Palmitoylation Regulates 17β-Estradiol-Induced Estrogen Receptor-α Degradation and Transcriptional Activity

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The estrogen receptor-α (ERα) is a transcription factor that regulates gene expression through the binding to its cognate hormone 17β-estradiol (E2). ERα transcriptional activity is regulated by E2-evoked 26S proteasome-mediated ERα degradation and ERα serine (S) residue 118 phosphorylation. Furthermore, ERα mediates fast cell responses to E2 through the activation of signaling cascades such as the MAPK/ERK and phosphoinositide-3-kinase/v-akt murine thymoma viral oncogene homolog 1 pathways. These E2 rapid effects require a population of the ERα located at the cell plasma membrane through palmitoylation, a dynamic enzymatic modification mediated by palmitoyl-acyl-transferases. However, whether membrane-initiated and transcriptional ERα activities integrate in a unique picture or represent parallel pathways still remains to be firmly clarified. Hence, we evaluated here the impact of ERα palmitoylation on E2-induced ERα degradation and S118 phosphorylation. The lack of palmitoylation renders ERα more susceptible to E2-dependent degradation, blocks ERα S118 phosphorylation and prevents E2-induced ERα estrogen-responsive element-containing promoter occupancy. Consequently, ERα transcriptional activity is prevented and the receptor addressed to the nuclear matrix subnuclear compartment. These data uncover a circuitry in which receptor palmitoylation links E2-dependent ERα degradation, S118 phosphorylation, and transcriptional activity in a unique molecular mechanism. We propose that rapid E2-dependent signaling could be considered as a prerequisite for ERα transcriptional activity and suggest an integrated model of ERα intracellular signaling where E2-dependent early extranuclear effects control late receptor-dependent nuclear actions. **(*Molecular Endocrinology* 26: 762–774, 2012)**

NURSA Molecule Pages: Nuclear Receptors: ER-α; **Ligands:** 17β-estradiol.

The sex hormone 17β-estradiol (E2) is one of the pivotal regulators of female and male physiology because it controls the homeostasis of reproductive tissues and exerts a myriad of effects in nonreproductive organs. These pleiotropic hormone actions depend on E2 signaling that differentially directs proliferation, apoptosis, and differentiation of E2-responsive cells. These physiological functions of E2 occur because E2 engages the estrogen receptors (*i.e.* ERα and ERβ), which are nuclear receptors that work as ligand-activated transcription factors (1).

Several lines of evidence identify also a pool of the same nuclear ERα at the cell plasma membrane (1, 2). It is now clear that E2 rapidly activates many signal transduction cascades in the extranuclear compartment (*i.e.* ex-

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Abbreviations: Ai, AKT inhibitor; AKT, v-akt murine thymoma viral oncogene homolog 1; DTT, dithiothreitol; E2, 17β-estradiol; EGF, epidermal growth factor; ER, estrogen receptor; ERE, estrogen-responsive element; Ly, Ly 294002; PAT, palmitoyl-acyl-transferase; PD, PD 98059; PI3K, phosphoinositide-3-kinase; qPCR, quantitative PCR; wt, wild type.

tranuclear signaling) through the engagement of a plasma membrane-localized ERα (2). Palmitoylation, a dynamic posttranslational modification that increases protein hydrophobicity and membrane association of proteins (3), occurs on the ERα cysteine (C) residue 447 through the action of two palmitoyl-acyl-transferases (PAT) (4). The mutation of the C447 to A and the chemical inhibition of PAT activity with 2-bromo-hexadecanoic acid (2-Br) prevent ERα palmitoylation, plasma membrane localization, and E2-evoked extranuclear signaling (5–8). The use of both the palmitoylation-defective ERα C447A mutant, the PAT inhibitor, and exogenous ERα ligands demonstrated that cellular functions, such as the balance between proliferation/apoptosis or proliferation/differentiation, in E2-responsive cells depend on the activation of the plasma membrane-localized ERα (2, 9–11).

However, E2-induced cellular responses also include the regulation of ERα stability (12); the ERα half-life is 3–4 h in the absence of ligands, whereas E2 decreases it to about 2 h through the action of the 26S proteasome. This feature is directly correlated with ERα transcriptional activity. Upon E2 binding, ERα starts to activate transcription within 1 h through its translocation and direct physical association with estrogen-responsive elements (ERE) located in the promoter regions of E2 target genes (*e.g.* presenelin 2 and *pS2/TFF1*) (13, 14). The E2-ERα bound to the ERE further recruits transcriptional cofactors and the basal transcriptional machinery in an ordered manner, thus initiating mRNA synthesis. After productive gene transcription, activated ERα becomes polyubiquitinated, dissociates from ERE, and transiently accumulates in the nuclear matrix to be subsequently degraded by the action of the 26S proteasome (13–15). As a consequence, ERE-containing promoters are again available for a subsequent cycle of E2-ERα-dependent gene transcription (13, 14). In addition to receptor degradation, activation of E2-induced target gene transcription also requires ERα to become phosphorylated on the serine (S) residue 118. Although the role of ERα S118 phosphorylation in E2-induced receptor breakdown is not firmly established (12, 16, 17), it is clear that E2 evokes a rapid (20 min) and sustained (up to 24 h) ERα S118 phosphorylation (18). This posttranslational modification determines an increased association of the phosphorylated ERα with ERE-containing promoters (*e.g. pS2/TFF1*) (17) and an enhanced recruitment of transcriptional cofactors in the cell nucleus (19). In this way, E2-induced receptor degradation and S118 phosphorylation synchronize ERα transcriptional activity and coordinate the physiological responses to the hormone (13, 14).

Although several kinases rapidly activated by the E2-ERα complex have been implicated in receptor S118 phosphorylation (18, 20), the impact of ERα palmitoylation on these molecular circuitries is unknown. Thus, we sought to determine the contribution of ERα palmitoylation on the ability of E2 to control ERα degradation and S118 phosphorylation as well as ERα transcriptional activity. Our results indicate that ERα palmitoylation is required for fast E2 actions that regulate ERα transcriptional activity. Consequently, we suggest that rapid E2-dependent extranuclear signaling could be a prerequisite for ERα nuclear actions.

Results

Palmitoylation affects E2-induced ERα degradation

Treatment of many ERα-expressing cell lines, including ductal carcinoma cells (MCF-7), with the PAT inhibitor 2-bromo-hexadecanoic acid (2-Br) prevents ERα palmitoylation (Fig. 1A) and thus receptor plasma

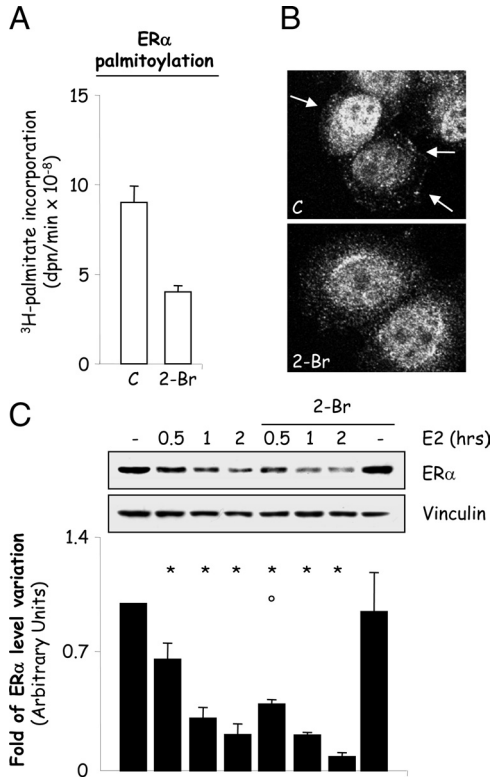

FIG. 1. Role of ERα palmitoylation on E2-induced receptor degradation in MCF-7 cells. Panels A and B, [^{3}H]Palmitate incorporation (A) and ERα immunofluorescence staining (B) in MCF-7 cells treated with the PAT inhibitor 2-Br (10 μM, 30 min); panel C, Western blot analysis of ERα cellular levels in MCF-7 cells treated with E2 (10 nM) at different time points in the presence of 30 min pretreatment with the PAT inhibitor 2-Br (10 μM) before E2 administration. Inhibitor alone was administrated for 2 h 30 min. Loading control was done by evaluating vinculin expression in the same filter. *, Significant differences with respect to the relative control sample; °, significant differences with respect to the corresponding E2 sample ($P < 0.05$). Representative blots are shown. *Arrows* indicate membrane ERα. C, Control.

membrane localization (Fig. 1B) (2, 6, 7). Therefore, to begin to unravel the potential influence of ERα palmitoylation on the E2-dependent regulation of receptor intracellular levels, E2-induced ERα degradation was analyzed in MCF-7 cells both in the presence and in the absence of the PAT inhibitor 2-Br. Cell treatment with E2 induced a time-dependent reduction (70%) in ERα cellular content within the first 2 h (Fig. 1C), whereas 24 h E2 treatment did not further enhance receptor elimination (21) (data not shown). A significant reduction (40%) in ERα cellular levels was already detected in MCF-7 cells after 30 min of E2 administration (Fig. 1C), whereas pretreatment of MCF-7 cells with 2-Br for 30 min further enhanced (70%) the E2 effect on ERα cellular levels (Fig. 1C). These data suggest that palmitoylation influences the E2-dependent regulation of ERα cellular levels in MCF-7 cells.

