The Hormonal Response of Estrogen Receptor β Is Decreased by the Phosphatidylinositol 3-Kinase/Akt Pathway via a Phosphorylation-dependent Release of CREB-binding Protein

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The hormonal response of estrogen receptors (ER) α and ERβ is controlled by a number of cofactors, including the general transcriptional coactivator CREB-binding protein (CBP). Growing evidence suggests that specific kinase signaling events also modulate the formation and activity of the ER coactivation complex. Here we show that ERβ activity and target gene expression are decreased upon activation of ErbB2/ErbB3 receptors despite the presence of CBP. This inhibition of ERβ involvement activation of the phosphatidylinositol 3-kinase/Akt pathway, abrogating the potential of CBP to facilitate ERβ response to estrogen. Such reduced activity was associated with an impaired ability of ERβ to recruit CBP upon activation of Akt. Mutation of serine 255, an Akt consensus site contained in the hinge region of ERβ, prevented the release of CBP and rendered ERβ transcriptionally more responsive to CBP coactivation, suggesting that Ser-255 may serve as a regulatory site to restrain ERβ activity in Akt-activated cells. In contrast, we found that CBP intrinsic activity was increased by Akt through threonine 1872, a consensus site for Akt in the cysteine- and histidine-rich 3 domain of CBP, indicating that such enhanced transcriptional potential of CBP did not serve to activate ERβ. Interestingly, nuclear receptors sharing a conserved Akt consensus site with ERβ also exhibit a reduced ability to be coactivated by CBP, whereas others missing that site were able to benefit from the activation of CBP by Akt. These results therefore outline a regulatory mechanism by which the phosphatidylinositol 3-kinase/Akt pathway may discriminate nuclear receptor response through coactivator transcriptional competence.

Estrogen mediates many aspects in growth, development, and reproduction, through its interaction with estrogen receptors ERα and ERβ. Although encoded by unique genes, the two ERs share the functional domains characteristic of the nuclear hormone receptor family (1). These consist of an N-terminal region (also termed AB region), which confers ligand-independent activation of ERs through its activation function (AF)-1, a highly conserved DNA-binding domain (C) that allows specific binding to genomic response elements, a flexible hinge region (D) that includes signals for nuclear localization and the binding of heat shock proteins, and finally a C-terminal region (EF) that contains the ligand binding domain, and the AF-2 function that mediates hormone-dependent activation.

Increasing evidence suggests that, beside hormonal activation, ER function can be modulated by phosphorylation-dependent mechanisms, involving a wide variety of protein kinases that mostly target the AF-1 domain (2, 3). In particular, direct phosphorylation of ERα AF-1 by MAPK/ERK in response to EGF was shown to induce ERα transactivation in the absence of ligand (4, 5). Similarly, phosphorylation of Ser-167 by pp90RSK1 was described to promote ERα AF-1 activity (6). Activation of phosphatidylinositol 3-kinase (PI3K) and Akt/protein kinase B also contributed to phosphorylate ERα and mediate its ligand-independent activation, an effect shown to oppose the tamoxifen-induced apoptosis in breast cancer cells (7). Although phosphorylation of ERβ has not been examined in detail, ERβ has been proposed as a potential target for intracellular kinases that modulate its transactivation properties. It was found that the ability of EGF and the oncogene Ras to activate ERβ resulted from the MAPK-directed phosphorylation of Ser-106 and Ser-124 within the AF-1 domain leading to favored recruitment of coactivators SRC-1 and CBP (8, 9). Furthermore, the ligand-dependent activation of ERβ by the protooncogene Brx was shown to involve phosphorylation of ERβ in a p38-dependent manner, although the exact site(s) were not described (10). More recently, we reported that activation of ErbB2 and ErbB3, which belong to the EGFR/ErbB receptor...
tyrosine kinase family, by growth factor heregulin resulted in a decrease in the estrogen-dependent cell growth and activity of ERα and ERβ in breast cancer cells (11). However, unlike ERα, this transcriptional repression of liganded ERβ by heregulin was dependent upon ERβ AF-1 function, thereby supporting a repressive role for kinase-mediated pathways in regulating ERβ AF-1 and AF-2 functions. Taken together, the regulation of estrogen receptor activity by phosphorylation is intricate and could dictate receptor function, whether it involves activation or repression.

Recent evidence has emerged suggesting that nuclear receptor coactivators may also serve as points of convergence between the ER and growth factor signaling pathways. Phosphorylation of SRC coactivators has been described to modulate their intrinsic activities in mediating nuclear receptor transcription (12). Coregulatory proteins are often present in limiting concentrations in the nucleus so that modifications of their level of expression as well as their activity can lead to alterations in nuclear receptor signaling. The transcriptional coactivators CREB-binding protein (CBP) and p300 are evolutionary highly conserved proteins, and genetic evidence supports their availability to be critical. In humans, loss of one functional copy of cebp leads to Rubenstein-Taybi syndrome, a haploinsufficiency disorder resulting in mental retardation (13). Through their extremely versatile ability in bridging numerous transcription factors, including most nuclear receptors, with the basal transcription machinery, recruitment of CBP/p300 is important to maintain appropriate transcriptional events (14). One of the likely mechanisms responsible for CBP/p300 recruitment involves phosphorylation. It was reported that phosphorylation of CBP promotes its interaction with several transcription factors, including CREB, Smad3, NFκB p65 subunit, and p53 (15, 16). We have recently shown that MAPK-dependent phosphorylation of ERβ also facilitates the recruitment of CBP to potentiate the ligand-independent activation of ERβ in response to growth factors (9). Given such diversity in the signaling pathways integrated by CBP, it is believed that phosphorylation-mediated events may compete at various levels for the limited availability of CBP.

