



Published in final edited form as:

J Biol Chem. 2001 December 14; 276(50): 46792–46797. doi:10.1074/jbc.M106927200.

The Proto-oncoprotein Brx Activates Estrogen Receptor β by a p38 Mitogen-activated Protein Kinase Pathway*

Paul H. Driggers^{‡,||,§}, James H. Segars[¶], and Domenica M. Rubino[‡]

[‡]Department of Obstetrics and Gynecology, Uniformed Services University of the Health Sciences, Bethesda, Maryland 20814

^{||}United States Military Cancer Institute, Bethesda, Maryland 20814

[¶]Pediatric and Reproductive Endocrinology Branch, NICHD, National Institutes of Health, Bethesda, Maryland 20892

Abstract

The estrogen receptors (ERs) are ligand-inducible transcription factors that play key roles in the control of growth and differentiation in reproductive tissues. We showed that the novel Dbl family proto-oncoprotein Brx enhances ligand-dependent activity of ER α via a Cdc42-dependent pathway. Brx also significantly enhances ligand-dependent activity of ER β . This enhancement is not affected by inhibition of p44/42 mitogen-activated protein kinase (MAPK) activation by PD98059. However, addition of the p38 MAPK inhibitor SB202190 abrogates the enhancement of ER β activity by Brx, showing that p38 MAPK activity is required for the enhancement of ER β function by Brx. In COS-7 cells, transfection of Brx leads to activation of endogenous p38 MAPK activity. Co-expression of the $\beta 2$ isoform of human p38 MAPK and a constitutively active form of the p38 MAPK kinase MKK6 (MKK6-EE) synergistically augments ligand-dependent activity of ER β . Our findings suggest that p38 MAPKs may be important regulators of ER β activity.

The steroid hormone estrogen plays a critical role in the regulation of growth and development of reproductive tissues. The effects of estrogen in these tissues are due to changes in gene expression modulated by the estrogen receptors (ERs)¹ that are members of the nuclear hormone receptor superfamily of transcription factors. After being activated by binding estrogen, the ER activates transcription of hormone-responsive genes. Prior to 1996 estrogen-dependent effects were thought to be mediated by a single estrogen receptor molecule, now designated ER α . Another estrogen receptor, ER β , was cloned from human testis (1, 2). The ERs are modular transcription factors containing two transcription activation functions, AF-1 located in the N-terminal A/B domain, and AF-2 located within

*This work was supported by grants from the Uniformed Services University of the Health Sciences, the Elsa U. Pardee Foundation, and the Department of Defense Breast Cancer Research program.

[§]First and senior author. To whom correspondence should be addressed: Dept. of Obstetrics and Gynecology, Uniformed Services University of the Health Sciences, 4301 Jones Bridge Rd., Bethesda, MD 20814. Tel.: 301-295-3777; Fax: 301-295-6774; pdriggers@rocketmail.com.

¹The abbreviations used are: ER, estrogen receptor; MAPK, mitogenactivated protein kinase; ERK1/2, extracellular signal-regulated kinases 1 and 2; MEK, MAPK/ERK kinase; MKK, MAPK kinase; GEF, guanine nucleotide exchange factor; Brx, breast cancer nuclear hormone receptor auxiliary factor; RSV, Rous sarcoma virus; HRP, horseradish peroxidase; JNK, c-Jun N-terminal kinase; SRC-1, steroid receptor co-activator 1; DBD, DNA-binding domain; SRE, serum response element.

the C-terminal ligand-binding domain. Although AF-2 function is dependent upon ligand binding, AF-1 functions independently of ligand binding (reviewed in Ref. 3) but synergizes with AF-2 in the promotion of ligand-dependent transcription activation by the receptor (4).

The ER can also be activated by ligand-independent pathways involving signals originating from growth factor receptors. This pathway involves signaling from cell surface receptors and results in phosphorylation of the estrogen receptor (reviewed in Refs. 5, 6). Activation of ER α by epidermal growth factor was shown to involve phosphorylation of serine 118 in the AF-1 region of ER α through a Ras-Raf-MAPK signaling pathway (7, 8). Both ER α and ER β are activated in transient transfection systems by co-expression of the oncogenic V12 mutant of Ras (RasV12). In both cases the activation depends on phosphorylation of target serine residues located within the receptors' AF-1 domains by p44/42 MAPK (ERK1/2) (9). Ras-dependent phosphorylation of the AF-1 domain of ER β is thought to enhance ligand-independent transcriptional activation by enhancing recruitment of transcription co-activators such as SRC-1 (10).

We previously described the cloning and characterization of a cDNA encoding a protein Brx (breast cancer nuclear hormone receptor auxiliary factor) that interacts with ER α , is preferentially expressed in reproductive and immune tissues, and augments ligand-dependent transcriptional activation by ER α (11). Sequence analysis showed that Brx belongs to the Dbl family of proto-oncogenes (reviewed in Ref. 12) that function as guanine nucleotide exchange factors (GEFs) for Rho-GTPases, small Ras-related cytoplasmic proteins involved in signal transduction pathways governing growth and morphogenic transformation. Proteins acting as GEFs shift the equilibria of small GTPases toward an active GTP-bound state. We found that activation of ER α by Brx in transient transfection assays involves the Rho-related GTPase Cdc42. Our findings demonstrated for the first time that ER α function might be modulated by pathways dependent on small GTPases other than Ras.

Brx mRNA is highly expressed in the same human tissues that express ER β mRNA. These include, specifically, ovary, uterus, testis, spleen, thymus, peripheral blood leukocytes, and specific areas of the brain, including the amygdala and hypothalamus,² which are thought to be regulated by estrogen. The present study was performed to investigate the effect of Brx on ER β . We show that Brx enhances ligand-dependent activity of ER β . Furthermore, we demonstrate that Brx sends signals to ER β through a pathway involving p38 MAPKs.

