p38γ Mitogen-activated Protein Kinase (MAPK) Confers Breast Cancer Hormone Sensitivity by Switching Estrogen Receptor (ER) Signaling from Classical to Nonclassical Pathway via Stimulating ER Phosphorylation and c-Jun Transcription

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Background: ER signals through binding to estrogen-responsive elements and interacting with c-Jun.
Results: p38γ phosphorylates ER at Ser-118. This increases ER-c-Jun binding, promotes AP-1-dependent transcription, and confers breast cancer hormone sensitivity.
Conclusion: p38γ increases breast cancer hormone sensitivity by regulating ER signaling between classical and nonclassical pathways.
Significance: Regulating p38γ activity may be a new approach to increase breast cancer hormone sensitivity.

Estrogen receptor (ER) α promotes breast cancer growth by regulating gene expression through classical estrogen response element (ERE) binding and nonclassical (interaction with c-Jun at AP-1 sites) pathways. ER is the target for anti-estrogens such as tamoxifen (TAM). However, the potential for classical versus nonclassical ER signaling to influence hormone sensitivity is not known. Moreover, anti-estrogens frequently activate several signaling cascades besides the target ER, and the implications of these “off-target” signaling events have not been explored. Here, we report that p38γ-MAPK is selectively activated by treatment with TAM. This results in both phosphorylation of ER at Ser-118 and stimulation of c-Jun transcription, thus switching ER signaling from the classical to the nonclassical pathway leading to increased hormone sensitivity. Unexpectedly, phosphorylation at Ser-118 is required for ER to bind both p38γ and c-Jun, thereby promoting ER relocation from ERE to AP-1 promoter sites. Thus, ER/Ser-118 phosphorylation serves as a central mechanism by which p38γ regulates signaling transduction of ER with its inhibitor TAM.

Estrogen receptor α (ER)2 signals directly by binding to estrogen response elements (EREs) on DNA (the classical pathway or signaling) and indirectly through interactions with c-Jun at AP-1 sites (the nonclassical pathway or signaling) (1). Using an ERE-binding-deficient ER mutant, it has been demonstrated that ER affects 268 target genes through the nonclassical pathway (2). However, the factors that determine whether ER signals through the classical versus the nonclassical pathway remain completely unknown.

ER is expressed in about 70% of breast cancers and regulates the expression of genes important for breast cancer growth. ER is the therapeutic target of selective ER modulators (SERMs) such as tamoxifen (TAM) (1, 3). However, approximately 50% of ER-positive (ER+) breast cancers are refractory to TAM therapy, and strategies to improve this response are therefore urgently needed (4). SERMs are believed to exert their growth-inhibitory activity through acting as antagonists of ER (4). However, SERMs can also activate other signaling cascades (5), and the implications of these “off-target” effects on hormone sensitivity have not been explored. Moreover, approximately one-third of the genes regulated by ER do not contain ERE in their promoters, and the contributions of nonclassical ER signaling to hormone sensitivity have not been demonstrated (6). AP-1 is a central transcription factor downstream of MAPKs (mitogen-activated protein kinases) and is often activated concomitantly with the classical ER pathway (7). Because SERMs frequently activate MAPKs (8, 9), there may exist a fundamental mechanism that determines breast cancer hormone sensitivity by regulating the ER signaling distribution between the classical and nonclassical pathways.