However, although 2-Br is a specific inhibitor for PAT (22), many signal transduction proteins are palmitoylated (3) and thus their depalmitoylation could contribute to ERα degradation. Therefore, to better understand the role of ERα palmitoylation in E2-induced receptor degradation, we produced cell lines stably expressing the nonpalmitoylable form of the ERα (*i.e.* ERα C447A) (5–7). For this purpose, HEK293 cells were chosen (23). To characterize this experimental model, we first determined the ability of E2 to induce cell proliferation in HEK293 cells stably expressing the wild type (wt) or the C447A mutated ERα. As expected (5–7), E2 treatment increased the cell number in the stable wt ERα HEK293 cells but not in the C447A mutant receptor-expressing cells (Fig. 2A). These data confirm that ERα palmitoylation is required for E2-induced proliferative signals (5–7) and further indicate that the effects of the mutation at ERα palmitoylation site in E2 signaling can be studied also on E2 signaling in stable HEK293 cells (23). Prompted by these results, we evaluated the ability of E2 to induce ERα degradation in stable expressing HEK293 cells. The dose-response curve revealed that a reduction in ERα cellular levels could be achieved in both wt as well as C447A mutated ERα when the cells were treated with 10 nM E2 (Fig. 2B). In HEK293 cells stably expressing wt ERα, E2 was capable of inducing a significant reduction in ERα cellular content within the first 4 h (Fig. 2C). Longer E2 treatment (8 h) did not further enhance receptor degradation (Fig. 2C). Notably, the difference in the time-dependent E2-mediated receptor degradation between MCF-7 cells and HEK293 stable cell lines could be ascribed to ERα overexpression in the latter cell line (data not shown). On the contrary, in HEK293 cells stably expressing the C447A mutant ERα, 2 h of E2 administration were sufficient to determine a significant reduction in ERα levels (Fig. 2C). To further demonstrate the impact of ERα palmitoylation on receptor degradation, we analyzed the time course of E2-dependent ERα breakdown in HEK293 stably expressing the wt ERα both in the presence and in the absence of the PAT inhibitor 2-Br. Figure 2D shows that under 2-Br pretreatment, E2 induced an higher reduction of ERα cellular levels than the one observed in the absence of the PAT inhibitor,

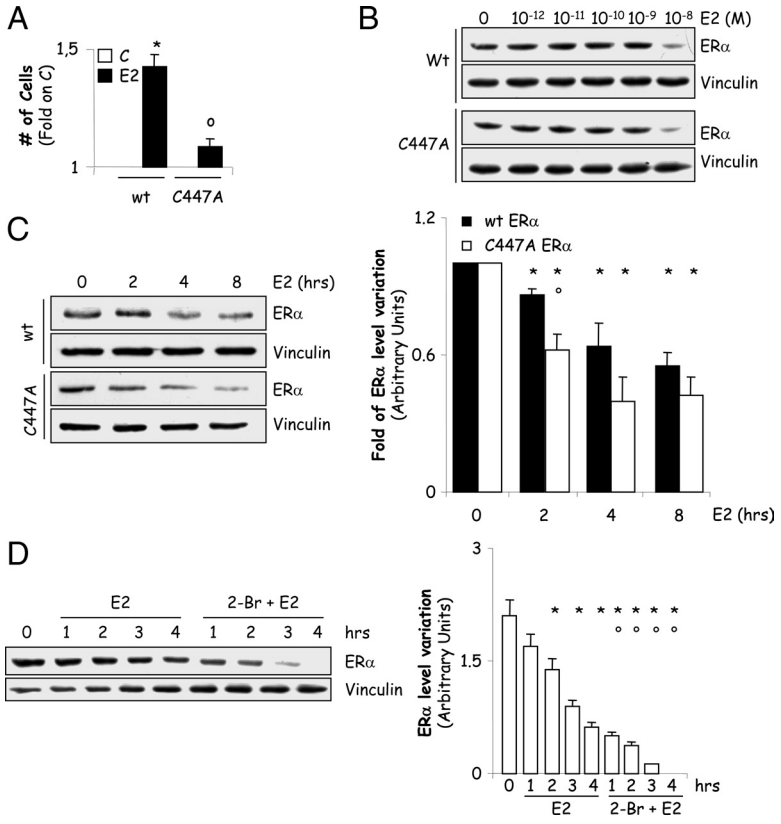

FIG. 2. Role of ERα palmitoylation on E2-induced receptor degradation in stable HEK293 cells. Panel A, Number of the HEK293 stable cells expressing the pcDNA flag-ERα (wt) and the pcDNA flag-ERα C447A (C447A) mutant was assayed either in the presence or in the absence of E2 (48 h); panel B, Western blot analysis of ERα cellular levels in pcDNA flag-ERα (wt) and the pcDNA flag-ERα C447A stably expressing clones treated with E2 at the indicated doses for 24 h; panel C, Western blot analysis of ERα cellular levels in HEK293 cells stably expressing the pcDNA flag-ERα (wt) (panels C and D) and the pcDNA flag-ERα C447A (C447A) (panel C). Where indicated, cells were treated for 30 min with the PAT inhibitor 2-Br (10 μM) before E2 administration. Loading control was done by evaluating vinculin expression in the same filter. *, Significant differences with respect to the relative control sample; °, significant differences with respect to the corresponding E2 sample ($P < 0.01$ for growth curves; $P < 0.05$ for Western blots). Representative blots are shown. C, Control.

whereas 2-Br alone did not modify the basal ERα cellular content of stable HEK293 cells (data not shown). These data demonstrate that inhibition of PAT activity as well as mutation of the ERα palmitoylation site determine a receptor pool that undergoes a faster elimination in response to E2 in endogenous as well as in stable expressing ERα cells, thus indicating that ERα palmitoylation protects the receptor from E2-dependent degradation.

Extranuclear E2 signaling influences ERα degradation

We and others have previously demonstrated that ERα palmitoylation is required for the activation of the rapid E2 extranuclear signaling (5–7). Accumulating evidence identifies the ERK/MAPK and phosphoinositide-3-kinase (PI3K)/v-akt murine thymoma viral oncogene homolog 1 (AKT) pathways as the principal transduction cascades activated by E2 in many different cell contexts (9). In line with these notions, time-course analysis revealed that E2 induces a rapid increase in ERK1/2 and AKT phosphorylation in the wt ERα-expressing HEK293 cells, whereas the hormone fails to trigger it in the C447A mutant receptor-expressing cells (Fig. 3A). 2-Br treatment also dampened E2-induced ERK1/2 and AKT phosphorylation in MCF-7 cells (Fig. 3B). The basal ERK1/2 activation was increased and the basal AKT phosphorylation was reduced when the cells were transfected with the C447A mutant receptor with respect to the wt ERα (Fig. 3A), possibly because of compensatory mechanisms due to the introduction of the exogenous mutated receptor. Thus, to exclude that the lack of the E2-dependent ERK1/2 and AKT activation observed in the presence of both the PAT inhibitor 2-Br and the ERα palmitoylation site mutant C447A could depend on a nonspecific impairment in the activation of the signaling kinases, the effect of epidermal growth factor (EGF) on the activation of ERK1/2 and AKT was assessed in MCF-7 cells and in HEK293 clones. Time-course analyses revealed that EGF treatment triggers the rapid phosphorylation of both ERK1/2 and AKT in MCF-7 cells (Fig. 3C) and in HEK293 cells stably expressing wt ERα (Fig. 3D), both in the presence and in the absence of the PAT inhibitor 2-Br, whereas the inhibitor alone did not modify the basal level of kinase phosphorylation. EGF treatment also induced ERK1/2 and AKT activation in HEK293 cells stably expressing C447A mutant ERα (Fig. 3E). Furthermore, ERα membrane localization was detected in HEK293 cells stably expressing wt ERα but not in C447A mutant-expressing cells (Fig. 3F). These data demonstrate that inhibition of ERα palmitoylation (*i.e.* lack of ERα membrane localization) affects only E2-depedent ERK1/2 and AKT activation, whereas the ability of cells to activate signaling cascades in response to EGF remains intact.