Here we describe a molecular mechanism by which ErbB2/ErbB3 and PI3K/Akt signaling impairs the activity of ERβ by reducing its ability to recruit and use CBP as a coactivator. The repression by Akt was also found for other nuclear receptors, for which a conserved Akt site may also participate in a manner similar to ERβ. In contrast, nuclear receptors that do not share such homology yielded increased responsiveness to CBP and benefit from the enhanced intrinsic activity of CBP by Akt.

**EXPERIMENTAL PROCEDURES**

**Plasmid Constructs**—Expression of pCMX plasmids coding for ERα, ERβ, CBP, ErbB2, its constitutive variant V659E and ErbB3 receptors, and luciferase reporter constructs vitA2-ERE, pDBx, and UAStkLuc have been described previously (8, 9, 11). ERβ fragments corresponding to the AB (amino acids 1–167) and DEF (amino acids 234–549) regions were obtained by PCR amplification and fused in-frame with the Gal4 DNA binding domain. The ERβ Ser-255 to alanine and the CBP Thr-1872 to alanine mutants were generated by PCR mutagenesis using Pfu polymerase (Stratagene). All constructs were verified by automated sequencing. The expression plasmid coding for the constitutively active PI3K p110α catalytic subunit was a kind gift from J. Downward, and plasmids expressing Akt and K179M kinase dead Akt were generously provided by T. Chan and P. Tsichlis.

**Cell Culture, DNA Transfection, and Luciferase Assay**—Human embryonic kidney 293T cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 5% fetal bovine serum (FBS). The cells were maintained at 37 °C in a humidified atmosphere with 5% CO₂. For transient transfection, cells were seeded in phenol red-free DMEM supplemented with 5% charcoal dextran-treated FBS, and plasmid constructs were introduced into cells using the calcium phosphate precipitation method as described (11). Typically, 50–60% confluent cells were transfected with 2 µg of DNA per well, which include 500 ng of reporter plasmid, 100 ng of receptor expression vector, 250 ng of CMX-βgal, 100 ng each of PI3K and Akt expression vector, and 30 ng of CBP plasmid when indicated. After 5–8 h, the medium was changed, and cells were stimulated with 10 nM estradiol (E2; Sigma) and/or 50 ng/ml heregulin-β (R&D Systems) for 16–20 h or left untreated. For luciferase assay, cells were lysed in potassium phosphate buffer containing 1% Triton X-100, and light emission was measured using a luminometer (Wallac) after the addition of luciferin. Luciferase assays were performed in duplicate from at least three independent experiments, and values were expressed as relative light units normalized to the β-galactosidase activity of each sample.

**Western Analysis and Immunoprecipitation Assay**—Western analysis for the determination of phosphorylated and total Akt was performed as described with minor modifications (11). Briefly, transfected 293T cells were treated with 50 ng/ml heregulin-β for 20 min, washed in ice-cold PBS, and lysed in PBS containing 0.5% sodium deoxycholate, 0.1% SDS, 1% Triton X-100, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitors (Roche Applied Science). Cell lysates were then subjected to SDS-PAGE and proteins transferred to nitrocellulose for immunoblotting. Membranes were incubated at 4 °C with blocking reagent (Roche Applied Science) in TBS, probed with either a rabbit polyclonal antibody against phosphorylated Akt (Santa Cruz Biotechnology) or a mouse anti-Akt monoclonal antibody (Cell Signaling Technology), and signals revealed by ECL using appropriate horseradish peroxidase-conjugated secondary antibodies. The same procedure was used to determine the levels of ERβ, except that cells were transfected with HA-tagged ERβ (WT or S255A) and analyzed by Western using an anti-HA antibody (12CA5). For immunoprecipitation assay, transfected cells were washed in ice-cold PBS and lysed as described above. Cell lysates were precleared before incubation with an anti-CBP antibody (Santa Cruz Biotechnology) and protein A-Sepharose beads at 4 °C. Immunoprecipitates were then washed in lysis buffer, resolved by SDS-PAGE, and analyzed by Western blotting using an anti-HA antibody. Membranes were also probed with an anti-CBP antibody for standardization of CBP levels in each well.
CBP-mediated Regulation of ERβ by Akt

Generation of Hs-ER-stable Clones and RT-PCR—ER-negative Hs578t breast cancer cells were maintained in DMEM containing 10% FBS and transfected with expression vectors for ERα and ERβ as described previously (11), and resistant clones were isolated in the presence of G418 (0.6 mg/ml; Invitrogen) to generate Hs-ERα and Hs-ERβ cell lines, respectively. Stable clones were functionally validated for their respective expression of ERα or ERβ by Western analysis and for their estrogenic response by luciferase assay, compared with mock-transfected Hs-578t cells. Total RNA was isolated from cells using TRIzol reagent (Invitrogen), and RT-PCR analysis was performed as described (17). The relative signal intensity was analyzed (Alpha Innotech, San Leandro, CA) from three separate experiments.

In Vitro Phosphorylation Assay—Bacterially expressed and purified GST fusions of wild type and S255A mutated ERβ were prepared as described (18). For in vitro phosphorylation assay, GST-ERβ fusions immobilized on glutathione-Sepharose 4B beads were resuspended in kinase buffer containing \([\gamma-32P]ATP\) (Amersham Biosciences) and active Akt1 (Cell Signaling) and incubated at 30 °C for 30 min according to the manufacturer’s instructions. Beads were then washed twice in kinase buffer and twice in PBS, and \(32P\) incorporation was determined following SDS-PAGE and autoradiography. Gels were stained with Coomassie Blue to monitor for equal loading.