ER is phosphorylated at Ser-118 by ERKs (10) and other kinases (11). This phosphorylation can occur in response to estrogens and SERMs (11, 12) and is required for ER regulating gene expression (13). Increased levels of p-ER/Ser-118 in primary breast cancer correlate with a better clinical response to...
TAM therapy (14), indicating that p-ER/Ser-118 is a determinant factor in breast cancer hormone sensitivity. Although ERK is the most established kinase for ER/Ser-118 (10, 15), increases in p-ERK can be associated with either increased (14) or decreased (16) clinical response to the hormone therapy. This indicates that there are additional unrecognized kinase(s) that can generate p-ER/Ser-118. This notion is further supported by a recent observation that inhibition of ERK does not affect the amount of p-ER/Ser-118 expression (17). Because c-Jun is a major component of the ER nonclassical pathway (18) and is a recent observation that inhibition of ERK does not affect the can generate indicates that there are additional unrecognized kinase(s) that decreased (16) clinical response to the hormone therapy. This is the most established kinase for ER/Ser-118 (10, 15), increases nant factor in breast cancer hormone sensitivity. Although ERK can phosphorylate ER/Ser-118 in breast cancer (22). Moreover, the proteasome-dependent degradation of ER. Moreover, we showed that p38γ phosphorylates ER at Ser-118 in vitro and in vivo. This leads to the proteasome-dependent degradation of ER. Moreover, we showed that p38γ is activated by treatment of breast cancer cells with TAM. In addition, we found that p38γ-induced ER/Ser-118 phosphorylation is both important for p38γ regulation of ERE- and AP-1-dependent transcription through enhancing ER binding with c-Jun and/or p38γ at the AP-1 DNA, and for TAM-induced breast cancer growth inhibition. Together, these results indicate that p38γ actively regulates the signal transduction of ER with TAM by switching the ER signaling from the classical to the nonclassical pathway. This occurs because of the ability of p38γ to phosphorylate ER at Ser-118 and to stimulate c-Jun expression, thus resulting in ER signaling switching from the classical to the nonclassical pathways.

EXPERIMENTAL PROCEDURES

Cell Culture, Antibodies, and Other Reagents—Minimum Eagle’s medium and other reagents for cell culture were purchased from Invitrogen. A mouse monoclonal antibody against ERα (F10) was obtained from Santa Cruz Biotechnology. Antibodies to p38α and p38γ were purchased either from Santa Cruz or R&D Systems, whereas p-p38, p-ERK, and p-ER/Ser-118 antibodies were from Cell Signaling. GAPDH, c-Jun, Pan-Ras, Raf-1, EGFR, ERK1/2, and cyclin D1 antibodies were from Sigma. Cycloheximide (CHX), the proteasome inhibitor MG132, TAM, estrogen (E2), and the p38γ inhibitor pirfenidone (PFD) were all purchased from Sigma. All human breast cancer cell lines (ERγ: MCF-7, T47D, BT474 (474), and ZR-75–1; ERβ: MDA-MB-231 (231), MDA-MB-468 (468), and MDA-MB-578 (578)) and 293T cells were obtained from ATCC and maintained in minimum Eagle’s medium containing 10% FBS and antibiotics at 37 °C in a humidified atmosphere containing 5% CO2.

Transfection, Viral Infection, and Colony Formation—The constructs for expressing p38γ and its nonphosphorable mutant p38γ/AGF (by changing TGY to AFG) were provided by Dr. J. Han (34). The V5-tagged pcDNA3 vector (Invitrogen) was used to transiently express ER and its mutants (35). The vectors expressing the fusion protein constructs including the constitutively active (CA) p38γ (MKK6-p38γ), its nonphosphor-dominant negative mutant (MKK6-p38γ/AGF), and their p38α counterparts were described previously (24). The tetracycline-inducible system (Tet-on; Invitrogen) was used to express the MKK6-p38γ fusion protein in MCF-7 cells and to express ER and ER/S118A in 231 cells, as described previously (23, 29, 35). To deplete p38γ expression, lentiviral vectors expressing shp38γ or the control shLuc were transfected into packaging cells, and supernatants were collected for infecting target cells, followed by an antibiotic selection (25). To overexpress p38γ or its AGF mutant, adenoviral mediated gene delivery was used as described previously (23). For colony formation, cells (1000 cells/well) were seeded in 6-well plates in the absence and presence of TAM, and colonies formed were stained and manually counted as described previously (36, 37).