Experimental Procedures

Cell Culture and Transient Transfection Studies

Ishikawa human endometrial adenocarcinoma cells were cultured in phenol red-free Dulbecco's modified Eagle's medium/F-12 (Life Technologies, Inc.) supplemented with 5% charcoal-stripped fetal bovine serum (HyClone). For experiments examining the activation of the *c-fos* SRE, cells were serum-starved overnight prior to transfection. COS-7 cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. For

²D. M. Rubino, unpublished.

experiments examining the activation of endogenous p38 MAPK activity, COS-7 cells were plated onto 100-mm dishes and transfected with 15 μ g of expression vector for Brx, pBK-RSV, or MKK6-EE using FuGENE-6 (Roche Molecular Biochemicals) as described by the manufacturer. Twenty-four hours after transfection, cells were subjected to overnight serum starvation prior to lysis and immunoblotting. For transient transfection studies Ishikawa cells were plated onto 12-well plates and 100 ng of pRSV-ER β , 1 μ g of pRSV-Brx, 500 ng of GAL4-Elk-1, 500 ng of GAL4-ER β or empty vector, and 1.5 μ g of pBK-CMV, 1.0 μ g of (ERE)₂-tk-luciferase, 1.0 μ g of SRE-tk-luciferase or 1.0 μ g of G4E1b-luciferase were added to cells with FuGENE-6 as per the manufacturer's instructions. Cells were harvested 20 h after transfection, and luciferase assays were performed and normalized as described previously (11). In all transfection experiments, total amounts of DNA were made constant by the addition of empty vectors. 17- β -Estradiol (10 nM), 4-hydroxy tamoxifen (40 nM), PD98059 (10 μ M, Cal-biochem), SB202190 (2 μ M, Calbiochem) were added as described.

Plasmids

Expression vectors for Brx, (ERE)₂-tk-luciferase, SRE-tk-luciferase, and MEKE, were described previously (11, 13–15). A full-length cDNA-encoding human ER β (2) was amplified from a human prostate Marathon-Ready cDNA library (CLONTECH). The cDNA was subcloned into pBK-RSV (Stratagene) and sequenced to make RSV-ER β . The ER β cDNA beginning at the second codon was subcloned into the pM expression vector (CLONTECH) containing the GAL4 DNA binding domain to make GAL4-ER β . A full-length cDNA encoding Elk-1 was amplified from a human peripheral blood leukocyte cDNA library (CLONTECH), subcloned into pM, and sequenced to make GAL4-Elk-1. Expression plasmids for MEKE and wild type MKK6 were kindly provided by Dr. Silvio Gutkind. MKK6-EE containing Ser to Glu and Thr to Glu mutations at codons 207 and 211, respectively, were derived from wild-type MKK6 by overlap amplification with primers containing site-specific mutations. The resulting cDNA was subcloned into pBK-RSV, and individual clones were sequenced to verify the presence of the mutations. Full-length cDNA encoding human p38 β 2 was amplified from a brain cDNA library (CLONTECH), subcloned into pBK-RSV, and sequenced.

Immunoblotting

Transfected COS-7 cells were harvested at 4 °C and lysed in SDS sample buffer by sonication. Following centrifugation, proteins were resolved by SDS-polyacrylamide gel electrophoresis and transferred to BA85 nitrocellulose (Schleicher and Schuell) by semi-dry electroblotting. Membranes were blocked in Tris-buffered saline containing 0.05% Tween-20 and 5% nonfat dry milk. Phospho-p38 MAPK primary antibody and HRP-conjugated anti-rabbit secondary antibody (both from New England BioLabs) were used and detected per the manufacturer's instructions. Epitope-tagged Brx and GAL4-ER β were detected with HRP-conjugated anti-FLAG M2 monoclonal antibody (Sigma Chemical Co.) and HRP-conjugated GAL4 (DBD) monoclonal antibody (Santa Cruz Biotechnology, Inc.) per the manufacturers' instructions.

Protein Phosphorylation

Proteins were phosphorylated with active recombinant human p38 β (Upstate Biotechnology Inc.) per the manufacturer's instructions. Twenty milligrams of protein substrates were incubated with 100 ng of active p38 β in the presence of [γ -³²P]ATP (1 mCi/ml) at 30 °C for 15 min with rotation in a final reaction volume of 40 μ l. Reactions were stopped by addition of SDS sample buffer and heating to 95 °C for 5 min. Labeled proteins were resolved on 10% SDS-polyacrylamide gels and detected by autoradiography.

Results

Brx Enhances Ligand-dependent Transcriptional Activation by Estrogen Receptor β

Given our previous observation that Brx enhances ligand-dependent transcriptional activation by ER α in transiently transfected Ishikawa endometrial cells, we wanted to test whether Brx expression could also affect the ability of ER β to activate a reporter. Transfection of ER β resulted in severalfold ligand-dependent activation of the G4E1b-luciferase reporter (Fig. 1A). Co-transfection of an expression vector for full-length Brx enhanced ligand-dependent trans-activation by ER β ~ 10-fold.

Although tamoxifen acts as an estrogen antagonist in the breast, in the uterus it acts as an estrogen agonist, and long term treatment of breast cancer with tamoxifen results in an increased risk of endometrial cancer (16, 17). We found that in Ishikawa cells 40 nM 4-hydroxytamoxifen blocked ligand-dependent activity of ER β (Fig. 1A). Addition of 40 nM 4-hydroxytamoxifen completely eliminated ligand-dependent activation of the reporter by ER β in the absence of Brx and greatly reduced ligand-dependent activity of the receptor in the presence of Brx. These results suggest that the full level of activity of ER β in the presence of Brx was dependent on activation of the receptor by ligand binding. In our previous study we reported an antagonistic effect of tamoxifen on the ligand-dependent activity of ER α in Ishikawa cells (11).