These results open the possibility that E2-dependent ERK/MAPK and

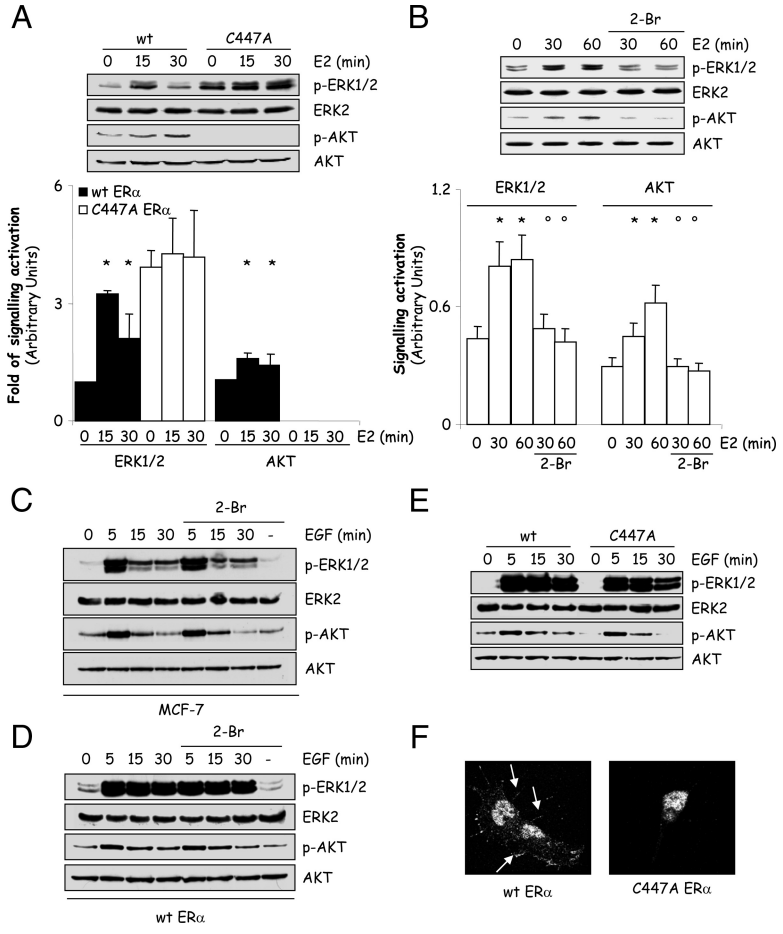

FIG. 3. Role of ERα palmitoylation on E2 and EGF signaling. Western blot analysis of ERK1/2 and AKT phosphorylation in HEK293 cells stably expressing the pcDNA flag-ERα (wt) (A, D, and E) and the pcDNA flag-ERα C447A (C447A) (A and E) and in MCF-7 cells (B and C) treated with E2 (10 nM) or EGF (1 μg/ml) at different time points. Where indicated, cells were treated for 30 min with the PAT inhibitor 2-Br (10 μM). Inhibitor alone was administrated for 1 h. The filter was reprobed with anti-ERK2 and anti-AKT antibodies. Loading control was done by evaluating vinculin expression in the same filter. *, Significant differences with respect to the relative control sample; °, significant differences with respect to the corresponding E2 sample ($P < 0.05$). Representative blots are shown. F, Confocal microcopy analysis of the HEK293 stable clones expressing pcDNA flag-ERα (wt) and the pcDNA flag-ERα C447A (C447A) mutant kept in growing conditions and stained for flag. *Arrows* indicate membrane ERα. p-, Phospho-.

PI3K/AKT pathways may be involved in the E2-dependent control of ERα levels. To test this hypothesis, the effect of E2 in reducing ERα cellular content was evaluated with a set of pharmacological inhibitors that block PI3K [Ly 294002 (Ly)] or AKT [AKT inhibitor (Ai)] as well as ERK1/2 kinase activity [PD 98059 (PD)]. In MCF-7 cells, dose-response curves showed that basal ERα cellular levels were unaffected and the E2-induced activation of both ERK/MAPK and PI3K/AKT pathways was prevented when the cells were treated with 10 μM PD (Fig. 4A) or with 1 μM Ly (Fig. 4B) and 5 μM Ai (data not shown), respectively. As shown in Fig. 4, C and D, incubation of MCF-7 cells with either Ly or Ai induced an increase in the time-dependent E2-evoked reduction of ERα cellular amount with a statistically significant maximum effect (70%) occurring after 30 min of E2 stimulation (Fig. 4F). On the contrary, PD administration did not change the ability of E2 to induce the reduction of ERα cellular levels (Fig. 4, E and F). These data demonstrate that inhibition of the PI3K/AKT pathway sensitizes ERα to E2-dependent removal, thus indicating that the E2-dependent PI3K axis activation defends the receptor from hormone-mediated degradation.

Palmitoylation controls ERα Ser118 phosphorylation

A role for the ERα serine (S) residue 118 in the modulation the E2-dependent receptor degradation has been previously reported (16). Because ERα palmitoylation is involved in the process of E2-evoked ERα elimination (Figs. 1 and 2), we sought to determine the impact of ERα palmitoylation on the E2-evoked S118 phosphorylation. MCF-7 cells were pretreated with the PAT inhibitor 2-Br and then time-course analysis of S118 phosphorylation was performed under E2 stimulation. However, because E2 determines a reduction in ERα cellular content both in the presence and in the absence of 2-Br (Fig. 1C and 5A), the receptor S118 phosphorylation was analyzed by quantifying the fraction of the modified ERα with respect to the total receptor quantity. Figure 5A shows that E2 induced an increase in the amount of the S118-phosphorylated pool of the ERα within the first 30 min of hormone administration. Although total receptor cellular levels were reduced by E2, the amount of the S118-phosphorylated ERα remained constant for the next 2 h of E2 administration (Fig. 5D). 2-Br reduced the amount of the S118-phosphorylated ERα in response to E2 without changing the basal ERα S118 phosphorylation levels (Fig. 5A). In HEK293 cells, E2 increased in a time-dependent manner the phosphorylation of the S118 residue in stable wt ERα-expressing cells but not in those stably expressing the C447A mutated receptor (Fig. 5B). These data indicate that ERα palmitoylation is required for the E2-dependent phosphorylation of the ERα on the S118 residue.

We next evaluated the impact of the E2 extranuclear signaling cascades on the ERα S118 phosphorylation status. In MCF-7 cells, Ai but not PD pretreatment resulted in a reduction in the amount of the S118-phosphorylated ERα in response to E2 with respect to cells that were treated with the hormone alone (Fig. 5, C and D) without affecting the basal ERα S118 phosphorylation levels (data not shown). Notably, the overall E2-dependent ERα S118 phosphorylation kinetic was not changed under either inhibitor treatments (Fig. 5, C and D). These data indicate that ERα palmitoylation and the E2 extranuclear-activated PI3K/AKT pathway control S118 phosphorylation.