ER/Ser-118 Phosphorylation Assays in Vitro and in Vivo—HA-tagged CA p38s or Myc-tagged CA ERK proteins were expressed in 293T cells and were immunoprecipitated from cell lysates using a HA or Myc antibodies. Precipitates were then incubated in vitro with GST-ER or its mutant form (GST-ER/S118A), and the in vitro kinase assay was performed as described previously (38). Proteins were separated by SDS-PAGE, and blots were probed with a specific antibody against phosphorylated ER/Ser-118 (p-ER) as described (15). To measure in vivo ER phosphorylation, V5-tagged ER constructs were co-expressed with the indicated CA kinases in 293T cells, and phosphorylated ER/Ser-118 was assessed by direct Western blotting. Moreover, endogenous p-ER in MCF-7 cells was assessed by Western blotting of cells in which CA p38γ was expressed by the Tet-on system (29). Additional methods are described under supplemental Experimental Procedures.

Statistical Analysis—Experiments were repeated at least three times, and results were analyzed by Student’s t test otherwise specified.

RESULTS

p38γ phosphorylates ER at Ser-118 in Vitro and in Vivo and Increases ER Degradation through Ser-118 by E6AP/Proteasome-dependent Mechanisms—Previous studies showed that ERK2 can phosphorylate ER at Ser-118 independent of estro- gen (10). We first determined whether p38γ (ERK6) acts simi-
larly. Because there are no CA MAPKs available, we purified a HA-tagged MKK6-p38 (CA p38) and HA-tagged MKK6-p38 (CA p38/H9251) fusion proteins expressed in 293T cells using a Myc-tagged CA ERK2 as a positive control (39). Their activities to phosphorylate bacterially expressed GST-ER at Ser-118 were examined in vitro using a specific antibody. Results in Fig. 1A (left) show that in the absence of estrogen, CA p38, but not CA p38, phosphorylates ER at Ser-118 (p-ER), whereas there is no phosphorylation of ER/S118A. The phosphorylation of ER by p38 is considerably more robust than catalyzed by CA ERK2 (Fig. 1A, left). Similarly, ER is phosphorylated in 293T cells that were co-expressed with V5-ER and CA p38, whereas ER phosphorylation was not enhanced in cells with CA p38 (Fig. 1A, right). To demonstrate whether p38 phosphorylates ER in breast cancer cells, p38 was overexpressed in ER/H11001 T47D cells using adenoviral delivery. In ER/H11001 MCF-7 cells a tetracycline-inducible expression system (Tet-on) was used to express CA p38. The resulting effects on the ER phosphorylation were then assessed by analyzing ER immunoprecipitates and/or by direct Western blotting. Results in Fig. 1B (left) show that whereas levels of p-ER are modestly increased in whole cell lysates by Tet-inducible CA p38 expression in MCF-7 cells, analysis of ER immunoprecipitates showed its significant elevation in T47D cells in response to adenoviral mediated p38 overexpression. Thus, p38 phosphorylates ER at Ser-118 both in vitro and in vivo.

Several MAPKs including ERK1/2 (40) and ERK7 (41) can decrease ER levels, but the underlying mechanisms have not been determined. Mutation of the Ser-118 to S118A protects ER from estrogen-induced proteolysis through proteasomal pathways (12). We explored whether the p38 phosphorylation of ER at Ser-118 promotes ER degradation. Results in Fig. 1B (right) showed that the inducible expression of CA p38 (but not its nonphosphorable AGF variant) decreases levels of ER expression after prolonged incubation with Tet in MCF-7 cells. A similar effect was observed in T47D cells in which the ER target PR (progesterone receptor) is also down-regulated by p38 overexpression (Fig. 1B, left, Input). Together, these

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**FIGURE 1.** p38γ phosphorylates ER/Ser-118 in vitro and in vivo and increases ER degradation by E6AP/proteasome-dependent pathways. A, left, Western blots with the indicated antibodies show that in vitro, CA p38γ, but not CA p38α, phosphorylates ER at Ser-118 but not ER/S118A. A, right, in 293T cells co-transfected with V5-ER, CA p38γ (but not CA p38α) induces the phosphorylation of ER. The CA ERK2 is included as a positive control for both in vitro and in vivo ER phosphorylation. B, left, p38γ overexpression enhances ER phosphorylation in T47D and MCF-7 cells and decreases the levels of ER and PR. B, right, Tet-induced expression of CA p38γ in MCF-7 cells decreases levels of ER, whereas the inactive p38γ/AGF variant does not alter ER levels. C, left, p38γ increases ER degradation through E6AP via proteasomal pathways. Tet-on MCF-7 cells were cultured with and without Tet overnight and treated further with and without CHX (100 μg/ml, 2 h) and/or MG132 (10 μM, 6 h) before Western blot analysis. C, right, 293T cells were transfected as indicated and then treated with and without MG132 (10 μM, 6 h) before Western blot analysis.
**p38γ Regulates ER Signaling Distribution**