We tested the possibility that Brx augments receptor activity through an effect on receptor expression levels. COS-7 cells were transfected with expression vectors for Brx and GAL4-ER β . Western analyses showed that co-expression of Brx did not affect the level of GAL4-ER β expression (Fig. 1B), suggesting that the observed augmentation of ligand-dependent activity of ER β by Brx is not simply due to an increase in the amount of receptor protein expressed. We also tested the specificity of Brx action by assessing the ability of Brx to activate a chimeric GAL4-VP16 transcription factor. Under conditions in which Brx enhances the ligand-dependent activity of ER β , Brx failed to enhance the activity of GAL4-VP16 (Fig. 1C). Brx expression also did not affect the activity of the G4E1b-luciferase reporter.

Brx Signaling to ER β Does Not Involve p44/42 MAPK (ERK1/ERK2)

ER β has been shown to be activated independently of ligand binding by a mechanism involving phosphorylation of Ser residues in AF-1 through the Ras-Raf-p44/42 MAPK (ERK1/ERK2) signaling pathway (9). It has also been shown that Cdc42 can activate ERK2 in cooperation with Raf (18). We therefore tested whether activation of ER β by Brx involves

p44/42 MAPK. Dual specificity mitogen-activated protein kinase kinases (MEK) activate p44/42 MAPK by phosphorylating specific Thr and Tyr residues. The inhibitor PD98059 is thought to block activation of p44/42 MAPK (19) by binding to inactive MEK1 and blocking activation by upstream kinases (20). In these experiments Brx only modestly affected activity of the receptor. Addition of 10 mM PD98059 did not affect the enhancement of ligand-dependent activity of ER β by Brx (Fig. 2A). Addition of the inhibitor also did not affect ligand-dependent activity of ER β in the absence of Brx. In control experiments the same concentration of PD98059 effectively inhibited activation of a SRE-*tk*-Luc reporter by mutationally activated MEK1 (21) (Fig. 2B).

Brx Activates ER β by a p38 MAPK-dependent Pathway

We previously showed that Brx enhances ligand-dependent activity of ER α by a Cdc42-dependent pathway. Because activation of ER β by Brx did not appear to involve p44/42 MAPK pathways, and because Cdc42 has been shown to activate p38 MAPKs (22), we tested whether the enhancement of ER β activity by Brx could involve a p38 MAPK pathway. The pyridinyl imidazole compound SB202190 inhibits the α and β isoforms of p38 MAPKs by a mechanism involving direct binding to the kinases (23). In our transient transfection system using ER β expressed as a GAL4 DBD fusion protein (GAL4-ER β) and the G4E1b-luciferase reporter, transfection of GAL4-ER β activated luciferase expression severalfold in the presence of ligand compared with control transfections lacking ligand. Co-transfection of Brx enhanced ligand-dependent activity of GAL4-ER β ~10-fold (Fig. 3A). Addition of 2 μ M SB202190 inhibited activation of ER β by Brx by ~70%, suggesting that p38 α or p38 β activity is required for enhancement of ligand-dependent activity of ER β by Brx. SB202190 did not inhibit the activity of a chimeric GAL4-VP16 transcription factor (Fig. 3B).

Because our results suggested that p38 MAPK activity is required for augmentation of receptor activity by Brx, we tested whether p38 activation affects activity of ER β in Ishikawa cells. The p38 MAPKs can be activated by several dual-specificity MAPK kinases, including MKK3, MKK6, MKK4, and MKK7 (24–27). MKK4 and MKK7 also efficiently activate JNK, and MKK3 activates only p38 α , γ , and δ . Each of the p38 MAPKs (α , β , γ , and δ) has been shown to be phosphorylated and activated by MKK6 (28). A constitutively active mutant form of MKK6 (MKK6-EE,) contains Ser to Glu and Thr to Glu mutations at amino acid positions 207 and 211, respectively, and activates p38 MAPKs (29).

Transfection of Ishikawa cells with an expression vector for the β 2 isoform of p38 MAPK or MKK6-EE alone did not activate GAL4-ER β , but co-transfection of both synergistically enhanced ligand-dependent activity of GAL4-ER β (Fig. 4A). The transcription factor Elk-1 is thought to be a physiological substrate of p38 MAPK (29). We therefore performed control experiments to test for activation of Elk-1 by MKK6-EE and p38 β 2 in Ishikawa cells. As we observed for ER β , transfection of either p38 β 2 or MKK6-EE was not sufficient to activate a control GAL4-Elk-1 construct. Co-transfection of Ishikawa cells with both p38 β 2 and MKK6-EE synergistically activated GAL4-Elk-1 (Fig. 4B). To test directly whether Brx activates p38 MAPK, we transfected COS-7 cells and assessed the effect of Brx expression on p38 MAPK activity by immunoblotting. Transfection of Brx increased

endogenous p38 MAPK activity approximately 3-fold (Fig. 4C). Transfection of MKK6-EE also activated endogenous p38 MAPKs severalfold in COS-7 cells (Fig. 4C). A relatively low transfection efficiency has prevented us from performing a similar analysis in Ishikawa cells. We tested whether p38 MAPK can utilize ER β as a phosphorylation substrate. Active recombinant human p38 β 2 MAPK efficiently phosphorylated recombinant ER β (Fig. 4D), suggesting that ER β is a potential target substrate of the kinase.