Fluorescence Microscopy—Cells were seeded on coverslips in a 6-well plate overnight prior to transfection in phenol red-free DMEM supplemented with 5% charcoal dextran-treated FBS. Transient transfections were carried as above using the expression plasmids YFP-CBP and CFP-ERβ. 20 h after transfection, cells were washed twice with cold PBS and fixed in 4% formaldehyde. The coverslips were mounted on microscope slides and examined in fluorescence with excitation/ emission filters of 435/470 nm (for CFP) and 480/535 nm (for YFP) using a Nikon TE-2000 inverted microscope.

RESULTS

ErbB2/ErbB3 Receptor Dimer Activation Impairs the Hormonal Response and Coactivation of ERβ by CBP—Activation of the epidermal growth factor receptor EGFR/ErbB1, a member of the ErbB receptor tyrosine kinase family, is well recognized the epidermal growth factor receptor EGFR/ErbB1, a member of the ErbB receptor tyrosine kinase family, is well recognized.

Coactivation of ERβ by CBP was determined following SDS-PAGE and autoradiography. Gels were stained with Coomassie Blue to monitor for equal loading.

**FIGURE 1.** ErbB2/ErbB3 signaling impairs the hormonal response and coactivation of ERβ by CBP. A, 293-T cells were transfected with an ERβ plasmid and an ErbBLuc reporter, and expression vectors encoding ErbB2 and ErbB3, or a constitutively active ErbB2 (V659E) mutant in the presence of CBP. Cells were then treated with 10 nM E2 and/or 50 ng/ml heregulin (Hrg-β) for 20 h and harvested for transcriptional activity. Luciferase values were normalized to β-galactosidase activity and expressed as fold activation compared with untreated cells set at 1.0. B, 293-T cells were transfected with ERβ plasmids and treated with 50 ng/ml heregulin for 20 min as indicated. Total cell extracts were analyzed by electrophoresis, and endogenous Akt phosphorylation was monitored by Western blotting using a specific anti-phospho Akt. Cell lysates were also analyzed for Akt content using an anti-Akt antibody.

Transactivation of ERβ in control cells, it is unable to prevent the inhibition of the hormonal response of ERβ when the ErbB2/ErbB3 heterodimer is not only expressed but is also stimulated by heregulin-β (Fig. 1A). Despite the presence of CBP, the transcriptional activity of ERβ was decreased in both a hormone-independent and -dependent manner. This inhibition was even more pronounced in cells expressing the constitutive ErbB2 V659E mutant.

Signaling of the EGFR/ErbB family members involves the activation of a variety of kinase pathways. More specifically, activation of the ErbB2/ErbB3 heterodimer has been shown to efficiently couple with the PI3K/Akt pathway, mainly through the intrinsic ability of numerous Src homology 2-binding motifs within ErbB3 that recognize the p85 regulatory subunit of PI3K (20, 21). To evaluate the impact of ErbB2/ErbB3 activation on the Akt pathway, the activity of endogenous Akt was determined by Western analysis using a phospho-specific antibody against Ser-473. Although treatment of mock-transfected 293T cells with heregulin-β did not lead to activation of Akt, indicating that endogenous expression of ErbB3 is negligible, if not absent, an increase in phosphorylated Akt was observed in cells expressing ErbB2/ErbB3 and treated with heregulin-β (Fig. 1B). Similarly, cells expressing the ErbB2 V659E variant in the presence of ErbB3 also showed increased levels of phosphorylated Akt.
Activation of the PI3K/Akt Pathway Mimics the Inhibition of ERβ Response to Hormone in the Presence of CBP through the C-terminal Region of ERβ—The possibility that ErbB2/ErbB3 activation by heregulin-β decreases ERβ activity and its coactivation by CBP by enhancing the activity of Akt was further tested by transient expression of a membrane-bound and constitutively active p110α subunit of PI3K, as indicated. Cells were then treated with 10 nM E2 for 20 h and harvested for transcriptional activity. Luciferase values were normalized to β-actin expression and presented as fold activation compared to untreated cells set at 1.0. Western analysis of ERβ in response to Akt activation. 293T cells were transfected with ERβ in the absence or presence of p110α PI3K and Akt plasmids to trigger the Akt pathway. Cells were then treated with 10 nM E2 or left untreated for 20 h and harvested for Western analysis using an anti-ERβ antibody. Loading was monitored with β-actin for each sample. Cells were transfected with a UAStkLac reporter and truncated forms of ERβ corresponding to the N-terminal or AB region (left) or the C-terminal or DEF region (right) fused to the Gal4 DNA binding domain. Cells were also transfected with p110α and Akt plasmids and treated with 10 nM E2 for 20 h prior to luciferase assay. Luciferase values are expressed as in A.

CBP-mediated Regulation of ERβ by Akt

Effects of activated PI3K on ERβ mainly transit through Akt (Fig. 2A). We next performed Western analysis to ascertain whether the modulation of ERβ activity was not a direct effect of its protein concentration under the conditions used. As shown in Fig. 2B, activation of the Akt pathway led to an accumulation of ERβ in untreated cells. A similar increase was also observed in the presence of estrogen, although the levels of ERβ were slightly lower compared with untreated cells, probably reflecting an increase in ER turnover in response to hormone as reported previously (23). These results suggest that the inhibition in ERβ activity to Akt activation is not related to a decrease in ERβ protein levels.