**A p38γ expression in primary breast cancer**

FIGURE 2. p38γ is overexpressed in primary breast cancers, and levels of its expression are further elevated in ER-/PR- breast cancer tissues and cell lines. A, p38γ levels are increased in primary breast cancer tissues, and there is an inverse correlation with ER-/PR- phenotype. Representative p38γ staining pictures (1 × 200) are presented at left, and the summarized results from 81 cases of specimens are displayed at right (the number of cases varies as indicated reflecting specimen availability for each category or parameter analyzed). Results shown are the mean scores (intensity × area) from the tumors minus those from the nearby normal tissues. The p values were calculated by Kruskal-Wallis test as described previously (37). B, levels of p38γ protein are also increased in a group of ER-/PR- breast cancer cell lines relative to their ER+ counterparts.

results indicate that p38γ decreases ER and/or PR expression through phosphorylation of ER.

To demonstrate whether high p38γ activity correlates with decreased ER expression in primary breast cancer, we analyzed a group of pathological specimens by immunohistochemistry as described previously (37). Results in Fig. 2A show an increased p38γ expression in tumor tissues compared with normal tissues (the score numbers are staining intensity (0–3 scales) × staining area (0–4 scales) of tumors minus those from the nearby normal tissues) as defined previously (42). In this cohort of 81 breast cancer specimens, 70.5% of tumors had increased p38γ expression, 21.3% had no change, and 8.2% had decreased p38γ expression relative to the matched controls. These results are consistent with previous reports from us and others (23, 31, 32). Importantly, levels of p38γ were increased significantly in ER-negative (ER−) and in PR-negative (PR−) specimens compared with their receptor-positive counterparts (Fig. 2A). This inverse relationship also exists in a panel of breast cancer cell lines (Fig. 2B). Moreover, increased levels of p38γ in ER− breast cancer cell lines appear to correlate with those of Ras, Raf-1, and p-ERK (Fig. 2B). These results are consistent with our previous findings that p38γ signals downstream of Ras to stimulate breast cancer invasion (23). Together, these results together indicate that high p38γ activity may drive the down-regulation of ER and its target PR, in primary tissues and established cell lines, suggesting a critical role for p38γ regulating ER activity.