Discussion

The estrogen receptors (ERs) are modular ligand-inducible transcription factors that play a key role in the regulation of growth and differentiation of reproductive tissues. An important issue in the study of ER signal transduction pathways is that of ligand-independent activation (5). Studies have documented effects upon ER-mediated gene activation by epidermal growth factor and other growth factors acting as ligand-independent receptor activators. Both ER α and ER β can be activated independently of ligand binding by a Ras-Raf-p44/42 MAPK signaling pathway involving the phosphorylation of specific Ser residues in the receptors' AF-1 domains (9). In the case of ER β , modification of the receptor was proposed to affect transcription by enhancing the recruitment of steroid receptor co-activator-1 (SRC-1) (10). The demonstration of signaling pathways linking Ras to regulation of ER activity was an important observation, because Ras has a critical role in cellular proliferation and oncogenesis.

Members of the Ras-related Rho family of small GTPases, including Rho, Rac, and Cdc42, regulate a variety of crucial cellular processes, including cytoskeletal organization, gene expression, cell cycle progression, cell adhesion, and intracellular membrane trafficking (30, 31). These proteins are regulated by GTP binding and are activated by guanine nucleotide exchange factors (Rho-GEFs) that catalyze the exchange of GDP for GTP. Rho-GEFs constitute a group of oncoproteins containing Dbl homology and pleckstrin homology domains that are required for the nucleotide exchange function (12). Different Rho-GEFs possess a variety of structural motifs presumably regulating function and specific interactions with other proteins. Although the Rho-GEFs are thought to be important targets of upstream activators of Rho GTPases, relatively little is known about the regulation of function of most of the GEFs. We recently described the cloning of *brx*, a novel Dbl family proto-oncogene, encoding Brx, a protein that enhances ligand-dependent activity of ER α by a Cdc42-dependent pathway (11). Our report was the first to implicate Rho GTPases in the regulation of ER function. Recently, Su *et al.* (32) demonstrated enhancement of ER α activity by the guanine nucleotide dissociation inhibitor GDI α , a finding that provided further support for a role for Rho GTPases in the regulation of ER activity. In this report we demonstrate that Brx enhances ligand-dependent activity of ER β . The mechanism appears not to involve the p44/42 MAPK pathway, but instead involves activation of a p38 MAPK pathway (Fig. 5). This report represents the first report implicating p38 MAPK activation in the regulation of ER β function.

Brx mRNA is highly expressed in human tissues that express ER β mRNA, specifically including ovary, uterus, testis, spleen, thymus, peripheral blood leukocytes, and specific areas of the brain, including the amygdala and hypothalamus, which are thought to be

regulated by estrogen (11).² Immunohistochemical staining of human ovarian tissues showed high levels of Brx protein expression in granulosa cells of follicles (33). These data suggest that Brx is co-expressed in tissues expressing ER β . We also found that Brx interacts with ER β in binding assays *in vitro*.² Given these findings, we wanted to know whether Brx affects ER β function. In transient transfection assays using Ishikawa human endometrial adenocarcinoma cells, ligand-dependent activity of ER β was markedly enhanced by co-transfection of a Brx expression vector. The full level of reporter activity required Brx, ER β , the ERE, and estrogen. Addition of tamoxifen totally eliminated ligand-dependent activity of ER β . In control experiments we demonstrated that activation of Brx is selective, because Brx did not affect the activity of the chimeric transcription factor GAL4-VP16. Additionally, analysis of protein expression levels in transfected COS-7 cells showed that Brx did not affect the level of GAL4-ER β protein expression, a finding that suggests Brx does not augment ligand-dependent receptor activity simply by increasing receptor expression. Taken together, these results suggest that Brx affects the ligand-dependent activity of ER β itself.

We examined the possibility that Brx enhances ER β activity through a Ras-p44/42 MAPK pathway. Inhibition of MAPK activation by PD98059 did not affect the observed enhancement of ligand-dependent ER β activity by Brx, suggesting that Brx affects ER β function by a pathway that does not depend on activation of MAPK. We have also found that enhancement of ER α activity by Brx involves neither p44/42 MAPK nor phosphorylation of Ser-118.² These findings showed that Brx must affect ER β by signaling pathways distinct from the previously described Ras and p44/42 MAPK-dependent pathways (9).

We previously demonstrated that Brx augments ligand-dependent activity of ER α by a Cdc42-dependent pathway (11). Mutationally activated Cdc42 also strongly activates JNK and p38 MAPK (14, 22). One possibility, therefore, is that Brx activates a JNK or p38 MAPK that in turn augments ligand-dependent function of ER β either directly or indirectly. We therefore tested for an effect of the p38 MAPK inhibitor SB202190 on ER β activation by Brx and found that 2 μ M SB202190 inhibited enhancement of ER β activity by Brx by ~70%, suggesting that p38 MAPK activity is required for activation of ER β by Brx. We next tested directly whether a specific p38 MAPK could activate ER β . Although transient transfection of either p38 β 2 or MKK6-EE was insufficient to activate the receptor, transfection of both synergistically activated the receptor. Similarly, for the GAL4-Elk-1 transfection control experiments, we found that the combination of both p38 β 2 and MKK6-EE cooperatively activated the luciferase reporter. We do not know why transfection of MKK6-EE was insufficient to activate ER β . It is possible that MKK6-EE was unable to activate cellular p38 MAPK in Ishikawa cells, although it was able to do so in COS-7 cells in our control experiment. Unfortunately, our transfection efficiency for the Ishikawa cells has been too low to permit a similar analysis. Selectivity of p38 MAPK isoform activation by MKK6 has been described by Alonso (34). This group showed that, at relatively low concentrations both *in vitro* and *in vivo*, wild-type MKK6 activated p38 α but failed to activate p38 γ . At present, we do not know which p38 MAPK isoforms are expressed in Ishikawa cells and we have not yet determined whether the enhancement of ER β activity by

