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AIB1 Is a Conduit for Kinase-Mediated Growth Factor Signaling to the Estrogen Receptor

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Growth factor modulation of estrogen receptor (ER) activity plays an important role in both normal estrogen physiology and the pathogenesis of breast cancer. Growth factors are known to stimulate the ligand-independent activity of ER through the activation of mitogen-activated protein kinase (MAPK) and the direct phosphorylation of ER. We found that the transcriptional activity of AIB1, a ligand-dependent ER coactivator and a gene amplified preferentially in ER-positive breast cancers, is enhanced by MAPK phosphorylation. We demonstrate that AIB1 is a phosphoprotein in vivo and can be phosphorylated in vitro by MAPK. Finally, we observed that MAPK activation of AIB1 stimulates the recruitment of p300 and associated histone acetyltransferase activity. These results suggest that the ability of growth factors to modulate estrogen action may be mediated through MAPK activation of the nuclear receptor coactivator AIB1.

The estrogen receptor (ER) is a member of the class I family of nuclear receptors (NRs) (for a review, see reference 24). It contains three major functional domains: an N-terminal activation domain (AF-1), a DNA-binding domain (DBD) highly conserved among other NRs, and a C-terminal hormone-binding domain which contains a second activation domain (AF-2) (19, 38). Although AF-1 and AF-2 contribute synergistically to the transcription of targeted genes, they have different mechanisms of activation. AF-1 activity is highly dependent on phosphorylation of serine 118 by mitogen-activated protein kinase (MAPK) (18). In contrast, the more potent AF-2 is activated by the binding of estrogenic ligands (3).

ER-mediated gene transcription is regulated at yet another level depending on the ligand; ER interacts with corepressors or coactivators that inhibit or enhance its activity on target genes. In the absence of ligand, ER is sequestered in the nucleus by interaction with heat shock proteins (29, 33). When activated by agonist ligand binding, ER exerts its action by promoting chromatin remodeling and stimulating the basal transcriptional machinery through interaction with a variety of coactivators (1, 14–16, 23, 27, 28, 34). One of the best-characterized groups of NR coactivators is the p160 family. When bound to agonists such as estradiol, ER AF-2 engages signature motifs (LXXLL) in the center of the p160 molecule (11). Recent studies have shown that the AF-1 domain of ER also interacts with p160 coactivators (40, 42), though the interaction seems to occur at a different site found in the C terminus of the p160 molecule. The p160 coactivators also contain two activation domains, AD1 and AD2, which are localized in the C terminus (6, 7, 41) and bind the secondary coactivators p300/CREB binding protein (CBP) and coactivator-associated arginine methyltransferase 1 (CARM1), respectively. Thus, AD1 and AD2 act as signal output domains in the process of transcriptional activation (22). Mutations in the AD1 region greatly reduced or eliminated the ability of p160 proteins to bind CBP or p300 and to serve as coactivators for NRs (7, 41),

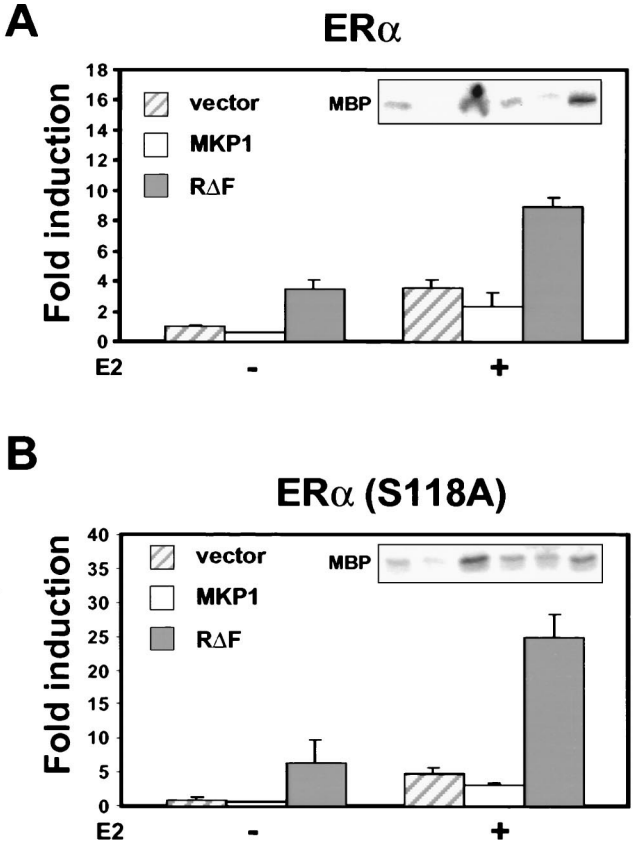

FIG. 1. The MAPK pathway promotes ER-signaling. (A) COS cells were transiently cotransfected with the wild-type human ERα and either MKP1 or constitutively activated MEK1 (RΔF). The transfection also included the luciferase reporter under the control of the ERE2 and tk-lacZ as an internal control. One day following transfection, cells were stimulated with 10 nM 17-β-estradiol (E2) (+) or with ethanol alone (−). On the third day, cells were lysed and assayed for the reporter activity. Values represent the ratio between luciferase units and β-galactosidase, relative to the basal activity of either ER or ER(S118A) in the absence of estrogen. (B) ERα containing the S118A mutation was cotransfected into COS cells as described above. Autoradiographs in the insets demonstrate the MAPK activity for each of the transfection conditions, using MBP as a substrate.