CBP is known to transactivate estrogen receptors through both its AF-1 and AF-2 activities (9, 24). In an attempt to identify the functional domain within ERβ responsible for its impaired ability to be coactivated by CBP in response to Akt, we used Gal4 fusions of truncated forms of ERβ for which each respective AF-containing domain has been removed. Fig. 2C shows that in Akt-activated cells, the activation of a Gal4-ABβ (corresponding to ERβ amino acids 1–167) on a UAStkLuc reporter was further enhanced by CBP, reaching a near 5-fold increase compared with control cells. The N-terminal domain of ERβ is known to contain several serine residues that are conserved within the recognition motifs for Ser/Thr kinases of the MAPK family, and phosphorylation of specific residues was shown to allow for coactivators such as CBP to be recruited and to potentiate ERβ AF-1 activity (8, 9). However, none of the potential phosphorylation sites within ERβ AB region belongs to a consensus Akt site, suggesting that the enhanced activity of ERβ AF-1 by CBP in response to Akt might possibly result from other kinase pathways activated by Akt or direct effects on CBP itself. We next tested the role of the C-terminal region of ERβ in the same conditions. Cells transfected with a Gal4-DEFβ (amino acids 234–549) showed a reduced hormone-dependent activity to Akt activation in the presence of CBP, mimicking the response observed with full-length ERβ (Fig. 2C, right panel). These results indicate that the repressive effect of activated Akt on CBP-mediated transactivation of ERβ is mediated through a region contained in the C-terminal portion of ERβ, which in the context of the full-length receptor seems to counteract the positive effect on the AF-1 activity.

Serine 255 in the Hinge Region Mediates ERβ Inhibition to ErbB2/ErbB3 Signaling—Our examination of the C-terminal sequence of mouse ERβ revealed a consensus sequence RQRAS255 in the hinge region of ERβ that corresponds to the recognition motif RXRSS(S/T) for the kinase Akt (Fig. 3A). To determine whether Ser-255 is a direct target for Akt-mediated phosphorylation, we used site-directed mutagenesis to convert the serine at position 255 into an alanine and performed an in vitro kinase assay. Fig. 3B shows that disruption of Ser-255 strongly abolished the phosphorylation of ERβ by Akt compared with wild type, indicating that Ser-255 can be efficiently phosphorylated by Akt. We then tested whether Ser-255 was involved in the inhibition of ERβ activity to ErbB2/ErbB3 activation as observed in Fig. 1A. Using the S255A mutant in luciferase assay, we found that the inhibition observed for WT ERβ by either ErbB2/ErbB3 dimer expression or its activation with heregulin-β was completely abrogated by disruption of Ser-255.
CBP-mediated Regulation of ERβ by Akt

(compare Figs. 1A and 3C). Noticeably, the hormonal response of S255A was enhanced upon ErbB2/ErbB3 activation and further potentiated by CBP. This enhanced response to hormone by the S255A mutant was also observed in response to Akt by the S255A mutant was also observed in response to Akt potentiating by CBP. This enhanced response to hormone and potentiating by CBP. This enhanced response to hormone by the S255A mutant was also observed in response to Akt activation using the constitutively active p110α PI3K construct in transfection (Fig. 3C). Therefore, the results indicate the hinge region of ERβ contains a specific site that not only can be targeted by Akt but also dictates responsiveness of ERβ to CBP coactivation in response to Akt signaling pathway. To determine whether Ser-255 is involved in the regulation of ERβ in terms of protein levels, we next performed Western analysis on cells expressing the ERβ S255A mutant. As compared with wild type ERβ (Fig. 2B), the disruption of Ser-255 completely abrogated the accumulation of ERβ in response to Akt activation (Fig. 3D), indicating that Ser-255 is a critical site in the regulation of ERβ levels by the PI3K/Akt pathway.

ERβ Modulates the Intranuclear Behavior of CBP in an Akt-dependent Manner through Serine 255—Studies using fluorescently tagged proteins have demonstrated that expression of the estrogen receptor, in particular ERα, affects the intranuclear organization of coactivators of the SRC/p160 family in response to hormone or anti-estrogens (25–27). Based on our results on the transcriptional response of ERβ to CBP in Akt-activated cells, we investigated whether ERβ could modulate the intranuclear behavior of CBP in response to Akt activation. Expression plasmids encoding a YFP-tagged full-length CBP and a CFP-fused ERβ were generated and functionally validated in luciferase coactivation assay (data not shown). We first determined the intranuclear distribution of CBP by transfecting cells with YFP-CBP in the absence or presence of CBP-ERβ S255A plasmids. Akt was activated by cotransfecting cells with p110α and Akt plasmids. Fluorescence signals were visualized using filters for YFP and CFP shown alone and merged. Cell nuclei were also stained with 4,6-diamidino-2-phenylindole (DAPI). B, CFP is released from ERβ through Ser-255 in Akt-activated cells. Cells were cotransfected with HA-tagged WT or S255A ERβ with HA-actin for each sample. Loading was monitored with HA antibody. Western analysis of ERβ and Akt was analyzed by Western blot. CBP was also monitored in each sample by Western analysis of ERβ and Akt. The particular behavior of CBP has been observed in different cell types under basal or nonactivated conditions, and although not fully characterized, such a pattern was associated to poorly transcribing or transcriptionally inactive compartments devoid of nascent mRNA transcription and active RNA polymerase II (28–30). Given our results on the effects of CBP on ERβ activity, we tested whether ERβ could modulate the intranuclear distribution of CBP by cotransfecting cells with YFP-CBP and CFP-ERβ. Both proteins were shown to colocalize to the nucleus, but the ectopic expression of ERβ strongly diminished the formation of CBP-related speckles, resulting in a more dispersed distribution of CBP throughout the nucleus (Fig. 4A). Interestingly, when the Akt pathway was activated in cells expressing both YFP-CBP and CFP-ERβ, CBP appeared to readopt the formation into speci-
les, whereas the dispersion of ERβ remained unaffected, indicating that Akt can induce a relocation of CBP within the nucleus in the presence of ERβ (Fig. 4A). Given the role of ERβ Ser-255 to impair CBP-mediated coactivation of ERβ in response to Akt, we next tested a CFP-ERβ S255A construct on CBP intranuclear distribution. We observed that as opposed to WT ERβ, expression of the S255A mutant did not favor CBP to fully reform into speckles, but instead CBP remained in a more diffuse pattern (Fig. 4A). This distinct behavior of CBP in response to WT versus S255A ERβ expression was also observed in the presence of hormone (data not shown), indicating that both the unliganded and liganded receptor affect CBP nuclear distribution to Akt activation in a similar manner. These results suggest that CBP relocates within the nucleus in response to Akt activation and that this behavior depends on the presence of ERβ in a manner specific to Ser-255.