Brx exhibits selectivity for p38 MAPK isoforms. It is also possible that MKK6 is not involved in activation of ER β by Brx in the Ishikawa cells. Our findings do suggest, however, that endogenous p38 MAPK activity in Ishikawa is required for activation of ER β by Brx and that activation of p38 β 2 MAPK by MKK6-EE effectively enhances ligand-dependent activity of the receptor. We showed that Brx expression results in the activation of endogenous p38 MAPK in COS-7 cells.

p38 MAPKs have well established roles in the regulation and function of pro-inflammatory cytokines such as tumor necrosis factor- α and interleukin-1; they are also activated in cultured cells in response to environmental stresses such as exposure to UV radiation and hyperosmotic conditions (reviewed in Ref. 35). Recent experiments suggest that activation of p38 MAPK pathways may also play important roles in cellular differentiation processes. Differentiation *in vitro* of several cell lines has been shown to depend on p38 MAPK activity (reviewed in Ref. 36). It was recently reported that activation of p38 MAPKs is deficient in rhabdomyosarcoma cells (37). Activation of p38 MAPKs in these cells by ectopic expression of MKK6-EE resulted in growth arrest and terminal differentiation. Our findings suggest that p38 MAPKs may also play a role in the regulation of estrogen receptors, which are in turn key regulators of growth and differentiation in reproductive tissues.

During the preparation of this manuscript, Lee *et al.* (38) published a report showing activation of ER α by constitutively active MEKK1 in Ishikawa cells. These authors proposed that MEKK1 activates ER α through activation of JNK and p38 MAPK. They also demonstrated phosphorylation of ER α by p38 MAPK *in vitro*. These findings, considered together with our own results, suggest that p38 MAPKs may be important regulators of both forms of ER.

We do not know the mechanism for enhancement of ER β activity by p38 MAPK and whether direct phosphorylation of the receptor by p38 MAPK is involved. However, we observed that p38 MAPK efficiently phosphorylated ER β *in vitro*. By analogy with p44/42 MAPK-dependent activation of ER β , direct phosphorylation of the receptor could affect the ability of liganded receptor to interact with or recruit a transcriptional co-activator such as SRC-1. Alternatively, p38 MAPK may indirectly affect the activity of ER β by phosphorylating other factors required for ER β function such as SRC-1 or p300.

Acknowledgments

We are grateful to Dr. Silvio Gutkind for the kind gifts of COS-7 cells and expression vectors for MKK6 and MEKE. We thank Drs. William Haffner and Prabir Chakraborty for their support during this project.

References

1. Kuiper GGJM, Enmark E, Peltö-Huikko M, Nilsson S, Gustafsson JA. *J Biol Chem.* 1996; 93:5925–5930.
2. Mosselman S, Polman J, Dijkema R. *FEBS Lett.* 1996; 392:49–53. [PubMed: 8769313]
3. Tsai M, O'Malley BW. *Annu Rev Biochem.* 1994; 63:451–486. [PubMed: 7979245]
4. Tora L, White J, Brou C, Tasset D, Webster N, Scheer E, Chambon P. *Cell.* 1989; 59:477–487. [PubMed: 2805068]

5. O'Malley BW, Schrader WT, Mani S, Smith C, Weigel NL, Conneely OM, Clark JH. *Recent Prog Horm Res.* 1995; 50:333–347. [PubMed: 7740165]
6. Kato S, Masuhiro Y, Watanabe M, Kobayashi Y, Takeyama K, Endoh H, Yanagisawa J. *Genes to Cells.* 2000; 5:593–601. [PubMed: 10947845]
7. Kato S, Endoh H, Masuhiro Y, Kitamoto T, Uchiyama S, Sasaki H, Masushige S, Gotoh Y, Nishida E, Kawashima H, Metzger D, Chambon P. *Science.* 1995; 270:1491–1494. [PubMed: 7491495]
8. Bunone G, Briand PA, Miksicek RJ, Picard D. *EMBO J.* 1996; 15:2174–2183. [PubMed: 8641283]
9. Tremblay GB, Tremblay A, Copeland NG, Gilbert DJ, Jenkins NA, Labrie F, Giguere V. *Mol Endocrinol.* 1997; 11:353–365. [PubMed: 9058381]
10. Tremblay A, Tremblay G, Labrie F, Giguere V. *Mol Cell.* 1999; 3:513–519. [PubMed: 10230404]
11. Rubino D, Driggers P, Arbit D, Kemp L, Miller B, Coso O, Pagliai K, Gray K, Gutkind J, Segars J. *Oncogene.* 1998; 16:2513–2526. [PubMed: 9627117]
12. Cerione RA, Zheng Y. *Curr Opin Cell Biol.* 1996; 8:216–222. [PubMed: 8791419]
13. Nunez S, Medin J, Braissant O, Kemp L, Wahli W, Ozato K, Segars J. *Mol Cell Endocrinol.* 1997; 127:27–40. [PubMed: 9099898]
14. Coso O, Chiariello M, Yu J, Teramoto H, Crespo P, Xu N, Miki T, Gutkind J. *Cell.* 1995; 81:1137–1146. [PubMed: 7600581]
15. Fromm C, Coso O, Montaner S, Xu N, Gutkind J. *Proc Natl Acad Sci U S A.* 1997; 94:10098–10103. [PubMed: 9294169]
16. Rutqvist LE, Johansson H, Signomklao T, Johansson U, Fornander T, Wilking N. *J Natl Cancer Inst.* 1995; 87:645–651. [PubMed: 7752269]
17. Fisher B, Costantino JP, Wickerham DL, Redmond CK, Kavanah M, Cronin WM, Vogel V, Robidoux A, Dimitrov N, Atkins J, Daly M, Wieand S, Tan-Chiu E, Ford L, Wolmark N. *J Natl Cancer Inst.* 1998; 90:1371–1388. [PubMed: 9747868]
18. Frost JA, Steen H, Shapiro P, Lewis T, Ahn N, Shaw PE, Cobb M. *EMBO J.* 1997; 16:6426–6438. [PubMed: 9351825]
19. Pang L, Sawada T, Decker SJ, Saltiel A. *J Biol Chem.* 1995; 270:13585–13588. [PubMed: 7775407]
20. Alessi DR, Cuenda A, Cohen P, Dudley DT, Saltiel AR. *J Biol Chem.* 1995; 270:27489–27494. [PubMed: 7499206]
21. Cowley S, Paterson H, Kemp P, Marshall CJ. *Cell.* 1994; 77:841–852. [PubMed: 7911739]
22. Minden A, Lin A, Claret FX, Abo A, Karin M. *Cell.* 1995; 81:1147–1157. [PubMed: 7600582]
23. Lee JC, Laydon JT, McDonnell PC, Gallagher TF, Kumar S, Green D, McNulty D, Blumenthal MJ, Heys JR, Landvatter SW. *Nature.* 1994; 372:739–746. [PubMed: 7997261]
24. Sanchez I, Hughes RT, Mayer BJ, Yee K, Woodgett JR, Avruch J, Kyriakis JM, Zon LI. *Nature.* 1994; 372:794–798. [PubMed: 7997269]
25. Lin A, Minden A, Martinetto H, Claret FX, Lange-Carter C, Mercurio F, Johnson GL, Karin M. *Science.* 1995; 268:286–290. [PubMed: 7716521]
26. Derijard B, Raingeaud J, Barrett T, Wu IH, Han J, Ulevitch RJ, Davis RJ. *Science.* 1995; 267:682–685. [PubMed: 7839144]
27. Hu MCT, Wang YP, Mikhail A, Qiu WR, Tan TH. *J Biol Chem.* 1999; 274:7095–7102. [PubMed: 10066767]
28. Keesler GA, Bray J, Hunt J, Johnson DA, Gleason T, Yao Z, Wang SW, Parker C, Yamane H, Cole C, Lichenstein HS. *Protein Expr Purif.* 1998; 14:221–228. [PubMed: 9790884]
29. Raingeaud J, Whitmarsh AJ, Barrett T, Derijard B, Davis RJ. *Mol Cell Biol.* 1996; 16:1247–1255. [PubMed: 8622669]
30. Van Aelst L, D'Souza-Schorey C. *Genes Dev.* 1997; 11:2295–2322. [PubMed: 9308960]
31. Kjoller L, Hall A. *Exp Cell Res.* 1999; 253:166–179. [PubMed: 10579921]
32. Su LF, Knoblauch R, Garabedian MJ. *J Biol Chem.* 2001; 276:3231–3237. [PubMed: 11060289]
33. Miller B, Rubino D, Driggers P, Haddad B, Cisar M, Gray K, Segars J. *Am J Obstet Gynecol.* 2000; 182:286–295. [PubMed: 10694326]