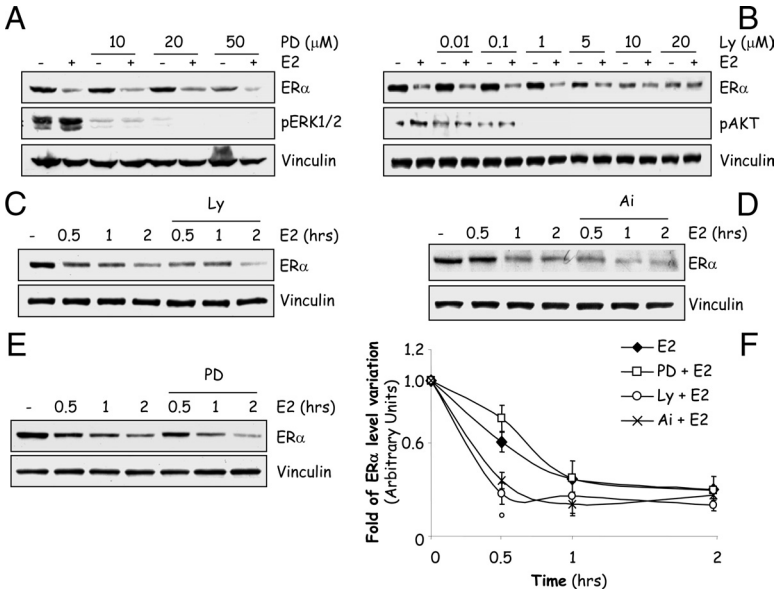

FIG. 4. Role of ERα palmitoylation-dependent extranuclear E2 signaling on receptor degradation: Western blot analysis of ERα cellular levels, ERK1/2, and AKT phosphorylation in MCF-7 cells treated with E2 (10 nM) for 2 h. A and B, Where indicated, cells were treated with different doses for 1 h either with the ERK1/2 inhibitor PD (A) or with the PI3K inhibitor Ly (B) before E2 administration; C–F, Western blot analysis of ERα cellular levels in MCF-7 cells treated with E2 (10 nM) at different time points. Where indicated, cells were treated for 1 h with the PI3K inhibitor Ly (1 μM) (C), with the Ai (5 μM) (D), or with the ERK1/2 inhibitor PD (10 μM) (E) before E2 administration. Loading control was done by evaluating vinculin expression in the same filter. *, Significant differences with respect to the relative control sample; °, significant differences with respect to the corresponding E2 sample ($P < 0.05$). Representative blots are shown. p, Phospho-.

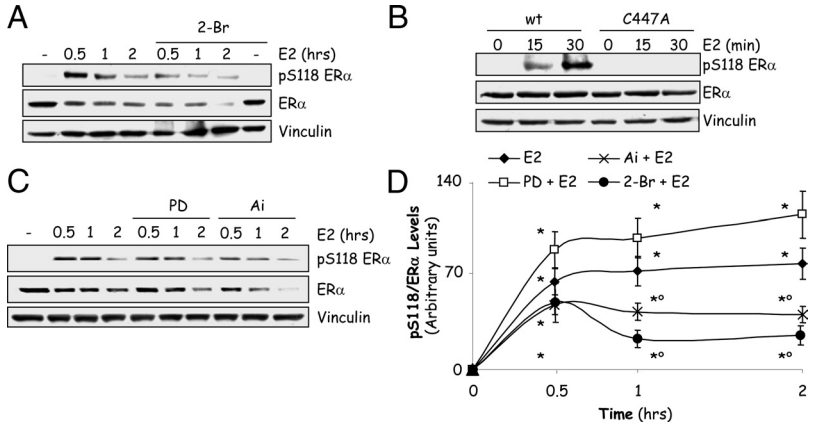

FIG. 5. Role of ERα palmitoylation on ERα S118 phosphorylation. A–C, Western blot analysis of ERα S118 phosphorylation (pS118) in MCF-7 cells (A and C) and in HEK293 cells stably expressing the pcDNA flag-ERα (wt) and the pcDNA flag-ERα C447A (C447A) (B) treated with E2 (10 nM) at different time points. Where indicated, cells were treated for 30 min with the PAT inhibitor 2-Br (10 μM) (A) or for 1 h either with the Ai (5 μM) (C) or with the ERK1/2 inhibitor PD (10 μM) (C) before E2 administration. The same filter was reprobed with anti-ERα antibody. Loading control was done by evaluating vinculin expression in the same filter. *, Significant differences with respect to the relative control sample; °, significant differences with respect to the corresponding E2 sample ($P < 0.05$). Representative blots are shown.

Palmitoylation is necessary for ERα transcriptional activity

It is well known that ERα S118 phosphorylation is required for full ERα transcription of the ERE-containing genes (18, 20). Because the lack of ERα palmitoylation prevents ERα S118 phosphorylation, we next studied its impact on E2-dependent ERα transcriptional activity. Real-time quantitative PCR (qPCR) analysis revealed that in MCF-7 cells, pretreatment with the PAT inhibitor 2-Br prevents the increase in the amount of the E2-responsive ERE-containing gene presenelin 2 (*pS2/TIFF*), cathepsin D, and progesterone receptor (*PR*) mRNA levels observed after 2 h of E2 administration (Fig. 6, A and B). The cell pretreatment with either the AKT inhibitor Ai or the ERK1/2 inhibitor PD also dampened the E2-induced increase in the *pS2* mRNA cellular content (Fig. 6A), thus sustaining the notion that rapid E2 extranuclear signaling contributes to ERα transcriptional activity (24). Incubation of MCF-7 cells with the inhibitors alone did not affect the total content of *pS2/TIFF*, cathepsin D, or *PR* mRNA.

These data also suggest that ERα palmitoylation rather than S118 phosphorylation is important for ERα-regulated ERE-containing gene expression. Therefore, to dissect the relative contribution of ERα palmitoylation and S118 phosphorylation on the E2-dependent ERα-mediated transcriptional activity, mutation of the S118 residue to A was first introduced both in the wt and in the non-palmitoylable C447A mutant ERα, and then the ability of the wt and mutant receptors to modulate E2-dependent ERE-based transcriptional activation was assayed in transiently transfected HeLa cells. As shown in Fig. 6C, 24 h of E2 treatment was able to trigger the activation of the artificial promoter containing three repetitions of the ERE sequence (*i.e.* 3×ERE-TATA, pERE) in the presence of both wt ERα and all the mutant receptors. Although the E2-ERα-mediated activation of the pERE promoter was significantly reduced (40%) in the presence of the S118A ERα mutant with respect to the wt receptor, when HeLa cells were transfected with either the C447A mutant ERα or with the S118A/C447A double-mutant receptor, the E2-induced pERE promoter activity was 70% and 50% less stimulated than the one in wt or S118A ERα-containing HeLa cells, respectively (Fig. 6C). Therefore, these data demonstrate a prevalent role of ERα palmitoylation with respect to ERα S118 phosphorylation for receptor transcriptional activity.

Palmitoylation controls E2-induced ERα promoter and nuclear matrix association

As a transcription factor, ERα cycles on and off its ERE-containing promoters with a frequency of about 30 min. E2 rapidly enhances the amount of the ERα associated with its responsive promoters and prolongs the frequency of the ERα-promoter association to about 60 min (13, 14). The data presented above suggest that ERα palmitoylation could be a prerequisite for E2-activated ERα ERE-containing gene expression (Fig. 6). Therefore, it is possible that lack of ERα palmitoylation may impair E2-activated ERα-promoter association. To test this hypothesis, we coupled chromatin immunoprecipitation assays with real-time qPCR analysis in MCF-7 cells to analyze the E2-dependent recruitment of ERα to the *pS2/TIFF* promoter region both in the presence and in the absence of the PAT inhibitor 2-Br. In MCF-7 cells, time-course analysis confirmed that E2-activated ERα cycles on and off to the *pS2/TIFF* promoter with a rapid receptor recruitment on the promoter occurring 30 min after hormone administration and a maximal level of promoter occupancy after 1 h of E2 administration (Supplemental Fig. 1, published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org) (13, 14, 25). Interestingly, 2-Br administration completely prevented the E2-induced ERα recruitment to *pS2/TIFF* promoter without affecting the basal ERα-promoter association (Fig. 7A). The specificity of the binding of ERα to