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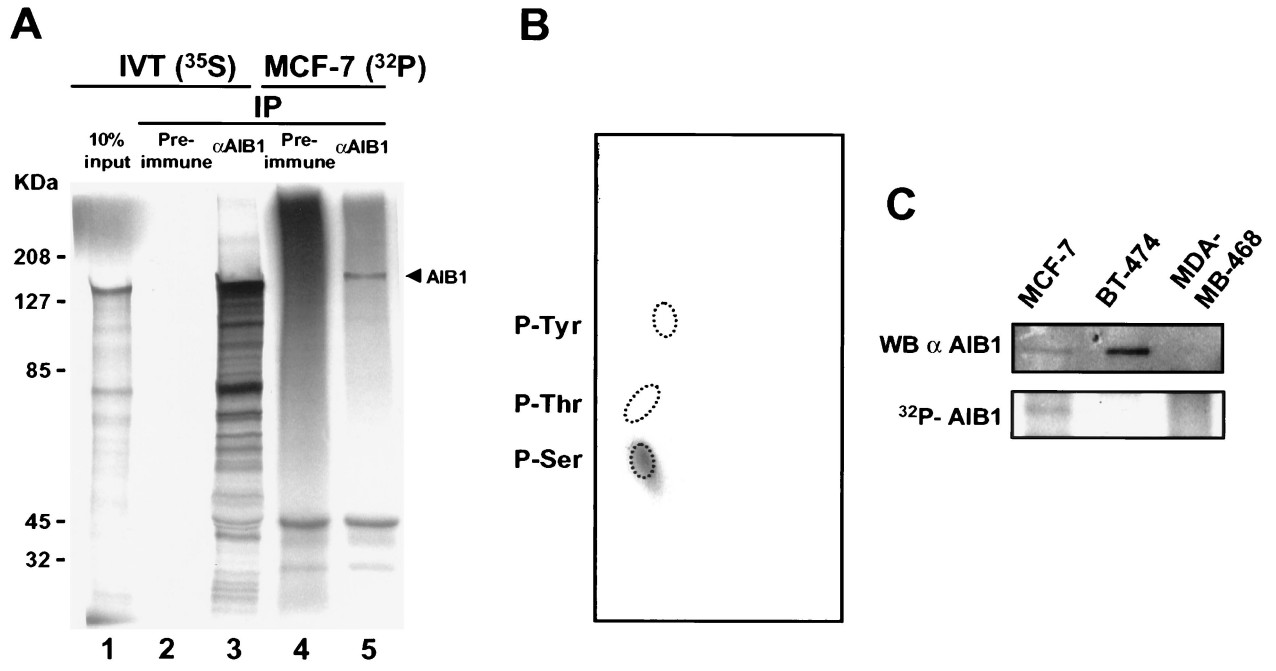

FIG. 2. AIB1 is phosphorylated in vivo. (A) In vitro translated (IVT) AIB1 was labeled with [^{35}S]Met and immunoprecipitated (IP) with either preimmune serum (lane 2) or anti-AIB1 antibodies (lane 3). Subconfluent MCF-7 cells were labeled with [^{32}P]orthophosphate for 6 h and immunoprecipitated with either preimmune serum (lane 4) or anti-AIB1 antibodies (lane 5). Immune complexes were resolved by SDS–8% PAGE. The gel was dried and exposed for autoradiography. The figure is representative of four independent experiments. (B) The band corresponding to AIB1 was excised from the gel and treated with HCl to total hydrolysis, analyzed by TLC together with phosphoamino acid markers, developed with ninhydrin to reveal the mobility of nonradioactive phosphoaminoacids (indicated by dashed circles), and subsequently exposed for autoradiography. (C) Breast cancer cell lines MCF-7, BT-474, and MDA-MB-468 were analyzed by Western blotting (WB) for the expression of AIB1. Each well was loaded with 30 μg of total protein from whole-cell lysates (upper panel). In parallel, cells were labeled with [^{32}P]orthophosphate for 6 h, and 0.8 mg of total protein from each cell line lysate was immunoprecipitated with anti-AIB1 antibodies (lower panel).

suggesting that AD1 is the principal coactivator domain responsible for downstream signaling through p300/CBP. In addition, the DRIP-ARC-TRAP-SMCC complex has also been implicated in activation mediated by several NRs, including the thyroid hormone and vitamin D receptors (17, 31). However, the exact role this complex plays in ER-mediated activation remains to be determined.

AIB1 (also named RAC3, ACTR, SRC-3, or p/CIP in mice) (7, 21, 36, 39) is amplified in certain breast and ovarian cancers (1) and is a member of the p160 family of coactivators (1, 21). Furthermore, AIB1 amplification is preferentially found in ER- and progesterone receptor-positive breast cancers (2). These findings suggest that AIB1 may play a critical role in steroid receptor signaling and breast cancer development. Other members of this family include SRC-1 and TIF2 (also named GRIP1); however, there is as of yet no evidence that they play an important role in human breast cancer.

