C and subjected to two-dimensional separation. The two major peptides were identified as A-1 and B-1. Note the presence of tryptic peptides A and B due to incomplete Glu-C digestion. (e and f) Edman degradation of phosphopeptides after double digestion. (g) Amino acid sequence of ER α tryptic peptide containing Thr³¹¹.

an effect of the mutation on hormone binding activity, the decreased receptor activity caused by the mutation was not recovered by treatment with excess amounts of 17 β -estradiol (Fig. 7b).

Regulation of ER α nuclear export by Thr³¹¹ phosphorylation. Nucleus-cytoplasm shuttling of proteins, including nuclear hormone receptors and their cofactors, has been shown to be regulated by phosphorylation (15, 18, 27, 47). In ER α , Thr³¹¹ appears to be located adjacent to the third nuclear localization signal (amino acids 299 to 303) (44) and within a putative nuclear export signal similar to that described for the inhibitor of protein kinase A (42) and p53 (46). The putative

nuclear export signal is conserved in ER α from different species (Fig. 8a). Accordingly, it is possible that Thr³¹¹ phosphorylation controls the nuclear localization of ER α .

To address this possibility, ER α -negative Ishikawa cells were transfected with ER α vector and treated with SB 203580, and the nuclear-cytoplasmic distribution of the ER α was analyzed. As shown in Fig. 8b(1), poly(ADP-ribose) polymerase (PARP) and Hsp60 were clearly separated into the nuclear and cytosolic fractions under our conditions, demonstrating the efficiency of our fractionation procedure. As can be seen in Fig. 8b(2), in the absence of estrogens, ER α was evenly distributed between the nucleus and the cytoplasm, which is consistent

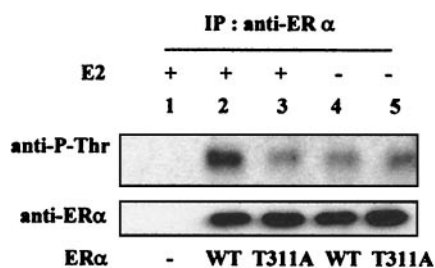


FIG. 5. Thr³¹¹ is phosphorylated in vivo. ER α -negative Ishikawa cells were transfected with 0.5 μ g of pLEN-ER α expressing either wild-type (WT) or T311A mutant ER α , as indicated, and treated with 17 β -estradiol (E2) or vehicle. The level of Thr phosphorylation was determined as for Fig. 2c. WT, wild-type ER α ; T311A, ER α in which Thr³¹¹ was replaced with an alanine. IP, immunoprecipitation.

with our data that p38 activity is induced by 17 β -estradiol. No ER α signal was detected in cells transfected with the control vector, showing the ER α -negative status of the cells as well as the specificity of the antibody for the ER α . After 17 β -estradiol treatments, the receptor was located in the nucleus, as shown in Fig. 8b(3), lanes 1 and 2, whereas it remained in the cytoplasm after cotreatment with the p38 inhibitor, as shown in Fig. 8b(3), lanes 3 and 4. This demonstrates that p38 is involved in ER α nuclear localization.

The p38 MAPK may regulate the nuclear localization of ER α by either increasing nuclear import or decreasing export. In order to distinguish between these possibilities, ER α -positive Ishikawa cells were treated with leptomycin B, a known exportin inhibitor, prior to analysis of the cellular distribution of endogenous ER α . As shown in Fig. 8b(3), lanes 5 and 6, leptomycin B restored the 17 β -estradiol-induced ER α nuclear localization in the presence of the p38 inhibitor, demonstrating that phosphorylation mediated through p38 inhibits Crm1-dependent ER α nuclear export. Accordingly, mutation of Thr³¹¹ to alanine blocked estrogen-induced nuclear localization, as shown in Fig. 8b(3), lanes 7 and 8, which was restored by leptomycin B treatment, as shown in Fig. 8b(3), lanes 9 and 10. It appears, therefore, that it is nuclear export that is controlled by Thr³¹¹ phosphorylation of ER α mediated through the p38 MAPK pathway.