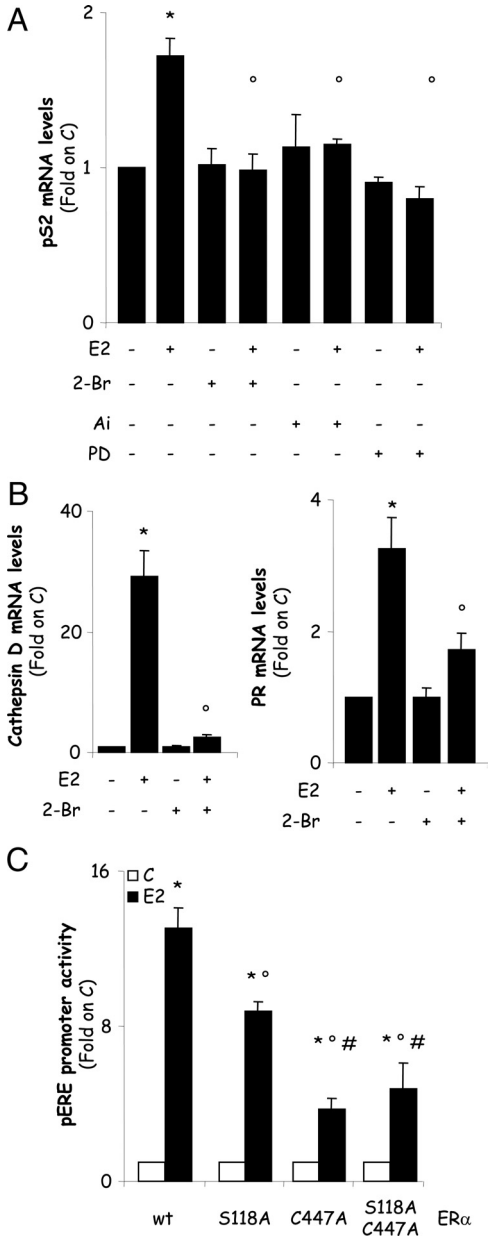

FIG. 6. Effect of extranuclear E2 signaling on ERα transcriptional activity. Panels A and B, RT-qPCR analysis of *pS2/TIFF* (panel A), cathepsin D (panel B, *left*), and progesterone receptor (*PR*) (panel B, *right*) mRNA expression normalized on the *GAPDH* mRNA expression in MCF-7 cells treated with E2 (10 nM) for 2 h. Where indicated, cells were treated for 30 min with the PAT inhibitor 2-Br (10 μM) or for 1 h either with the Ai (5 μM) or with the ERK1/2 inhibitor PD (10 μM) before E2 administration. *, Significant differences with respect to the relative C sample ($P < 0.01$). ° indicates significant differences with respect to the E2 sample ($P < 0.01$). Panel C, Luciferase assay detection on HeLa cells transiently cotransfected with the reporter plasmid 3×ERE-TATA and with the pcDNA flag-ERα (wt), pcDNA flag-ERα S118A (S118A), pcDNA flag-ERα C447A (C447A), or the pcDNA flag-ERα S118A C447A (S118A C447A) expression vectors and then treated 24 h with E2 (10 nM). *, Significant differences with respect to the relative C sample ($P < 0.01$); °, significant differences with respect to the wt E2 sample ($P < 0.01$); #, significant differences with respect to the S118A E2 sample ($P < 0.01$). C, Control samples.

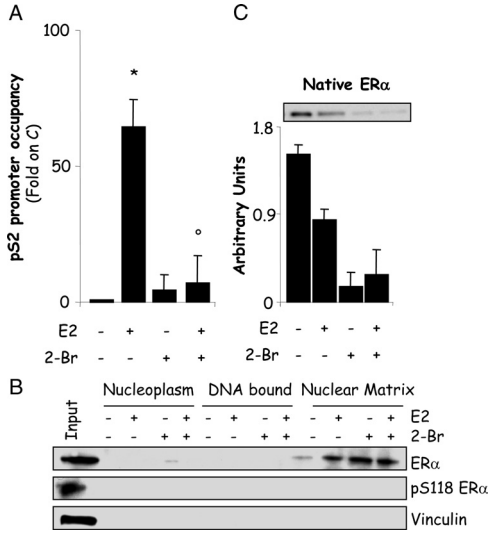

FIG. 7. Role of ERα palmitoylation on E2-induced receptor intranuclear dynamic. Panel A, Chromatin immunoprecipitation analysis of ERα *pS2/TIFF* promoter occupancy normalized on input DNA in MCF-7 cells treated with E2 (10 nM) for 1 h. *, Significant differences with respect to the C sample ($P < 0.01$); °, significant differences with respect to the E2 samples ($P < 0.01$). Panel B, Western blot analysis of ERα, ERα S118 phosphorylation (pS118), and vinculin in subnuclear fractions in MCF-7 cells treated with E2 (10 nM) for 2 h. Where indicated, cells were treated for 30 min with the PAT inhibitor 2-Br (10 μM). Panel C, Influence of 2-Br on basal rates of ERα synthesis. To assess ERα synthesis, cells were incubated with 10 nM [^{35}S]methionine in the absence or presence of 10 μM 2-Br and than treated with E2 (10 nM) for 2 h. Immunoprecipitated ERα from cell extracts were then submitted to SDS-PAGE and revealed by autofluorography. The quantitative data gave the level of ^{35}S-labeled ERα measured by scintillation counting. Blots are representative of two independent experiments, which gave similar results. C, Control samples.

the *pS2/TIFF* promoter was determined by using a primer set 1 kb upstream of the ERE in *pS2/TIFF*, which served as a negative control (data not shown).

After productive gene transcription, E2-ERα complexes are addressed to the nuclear matrix and are next degraded by the 26S proteasome (13–15). Because the lack of ERα palmitoylation fastens E2-evoked receptor degradation (Figs. 1 and 2) and prevents ERE-containing gene transcription (Fig. 6), we next evaluated the role of palmitoylation in E2-dependent ERα nuclear matrix association. As expected (13), fractionation analysis revealed that 2 h of E2 administration increased the amount of the receptor associated in the nuclear matrix compartment. Surprisingly, incubation of MCF-7 cells with the PAT inhibitor 2-Br determined a constitutive and E2-insensitve increase in the basal association of the ERα with nuclear matrix (Fig. 7B, *upper blot*). We then asked whether the receptor in the nuclear matrix subnuclear compartment was phosphorylated in the S118 residue. As shown in Fig. 7B (*middle panel*), the ERα associated with nuclear matrix was not S118 phosphorylated. The pres-

ence of vinculin in the cellular fractionations confirms that different nuclear fractions were not contaminated by cytosolic proteins and that redistribution of protein to the nuclear matrix was not an E2-regulated general event (Fig. 7B, *lower panel*). Altogether, our data demonstrate that ERα palmitoylation, and thus, E2 extranuclear signaling controls both the rapid E2-triggered recruitment of the ERα to E2-responsive ERE-containing promoters and intranuclear dynamics.

The fact that the treatment of MCF-7 cells with the PAT inhibitor 2-Br fastens E2-induced receptor degradation but constitutively addresses ERα to the nuclear matrix for subsequent proteolytic destruction suggests a potential impact of palmitoylation in ERα maturation process. Therefore, we finally studied the role of PAT inhibition on native ERα through [^{35}S]methionine labeling (12) to understand how 2-Br treatment constitutively drives a large amount of ERα to the nuclear matrix subnuclear compartment even in the absence of E2. As expected (12), 2 h E2 treatment reduced the levels of neosynthesized (*i.e.* native) ERα (Fig. 7C). On the contrary, irrespectively of E2 treatment, 2-Br completely eliminated the detection of any native receptor (Fig. 7C).

Discussion

Many effects of the sex steroid E2 have been increasingly documented and ascribed to membrane-localized ER (1, 2, 20). Diverse experimental approaches have shown that the same nuclear ERα associates also with the plasma membrane of normal and transformed cell lines including ductal carcinoma cells (*i.e.* MCF-7) (26) as well as many other tissues (9). The mechanism that triggers ERα plasma membrane localization and trafficking has been disputed, but it is now clear that the dynamic posttranslational modification of the ERα with palmitic acid (*i.e.* palmitoylation) addresses ERα to the cell plasma membrane (2, 9). Structure/function studies revealed that ERα is palmitoylated on the cysteine residue 447 (C447) by the action of two PAT and that the PAT-dependent enzymatic palmitoylation is required for ERα to associate with caveolin-1 and to mediate E2 extranuclear signaling (Figs. 1B and 3, A, B, and F) (4, 6, 7). Our research group has also indicated that E2 binding determines ERα depalmitoylation and dissociation from caveolin-1, a series of mechanistic events that facilitate receptor movements within membrane subdomains (6). As a consequence, E2 activation of the extranuclear signaling kinase cascades (*e.g.* ERK/MAPK and PI3K/AKT pathways) occurs and regulates several different physiological processes (*i.e.* proliferation, apoptosis, and differentiation) (9).