Growth factors of the insulin-like growth factor (IGF) and epidermal growth factor (EGF) family and their receptors have also been implicated in the development and progression of breast tumors (4, 20, 30). These molecules signal by triggering a cytosolic kinase cascade, including the activation of MAPK. In mice lacking ERα, both estrogen and EGF stimulation of uterine growth is disrupted (10). Thus, ER may mediate the transcription of target genes by integrating different signals from growth factor-activated kinases and the binding of steroid hormones. Accordingly, activation of cytosolic kinases by growth factors may constitute a mechanism for regulating NR responsiveness. We hypothesized that ligand-dependent coactivators such as AIB1 may mediate one level of the cross talk between growth factors and ER. To test this hypothesis, we evaluated the role of MAPK in AIB1-mediated coactivation. Our results suggest a novel mechanism by which the MAPK signaling pathway is coupled to the regulation of gene transcription by modulation of AIB1 transactivation capacity.

MATERIALS AND METHODS

Cell culture and transfection. MCF-7 and MDA-MB-468 human breast cancer cells, COS African green monkey kidney cells, and BOSC fetal human kidney cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). BT-474 human breast cancer cells were cultured in RPMI 1640 supplemented with 10% FBS. Transfections were performed with FuGene 6 reagent (Boehringer Mannheim) according to company specifications. For estrogen stimulation (10 nM for 24 h), cells were seeded in DMEM phenol-red-free medium before transfection. The medium also included 10% charcoal- and dextran-treated FBS (HyClone) to remove estrogens and glucocorticoids. For reporter assays, cells were plated at 1.5×10^5/well in six-well dishes. To assay ER-dependent reporter transactivity, the following amounts of plasmids were used: ERα or ER(S118A), 30 ng; ERE2-tk-luciferase, 500 ng; tk-lacZ, 20 ng; AIB1, 700 ng; MKP1, 500 ng; and RΔF, 900 ng. In Gal4-DBD-dependent reporter assays, the plasmid amounts used were 25 ng of Gal4-DBD or an equimolar ratio of Gal4-DBD AIB1 constructs, 500 ng of luciferase reporter containing five copies of upstream activation sequence (UAS), and 20 ng of tk-lacZ. In all cases, transfections were compensated with empty vector pcDNA3.1 to yield equimolar concentrations of plasmid DNA. The association of p300 and AIB1 was studied in BOSC cells. Sixty-millimeter culture dishes were seeded with 2.5×10^6 cells that were transfected on the next day with 4 μg of pCIp300, 4 μg of Gal4-DBD constructs, and either 2 μg of RΔF or 750 ng of pcDNA3.1. The MEK inhibitor U0126 was purchased from Promega (catalog no. 1121).

Plasmids. The ER S118A mutation was created in the pcDNA3.1− vector using the oligonucleotides 5′CCCGCCGCCGCAGCTGGCGCCTTTCCTGCAGCCCC3′ and 5′GGGGCTGCAGGAAAGGCGCCAGCTGCGGCGGCGGG3′ by PCR (QuickChange site-directed mutagenesis kit; Stratagene). pcDNA3.1+AIB1 was provided by Paul Meltzer (National Institutes of Health, Bethesda, Md.). Full-length pCMX Gal4-DBD AIB1 and AIB1 fragment 556-1420 were constructed by subcloning as *Nhe*I and *Ssp*I/*Nhe*I fragments, respec-

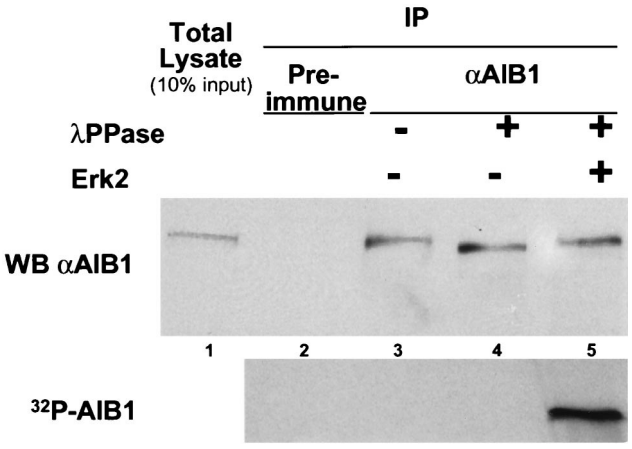

FIG. 3. AIB1 is a substrate of MAPK. Proliferating MCF-7 cells were lysed, and aliquots containing 3 mg of total protein were used for immunoprecipitation (IP) and kinase reactions. Lysates were immunoprecipitated with preimmune serum (lane 1) or with immunopurified anti-AIB1 polyclonal antibodies (lanes 3 to 5). AIB1 immune complexes were split into thirds. One third received no further treatment (lane 3). The other two thirds were treated with protein λ-phosphatase (λPPase) (lane 4), and after a washing, one third was further resuspended in 30 μl of buffer containing 100 μM ATP and treated with 0.1 μg of active Erk2 (Upstate Biotechnology) for 1 hour at 30°C (lane 5). In parallel, the other sample was treated with 0.1 μCi of [^{32}P]ATP and resolved independently. Gels were either Western immunoblotted (WB) with AIB1 antibodies (upper panel) or dried on Whatman paper and exposed for autoradiography (lower panel). Similar results were obtained in three independent experiments.

tively. Other AIB1 fragments were generated by adding appropriate flanking restriction sites by PCR, subcloning into the pCMX Gal4-DBD vector, and sequencing to confirm orientation.