Since the separation of nucleus and cytoplasm by fractionation is considered to determine only nuclear retention, the cellular distribution of the wild-type and T311A mutant ER α forms was analyzed by direct immunofluorescence staining. As shown in Fig. 8c, treatment with 17 β -estradiol induced the nuclear distribution of the wild-type receptor but not the T311A mutant. By comparing the FITC and DAPI signals, T311A in the presence of estrogens appeared to accumulate in the areas surrounding the nuclei. The exact meaning of this

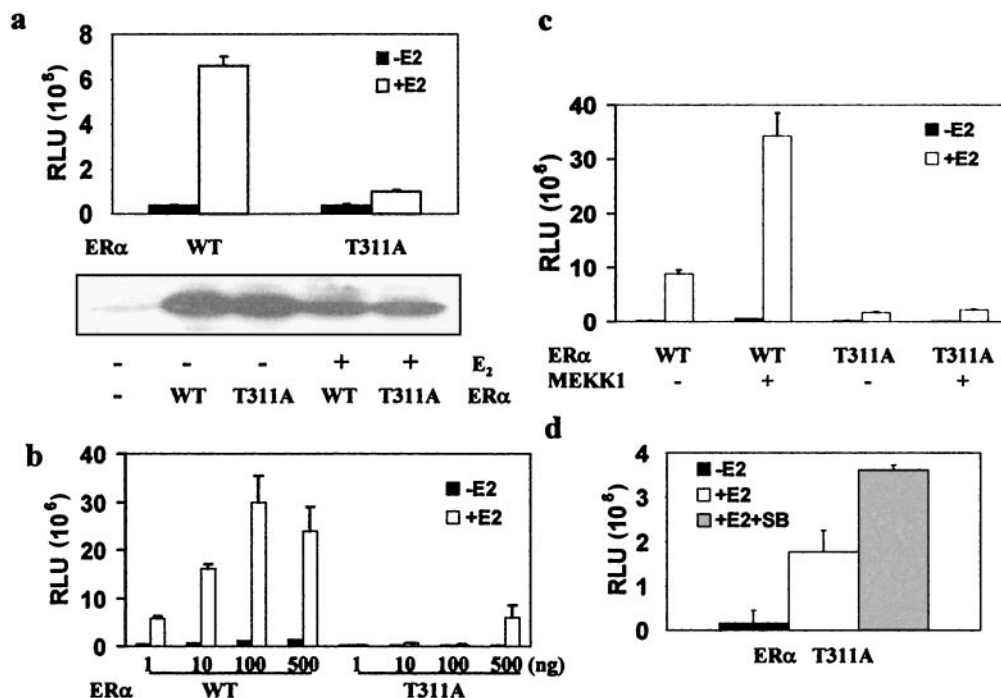


FIG. 6. Mutation of Thr³¹¹ to alanine decreases the transcriptional activity of ER α . (a) Lack of T311A activation by 17 β -estradiol. ER α -negative Ishikawa cells were transfected with 0.1 μ g of either wild-type (WT) or mutant (T311A) pLEN-hER α together with 0.5 μ g of pLEN β Gal and 0.5 μ g of EREe1bLuc and then treated overnight with 17 β -estradiol (+E2) or vehicle (-E2). ER α transcriptional activity was normalized to β -galactosidase activity and is expressed as relative luciferase units (RLU) (upper panel), and the level of ER α protein was analyzed in parallel (lower panel). (b) Transcriptional activity of the T311A mutant at different dosages. Cells were transfected with 0.5 μ g of EREe1bLuc, 0.1 μ g of pLEN β Gal, and different amounts of ER α plasmid as indicated. ER α activity was determined as for panel a. (c) Lack of T311A activation by MEKK1. Cells were transfected with 0.5 μ g of EREe1bLuc, 0.1 μ g of pLEN-hER α , 0.5 μ g of pLEN β Gal, and 0.2 μ g of SR α MEKK1(CT). ER α activity was determined as for panel a. (d) Effect of SB 203580 on T311A. Cells were transfected with 0.5 μ g of EREe1bLuc, 0.5 μ g of pLEN β Gal, and 0.1 μ g of pLEN-hER α expressing T311A and treated with 10⁻⁸ M 17 β -estradiol and 50 μ M SB 203580 or vehicle. ER α activity was determined as for panel a.