To determine whether p38γ decreases ER protein levels through proteasome-dependent mechanisms, we cultured MCF-7 cells containing Tet-on CA p38γ with and without several concentrations of Tet, followed by treatment with and without MG132, a proteasome inhibitor. Results in supplemental Fig. S1A show that levels of ER are decreased by the p38γ induction through increasing doses of Tet and that MG132 prevents the decreases in ER. In addition, MG132 reverses the declines in ER levels that are seen with the protein synthesis inhibitor CHX or with the induction of CA p38γ (Fig. 1C, left). Co-expression analysis further shows that the p38γ-induced proteasome-dependent ER degradation requires E6AP (Fig. 1C, right), an E3 ligase known for its involvement in ER degradation (43). This was demonstrated by showing that the co-expression of catalytically deficient E6AP (mE6AP) (43) blocks ER degradation induced by CA p38γ. To show whether Ser-118 is required for ER degradation, wild-type (WT) ER and its S118A mutant proteins were expressed in ER− 231 breast cancer cells under control of the Tet-on system (23). The stability of these ER proteins was assessed by Western blotting after incubation with CHX ± MG132 for various times. Results in Fig. S1B show a similar degradation of the expressed WT and mutant ER proteins following treatment with CHX, and that this degradation was partially blocked by MG132. Thus, the Ser-118 residue of ER is not required for proteasome-dependent ER degradation. However, co-expression experiments with 293T cells show that CA p38γ appears to increase the degradation of WT ER without noticeable effects on mutant ER (Fig. S1C), suggesting that p38γ activation requires the Ser-118 residue to trigger ER degradation. Together, these results indicate that p38γ requires E6AP to stimulate the proteasome-dependent degradation of ER in a Ser-118-dependent manner. This may explain the inverse relationship between ER and p38γ in primary breast cancer.
ER/Ser-118 Is Required for p38γ and c-Jun Binding to the cyclin D1 promoter. A and B, p38γ stimulates AP-1 and inhibits ERE reporter activity independent of estrogen but dependent on Ser-118. Cells were transfected with the indicated constructs and assessed for luciferase activity 48 h later with E2 (100 nM) added to one group for the last 24 h. Results shown are relative to the vector controls (mean ± S.D. (error bars), n = 3, *p < 0.05 versus respective controls). C, p38γ increases c-Jun protein and cyclin D1 RNA/protein expression. MCF-7 cells were collected for protein expression 24 h after incubation with and without Tet and 48 h after adenoviral mediated p38γ expression in T47D cells (bottom). For RNA expression, total RNAs were prepared and analyzed by quantitative RT-PCR (right, results normalized to β-actin and expressed as relative to its control, mean ± S.D., n = 3, *p < 0.05). D, p38γ expression increases ER/c-Jun recruitment to the AP-1 sites on the cyclin D1 promoter, as determined by ChIP assays. Cells were infected with control (Ad-β-Gal) and p38γ-expressing adenovirus (Ad-p38γ) for 48 h with and without E2 added to the last 24 h, and DNA precipitated with the indicated antibodies was analyzed by PCR using primers covering the AP-1 region of the cyclin D1 promoter as described (33).

ER/Ser-118 Is Required for p38γ Both to Inhibit ERE- and to Stimulate AP-1-dependent Transcription, and p38γ Stimulates Cyclin D1 Expression through Increasing ER/c-Jun Binding to Its Promoter—ER regulates gene expression through the classical ERE and the nonclassical AP-1 pathways (1). Because p38γ stimulates c-Jun/AP-1 (33) and phosphorylates ER at Ser-118, we next explored whether p38γ distinctively regulates ERE- and AP-1-dependent transcription. In this regard, ER+ T47D breast cancer cells were transiently expressed with p38γ and c-Jun together ERE (ERE-Luc) and AP-1 (AP-1 Luc) luciferase reporter constructs. Results in Fig. 3, A and B (left) showed that p38γ increases the AP-1-dependent activity but suppresses the ERE-dependent transcription, whereas c-Jun only significantly stimulates the AP-1 reporter activity. Incubation with E2 only reverses the ERE inhibition by p38γ, likely as a result of additional signaling events involved. To show whether the Ser-118 of ER has a role in these events, WT ER and its S118A mutant were co-expressed with p38γ and the luciferase reporters in ER+ T47D cells. To our surprise, the S118A expression blocks both the ERE-inhibitory and the AP-1-stimulatory activities of p38γ compared with the WT ER, whereas it only abolishes the c-Jun-induced ERE stimulation (Fig. 3, A and B, right). Together, these results indicate that ER/Ser-118 phosphorylation is critical for p38γ both to stimulate AP-1-dependent transcription and to inhibit ERE-dependent activity. This suggests that p38γ causes a redistribution of ER signaling from the classical to the nonclassical pathway.

Additional studies were conducted to demonstrate further whether p38γ stimulates AP-1 target gene expression. Both c-Jun (44) and cyclin D1 (45) promoters contain the functional AP-1 binding sites and are therefore considered typical AP-1 targets. Results in Fig. 3C (left) show that levels of c-Jun and cyclin D1 protein expression are increased by p38γ in both
MCF-7 and T47D cells. Increases in cyclin D1 expression were further demonstrated by quantitative RT-PCR (Fig. 3, right). These results, together with its c-Jun stimulatory activity (33), indicate that p38γ increases cyclin D1 expression via a c-Jun/AP-1-dependent pathway. Because p38γ and c-Jun form a functional complex at the AP-1 site of the matrix metalloproteinase 9 promoter (33), we used chromatin immunoprecipitation (ChIP) assays to determine whether p38γ affects the binding of ER to c-Jun. Indeed, although there was no substantial change in the binding of p38γ to the cyclin D1 promoter, the occupancy of both ER and c-Jun on this promoter was significantly increased by p38γ (Fig. 3D). Together, these results indicate that p38γ may act as a critical co-factor for c-Jun and ER through a mechanism involving ER/Ser-118 phosphorylation resulting in activation of the nonclassical pathway and increased expression of AP-1 target genes.