Antibodies. Monoclonal antibodies against p300 (clone RW128) were kindly provided by David Livingston (Dana-Farber Cancer Institute, Boston, Mass.). Polyclonal antibodies against Gal4-DBD were purchased from Santa Cruz Biotechnology (no. sc-577) and used to immunoprecipitate Gal4-DBD–AIB1 proteins from transient transfected cells. Western blot analysis of Gal4-DBD proteins was performed with a monoclonal antibody against Gal4-DBD (Santa Cruz Biotechnology; no. sc-510). To test whether AIB1 was a phosphoprotein, we first developed polyclonal antibodies against glutathione *S*-transferase (GST)-AIB1 (amino acids 695 to 933). The rabbit serum was then affinity purified using GST-AIB1 which had been cleaved by thrombin and covalently immobilized to Affi-Gel 15 (Bio-Rad). The preimmune serum was also purified in the same manner.

Immunoprecipitations and Western blotting. Cells were lysed in a buffer containing 20 mM HEPES buffer (pH 7.5), 150 mM NaCl, 10 mM EGTA, 40 mM β-glycerophosphate, 1% NP-40, 2.5 mM $MgCl_2$, 2 mM orthovanadate, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10 μg of aprotinin/ml and 10 μg of leupeptin/ml. Following centrifugation at 15,000 × *g* for 10 min at 4°C, supernatants were recovered.

AIB1 immune complex (from 3 mg of MCF-7 whole-cell lysate) was treated with 1,000 U of protein λ-phosphatase (New England BioLabs; no. 753S) in 30 μl of the supplied buffer. After five washes with the lysis buffer and one with MAPK buffer, the immune complex was divided into halves. One half was further treated with 0.1 μg of active Erk2 (Upstate Biotechnology) in 30 μl of a buffer containing 12.5 mM MOPS (morpholine propanesulfonic acid) (pH 7.5), 12.5 mM β-glycerophosphate, 7.5 mM $MgCl_2$, 0.5 mM EGTA, 0.5 mM NaF, 0.5 mM orthovanadate, and either 100 μM cold ATP or 1 μCi of [γ-^{32}P]ATP plus 20 μM cold ATP as indicated. After 1 h at 30°C, the reaction was stopped by adding 10 μl of 5× Laemmli buffer and heating at 95°C for 5 min. Samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). For the analysis of AIB1 bandshift, we used a 10% acrylamide gel containing 26% urea. After SDS electrophoresis, proteins were transferred to Immobilon-P membranes (Millipore, Bedford, Mass.) and blotted with the indicated antibodies.

MAP kinase activity. Lysates containing 1 mg of total protein were immunoprecipitated with 0.5 μg of anti-Erk2 (C14) antibody (sc-154; Santa Cruz Biotechnology). Immunoprecipitates were washed three times with lysis buffer, once with 0.5 M LiCl–100 mM Tris-HCl (pH 7.5), and once with MAPK assay buffer containing 12.5 mM MOPS (pH 7.5), 12.5 mM β-glycerolphosphate, 7.5 mM $MgCl_2$, 0.5 mM EGTA, 0.5 mM NaF, and 0.5 orthovanadate. Finally, they were resuspended in 30 μl of MAPK assay buffer containing 1 μCi of [γ-^{32}P]ATP, 20 μM cold ATP, 3.3 μM dithiothreitol, and 1.5 mg of myelin basic protein (MBP) (M-1891; Sigma) per ml. After 20 min at 30°C, the reaction was stopped with Laemmli buffer. Samples were analyzed by SDS-PAGE.

HAT activity. Whole-cell lysates were incubated with polyclonal antibodies against Gal4-DBD for 2.5 h at 4°C with protein G-Sepharose beads. Immunoprecipitates were washed three times with the lysis buffer and used for histone acetyltransferase (HAT) assays in solution with histones (Boehringer Mannheim) as substrates (5).

In vitro translation assays. In vitro translation assays were performed by using the Promega TNT kit. AIB1 was transcribed with the T7 polymerase.

Phosphoamino acid analysis. MCF-7 cells grown in DMEM–10% FBS were changed to a phosphate-free medium and labeled for 6 h with [^{32}P]orthophosphate (0.2 mCi/ml; 4 ml). Cells were lysed and immunoprecipitated with anti-AIB1 or preimmune antibodies. Immune complexes were resolved by SDS–8% PAGE. The gel was dried on a 3-mm Whatman paper filter and exposed for autoradiography. BT-474 and MDA-MB-468 cells were labeled in vivo with [^{32}P]orthophosphate as described above for MCF-7 cells.