Here, we demonstrate additional functions of ERα palmitoylation by showing that this receptor posttranslational modification is involved in the regulation of ERα stability. In particular, the use of the PAT inhibitor 2-Br in breast cancer cells (*i.e.* MCF-7) and the stable insertion of the nonpalmitoylable ERα mutant C447A in the ERα-devoid HEK293 cells have allowed to discover a previously unrecognized pathway in which ERα palmitoylation is the upstream structural determinant that guarantees the physiological balance of the ERα protein levels (Fig. 8). Steady-state ERα cellular content is under the control of the 26S proteasome activity, which affects both the unliganded and the E2-activated ERα (12). It has been shown that both the pool of the neosynthesized receptor and the mature ERα fraction can be targeted for proteolytic destruction (12). Transport to the nuclear matrix sub-nuclear compartment for both the apo-receptor and for the E2-bound ERα appears to be a required step for subsequent receptor 26S proteasome degradation (13, 15). We found that, in the absence of E2, lack of ERα palmitoylation does not affect total ERα protein content, whereas, in the presence of E2, it causes faster receptor degradation (Figs. 1C and 5A). In parallel, irrespective of E2 treatment, inhibition of ERα palmitoylation constitutively addresses ERα to the nuclear matrix and induces the basal degradation of the neo-synthesized ERα (Fig. 7C and Supplemental Fig. 2). Because we also observed that inhibition of PAT activity does not change basal and E2-regulated ERα mRNA content (data not shown), we conclude that in the absence of E2, the native ERα pool requires palmitoylation for stabilization. Our data additionally indicate that inhibition of PAT activity prevents protein ERα neosynthesis, thus further suggesting that palmitoylation is required for receptor maturation of native ERα. In support of this, previous work had already demonstrated that any other treatment that interferes with ERα maturation or nuclear transport induces degradation of the neosynthesized receptor (27, 28). In parallel, in the presence of E2, the reduction in ERα palmitoylation (6) determines a receptor that undergoes faster proteolytic breakdown (Supplemental Fig. 2); thus, palmitoylation controls overall ERα turnover.

Here, we present the first evidence that E2-induced membrane ERα-dependent extranuclear signaling modulates ERα degradation. Evaluation of the potential implication of membrane E2-binding sites on intracellular ERα level regulation has been previously performed by using E2-BSA conjugates, which engage the membrane-localized ERα (28, 29). Although the fact that 3 h of E2-BSA MCF-7 cell incubation did not cause any lack in cellular E2-binding capacity suggested that membrane localization of ERα would not be implicated in E2-induced ERα

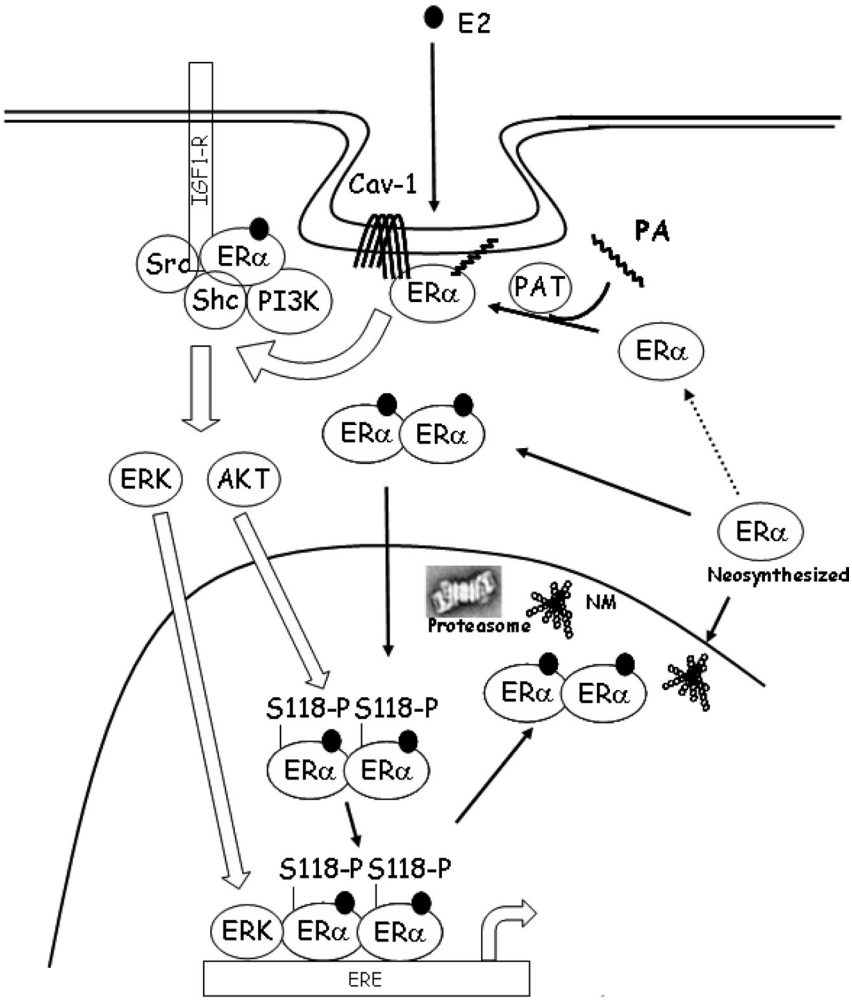

FIG. 8. An integrated model of the role of ERα palmitoylation on E2-induced receptor degradation. A, Neosynthesized ERα requires palmitoylation for plasma membrane association. E2 triggers rapid receptor depalmitoylation and activation of ERα-dependent rapid signaling that differentially affects E2-dependent S118 phosphorylation, receptor/promoter occupancy, and gene transcription. After productive mRNA synthesis, activated ERα is transiently addressed to the nuclear matrix, dephosphorylated, and then degraded by the 26S proteasome. For details and references, please see the text. *Solid lines* refer to observed evidence, whereas *dashed lines* are speculative conclusions. Cav-1, Caveolin-1; NM, nuclear matrix; PA, palmitic acid.

down-regulation (28, 29), the present data indicate that the use of reagents that impede ERα membrane localization (*i.e.* PAT inhibitor and C447A mutant ERα) (6, 7) influences E2-induced ERα down-regulation. These discrepancies can be reconciled by considering that the E2-binding capacity does not necessarily correlate to the total ERα content as assessed by Western blotting (30). Moreover, our time-course analysis in MCF-7 cells demonstrates that the impact of ERα membrane localization (6, 7) on the E2-modulated control of receptor intracellular content occurs rapidly (*i.e.* 30 min) and is not significantly apparent after 2 h of E2 administration (Figs. 1C and 4F).

Accordingly, we also show that the rapid E2-dependent activation of the PI3K/AKT pathway but not of the ERK/MAPK pathway regulates ERα cellular levels. Indeed, the effect of the lack of ERα palmitoylation on E2-evoked ERα degradation is mimicked by PI3K/AKT pathway inhibition and unaffected by ERK1/2 inhibitor. Signaling modulation of the ERα proteasomal-dependent pathway has not been analyzed in details, and only a little and divergent information is available about the identity of the E2-induced phosphorylation cascade that modulates ERα degradation (21, 31, 32). Pharmacological inhibition of the PI3K activity (*i.e.* Ly treatment, 20 μM) has been shown to impede E2-dependent receptor proteolytic destruction (21) (Fig. 4B). However, we further noticed a dose-dependent reduction both in the total amount of the cellular ERα content and in the ability of the cells to respond to E2 (Fig. 4B). Nevertheless, PI3K and AKT inhibitor doses, which prevent E2-induced AKT phosphorylation and do not affect total ERα content (Fig. 4B and data not shown), mimic the effect of the lack of ERα palmitoylation on the E2-dependent modulation of ERα degradation (Fig. 4, C and D). Thus, the PI3K/AKT pathway is involved in the regulation of the ERα cellular levels. Regarding the role of ERK/MAPK pathway, whereas some investigators showed that MAPK activation facilitates ERα degradation in breast cancer cells (21, 31), other evidence supports our observations (Fig. 4E) that this pathway does not affect E2-induced ERα breakdown (32). The mechanistic reasons underlying the different ability of the E2-induced ERK/MAPK and PI3K/AKT pathways in regulating ERα degradation became apparent with the analysis of the ERα phosphorylation status on the serine residue 118; the lack of the E2-dependent AKT activation prevents ERα S118 phosphorylation, whereas the blockade of the E2-induced ERK/MAPK pathway does not affect the receptor phosphorylation on this S residue (Fig. 5). This evidence is in line with the concept that, although the ERα S118 residue can be phosphorylated by ERK/MAPK *in vitro*, the E2-induced ERα S118 phosphorylation is ERK/MAPK independent in breast cancer cells (18, 20), thus supporting the notion that other pathways including the

PI3K/AKT pathway control the E2-dependent regulation of S118 phosphorylation (23, 33, 34).