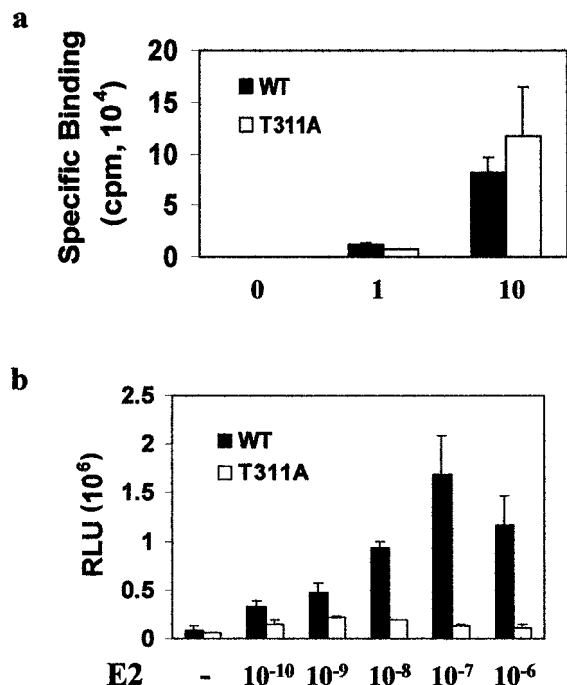


FIG. 7. Decreased transcriptional activity of T311A mutant ER α is not due to a change in hormone binding ability. (a) Similar hormone binding abilities of wild-type (WT) and T311A ER α s. ER α -negative Ishikawa cells at a density of 10^6 cells/100-mm dish were transfected with 5 μ g of wild-type or T311A mutant ER α . Forty-eight hours posttransfection, *in vivo* hormone binding was performed as described in the text. Specific binding was calculated by subtracting nonspecific binding ($[^3\text{H}]17\beta$ -estradiol plus a 200-fold excess of unlabeled DES) from total binding ($[^3\text{H}]17\beta$ -estradiol alone). The data are representative of three independent experiments, and each data point was analyzed in duplicate. (b) Transcriptional activity of the T311A mutant was not restored by treatment with excessive estrogen concentrations. ER α -negative Ishikawa cells were transfected as described for Fig. 6a and treated with 17β -estradiol (E2) at the indicated concentrations. ER α activity was determined as for Fig. 6a.

observation is unclear, but it may reflect signals from the newly exported mutant ER α which have not been fully dissociated from the exporting complex. The lack of FITC signal in cells transfected with the control vector demonstrated that the FITC signal is specific to the ER α .

If the lack of transcriptional activity of T311A were due to its cytoplasmic dislocation, its restored nuclear localization would be expected to restore its transcriptional activity. The transcriptional activities of the wild-type ER α and the T311A mutant were measured in the presence and absence of leptomycin B (Fig. 9). While leptomycin B did not affect the estrogen-induced activity of wild-type ER α , the activity of the T311A mutant was increased threefold. The decreased basal activity might be due to the cytotoxicity of the drug. The lack of an effect of leptomycin B on the activity of the wild-type receptor was expected because, with respect to the effect on ER α localization, leptomycin B and ER α Thr³¹¹ phosphorylation should be redundant.

Regulation of ER α interaction with steroid receptor coactivators by Thr³¹¹ phosphorylation. Although leptomycin B selectively increased the activity of the T311A mutant, the activity of the mutant in the presence of leptomycin B was still less

than that of the wild type, suggesting that the mutation affected other steps of the receptor activation process in addition to its effect on estrogen-induced nuclear targeting. Therefore, the ability of the wild-type and mutant ER α s to bind members of the p160 SRC family was compared in coimmunoprecipitation assays. As shown in the upper panel of Fig. 10, the wild-type ER α coprecipitated with both SRC1 and TIF2 in an estrogen-dependent manner, while the T311A mutant showed no binding to SRC1 and significantly reduced binding to TIF2. In parallel studies, the expression levels of the wild-type ER α and the T311A mutant were comparable (Fig. 10, lower panel), demonstrating that the decreased interaction with steroid receptor coactivators and the selective effect of Thr³¹¹ mutation on the interaction with SRC1 are not due to variation in the level of receptor protein expression or variation in the efficiency of the immunoprecipitations.

Regulation of biological activity of ER α in endometrial cancer cells by p38 MAPK. In endometrial cancer cells, estrogens have been shown to induce alkaline phosphatase (14). To demonstrate the significance of p38 phosphorylation on estrogen regulation of endogenous gene expression, alkaline phosphatase activity was measured in ER α -positive Ishikawa cells after estrogen treatment in the presence or absence of SB 203580. In the analysis, 17β -estradiol caused a threefold increase in alkaline phosphatase activity (Fig. 11a), which was blocked by SB 203580. The p38 inhibitor did not decrease the basal activity of alkaline phosphatase, indicating the absence of general cytotoxicity.

