Tyrosine Phosphorylation of the Nuclear Receptor Coactivator AIB1/SRC-3 Is Enhanced by Abl Kinase and Is Required for Its Activity in Cancer Cells^{∇†}

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Overexpression and activation of the steroid receptor coactivator amplified in breast cancer 1 (AIB1)/steroid receptor coactivator-3 (SRC-3) have been shown to have a critical role in oncogenesis and are required for both steroid and growth factor signaling in epithelial tumors. Here, we report a new mechanism for activation of SRC coactivators. We demonstrate regulated tyrosine phosphorylation of AIB1/SRC-3 at a C-terminal tyrosine residue (Y1357) that is phosphorylated after insulin-like growth factor 1, epidermal growth factor, or estrogen treatment of breast cancer cells. Phosphorylated Y1357 is increased in HER2/neu (v-erb-b2 erythroblastic leukemia viral oncogene homolog 2) mammary tumor epithelia and is required to modulate AIB1/SRC-3 coactivation of estrogen receptor alpha (ER α), progesterone receptor B, NF- κ B, and AP-1-dependent promoters. c-Abl (v-Abl Abelson murine leukemia viral oncogene homolog 1) tyrosine kinase directly phosphorylates AIB1/SRC-3 at Y1357 and modulates the association of AIB1 with c-Abl, ER α , the transcriptional cofactor p300, and the methyltransferase coactivator-associated arginine methyltransferase 1, CARM1. AIB1/SRC-3-dependent transcription and phenotypic changes, such as cell growth and focus formation, can be reversed by an Abl kinase inhibitor, imatinib. Thus, the phosphorylation state of Y1357 can function as a molecular on/off switch and facilitates the cross talk between hormone, growth factor, and intracellular kinase signaling pathways in cancer.

Coactivators significantly enhance the rate of transcription by binding to, and bringing together, components of the basal transcriptional machinery complex at gene promoters. A member of the p160 steroid receptor coactivator (SRC) gene family amplified in breast cancer 1 (AIB1) (also called steroid receptor coactivator-3 [SRC-3], TRAM1, RAC3, ACTR, and NCOA3) is amplified, and its corresponding mRNA and protein levels are overexpressed in multiple cancers (3, 20, 29, 43, 58). Overexpression of AIB1/SRC-3 is associated with markers of poor prognosis in breast cancer cells, including exhibiting increased p53 expression, being HER2 positive, and lacking estrogen receptor (ER) and progesterone receptor (PR) expression (5, 38). Phenotypic studies strongly argue that AIB1/SRC-3 has a role in both hormone- and growth factor-dependent gene expression. Cancer cell line studies demonstrate that AIB1 is critical for growth dependent on estrogen (28) and insulin-like growth factor 1 (IGF-1); it protects cells against apoptosis or anoikis (a form of apoptosis that is induced by anchorage-dependent cells detaching from the surrounding extracellular matrix) (37) and increases cell size and proliferation

(64). AIB1 also regulates epidermal growth factor (EGF) receptor tyrosine phosphorylation and the subsequent downstream EGF-induced activation of STAT5 and c-Jun N-terminal kinase (25). Targeted disruption of p/CIP (CREB-binding protein [CBP]-interacting protein), the mouse homologue of AIB1, demonstrates that AIB1 is critical for somatic growth (54, 59), energy balance (53), adipogenesis (30), and the rate of oncogene-induced (24) and carcinogen-induced (23) tumor formation. Overexpression of AIB1 or its naturally occurring isoform AIB1- Δ 3 in mice caused increased mammary gland size, increased mammary epithelial cell proliferation (50), and increased tumor incidence in multiple organs (51).

Site-specific phosphorylation and dephosphorylation are common posttranslational modifications utilized to control target protein functions. For AIB1, serine and threonine phosphorylation has been described (57) and can be an initiating modification that occurs before further posttranslational modifications, e.g., sumoylation (55), ubiquitylation (16, 32, 56), or methylation (13, 33). How tyrosine phosphorylation regulates the interactions of AIB1 with these other modifying enzymes or with other transcription cofactors and its relationship to pathway signaling are examined here for the first time. Our study documents that a single, site-specific AIB1 phosphorylation (at Y1357) can change the interaction of AIB1 with three proteins often found in transcription complexes bound to promoter elements: a methyltransferase (coactivator-associated arginine methyltransferase 1 [CARM1]), a histone acetyltransferase (p300), and a nuclear receptor (estrogen receptor alpha [ER α]). Dynamic simulations suggest a molecular mechanism for these changed interactions postphosphorylation.

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For the first time, we demonstrate a novel role for c-Abl (v-Abl Abelson murine leukemia viral oncogene homolog 1) (Abl) kinase in steroid receptor signaling via alteration of coactivator function. Abl kinase directly phosphorylated and bound to AIB1 via the Y1357 site. These results suggest that there is an on/off switch for coactivating ability and that cross talk between steroid and growth factor signaling can occur in breast cancer cells via modulation of AIB1 Y1357 phosphorylation. Furthermore, detection of phospho-Y1357 is potentially a response marker in cancer tissues for inhibitors of Abl, such as imatinib (Gleevec).