34. Alonso G, Ambrosino C, Jones M, Nebreda AR. *J Biol Chem.* 2000; 275:40641–40648. [PubMed: 11010976]
35. Paul A, Wilson S, Belham CM, Robinson CJM, Scott PH, Gould GW, Plevin R. *Cell Signal.* 1997; 9:403–410. [PubMed: 9376221]
36. Nebreda A, Porras A. *Trends Biochem Sci.* 2000; 25:257–260. [PubMed: 10838561]
37. Puri PL, Wu Z, Zhang P, Wood LD, Bhakta KS, Han J, Feramisco JR, Karin M, Wang JYJ. *Genes Dev.* 2000; 14:574–584. [PubMed: 10716945]
38. Lee H, Jiang F, Wang Q, Nicosia SV, Yang J, Su B, Bai W. *Mol Endocrinol.* 2000; 14:1882–1896. [PubMed: 11075819]

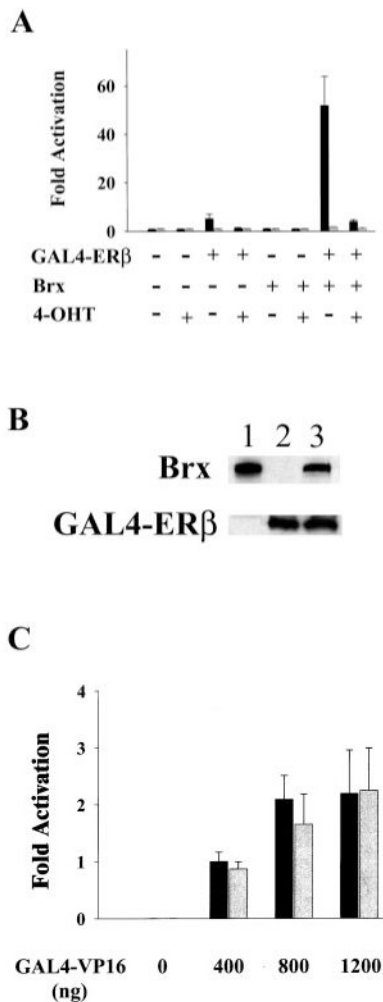


Fig. 1. A, Brx co-expression augments ligand-dependent activation of a G4E1b-luciferase reporter plasmid by GAL4-ER β . Augmentation of ER β -mediated activation of G4E1b-luciferase by Brx co-expression is inhibited by the addition of 4-hydroxy tamoxifen. Ishikawa cells were transfected with 1 μ g of G4E1b-luciferase and expression vectors for GAL4-ER β (500 ng) and Brx (1 μ g) or the respective amounts of empty vectors as control. Estradiol (10 nM, *solid bars*) or vehicle control (*gray bars*), and vehicle control or 4-hydroxytamoxifen (40 nM) was added to the cells as indicated. Cells were harvested after 20 h. Luciferase values represent - fold activation (mean) over control (RSV-0 with G4E1b-luciferase) in the absence of ligand, under each condition from three experiments performed in triplicate. *Error bars* represent standard deviations. B, Brx did not affect receptor expression levels. COS-7 cells were transfected with expression vectors for Brx (*lane 1*), GAL4-ER β (*lane 2*), or both (*lane 3*). Brx and GAL4-ER β were detected in lysates of transfected cells by Western blotting. Plasmid amounts were held constant by addition of empty expression vectors. C, Brx does not augment the activity of a chimeric GAL4-VP16 transcription factor. Ishikawa cells were transfected with 1 μ g of G4E1b-luciferase reporter plasmid and expression vectors for GAL4-VP16 (0, 400, 800, or 1200 ng, as indicated) and Brx (1 μ g, *gray bars*) or the same