The predicted AIB1 band was excised from the paper and treated with 200 μl of 6 M HCl at 100°C for 90 min. The pellet was washed twice with deionized water. The supernatants were pooled and dried by vacuum. The remaining pellet was resuspended in 5 ml of deionized water to which was added 1 μl of phosphoamino acid standards (10 mg/ml containing phosphoserine, -threonine, and -tyrosine). The resulting sample was spotted on a thin-layer chromatography (TLC) plate and resolved by ascendant chromatography with 70 ml of isopropanol, 15 ml of HCl and 15 ml of deionized water. Once chromatography was finished, the plate was dried and developed with a solution of ethanol containing 0.2% ninhydrin. The plate was heated in an oven at 100°C for 30 min and then exposed to autoradiography.

RESULTS

MAPK modulates estrogen signaling in the absence of S118. To study the role of MAPK in the regulation of ER signaling, we tested the effects of the MAPK phosphatase-1 (MKP1) and the constitutively activated MEK1 (RΔF) on ER-mediated transactivation. Transfection with RΔF induces the phosphorylation and activation of MAPK (13, 25), resulting in constitutive activation of the MAPK pathway. In contrast, MKP1 dephosphorylates MAPK and thus inactivates it, causing an overall reduction in MAPK signaling (37). Transfection with MKP1 reduced ER-dependent transcriptional activity approximately 40% (Fig. 1A). Addition of the MEK inhibitor U0126 produced an effect similar to that of MKP-1 (data not shown). Activation of MAPK by RΔF promoted significant increases in reporter activity. A fully activated ERα was potently enhanced by the activation of MAPK by RΔF, ninefold over the control and three times more than in the absence of the activated MEK1 (Fig. 1A). To confirm that MKP1 and RΔF altered MAPK activity, endogenous MAPK (Erk2) from the transfected cultures was immunoprecipitated and its activity was measured by using MBP as a substrate. As expected, MKP1 reduced and RΔF enhanced the overall MAPK activity in transfected cells (Fig. 1, insets). To test whether the effect of MAPK was on ERα alone or whether other factors involved in ER signaling might be involved, we abrogated the direct regulation of ERα by MAPK by introducing the S118A mutation. Ligand-independent activation mediated by the AF-1 domain is dependent on phosphorylation at this site by MAPK (18). This mutant exhibited a much lower basal transcriptional activity than the wild type (Fig. 1B). Thus, the induction in response to estrogen is much greater in the mutant than in the wild type. Interestingly, in the presence of estrogen, RΔF increased by 26-fold the activity of ERα(S118A), which was sixfold greater than in the absence of the activated MEK1 (Fig. 1B). These results suggest that factors involved in estrogen signaling other than ER itself may be modulated by activation of the MAPK pathway.

AIB1 is phosphorylated in vivo and can serve as a substrate of MAPK. Based on the ability of MAPK activation to augment ER(S118A) signaling, we questioned whether p160 coactivators such as AIB1 might also be a target of regulatory kinases. To test whether AIB1 is a phosphoprotein, we labeled subcon-