**Cellular Activation of Akt Releases CBP from ERβ through Serine 255**—The observation that CBP could relocalize within the nucleus in a manner dependent of ERβ, and that Ser-255 seems to modulate that behavior in response to Akt activation prompted us to determine the effect of activation of the PI3K/Akt pathway on the interaction of ERβ with coactivator CBP. We found that under basal conditions CBP potently coimmunoprecipitated with ERβ and that this interaction was further stabilized in the presence of estradiol (Fig. 4B), thus correlating with the enhanced activation of ERβ by hormone and CBP (Figs. 1A and 2A). However, such interaction was strongly disrupted in Akt-activated cells independently on the presence of hormone (Fig. 4B). We then tested the S255A mutant using similar conditions and found that, as opposed to WT ERβ, CBP could efficiently coimmunoprecipitate the mutant ERβ in the absence or in the presence of estradiol despite activation of Akt in cells (Fig. 4B). These results therefore provide a role for ERβ Ser-255 to induce a release of CBP from ERβ in response to Akt activation.

Akt Promotes the Intrinsic Transcriptional Activity of CBP through Thr-1872—CBP can be phosphorylated by several kinase signaling pathways, such as cyclin-dependent kinase or MAPK/ERK, leading to up-regulation of its histone acetyltransferase activity and therefore its intrinsic potential to activate transcription (31). To determine how CBP could affect transcription by ERβ in response to Akt, we generated a Gal4 fusion of full-length CBP which, by interacting onto a UAStkLuc reporter, allows us to monitor directly CBP transcriptional activity in a luciferase assay. Cells transfected with Gal4-CBP showed a 4-fold activation in luciferase activity compared with control, was observed upon expression of full-length CBP which, by interacting onto a UAStkLuc reporter and expression vectors encoding ERβ, ERβ S255A mutant, or ERα in the presence of CBP or T1872A plasmid. The p110α/Akt plasmids were used to trigger Akt in cells. After transfections, cells were treated with 10 nM E2 for 20 h or vehicle, and transcriptional activity was measured. Normalized luciferase values are expressed as fold activation compared with control cells set at 1.0.

**FIGURE 5. Akt promotes the intrinsic transcriptional activity of CBP through Thr-1872.** A, intrinsic ability of CBP to activate transcription was assessed by transfecting cells with a UAStkLuc reporter in the presence or absence of Gal4 fusions of CBP or CBP T1872A mutant and p110α/Akt plasmids. Cells were harvested 20 h after transfection and analyzed for luciferase activity. B, Western analysis of CBP and T1872A mutant in response to Akt activation. Loading was monitored with β-actin for each sample. C, Thr-1872 of CBP is not involved in the response of ERβ to Akt. 293T cells were transfected with a EREtkLuc reporter and expression vectors encoding ERβ, ERβ S255A mutant, or ERα in the presence of CBP or T1872A plasmid. The p110α/Akt plasmids were used to trigger Akt in cells. After transfections, cells were treated with 10 nM E2 for 20 h or vehicle, and transcriptional activity was measured. Normalized luciferase values are expressed as fold activation compared with control cells set at 1.0.

its activity. We found that not only was the response to Akt activation completely abrogated by the mutation, but the basal activity was also severely impaired (Fig. 5A), indicating that Thr-1872 is a crucial regulatory site for CBP activity. The T1872A mutation did not significantly affect the steady-state levels of CBP expressed in cells, and Akt activation did not modulate wild type or mutated CBP levels as shown by Western analysis (Fig. 5B). Given the ability of CBP to respond to Akt through Thr-1872, we next tested whether this site was involved in the response of ERβ and of ERα to Akt. Although the CBP T1872A mutant was less efficient in promoting ERβ response to estrogen, we found that it behaves similarly as WT CBP in the inhibition of ERβ by Akt, indicating that these effects were independent of CBP Thr-1872 (Fig. 5C). However, the activation of ERβ S255A by Akt in the presence of WT CBP was lost when CBP T1872A mutant was expressed in cells. Similar results were obtained with ERα (Fig. 5C), suggesting that in contrast to ERβ, ERα seems to benefit from the enhanced activity of CBP to Akt in a manner dependent on Thr-1872.