To further demonstrate the significance of p38 phosphorylation in the biological function of the ER α , the growth response of the ER α -positive Ishikawa cells to estrogens was assayed in the presence and absence of the p38 inhibitor. As shown in Fig. 11b, 17β -estradiol stimulated the growth of the endometrial cancer cells, which was blocked by SB 203580. Taken together with the data from Fig. 11a, these findings support the important role of the p38 MAPK as a determinant for the activation of transcriptional activity of endogenous genes through the ER α as well as its biological importance in mediating the regulation of endometrial cancer cell growth.

DISCUSSION

In summary, our studies demonstrate that the signaling pathway mediated through p38 MAPK plays an important role in ER action in endometrial cancer cells. The pathway exerts its effect by inducing ER α phosphorylation at Thr³¹¹, the first threonine residue reported to be phosphorylated in ER α in response to estrogens. Although located in helix 1 of the ligand-binding domain, phosphorylation does not affect the ability of the receptor to bind the natural ligand. Instead, it blocks ER α nuclear export and promote the interaction of ER α with p160 steroid receptor coactivators, which may explain both the observed effect of the p38 MAPK pathway on the transcriptional activity of ER α in regulating endogenous gene expression as well as the biological activity of estrogens in stimulating endometrial cancer cell growth.

Although our studies clearly suggest that the p38 MAPK induced by the natural ER α agonist mediates Thr³¹¹ phosphorylation, it appears unlikely that p38 is the kinase that directly phosphorylates ER α at Thr³¹¹. First, the Ser-Pro motif is a