p38γ Increases Binding of c-Jun to ER, and Phosphorylation of ER at Ser-118 Is Necessary and Sufficient for Formation of ER–p38γ–c-Jun Complex—The ER/c-Jun interaction is the foundation for ER signaling through the nonclassical pathway (1, 18). In addition to increasing c-Jun abundance through stimulating the amount of c-Jun RNA and protein (33) (Figs. 3C and 4, A and C), the phosphorylation of ER at Ser-118 by p38γ may increase the binding of ER to c-Jun. Consistent with this hypothesis, adenoviral mediated overexpression of p38γ increases the binding of ER to c-Jun in T47D cells (Fig. 4A) and in 231 cells expressing Tet-inducible ER but not ER/S118A (Fig. 4B, left). Because in ER/S118A-expressed 231 cells, p38γ fails to both stimulate AP-1 (Fig. 3B) and to increase the binding of c-Jun to ER compared with the ER-expressing cells (Fig. 4B, right), these results indicate an essential role for the Ser-118 of ER in the ability of p38γ to enhance the ER/c-Jun binding and to activate the nonclassical AP-1 pathway.
To show further the role of Ser-118 phosphorylation, V5-tagged WT ER and its V5-tagged mutants (S118A and the phosphomimetic S118E) were transiently transfected in 293T cells. V5 immunoprecipitates were examined for their binding activity to c-Jun and p38γ, as well as for their recruitment to AP-1 sites of the c-Jun promoter by the ChIP assay. To demonstrate further whether a change in the ER binding to the AP-1 promoter coupled with an alteration in its recruitment to an ERE promoter DNA, the ChIP assay was also conducted in parallel for its occupying the promoter of cathepsin D (CathD), an established ERE target gene (46). Results in Fig. 4D (bottom) show that relative to WT ER, ER/S118E increases the formation of ER–p38γ–c-Jun complexes, whereas S118A decreases these complexes. Most significantly, these binding properties correlate closely with the binding of ER to AP-1 sites on the c-Jun promoter, but not with the binding to the classical ERE site of the CathD promoter (Fig. 4D, top). Of particular interest, the decreased binding of ER/S118A to the c-Jun promoter is in contrast to its increased binding to the CathD promoter (relative to WT ER) (Fig. 4D, top). This further points to the ER signaling switch from the classical ERE/CathD to the nonclassical AP-1/c-Jun pathway in response to ER/Ser-118 phosphorylation. These results therefore show that ER/Ser-118 phosphorylation is necessary and sufficient for ER–p38γ–c-Jun complexes to signal through the nonclassical pathway. Together, these results indicate that Ser-118 phosphorylation may be the primary mechanism by which the binding of ER to c-Jun is increased, leading to activation of the nonclassical pathway (increased AP-1 target gene expression). The resulting increase in c-Jun levels may through a positive feedback loop, further stimulate the formation of ER–c-Jun complexes leading to even more ER signaling through the nonclassical pathway (see Fig. 6D).