Reduction in S118 phosphorylation correlates with a faster E2-induced receptor elimination and with an impairment in ERα-mediated gene transcription. We found that ERα palmitoylation controls all these processes. Although the role of ERα S118 phosphorylation in regulating E2-induced receptor breakdown is not clear, previous works suggested that S118 phosphorylation could be essential for ERα entry into the ubiquitin-proteasome pathway (16). S118 phosphorylation is required for full ERα transcriptional activity (18, 20) because S118-phosphorylated ERα translocates to E2-responsive promoters (17) and recruits transcriptional cofactors (19). Our data are in line with all these assumptions because E2 maintains both a constant level of S118 phosphorylation, whereas it triggers a significant reduction in total ERα content and a parallel increase in ERα gene transcription (Figs. 1, 5, and 6). In addition, we found that the receptor pool that is addressed to the nuclear matrix for subsequent degradation is non-S118 phosphorylated (Fig. 7B). Thus, a situation can be envisioned in which after E2-induced depalmitoylation (6), ERα becomes phosphorylated on the S118 through the activation of the PI3K/AKT pathway. S118-phosphorylated ERα is next necessary for ERα-promoter (17) and cofactor-promoter recruitment (19). After gene transcription activation, ERα loses S118 phosphorylation and is addressed to the nuclear matrix for subsequent proteolytic destruction (Supplemental Fig. 3).

One of the main findings in this study is the impact of ERα palmitoylation and of the E2 extranuclear signaling on the ERE-containing gene transcription (*e.g. pS2/TIFF*, cathepsin D, and progesterone receptor, *PR*). The ability of membrane-localized ERα and of the relatively rapid E2-induced extranuclear signaling (*e.g.* PI3K/AKT and the ERK/MAPK pathways) to modulate the nuclear ERα functions has been reported (24, 35, 36). Accordingly, the blockade of ERα palmitoylation leading to a fast reduction in the amount of the receptor recruited to the *pS2/TIFF* promoter and to a consequent rapid (2 h) decrease in the E2-induced accumulation of the *pS2/TIFF* mRNA unveils that the role of ERα palmitoylation in ERα transcriptional activity is to regulate the ERα-ERE-containing promoter interaction. In this respect, our data further indicate a selective role for the E2-activated PI3K/AKT and ERK/MAPK pathways in this process. Although the inhibition of ERK activity impedes E2 *pS2/TIFF* gene transcription most likely because it prevents ERK2 recruitment to the *pS2/TIFF* promoter together with ERα (36), the impairment of the PI3K/AKT pathway strongly reduces the E2-dependent ERα S118 phosphorylation, thus hampering the S118-phosphorylated receptor recruitment to the *pS2/TIFF* chromatin (17). The critical role for ERα palmitoylation in the regulation of E2-induced ERE-containing gene transcription is further demonstrated by the discovery that the mutation of the ERα palmitoylation site rather than the mutation of the major ERα phosphorylation site (*i.e.* S118) (18, 20) determines a drastic reduction in ERα transcriptional activity (Fig. 4B). The apparent paradox for which the PAT inhibitor completely blocks E2-induced *pS2/TIFF* and cathepsin D mRNA accumulation in MCF-7 cells and the C447A mutant ERα strongly reduces the E2-triggered activity of the 3×ERE-TATA promoter in transfected HeLa cells can be reconciled by considering the different duration of E2 cell stimulation (*i.e.* 2 h for *pS2/TIFF* and cathepisn D gene transcription and 24 h for ERα mutant-dependent 3×ERE-TATA promoter studies). Therefore, ERα palmitoylation appears to be required for the initial events of E2-induced activation of ERα transcriptional activity (*e.g.* cofactors and ERα promoter recruitment). This evidence together with the notion that ERα palmitoylation is necessary also for non-ERE-containing gene transcription (*e.g.* cyclin D1) (6) demonstrates that E2-induced extranuclear signaling cross talks with nuclear ERα transcriptional activity to preserve the pleiotropic E2 effects into the cell.

In conclusion, the discoveries reported here reveal a circuitry in which receptor palmitoylation links E2-dependent ERα degradation, S118 phosphorylation, and transcriptional activity in a unique molecular mechanism (Fig. 8). Although current data indicate a complicated pattern of ERα modifications in *in vitro* models and human tissues (20), evidence is accumulating for the presence of ERα signaling and extranuclear plasma membrane localization in breast tumor specimens (9, 20, 37, 38). Thus, understanding whether these molecular circuitries are conserved *in vivo* and possibly deregulated in cancer (*e.g.* breast cancer) will be required for pharmacological targeting of these pathways.

However, we propose that rapid E2-dependent signaling could be a prerequisite for ERα transcriptional activity and suggest an integrated model of ERα intracellular signaling where E2 early membrane-dependent extranuclear effects are in control of late receptor-dependent nuclear actions (Fig. 8).

Materials and Methods

Cell culture and reagents

Human ductal carcinoma cells (MCF-7) and human cervix carcinoma cells (HeLa) as well as stably transfected human embryonic kidney 293 cells (HEK293) were grown as previously

described (23, 39). E2, gentamicin, penicillin and other antibiotics, DMEM (with and without phenol red), charcoal-stripped fetal calf serum, and the PAT inhibitor 2-bromohexadecanoic acid (2-bromo-palmitate; 2-Br) (IC_{50} of ~4 μM) (22) were purchased from Sigma-Aldrich (St. Louis, MO). 9,10-[^{3}H]Palmitic acid (specific activity 57 Ci/mmol) was purchased from PerkinElmer Life and Analytical Sciences (Boston, MA). L-[^{35}S]Methionine (>100 mCi/mmol) was purchased from Amersham Biosciences (Buckinghamshire, UK). Lipofectamine reagent was obtained from Invitrogen (Carlsbad, CA). The luciferase kit was obtained from Promega (Madison, WI). Bradford protein assay was obtained from Bio-Rad (Hercules, CA). Specific antibodies against ERα (D12 mouse, MC-20 rabbit, and HC-20 rabbit), phospho-ERK1/2, anti-ERK2 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA); antibodies against flag epitope (M2) and vinculin were purchased from Sigma-Aldrich (St. Louis, MO). All other antibodies were purchased from Cell Signaling Technology Inc. (Beverly, MA). CDP-Star, chemiluminescence reagent for Western blot, was obtained from PerkinElmer. The MAPK cascade inhibitor PD, the PI3K inhibitor Ly, and the Ai were obtained from Calbiochem (San Diego, CA). All the other products were from Sigma-Aldrich. Analytical- or reagent-grade products, without further purification, were used.

Plasmids

The reporter plasmid 3×ERE-TATA and the pcDNA flag 3.1 C as well as the pcDNA flag-ERα were previously described (23, 39). The pcDNA flag-ERα C447A was obtained by subcloning the ERα C447A open reading frame from the pSG5-HE0 C447A (6) into the pcDNA flag 3.1 C. The pcDNA flag-ERα S118A and the pcDNA flag-ERα S118A C447A were obtained by site-directed mutagenesis of the relative templates by using the QuikChange kit (Stratagene, La Jolla, CA) and the following oligonucleotide: 5′-CACCCGCCGCCGCAGCTG*G*CGCCTT-TCCTGCAGCCCCAC-3′ (*bold underlined* nucleotides differ from the ERα open reading frame). Plasmids were than sequenced to verify the introduction of the desired mutations.

Cellular and biochemical assays

Before any cellular and biochemical assay, cells were grown in 1% charcoal-stripped fetal calf serum medium for 24 h and then stimulated with E2 at the indicated time points; where indicated, inhibitors were added 1 h (PD, Ly, and Ai) or 30 min (2-Br) before E2 administration. Cells were lysed in YY buffer [50 mM HEPES (pH 7.5), 10% glycerol, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA] plus protease and phosphatase inhibitors. Proteins were transferred onto a nitrocellulose membrane (GE Healthcare, Fairfield, CT). After blocking [1 h at room temperature in 5% nonfat dry milk Tris-buffered saline with Tween 20 (TBS-T) solution or in 5% BSA dissolved in TBS-T solution], filters were incubated with the appropriate primary antibody overnight at 4 C, followed by three washes of 10 min each in TBS-T and then incubated with the antimouse or antirabbit horseradish peroxidase-conjugated secondary antibody diluted in TBS-T for 60 min at room temperature. After incubation with the secondary antibody, the filter was washed three times in TBS-T (5 min each), and the bound secondary antibody was revealed using the enhanced chemiluminescence method (GE Healthcare). Growth curves were performed as previously reported (23, 39).