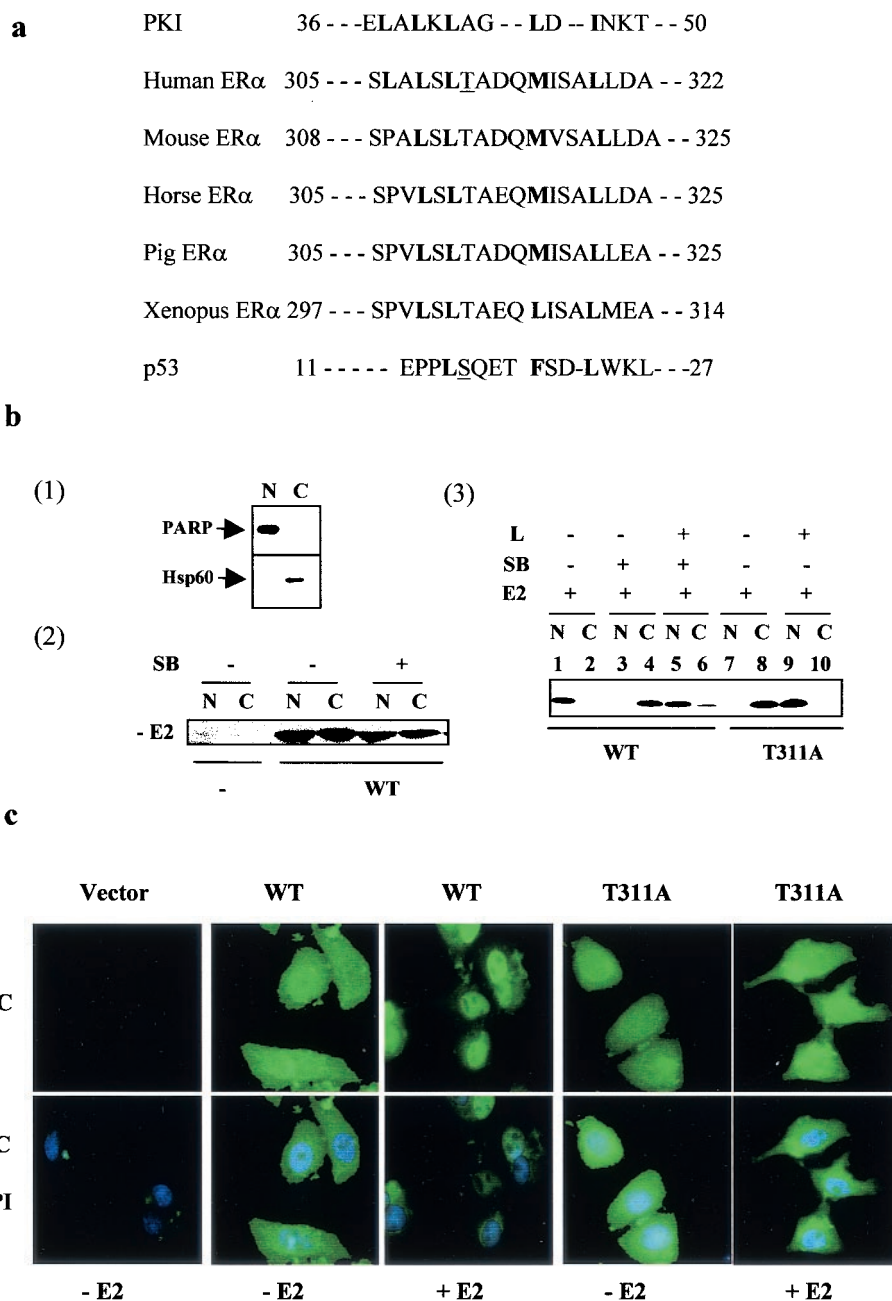


FIG. 8. Nuclear export of ER α is regulated by Thr³¹¹ phosphorylation. (a) The putative leucine-rich nuclear export signal sequence that is highly conserved among ER α s from different species. The amino acids critical for the nuclear export signal function of the protein kinase I (PKI) and conserved in ER α and p53 are bold. Note the close similarity between the conserved Thr³¹¹ and serine-15 of p53, which are both underlined. (b) Panel 1, separation of nuclear and cytosolic proteins. Nuclear (N) and cytosolic (C) extracts were separated as described in the text and confirmed by immunoblotting with antibodies against known nuclear (PARP) and cytoplasmic (Hsp60) markers. Panel 2, lack of effect of p38 inhibition on ER α nuclear localization in the absence of estrogens. ER α -negative Ishikawa cells were transfected with parental vector or pLEN-hER α and treated with vehicle or SB 203580 for 45 min. Cellular localization of ER α was determined with anti-ER α antibody. Panel 3, regulation of Crm1-dependent nuclear export of ER α by p38 MAPK-mediated Thr³¹¹ phosphorylation. Cells were transfected with pLEN-hER α and treated with 17 β -estradiol (E2), SB 203580 (SB), leptomycin B (L, 5 ng/ml), or a combination. Leptomycin B was added to the cells 30 min before treatment with 17 β -estradiol or SB 203580. (c) Visualization of subcellular distribution of wild-type (WT) and T311A mutant ER α s by direct immunofluorescence staining. ER α -negative Ishikawa cells were plated on coverslips and transfected with vector or wild-type or T311A mutant ER α DNA. Forty-eight hours posttransfection, cells were treated with 17 β -estradiol for 30 min and fixed. Following immunostaining as described in the text, ER subcellular distribution (FITC signal) and cell nucleus (DAPI signal) were visualized, and images were captured under a fluorescence microscope.

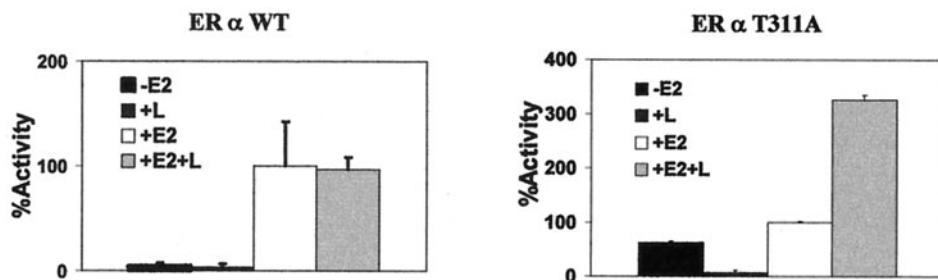


FIG. 9. Exportin inhibitor increases the activity of T311A but not the wild-type (WT) ER α . ER α -negative Ishikawa cells were transfected with 0.5 μ g of EREe1bLuc, 0.5 μ g of pLEN β Gal, and 0.1 μ g of pLEN-hER α expressing wild-type or T311A ER α and treated overnight with vehicle, 17 β -estradiol (E2), leptomycin B (L), or a combination. ER α activity was expressed as a percentage of the activity in cells treated with 10⁻⁸ M 17 β -estradiol but without leptomycin B.