p38γ Is Activated Selectively by Incubation with TAM Leading to Increased Formation of ER–p38γ–c-Jun Complexes and to ER/Ser-118-dependent Sensitization to TAM-induced Growth Inhibition—Previous clinical studies suggest a role of increased p38α expression (47) and/or phosphorylation (48) in breast cancer hormone resistance. Because we (24) and others (49) have shown opposing activities for p38α with p38γ, we next examined whether p38γ-mediated effects on ER signaling can affect response to E2 and sensitivity to the anti-estrogen TAM. To test this possibility, ER+ breast cancer cells were pulse-treated with E2 or TAM for 30 min, and all of the phosphorylated p38γ was captured by immunoprecipitation with a p-p38-specific antibody (reactive with all phosphorylated p38 isoforms). The precipitates were next examined by Western blotting with isoform-specific antibodies for phosphorylated p38α and p38γ, which are the two p38 isoforms expressed predominantly in breast cancer (23). Results in Fig. 5A show that TAM induces p38γ, but not p38α, phosphorylation, which correlates with the increased binding of p38γ to ER/p-ER, whereas E2 only had a moderate effect on promoting ER–p38γ complexes. Moreover, TAM, but not E2, significantly increases the binding activity of ER with both c-Jun and p38γ (Fig. 5B, left), an effect that phenotypically resembles that seen with ER/S118E (Fig. 4D, bottom). Furthermore, a prolonged incubation with TAM (2 h) also increases the levels of p38γ, but not p38α, in breast cancer cells (Fig. S1D). Together, these results indicate that TAM selectively activates p38γ, thereby facilitating its binding with ER and/or c-Jun, leading to activation of the nonclassical ER pathway.

Colony formation assays were next used to determine whether p38γ affects the ability of TAM to inhibit breast cancer cell growth (37). Results in Fig. 5B (right) show that adenviral mediated p38γ expression significantly increases the ability of TAM to inhibit the growth of T47D cells. Moreover, this sensitizing effect was observed after Tet-inducible expression of CA p38γ, but not CA p38α, in MCF-7 cells (Fig. 5C and supplemental Fig. S2A). Furthermore, experiments in Tet-on 231 cells show that p38γ expression increases the TAM sensitivity in cells expressing WT ER, but not ER/S118A (Fig. 5D), indicating a required role of Ser-118 in p38γ increasing breast cancer hormone sensitivity. These results, together with the selective TAM-induced activation of p38γ (Fig. 5A) and with the ability of p38γ to phosphorylate ER at Ser-118 (Fig. 1, A and B), indicate a specific and sequential signaling event from TAM treatment to p38γ activation and further to ER/Ser-118 phosphorylation, leading to increased TAM sensitivity (Fig. 6D).

**DISCUSSION**

ER plays a critical role in breast cancer growth, and accordingly, SERMs including TAM have been a major modality for the treatment of patients with ER+ breast cancer (4). However, in addition to targeting ER, SERMs regulate other signaling cascades (5) and the impact of these off-target effects on SERMs sensitivity has never been explored. Moreover, prior to the findings reported herein, there was little information on how differential ER signaling between the ERE-dependent classical and the AP-1-dependent nonclassical pathways impacts breast can-
Here, our studies provide several pieces of evidence that together indicate that p38\(\gamma\)/H9253 is a crucial ER/Ser-118 kinase in breast cancer and that it actively regulates the signal transduction of SERMs with their target ER by switching the classical to the nonclassical pathway leading to increased SERM sensitivity (Fig. 6D). First, p38\(\gamma\) phosphorylates ER/Ser-118 in vitro and in vivo, and this phosphorylation is necessary to inhibit the ERE signaling and to enhance ER degradation. Thus, active p38\(\gamma\) inhibits the classical ER pathway by phosphorylating ER at Ser-118 which increases E6AP/proteasome-dependent ER degradation. Second, p38\(\gamma\) increases the binding of both ER and c-Jun to the AP-1 sites of the cyclin D1 promoter. The Ser-118 of ER is required for the enhanced binding of ER to c-Jun and for p38\(\gamma\) to increase the AP-1 reporter activity. Third, an incubation of breast cancer cells with TAM selectively activates p38\(\gamma\), but not p38\(\alpha\), and increases the binding of ER to p38\(\gamma\) and/or c-Jun, leading to an ER/Ser-118-dependent sensitization of breast cancer cells. Together, these results indicate a positive feedback loop through which the p38\(\gamma\)-mediated off-target effect signals to switch ER signaling from the classical to the nonclassical pathway resulting in increased TAM sensitivity (Fig. 6D). Regulating p38\(\gamma\) expression and/or activity may consequently be a new approach to increase breast cancer hormone sensitivity.