**Estrogen-responsive Genes Are Regulated Differently by Heregulin in ER-expressing Stable Clones**—Based on our results on the apparent difference between the ERα and ERβ response to CBP when Akt is activated and to delineate each ER contribution, we generated ERα- and ERβ-expressing stable clones using ER-negative Hs578t breast cancer cells. Hs578t cells are an appropriate model to study the effect of Akt because they exhibit high basal Akt activity through ErbB receptor signaling and mutated active Ras (33, 34). In addition, Akt can be further activated by heregulin-β in each Hs-ER stable clone in a time-
dependent fashion (Fig. 6A), indicating that these cells maintain the ability to respond to heregulin-β (35). Stable expression of ERα or ERβ also confers enhanced estrogen-dependent activation of Akt compared with negative cells (Fig. 6A). Using RT-PCR analysis on cathepsin D1 (CatD1) and progesterone receptor (PR), two recognized estrogen-responsive genes, we found their expressions were enhanced by estradiol in both ER stable clones, compared with negative control cells (Fig. 6B). However, these increases were severely impaired by the addition of heregulin-β to Hs-ERα cells, therefore correlating with the results obtained in luciferase assays. In contrast, treatment of Hs-ERβ cells with heregulin-β further potentiated the estrogen-stimulated expression of both genes (Fig. 6B). This suggests that the regulation of CatD1 and PR expression by ERβ was more dependent on the effect of heregulin-β than the one through ERα (Fig. 6B). Under these conditions, the CBP steady-state levels were not significantly modified in Hs-ERα and Hs-ERβ cells (Fig. 6C).

A Conserved Akt Site Can Predict the Transcriptional Response of Nuclear Receptors to CBP—Based on our results on the critical role of Ser-255 in regulating the response of mouse ERβ to Akt and CBP coactivation, we checked whether the Akt motif containing Ser-255 was conserved within the nuclear receptor family. It should be noted that Ser-255 is located within the hinge region of ERβ, which is generally more conserved between ERs and orphan estrogen-related ERRs than with other nuclear receptors. As such, the sequence alignment in Fig. 7A showed that although ERα and all three isoforms of ERR contain the necessary arginine residue at position −3, and

![FIGURE 6. Estrogen-responsive genes are regulated differently by ERα and ERβ in response to heregulin-β. A, activation of Akt in stable Hs-ERα and Hs-ERβ clones in response to heregulin-β and estrogen. ERα and ERβ-expressing stable clones have been generated using ER-negative Hs578t cells (control) and were treated with 50 ng/ml heregulin-β for the indicated time or 10 nM E2 for 60 min. Endogenous Akt phosphorylation was monitored by Western blotting using a specific anti-phospho Akt. Cell lysates were also analyzed for Akt content using an anti-Akt antibody. B, RT-PCR analysis of ER-responsive genes from Hs-ER clones and Hs control cells treated with 10 ng/ml heregulin-β and/or 50 nM E2 for 20 h prior to RNA isolation. Representative images are shown from at least three separate experiments. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression was used to normalize samples. C, Western analysis of CBP in Hs-ER stable clones and Hs control cells treated as above. Samples were normalized for protein loading with β-actin.

![FIGURE 7. A conserved Akt site dictates the transcriptional response of nuclear receptors to CBP. A, sequence alignment for predicted Akt phosphorylation site of nuclear receptors. Shown are the predicted phosphorylated serines (arrow) with the obligatory arginine residues at position −3 and the less stringent arginine/lysine residues at position −5 of the receptor sequences aligned with mouse ERβ. The predicted Akt site is conserved in human and mouse ERRβ and GR. B, response of nuclear receptors to Akt and CBP coactivation. 293T cells were transfected with expression plasmids for the indicated nuclear receptors with their respective luciferase reporter as follows: EREtkLuc for ERα and the estrogen-related receptor ERR isoforms; GREtkLuc for GR and PR; PPREtkLuc for PPARγ; and CREtkLuc for the cAMP-responsive binding protein CREB. Cells were also transfected with CBP and/or p110α/Akt plasmids and treated with ligands as follows: 10 nM E2 (ERα), 10 nM dexamethasone (GR), 10 nM progesterone (PR), or 1 μM troglitazone (PPARγ) for 20 h prior to determination of luciferase activity. Normalized luciferase values are expressed as fold activation compared with untreated cells set at 1.0 for each receptor.]
the less stringent arginine/lysine residues at position −5 of the canonical site for Akt (36) in their respective hinge regions, only ERRβ possesses the expected phosphorylated serine (Fig. 7A). It is interesting to note that as opposed to the mouse and rat isoforms, human ERRβ does not contain a serine at the corresponding position but rather has a negatively charged aspartic acid residue. In addition, human, mouse, and rat forms of ERα do not share the conserved serine residue, having a glycine or leucine when aligned with ERβ Ser-255 (mERα is shown in Fig. 7A). Although no perfect consensus site for Akt could be found in glucocorticoid receptor (GR) and PR, a putative Akt site conserved in mouse and human GR was found with the required arginine residue at position −3 and was aligned with ERβ. To address how other nuclear receptors responded to Akt- and CBP-mediated coactivation and to find a possible correlation with respect to their sequence homology with ERβ Ser-255, we tested various nuclear receptors in the luciferase assay. Using an ERε-driven luciferase reporter known to bind and respond to ERRs as dimers (37), we found that coexpression of CBP increased the activity of the three ERR isoforms by 2–3-fold in 293 cells (Fig. 7B). Interestingly, when Akt activation was induced with p110α PI3K expression, CBP only failed to further transactivate ERRβ, whereas ERRα and ERRγ reached 3- and 5-fold activation, respectively, compared with controls. Under these conditions, the response of ERRβ to Akt activation and the inability of CBP to potentiate transactivation strongly correlate with what we observed with ERβ, and therefore point to a shared role for the putative Ser-191 Akt site of ERRβ that overlaps with Ser-255 of ERβ. This observation also applies to GR for which CBP-mediated coactivation was severely abrogated in response to Akt. Conversely, among the receptors tested that do not share homology with ERβ Ser-255, we found that ERα, PR, and peroxisome proliferator-activated receptor PPARγ were all further activated by CBP in Akt-activated cells in the presence of their respective ligand (Fig. 7B). CBP, which has originally been described to directly interact with the CAMP-responsive binding protein CREB, also potentiated CREB activation to Akt. Hence, the impaired ability of CBP to transactivate ERβ in response to Akt can be transposed to other receptors that share an apparent homology with the ERβ Ser-255-containing motif. Accordingly, at least for those receptors tested that do not fit into that category, they seem to benefit from the enhanced intrinsic activity of CBP in response to activation of the PI3K/Akt pathway.