feature shared by substrates for known MAPKs, and Thr³¹¹ is not followed by a proline. Second, SB 230580, when added directly to the kinase reactions, failed to block the in vitro phosphorylation of the recombinant ER α by the p38 immunocomplex (Fig. 2b), although the same inhibitor blocked estrogen stimulation of Thr³¹¹ phosphorylation in vivo and ATF-2 phosphorylation by purified human recombinant p38 kinase in vitro. Third, purified recombinant p38 did not phosphorylate ER α protein in in vitro kinase assays, whereas it phosphorylated ATF2 in parallel reactions (Fig. 2b). However, under the same conditions in which ER α phosphorylation by p38 immunocomplexes was demonstrated (Fig. 2a), large-scale affinity chromatography failed to detect any specific p38-bound protein after silver staining, even though the amount of purified p38 was readily detectable by Coomassie blue staining (data not shown). These studies suggest that the kinase phosphorylating Thr³¹¹ may be a member of the p38 family or a separate kinase that has a high specificity toward ER α but binds to p38 at a level too low to be detected by traditional protein-staining methods after affinity purification.

One of the main findings in these studies is that the transient ER α phosphorylation at Thr³¹¹ induced by the natural ligand is required for the stable localization of the ER α protein in the

nucleus. The transient phosphorylation may initiate a more stable secondary event that keeps the receptor in the nucleus. For example, Thr³¹¹ phosphorylation may only prolong the retention of the liganded ER α in the nucleus, permitting more persistent binding of the receptor to nuclear protein or DNA components, which then obviates the requirement for phosphorylation.

From the data in Fig. 8, it appears that, in the presence of the p38 inhibitor, 17 β -estradiol promotes the accumulation of ER α in the cytoplasm. It is conceivable that, in the absence of ligands, equilibrium between basal nuclear import and export determined the approximately equal distribution of ER α in the nucleus and cytoplasm in endometrial cancer cells. The binding of ligands accelerates both import and export activities, but nuclear export is blocked by the ligand-induced Thr³¹¹ phosphorylation. It is the suppression of nuclear export induced by ligand-activated receptor phosphorylation, instead of acceleration of import, that shifts the steady state in favor of the nuclear localization of the ER α . According to our data, the pure antagonist ICI 182,780 may target ER α to the cytoplasm because of the inability of the pure estrogen antagonist to activate the p38 MAPK.

A role of the p38 MAPK signaling pathway in promoting nuclear export of MAPK-AP kinase 2 (10) and the nuclear factor of activated T cells (NFAT) (7) has been described. On the other hand, p38 binds p53 and induces phosphorylation of serine-15 within the N-terminal nuclear export signal of p53 that blocks nuclear export (3, 46). As shown in Fig. 8, there is a close sequence homology between the leucine-rich nuclear export signal in the amino terminus of p53 and the putative nuclear export signal in the ER α containing Thr³¹¹. Therefore, the effect of Thr³¹¹ phosphorylation on the nuclear export of the ER α is likely due to an alteration in the function of the putative nuclear export signal. However, our data do not rule out the possibility that the phosphorylation may affect nuclear export through nuclear export signal-independent mechanisms. This is particularly true because the role of the putative nuclear export signal in the intracellular traffic of ER α has not been characterized, and the existence of a functional nuclear export signal in steroid receptors is controversial (23, 41). In the study of progesterone receptor nuclear export, sequences with homology to leucine-rich nuclear export signals do not appear to be functional (41). In addition, studies by Liu et al.

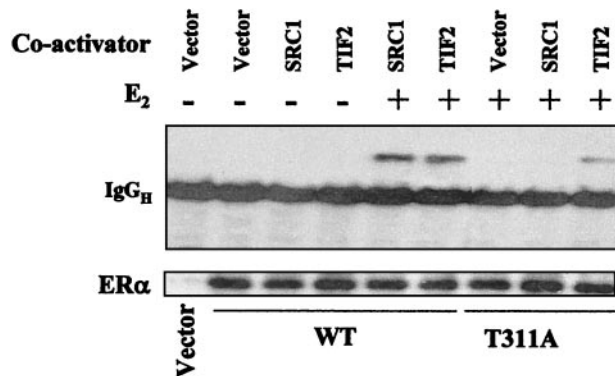


FIG. 10. Effect of T311A mutation on coactivator binding. Ishikawa cells were plated in 35-mm dishes at 3 \times 10⁵/dish and transfected with 0.5 μ g of pBind-SRC-1 or pBind-TIF2 and 0.5 μ g of wild-type (WT) or T311A mutant DNA. Cells were treated for 30 min with 17 β -estradiol (E₂) or not, and cellular extracts from duplicate dishes were pooled and subjected to immunoprecipitation with anti-ER α antibody, followed by immunoblotting with the anti-Gal4 DNA-binding domain (upper panel) or anti-ER α (lower panel) antibody.