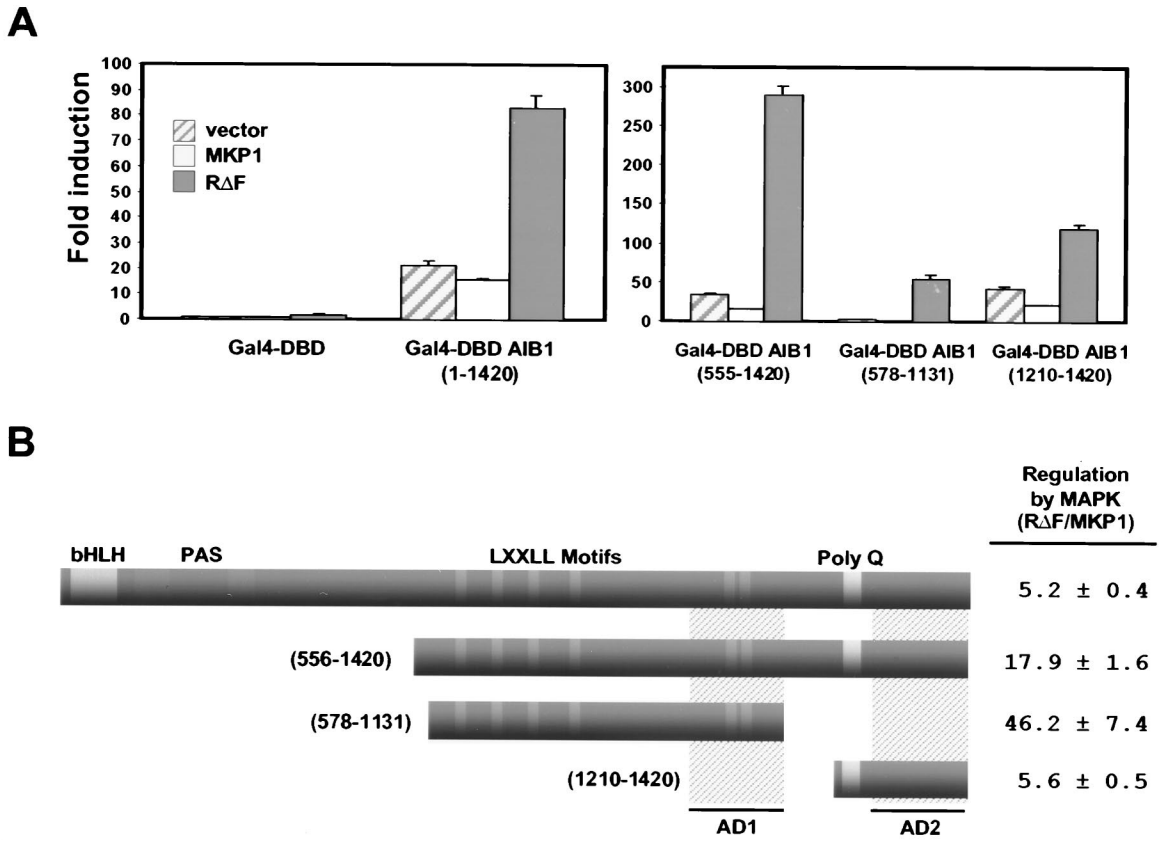

FIG. 4. MAPK stimulates AIB1 transcriptional activity. (A) COS cells were transiently transfected with Gal4-DBD fused to full-length or deletion mutants of AIB1, together with a luciferase reporter containing five copies of UAS. tk-lacZ was used as an internal control of the transfection. Transfections were performed in the presence of vector alone, MKP1, or activated MEK1 (RΔF). (B) Structural features of full-length AIB1 and the two deletion mutants. Values represent the ratio between the fold induction observed in the presence of a permanently activated MAPK (RΔF) and that observed in the presence of the phosphatase MKP1, as an indication of sensitivity to regulation by MAPK. Striped boxes indicate the two transactivation domains, AD1 and AD2; LXXLL, NR-interacting domains; Poly Q, stretch of polyglutamines.

fluent, proliferating MCF-7 cells with [^{32}P]orthophosphate. Cells were harvested and lysates were immunoprecipitated with preimmune or anti-AIB1 antibodies (Fig. 2A, lanes 4 and 5). Anti-AIB1 immunoprecipitates contained a unique radiolabeled band of the predicted AIB1 molecular weight, strongly suggesting that AIB1 is phosphorylated in vivo. The band was excised from the gel and subjected to phosphoamino acid analysis. The results revealed that AIB1 is predominantly phosphorylated on serine (Fig. 2B). Additionally, no phosphotyrosine-containing proteins were detected when anti-AIB1 immunocomplexes were probed by Western blotting with antiphosphotyrosine antibodies (data not shown). We also analyzed the phosphorylation of AIB1 in other cell lines, including BT-474 and MDA-MB-468 (Fig. 2C). Interestingly, while AIB1 expression was detected in both MCF-7 and BT-474 as previously reported (Fig. 2C, upper panel), significant levels of phosphorylation were detected only in MCF-7 cells (Fig. 2C, lower panel). We also examined the ER-negative breast cancer cell line MDA-MB-468; neither expression nor phosphorylation of AIB1 was observed in these cells.

The primary structure of AIB1 contains several potential phosphorylation sites for MAPK. Therefore, we also examined the ability of AIB1 to serve as a substrate for MAPK. For this purpose, we analyzed AIB1 phosphorylation status by alteration of its electrophoretic mobility as detected by Western blot analysis. Endogenous AIB1 was immunoprecipitated from MCF-7 cells and treated with protein λ-phosphatase. Phosphatase-treated AIB1 migrated faster than nontreated control immune complexes (Fig. 3, upper panel, lanes 3 and 4). This result further confirmed that AIB1 is a phosphoprotein. Dephosphorylated immune complexes were washed and subsequently treated with active MAPK Erk2 in the presence of ATP. Treatment with Erk2 restored the original mobility of AIB1 in immune complexes (Fig. 3, upper panel, lane 5), demonstrating that AIB1 can be phosphorylated in vitro by MAPK. In parallel, we also included [γ-^{32}P]ATP in Erk2 kinase reactions to demonstrate incorporation of radioactivity into AIB1, as detected by autoradiography (Fig. 3, lower panel).

AIB1 transactivation is stimulated by MAPK. We further investigated the role of the MAPK pathway in AIB1 function independent of ER. For that purpose, we fused AIB1 to Gal4-DBD and assayed this construct for transcriptional activity in transient cotransfections of COS cells with a reporter plasmid containing the GAL4 UAS (Fig. 4A, left panel). To determine the contribution of the MAPK pathway to AIB1 activity, we cotransfected Gal4-AIB1 together with MKP1 or with RΔF (Fig. 4A, left panel). In the absence of either MKP1 or RΔF, full-length Gal4-AIB1 exhibited potent transcriptional activation as expected. Cotransfection of MKP1 reduced somewhat the level of activation stimulated by Gal4-AIB1. More significantly, cotransfection of RΔF substantially augmented Gal4-