amounts of empty expression vectors (*solid bars*). Cells were harvested and assayed for luciferase activity after 20 h. Luciferase values represent means of-fold activation (with activation given by 400 ng of GAL4-VP16 arbitrarily assigned a value of 1.0) from two experiments performed in duplicate. *Error bars* represent standard deviations.

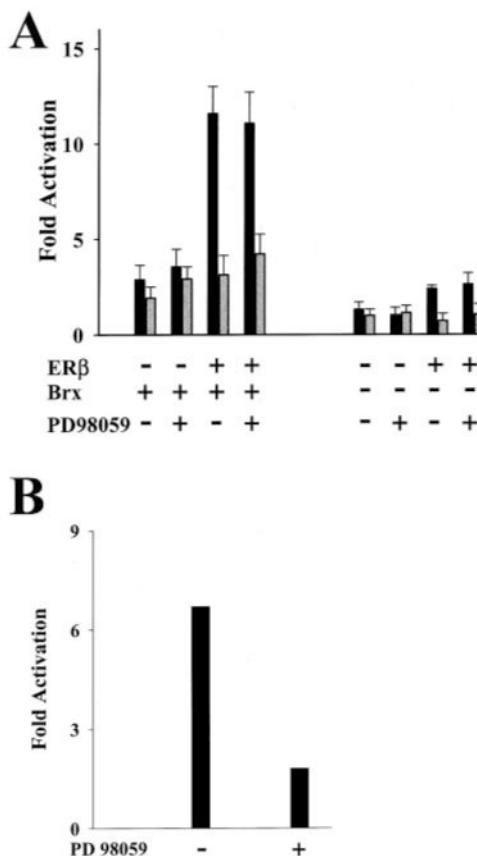


Fig. 2. A, augmentation of ERβ activity by Brx is not affected by the inhibition of mitogen-activated protein kinase (MAPK) activity. The two panels show activation of (ERE)₂-tk-Luc reporter plasmid by ERβ with co-expression of Brx (*left panel*) and without (*right panel*). Addition of PD98059, a mitogen-activated protein kinase kinase (MEK) inhibitor, did not attenuate the activation of ERβ with or without Brx co-expression. Ishikawa cells were transfected with 1 μg of (ERE)₂-tk-Luc and expression vectors for ERβ (100 ng) and Brx (1 μg) or the respective amounts of empty vectors as control. Estradiol (10 nM, *solid bars*) or vehicle control (*gray bars*) and PD98059 (10 μM) (+) or an equal amount of vehicle (-) was added to the cells as indicated. Cells were harvested after 20 h. Luciferase values represent fold activation (mean) over control (RSV-0 with (ERE)₂-tk Luc in the absence of ligand) from three experiments performed in triplicate. *Error bars* represent standard deviations. B, PD98059 inhibits MAPK in Ishikawa cells. Activation of a serum response element reporter in Ishikawa cells is attenuated by the addition of PD98059. Ishikawa cells were serum-starved overnight and transfected with 1.0 μg of a *c-fos* serum response element SRE-tk-luciferase reporter. The two columns show activation of the SRE-tk-Luc reporter by co-transfection of an expression vector encoding MEKE, a constitutively active mutant form of MEK. PD98059 (10 μM) (+) or an equal amount of vehicle (-) was added to the cells. Cells were harvested after 20 h. Luciferase values represent -fold activation compared with reporter activity after transfection of empty expression vector (not shown).