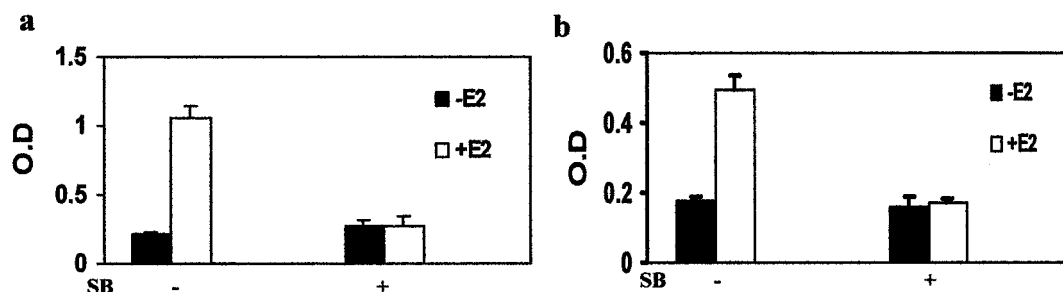


FIG. 11. p38 MAPK signaling pathway regulates the biological activity of ER α in endometrial adenocarcinoma cells. (a) Inhibition of estrogen induction of alkaline phosphatase by p38 inhibitor. ER α -positive Ishikawa cells were treated for 72 h with vehicle, 17 β -estradiol (E2), SB 203580 (SB), or a combination, and alkaline phosphatase activity was measured by colorimetric enzyme-linked immunosorbent assay. (b) Inhibition of estrogen stimulation of cell growth by the p38 inhibitor. ER α -positive Ishikawa cells were treated as for panel a for 4 days, and cell growth was measured by MTT assays. O.D., optical density.

(23) showed that glucocorticoid receptors did not utilize the exportin/Crm1 pathway for nuclear export.

Besides the effect on ER nuclear localization, our studies demonstrated that Thr³¹¹ phosphorylation of ER α regulates the receptor's interaction with steroid receptor coactivators. More interestingly, Thr³¹¹ phosphorylation preferentially affected the interaction with SRC-1, which has recently been shown to be expressed at a higher level in uterine than in breast cells and may contribute to the uterine-selective agonistic activity of tamoxifen (34). Our earlier studies demonstrated that MEKK1 increases the agonistic activity and decreases the antagonistic activity of 4-hydroxytamoxifen (20). Together with the stimulation of p38 activity by 4-hydroxytamoxifen shown in Fig. 1, our data support a role of the p38 MAPK signaling pathway in determining the tissue-selective agonistic activity of tamoxifen in endometrial cancer cells.

Our study is the first to link hormone, kinase pathway, specific receptor phosphorylation site, and receptor transcriptional activity with biological function in one comprehensive investigation. The activation of p38 by estrogens is fast and transient, obviously due to the nongenomic effect of the hormone. Taking all the data together, the study provides a good example of how the genomic and nongenomic effects of estrogens work together to fulfill biological functions. Consistent with our finding that the p38 pathway is positively involved in estrogen stimulation of endometrial cancer cell growth, studies by Razandi et al. (31) showed that estrogen activation of p38 is associated with protection of endothelial cells from hypoxia-induced apoptosis. However, Zhang et al. (45) reported that activation of p38 is coupled with ER-mediated apoptosis. The different data may be explained by the possibility that estrogen-induced p38 activity regulates estrogen action in different cells through distinct mechanisms. Consistent with this idea, studies by Razandi et al. (31) suggest that the protective effect of estrogen-activated p38 on vascular cells may be mediated through Hsp27 phosphorylation by MAPK-AP2, which is obviously different from the ER α phosphorylation by the p38 MAPK pathway proposed in our studies.

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