Stable transfection

Stably expressing wt ERα HEK293 cells were previously described (23). HEK293 cells stably expressing ERα C447A were generated by using G418 (400 μg/ml), as previously reported (23). For the ERα C447A, three individual clones were selected on the basis of the wt ERα expression levels and growth rate (Supplemental Fig. 4). Experiments are shown for one (clone 25) of each HEK293 clone.

Transient transfection and luciferase assay

HeLa cells were grown to 70% confluence and then transfected using Lipofectamine reagent according to the manufacturer's instructions. Three hours after transfection, the medium was changed, and 24 h later, the cells were serum starved for 24 h and then stimulated with E2 for 24 h. The cell lysis procedure as well as the subsequent measurement of luciferase gene expression was performed using the luciferase kit according to the manufacturer's instructions with a PerkinElmer Life and Analytical Sciences (Bad Wildbad, Germany) luminometer as previously described (23).

Subcellular protein extraction

The cellular components were sequentially extracted using a widely adopted biochemical fractionation and sequential extraction procedure (13, 14, 40) as cytoplasm (with Nonidet P-40 buffer) (not included in the blots) (13), nucleoplasm (with Triton X-100), DNA bound (with deoxyribonuclease treatment), and nuclear matrix protein fractions. Purity of the nuclear fraction was confirmed by the use of both nuclear (ERα) and cytoplasmic (vinculin) protein markers.

RNA isolation and qPCR analysis

The sequences for gene-specific forward and reverse primers were designed using the OligoPerfect Designer software program (Invitrogen). The following primers were used: for human *pS2*, 5′-CATCGACGTCCCTCCAGAAGAG-3′ (forward) and 5′-CTCTGGGACTAATCACCGTGCTG-3′ (reverse); for human cathepsin D, 5′-GTACATGATCCCCTGTGAGAAGGT-3′ (forward) and 5′-GGGACAGCTTGTAGCCTTTGC-3′ (reverse); for human progesterone, receptor (*PR*) 5′-AAATCATT-GCCAGGTTTTCG-3′ (forward) and 5′-TGCCACATGGTA-AGGCATAA-3′ (reverse); and for human *GAPDH*, 5′-CGAGATCCCTCCAAAATCAA-3′ (forward) and 5′-TGTGGTCATGAGTCCTTCCA-3′ (reverse). Total RNA was extracted from cells using TRIzol Reagent (Invitrogen) according to the manufacturer's instructions. To determine *pS2* gene expression levels, cDNA synthesis and qPCR were performed using the GoTaq two-step RT-qPCR system (Promega) in a ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Each sample was tested in triplicate and the experiment repeated twice. All primers used were optimized for real-time amplification in a standard curve amplification (>98% for each pair of primers) and verifying the production of a single amplicon in a melting curve assay. Results were normalized to the expression of *GAPDH* mRNA. The relative level for each gene

reported in arbitrary units, was calculated using the $2^{-\Delta\Delta Ct}$ method.

Chromatin immunoprecipitation

Chromatin immunoprecipitation assays were performed essentially as previously described (41). After starvation and ligand treatment, MCF-7 cells were cross-linked using 1% formaldehyde at 37 C for 10 min. Glycine (0.125 M) was then added for 5 min at room temperature. Cells were next washed twice with PBS and harvested in ice-cold PBS. Cell pellets were first resuspended in nuclei isolation buffer [50 mM Tris (pH 8.0), 60 mM KCl, 0.5% Nonidet P-40, protease inhibitor, and 10 mM dithiothreitol (DTT)], centrifuged at 3000 × *g* for 5 min, and resuspended in 200 μl lysis buffer [0.5% sodium dodecyl sulfate (SDS), 10 mM EDTA, 0.5 mM EGTA, 50 mM Tris (pH 8.0), protease inhibitor, and 10 mM DTT]. Nuclei were sonicated (Fisher Scientific; Sonic Dismembrator model 100) three times at 80% maximum power for 5 sec, and the sonicate was centrifuged at 14,000 × *g* for 10 min. The supernatant was diluted up to 500 μl with dilution buffer [1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris (pH 8), protease inhibitor, and 10 mM DTT] and 1/10 was taken aside as input for qPCR analysis. The samples were than precleared with 50 μl protein G beads for 1 h rotating at 4 C. After protein G beads removal, lysates were incubated at 4 C rotating overnight with 5 μg anti-ERα antibody (MC-20; Santa Cruz Biotechnology) and then pulled down at 4 C for 1 h with 50 μl protein G beads. After brief centrifugation, precipitates were sequentially washed twice with 1 ml washing buffer [0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.0), and 150 mM NaCl], once with 1 ml washing buffer II [1% Nonidet P-40, 1 mM EDTA, 20 mM Tris-HCl (pH 8.0), 250 mM LiCl], and twice with 1 ml of Tris EDTA [1 mM EDTA and 10 mM Tris-HCl (pH 8.0)]. Chromatin complexes were centrifuged and then eluted by incubating at room temperature for 30 min with the beads with 50 μl 1% SDS and 0.1 M $NaHCO_3$. After centrifugation, this step was repeated for 10 min at room temperature. The cross-linking was reversed by incubating at 65 C overnight with 200 mM NaCl and 200 mg/ml proteinase K (Invitroge). Ribonuclease A (1 mg/ml) was also added for 30 min at 37 C. DNA was next purified with QIAquick columns (QIAGEN). Real-time qPCR analysis was done with primers for the *pS2* gene (*pS2* promoter primers) −463 to −159 or 1 kb upstream of this element to serve as a negative control (*pS2* upstream primers) −1953 to −1651. The sequences of the *pS2* promoter primers were 5′-GAATTAGCTTAGGCCTAGACGGAATG-3′ and 5′-AGGATTTGCTGATAGACAGAGACGAC-3′. For the *pS2* upstream primers, the sequences were 5′-CTCCCTCTTCAGGCCTCTCT-3′ and 5′-TTCCCTGGTGTTGTCAAGTG-3′ (42).

Cell labeling with [^{3}H]palmitate or L-[^{35}S]methionine and immunoprecipitation

MCF-7 cells were incubated with 0.5 mCi/ml [^{3}H]palmitate at 37 C for 4 h. Where indicated, cells were treated with 2-Br (10 μM) for 30 min in the presence of [^{3}H]palmitate. The analysis of the [^{3}H]palmitate incorporation in the ERα was than performed as described elsewhere (5, 6, 43). For pulse-chase experiments, MCF-7 cells were plated in 60-cm^2 petri dishes (4.5×10^5 cells per dish). After 4 d of culture, they were fed with MEM devoid of L-methionine (GIBCO) and kept in that medium for 2 h before exposure to 10 nM [^{35}S]methionine under appropriate conditions for assessing the influence of 2-Br and E2 upon ERα synthesis (12). The analysis of the [^{35}S]methionine incorporation in the ERα was then performed as described previously (12).

Confocal microscopy analysis

MCF-7 cells were stained with rabbit anti-ERα antibody (Santa Cruz Biotechnology; HC-20, 1:30), and HEK293 cells were stained with anti-flag (1:10000) antibody as previously described (23). Briefly, cells were grown on 30-mm glass gelatin-coated coverslips and then fixed with paraformaldehyde (4%) for 1 h and permeabilized with Triton X-100 (0.1%) for 5 min. After the permeabilization process, cells were incubated with BSA (2%) for 1 h and then stained with the anti-ERα or anti-flag antibody for 1 h at room temperature. After that, cells were rinsed three times in PBS for 5 min and incubated with Alexa Fluor 546 donkey antirabbit secondary antibody (1:2000) or Alexa Fluor 488 donkey antimouse secondary antibody (Invitrogen) (1:400), respectively. After extensive washes, coverslips were mounted, and confocal analysis was performed using LCS (Leica Software, Heidelberg, Germany).

Statistical analysis

A statistical analysis was performed using the ANOVA test with the InStat version 3 software system (GraphPad Software Inc., San Diego, CA). Densitometric analyses were performed using the freeware software Image J by quantifying the band intensity of the protein of interest respect to the relative loading control band intensity. In all analyses, P values < 0.01 were considered significant, but for densitometric analyses, P was <0.05. Data are means of three independent experiments ± SD.

Acknowledgments

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