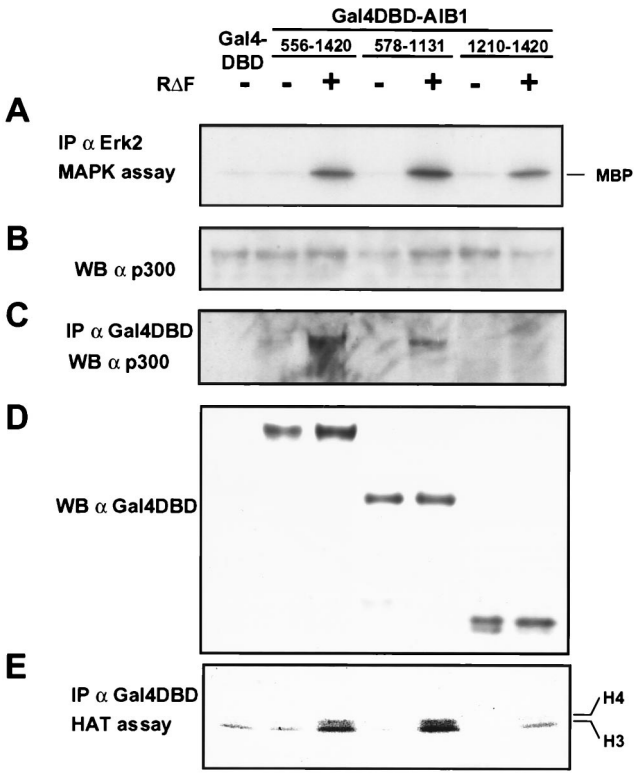

FIG. 5. Activation of MAPK recruits p300 to AIB1 complexes. Gal4-DBD alone or fused to AIB1 truncation mutants (amino acids 556 to 1420 or 578 to 1131) was transfected into BOSC cells along with pCIp300. The transfections also included vector alone (−) or vector containing activated MEK1 (RΔF). (A) After 48 h, endogenous Erk2 was immunoprecipitated to determine MAPK activity, using MBP as a substrate. (B) In parallel, 25 μg of whole-cell extract was analyzed for expression of p300 by Western blotting blot (WB). Lysates were further immunoprecipitated with a polyclonal antibody to Gal4-DBD. (C and D) Immune complexes were probed for p300 (C) and Gal4-DBD proteins (D). (E) In parallel, immune complexes were analyzed for HAT activity. Similar results were obtained in five independent experiments.

AIB1-mediated transactivation. Thus, Gal4-AIB1 was regulated over fivefold by alterations in MAPK signaling (Fig. 4B).

To isolate the domain of AIB1 responsive to MAPK, we tested three AIB1 deletion mutants as Gal4 fusions in this assay. Gal4-AIB1(556–1420) and Gal4-AIB1(578–1131) were both potently activated by MAPK (Fig. 4A, right panel; Fig. 4B). However, activity of the Gal4-AIB1(1210–1420) construct containing only the AD2 domain was much less dependent on the activity of the MAPK pathway. As Gal4-AIB1(578–1131) lacks AD2, this strongly implicates AD1 as the activation domain that is primarily responsive to MAPK.

Activation of AIB1 by MAPK leads to recruitment of p300. To further analyze the mechanism by which MAPK regulates AIB1, we focused on the AD1 domain. AD1 has been shown previously to function through the recruitment of p300/CBP (7). To test whether p300 was involved in MAPK activation of AIB1, we cotransfected p300 with the empty vector pCMX Gal4-DBD or with vector containing either AIB1 positions 556 to 1420, 578 to 1131, or 1210 to 1420. The first two truncations of AIB1 include AD1, whereas the later contains only the AD2 domain. As expected, transfection with RΔF potently activated MAPK as revealed by measuring endogenous MAPK activity using MBP as a substrate (Fig. 5A). Expression of p300 was similar in all samples, as detected in whole-cell extracts by immunoblot (Fig. 5B), and RΔF had no effect on the level of

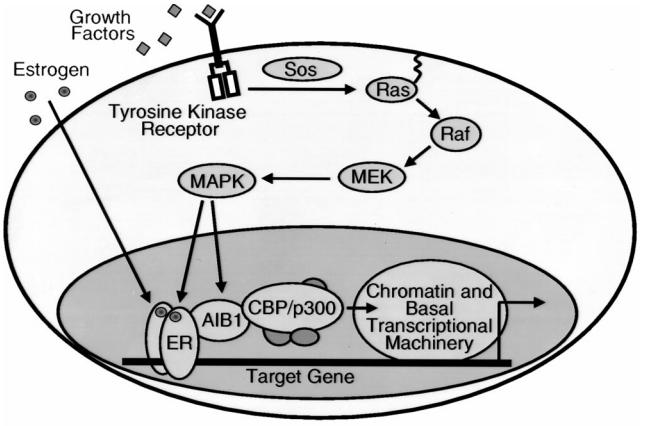

FIG. 6. Regulation of steroid receptor transcriptional complexes by MAPK activation. Growth factors such as EGF and IGF-1 stimulate the Ras-MAPK cascade via activation of their respective tyrosine kinase receptors. Activated MAPK may then phosphorylate nuclear targets including ER and AIB1 to modulate gene transcription in response to growth factor and/or steroid stimulation.