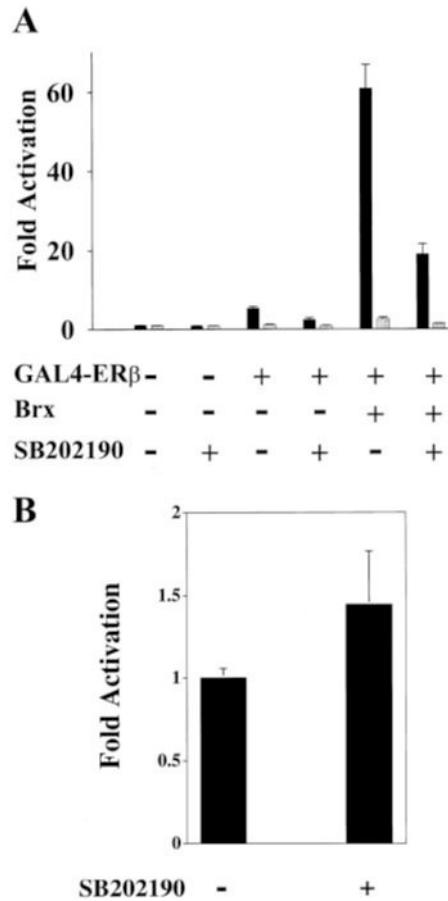


Fig. 3. Activation of ER β by Brx is dependent on p38 MAPK activity

A, activation of ER β by Brx is inhibited by the p38 MAPK inhibitor SB202190 (2 μ M). Ishikawa cells were transfected with 1 μ g of a G4E1b-luciferase reporter plasmid and expression vectors for GAL4-ER β (100 ng) and Brx (1 μ g) or the same amounts of empty expression vectors. Estradiol (10 nM, *solid bars*) or vehicle control (EtOH, *gray bars*) and SB202190 or control (SB202474) were added as indicated. Cells were harvested after 20 h. Luciferase values represent -fold activation (mean) over control (RSV0 with G4E1b-luciferase in the absence of ligand) from two experiments performed in duplicate. *Error bars* represent standard deviations. B, the p38 MAPK inhibitor SB202190 does not inhibit the activity of GAL4-VP16. Ishikawa cells were transfected with 1 μ g of a G4E1b-luciferase reporter plasmid and 400 ng of an expression vector for GAL4-VP16. SB202190 (3 μ M) was added as indicated. Luciferase values represent -fold activation (with activation given by 400 ng of GAL4-VP16 arbitrarily assigned a value of 1.0) from two experiments performed in duplicate. *Error bars* represent standard deviations.

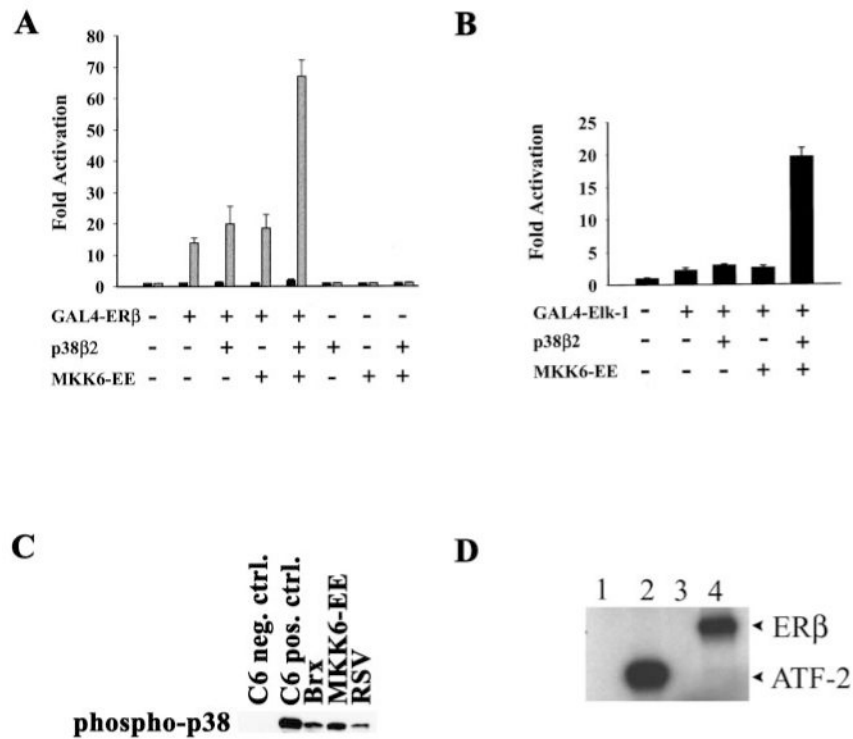


Fig. 4. *A* and *B*, MKK6-EE and p38 β 2 synergistically activate ER β (*A*) and Elk-1 (*B*) in Ishikawa. Cells were transfected with 1 μ g of G4E1b-luciferase reporter and 500 ng of expression vectors for GAL4-Elk-1 or GAL4-ER β , MKK6-EE, and p38 β 2 or the same amounts of empty expression vectors, as indicated. Cells were harvested for assay after 20 h. Values represent -fold activation (mean) over control (G4E1b-luciferase with empty vectors) from two experiments performed in duplicate. *C*, Brx activates endogenous p38 MAPK in COS-7 cells. Cells were transfected with expression vectors for Brx or MKK6-EE or with empty vector (RSV-0). Transfected cells were serum-starved overnight, and active p38 MAPK was detected in lysates of transfected cells by Western blotting. Positive and negative control lanes contained C-6 glioma cell extracts prepared with or without anisomycin treatment, respectively. *D*, ER β is efficiently phosphorylated by p38 β 2. Active recombinant human p38 β 2 MAPK was incubated *in vitro* with [32 P]ATP and substrate proteins for 15 min at 30 $^{\circ}$ C. Labeled proteins were resolved by electrophoresis and detected by autoradiography. Phosphorylation substrates were no protein (*lane 1*), recombinant GST-ATF-2 (*lane 2*), BSA (*lane 3*), and recombinant ER β (*lane 4*).

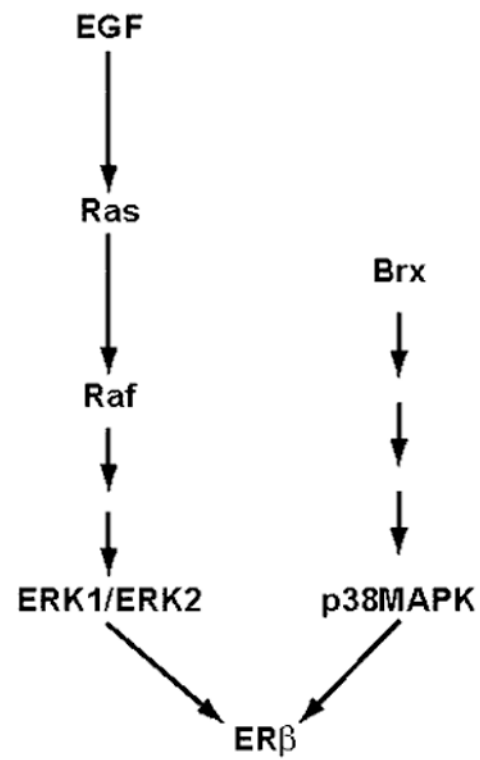


Fig. 5.
Brx augments ligand-dependent activity of ER β via a p38 MAPK pathway.