**DISCUSSION**

Increasing evidence suggests that besides ligand activation, nuclear receptors are responsive to kinase signaling mechanisms, and for estrogen-responsive tissues in particular, this may represent a mean to regulate the different ER-mediated transcriptional pathways (2, 3). More recently the idea that signaling pathways can also mediate transcriptional repression of estrogen receptors has led us to further investigate how these pathways are tightly controlled (11, 38). Here we show that activation of ErbB2/ErbB3 receptors and the PI3K/Akt pathway can impair the transcriptional response of ERβ to estrogen and its coactivation by CBP. The mechanism underlying ERβ inhibition involves Ser-255, which upon its phosphorylation by Akt prevents CBP from interacting with ERβ, therefore abrogating ERβ activity.

Dimerization of ErbB3 with its preferred partner ErbB2 is considered the most potent combination of ErbB receptors in terms of cellular growth and transformation (39). Deregulated signaling by ErbB2/ErbB3 has been associated with detrimental mitogenic potential in a number of reproductive cancers and the correlation of ErbB2 with ERα status has served as a predictive factor in endocrine-based therapy (40, 41). However, the response of ERβ to ErbB2/ErbB3 activation is not clearly defined, and the exact role of ERβ in tumorigenesis remains uncertain. We found that the transcriptional response of ERβ to estrogen was diminished upon activation of ErbB2/ErbB3 with ErbB3 ligand heregulin or the constitutive ErbB2 variant V659E derived from the Neu oncogene. These results have been transposed to ERβ-expressing stable breast cancer cells, therefore altering endogenous ER-responsive genes, as observed with the down-regulation of CatD1 and PR. Both conditions were associated with increased cellular activation of Akt.

Intriguingly, although the coactivator CBP potently contributes to enhance basal and estrogen-dependent response of ERβ, it became inefficient to render optimal activation of ERβ in Akt-activated cells. These effects seem to be specific to CBP because we showed that the coactivator SRC-1 was able to relieve ERβ inhibition to heregulin signaling (11). Based on our observations that SRC-1 and CBP can trigger ERβ response to growth factors in an AF-1-dependent manner (8, 9), it was predicted that both coactivators would behave similarly. In an attempt to delineate the role of the AF-1 domain, we found by using an N-terminal form of ERβ that CBP promoted ERβ activation to Akt, suggesting a positive effect of the Akt pathway that obviously did not correlate with the response of the full-length receptor. Although the N-terminal region of ERβ contains phosphorylation sites described to be directly phosphorylated by MAPK conferring AF-1 activity of the receptor in response to EGF or Ras (8, 18), it does not have a consensus site for Akt, and therefore up-regulation in AF-1 activity by Akt might relate to possible indirect effects, including activation of CBP itself, as predicted in Fig. 5A. Removal of the AF-1 region demonstrated a similar inhibitory pattern as observed with the full-length ERβ, and further identified Ser-255 as a functional site responsible for the inhibition of ERβ to ErbB2/ErbB3 and Akt signaling. Together, these findings clearly demonstrate that many signaling events converge to ERβ to regulate cofactor assembly and transcriptional activity either in a positive or negative manner.

Our observation that ERβ cellular levels were augmented by the PI3K/Akt pathway in the presence or absence of estrogen raised the possibility that ERβ turnover is regulated by Akt. Interestingly and consistent with this idea is the apparent opposite regulation of the S255A mutant in the same conditions, suggesting that Ser-255 is a determinant involved in ER recycling in response to Akt signaling. Studies using ERα have integrated the response to estrogen with the cellular degradation of the receptor, thus supporting a means by which target cells can sustain or limit a hormonal response through a continuous receptor turnover. ERα has been shown to be degraded through the proteasome pathway in a ligand-dependent manner (23,
CBP-mediated Regulation of ERβ by Akt

42), and blocking proteasome activity impaired the ability of ERα to mediate a transcriptional response to hormone (43−45), suggesting that ER turnover is necessary for receptor activity. Similarly, activation of the PI3K/Akt through platelet-derived growth factor stimulation of smooth muscle cells was shown to target CREB for degradation in a phosphorylation-dependent manner (46).