Gal4-AIB1 expressed (Fig. 5D). Lysates were immunoprecipitated with anti-Gal4-DBD and then probed for the presence of p300. Immunoblotting revealed the association of p300 with the AD1-containing fragments (AIB1 556 to 1420 and 578 to 1131) but not with AIB1 1210 to 1420, expressing only the AD2 domain. Significantly, appreciably more p300 was associated with these AIB1 fragments in the presence of RΔF, indicating that potent activation of MAPK enhances the association between AIB1 and p300 (Fig. 5C). In order to demonstrate that the increased association of p300 with AIB1 was functionally relevant, HAT activity associated with the Gal4-AIB1 complex was assayed. Significantly more H3/H4 acetyltransferase activity was associated with Gal4-AIB1 in the presence of activated MAPK (Fig. 5E). These results strongly suggest that MAPK activation augments ER-mediated transcription by enhancing the binding of the p160 coactivator AIB1 to p300. Thus, our observations provide a novel mechanism by which MAPK signaling is coupled to the regulation of estrogen signaling.

DISCUSSION

AIB1 is a member of the p160 family of steroid receptor coactivators (1), and it shares high homology with the other members of the family, SRC-1 (33% identity) and TIF2 (45%). AIB1 is highly expressed in the mammary gland, uterus, testis, pituitary gland, and muscle (36), suggesting that AIB1 may play a physiological role in these endocrine-responsive tissues. Interestingly, AIB1 is highly amplified in a proportion of breast and ovarian tumors (1), especially in ER-positive breast cancers (2). Here, we demonstrate that AIB1 is phosphorylated by MAPK in MCF-7 cells; activation of the MAPK pathway enhances the transactivation potential of this nuclear coactivator. Moreover, phosphorylation of AIB1 by MAPK recruits p300 and HAT activity to the AIB1 transcriptional complex. Thus, our observations suggest that the MAPK pathway, activated either by ER or growth factor signaling, may modulate AIB1 function to ultimately regulate gene transcription and cell growth.

The interaction between growth factors and estrogen signaling is complex and occurs at multiple levels. Previous work has implicated ER itself as a target of growth factor-signaling pathways involving MAPK. Conversely, activation of MAPK by estrogen and ER also occurs in various tissues and cell types,

though the exact mechanism remains to be determined (8, 12, 26, 35). These findings strongly suggest that ER is not only a ligand-induced transcriptional enhancer but also a mediator of common intracellular signaling pathways in multiple cell types (9).

We demonstrate that transcriptional activation mediated by AIB1 is enhanced by MAPK activation. In addition, we found that endogenous AIB1 is a phosphoprotein in MCF-7 breast cancer cells and that the MAPK Erk2 can phosphorylate AIB1 in vitro. Our results reveal a new level of complexity in the cross talk among growth factor-signaling pathways. In addition, we found that activation of AIB1 by MAPK phosphorylation increases its ability to recruit p300/CBP and its associated HAT activity, revealing a mechanism by which MAPK may modulate coactivator activity in the final response to growth factors. Taken together, these data implicate AIB1 as a molecular conduit for kinase-mediated growth factor signaling to the ER (Fig. 6) and presumably to other NRs. As both ER and AIB1 are substrates of MAPK, further studies will be required to determine how this complex regulation by phosphorylation ultimately regulates the transcriptional activation in ER-targeted genes. Phosphorylated AIB1 may also enhance the transcriptional activation of other NRs. Therefore, the physiological role of AIB1 activity and its phosphorylation may depend on the array of NRs and growth factor receptors expressed in a given cell type.

Recently it has been reported that recombinant SRC-1 overexpressed in COS-1 cells is a potential target of MAPK phosphorylation (32). Phosphopeptide mapping of SRC-1 revealed seven phosphorylation sites clustered in two regions of the protein. Sequence comparison between SRC-1 and AIB1 revealed that many of the phosphorylation sites found in SRC-1 are not found in AIB1. In SRC-1, only serines 395, 517, and 569 are also potentially conserved in AIB1 (serines 404, 505, and 551). However, the AIB1 S404 lacks an upstream proline, as is found in the SRC-1 S395 site and in other consensus MAPK phosphorylation motifs. Additionally, the presence of an acidic residue in AIB1, D549, at the −2 position of the S551 site makes it likely to be a poor substrate for MAPK. More importantly, all of these sites mapped in SRC-1 lie outside of the minimal region of AIB1 (578 to 1131) that we find responds to MAPK activation independently of ER and can be phosphorylated in vitro by MAPK (our unpublished data), suggesting potential coactivator-specific differences in the response to growth factor signaling.

We found that while AIB1 is highly expressed in the breast cancer cell line BT-474, it was not constitutively phosphorylated, as it is in MCF-7 cells, suggesting that these two cell lines may respond differently to hormone or growth factor stimulation. Interestingly, under standard culture conditions, BT-474 cells grow at a much lower rate than MCF-7 cells. We are currently investigating whether MAPK phosphorylation of AIB1 is tied to the growth state of the cells and whether AIB1 phosphorylation may contribute to a feed-forward loop in which growth factors such as IGF or transforming growth factor-α and estradiol act synergistically to promote cell cycle progression.

Our results provide new insights into how MAPK may contribute to the overall enhancement of transcriptional activity by promoting the activity of coactivators and by favoring the interaction among them. Furthermore, our results suggest that the ability of growth factors to augment estrogen action may be mediated, at least in part, through MAPK activation of AIB1 and this may explain the selection for AIB1 amplification during progression in ER-positive breast cancers.

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