p38\(\gamma\) may distinguish itself from other ER/Ser-118 kinases by its dual activity to stimulate c-Jun expression (33) and to phosphorylate ER/Ser-118 (Fig. 1) (23) thereby serving as a critical co-activator for the ER–c-Jun complex at AP-1-containing sites.,
promoters. Because c-Jun transcription is positively autoregulated (44) and p38γ stimulates c-Jun transcription through interacting with c-Jun (33), the resulting p38γ-mediated increases in c-Jun would further switch the ER signaling from the classical to the nonclassical pathway (Fig. 6D). In addition to p38γ promoting ER degradation, the “switching” mechanism may also explain why the increased ER degradation that results from Ser-118 phosphorylation inhibits the classical but not the nonclassical pathway. Although ERK phosphorylates ER/Ser-118 (10) and decreases ER expression (52), it has not been demonstrated whether this promotes the binding of ER to c-Jun. Moreover, ERK activation does not concurrently inhibit ERE-dependent transcription and stimulate AP-1-dependent signaling (52). An increased level of p-ERK can be associated with a decreased sensitivity of breast cancer cells to SERMs (9), which is in contrast to our findings here that increased p-p38γ enhances TAM sensitivity. Levels of c-Jun are decreased in TAM-resistant breast cancer (53), and an inhibition of c-Jun activity blocks TAM-induced apoptosis in breast cancer cells (19), indicating that c-Jun is required for breast cancer sensitivity to anti-estrogens. Although one recent study showed that CUEDC2, a ubiquitin-binding motif-containing protein, confers hormone resistance through stimulating ER degradation, it is unknown whether it also affects ER signaling through c-Jun/AP-1 (54). Here, we show that p38γ is up-regulated in primary breast cancer and that p38γ overexpression increases breast cancer sensitivity to TAM, whereas the inhibition or depletion of p38γ has the opposite effect. Therefore, the dual activity of p38γ in phosphorylating ER/Ser-118 and in stimulating c-Jun transcription appears to be essential for the stimulation of ER signaling through the nonclassical pathway and for sensitizing breast cancer cells to TAM. Further studies are warranted to determine whether patients with breast cancer that expresses high levels of both p38γ and p-ER achieve a greater benefit when treated with SERMs.

The observations that TAM selectively activates p38γ, but not p38α or ERKs, and that activated p38γ in phosphorylating ER/Ser-118 and in stimulating c-Jun transcription appears to be essential for the stimulation of ER signaling through the nonclassical pathway and for sensitizing breast cancer cells to TAM. Further studies are warranted to determine whether patients with breast cancer that expresses high levels of both p38γ and p-ER achieve a greater benefit when treated with SERMs.

FIGURE 6. p38γ activity and c-Jun are required to increase the TAM sensitivity. A, p38γ silencing with shRNA inhibits c-Jun expression (left) and decreases TAM sensitivity (middle). The forced expression of c-Jun restores the sensitivity of T47D cells in which p38γ has been suppressed (right). The cells were analyzed by Western blotting (left) or for sensitivity to TAM as determined by colony formation assays (mean ± S.D., error bars, n = 3, *, p < 0.05 versus shLuc (middle) or versus vector control (right, with protein expression shown as an inset)). B, forced expression of p38γ/AGF inhibits ER phosphorylation (left) and reduces the TAM sensitivity (right, mean ± S.D., n = 3, *, p < 0.05). C, incubation with the p38γ inhibitor PFD inhibits p-ER/c-Jun expression and reduces the TAM sensitivity. Cells were cultured for 24 h with and without a specific p38γ inhibitor PFD (20 μg/ml) in the presence and absence of Tet and analyzed by Western blotting (left) and colony formation in response to TAM (0.5 μM) (right, mean ± S.D., n = 3, *, p < 0.05 versus no PFD). D, experimental model illustrates that p38γ regulates signal transduction of TAM with ER thereby increasing breast cancer TAM sensitivity by switching ER signaling from the classical to the nonclassical pathway through its dual activity of directly phosphorylating ER/Ser-118 and stimulating c-Jun transcription.
both through stimulating the ER nonclassical pathway and through antagonizing p38α activity.

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