Recent studies derived from fluorescent-based approaches have revealed the dynamic nature of ERα within the nucleus and its behavior with transcriptional coactivators in response to hormonal stimuli (26, 44, 47, 48). Under basal conditions and in the absence of ERβ, CBP adopted a speckled pattern with a subpopulation being more diffuse within the nucleus. The reason for such behavior is unclear, but the ability of CBP to form speckles has been observed in different cell types under nonactivated conditions, and has been associated with poorly transcribing or transcriptionally inactive compartments devoid of nascent mRNA transcription and active RNA polymerase II (28−30). The speckled clustering of CBP has also been shown to not segregate with regions of histone hyperacetylation, suggesting a decreased activity of CBP (49). However, such compartmentalized pattern of CBP was not always related to transcriptional inactivity, as the promyelocytic leukemia protein was identified as a nuclear receptor coactivator that segregates CBP into nuclear bodies (50). Interestingly, the expression of ERβ resulted in a marked decrease in speckle formation and a more diffuse pattern of CBP throughout the nucleus that overlapped with the distribution of ERβ. This colocalization of ERβ and CBP occurred in the absence or presence of estrogen, therefore correlating with the enhanced activation of ERβ by CBP in luciferase assays. However, the activation of the PI3K/Akt pathway has the distinct effect of driving CBP to readopt a speckled pattern, whereas ERβ remained diffused, coinciding with a reduced ERβ activity. Although these studies did not allow assessing directly the interaction between CBP and ERβ, it is interesting to note that although the S255A mutant was tested, the formation of CBP-related foci was greatly reduced in Akt-activated cells. The expression of ERβ therefore allows for a redistribution of CBP in the nucleus, which implicates Ser-255 as a determinant in the response to Akt. Consistent with these observations, activation of Akt led to a release of CBP from ERβ even in the presence of estrogen as determined in the coimmunoprecipitation assay, whereas disruption of Ser-255 was found to stabilize such interaction. These observations emphasize the role of Ser-255 in mediating CBP release from ERβ in a phosphorylation-dependent process. Although phosphorylation provides an important mechanism by which steroid hormone receptors can be activated (3), increasing evidence suggests that phosphorylation also mediates nuclear receptor inhibition or repression involving various mechanisms and different kinase pathways. Phosphorylation of serine 236 by C/EBPα and Ser-112 by MAPK reduced the ligand binding affinity and activity of PPARγ (52). In the case of the androgen receptor, Ser-210 and Ser-790 were identified as phosphorylation sites for Akt, which inhibited the association of AR with coactivator ARA70 (53). Our results therefore provide a mechanism by which ErbB2/ErbB3 and Akt signaling impairs ERβ activity through a phosphorylation-dependent release of coactivator CBP.

CBP/p300 are general signal integrators common to many transcription factors, and evidence suggests that part of the mechanism that regulates their function involves direct phosphorylation (14). Interestingly, phosphorylation of Ser-1834 by Akt was shown to promote p300 histone acetyltransferase activity and its transcriptional potential (32). By mutating the corresponding site within CBP, we observed that Thr-1872 is essential to promote CBP enhanced transcriptional capacity in response to Akt activation. However, ERβ was not able to benefit from this improved activity as opposed to the S255A mutant, suggesting that phosphorylation of ERβ at Ser-255 may prevail in the response of ERβ to Akt. Indeed, phosphorylation of Ser-255 impaired CBP recruitment to ERβ and did not allow for proper CBP-mediated coactivation, therefore preventing any potential of CBP to activate ERβ. A similar mechanism was described in the inhibition of C/EBPβ-targeted gene expression by insulin, except that the phosphorylation of Ser-1834 in the C/H3 domain of p300 by Akt prevented p300 to interact with C/EBPβ (54). CBP Thr-1872 is also contained in the C/H3 domain, which is described to mediate the recruitment of many transcription factors to CBP/p300 (14). However, our results suggest that Thr-1872 of CBP does not regulate the transcriptional response of ERβ to Akt. A recent report has described the interaction of ERα with the CBP C/H3 domain in the presence of an anti-estrogen, as opposed to the previously recognized N-terminal interaction domain of CBP for agonist-bound nuclear receptors, but whether phosphorylation of CBP was involved has not been determined (55).

Genetic studies have established that the cellular availability of CBP is critical for normal physiologic functions, and as a coactivator that integrates the effects of several transcription factors, this may represent a mean by which CBP can discriminate between various regulatory pathways (16, 56). As such, while testing other members of the nuclear receptor family, we found that unlike ERβ, the activation of ERα by Akt was potentiated in the presence of CBP and further contributed to enhance the expression of known ER target genes such as those encoding CatD1 and PR, in stably ERα-expressing breast cancer cells. ERα does not contain the corresponding Ser-255 found in ERβ, but an Akt site within ERα AF-1 domain, which is absent in ERβ, has been described to functionally activate ERα (7, 57). Such isoform-selective coactivation of ERs by CBP may represent a mechanism by which CBP can discriminate between ERα- and ERβ-regulated pathways in response to Akt signaling. This mechanism can become important in pathologic conditions such as early breast cancer, in which activation of Akt is extremely frequent as a consequence of ErbB2 amplification (58). Clinically, Akt activation strongly correlates with ERα in breast tumors, whereas the prognostic value of ERβ is not established (40, 59). It therefore seems interesting to propose that the negatively charged aspartic residue that corresponds to mouse Ser-255 could predict a reduced response of human ERβ to CBP coactivation. Clearly, further studies are needed to unravel these distinctions.

The ER isoform-specific effect of CBP by the PI3K/Akt pathway has also been observed between ERR members. As opposed
to ERRs and ERRγ. ERRβ contains a consensus for Akt found within the same region as ERβ and was found negatively regulated by Akt in the presence of CBP. Although structurally closely related to the ERs, the ERRs do not exhibit estrogen binding and are still considered orphan receptors without a known endogenous ligand. However, our results predict that ERRs can be selectively regulated by kinase signaling pathways such as PI3K/Akt. With the emerging role of ERR isoforms in modulating ER functions and target gene expression (37, 60), it will be of interest to investigate whether such regulation might influence these aspects.

The present findings demonstrate a molecular mechanism by which the PI3K/Akt pathway may dictate the activity of ERβ and other nuclear receptors, through their selective ability to use CBP as a coactivator. With the impact of ErbB2 signaling and/or Akt activation pathways to also affect CBP intrinsic coactivation properties, elucidation of the various regulatory signals that dictate nuclear receptor-coactivator functions might provide insights into their integrative function.

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