MATERIALS AND METHODS

Plasmids and reagents. p300-HA, CARM1-HA, and c-Abl-AU5 plasmids were kindly provided by Maria L. Avantaggiati (Georgetown University), Michael R. Stallcup (University of Southern California), and J. Silvio Gutkind (NIH/NIDCR). AIB1-Δ3 plasmid was previously described (42). AIB1-Δ3-FLAG tag expression plasmids (wild-type, Y1357F, and S505A constructs) were made by PCR amplification of ACTR/AIB1-Δ3 cDNA (778 bp to 4,422 bp) to add a new 5' NotI site and a 3' BglII site. PCR product was cloned into p3XFLAG-CMV-10 (Sigma-Aldrich, Inc.). Imatinib (STI-571, Gleevec; Novartis, Inc.) was kindly provided by Jeffery A. Toretsky (Georgetown University). EGF was purchased from Roche Diagnostics Co. IGF-1 was purchased from R&D Systems.

Cell lines. MCF-7 and COS-7 cells were grown in Iscove modified Eagle medium (Invitrogen Co.) with 10% heat-inactivated fetal bovine serum (HI-FBS; Quality Biological Inc.). MDA-MB-231, A549, HeLa, and 293T cells were grown in Dulbecco modified Eagle medium (Invitrogen Co.) with 10% HI-FBS. CHO-K1 cells were grown in F12-Dulbecco modified Eagle medium (Invitrogen Co.) with 10% HI-FBS. Cells were hormone stripped in media containing 5% charcoal/dextran-stripped FBS (HyClone).

Immunoprecipitation (IP) and Western blot (WB) analysis. (i) **IP experiments with MCF-7, A549, and MDA-MB-231 cells.** Cells were grown to 80% confluence in 150-mm dishes, were serum starved for 24 h, and were untreated or treated with 50 ng/ml of IGF-1 or EGF for 10 min. Cells were washed with cold phosphate-buffered saline (pH 7.4) and harvested with 1% NP-40 lysis buffer containing 1 mM Na₂VO₄ and 1× Complete protease inhibitor cocktail (Roche Diagnostics Co.).

(ii) **IP experiments with 293T cells.** 293T cells were transfected with 4 μg of each plasmid. The antibodies used for IP were 4G10 phosphorylated tyrosine antibody (Ab) agarose conjugate (Upstate Biotech, Inc.), AIB1 monoclonal antibody (MAb) (BD Transduction Laboratories), phospho-Y1357 AIB1 polyclonal Ab (Pacific Immunology Co.), FLAG M2 affinity gel (Sigma-Aldrich, Inc.), hemagglutinin (HA) affinity matrix (Roche Diagnostics), AU5 (Covance Co.), Abl (BD Biosciences), and ERα Ab-7 (Lab Vision Co.). IP was performed as previously described (25). Protein lysates were subject to NuPAGE gel electrophoresis (Invitrogen Co.).

(iii) **WB analysis.** Western blot analysis was done as previously described (37). Additional antibodies used for WB were phospho-CrkL Y207 (Cell Signaling Co.), ERα Ab-15 (Lab Vision Co.), and actin (Millipore Co.), and HA (Roche Diagnostics Co.) antibodies.

Phosphorylation mapping. (i) **Sample preparation.** Serum-starved MCF-7 cells were treated for 10 min with 50 ng/ml IGF-1 or EGF (R&D Systems). Whole-cell lysates were harvested with 1% NP-40 lysis buffer, precleared, immunoprecipitated with anti-AIB1 MAb (BD Transduction Laboratories), and run on a 4 to 12% sodium dodecyl sulfate-polyacrylamide gel (Invitrogen Co.).

(ii) **Phosphorylation mapping by ProtTech Inc.** Sequence grade modified trypsin (Promega Co.) or Asp-N (Roche Diagnostics) was used for protein digestion reactions. For each digest, ~20 to 50% of the sample was used for phosphatase differential analysis. Two aliquots of peptide mixture were analyzed for each digestion: one unit of alkaline phosphatase (Roche Diagnostics) was added to the treated reaction mixture, while in the control reaction, heat-inactivated alkaline phosphatase was used. Both samples were commercially analyzed by matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry (MS) (Micromass Proteome Work System MALDI-TOF Reflectron mass spectrometer). α-Cyano-4-hydroxycinnamic acid was used as a matrix. Phosphopeptides were identified by manually comparing the spectra from phosphatase-treated and control samples.

Luciferase reporter assays. Luciferase assays were performed as previously described (42) using the luciferase assay system (Promega Co.). A total of 3 × 10⁴ hormone-stripped cells were plated in each well of a 24-well plate. Cells were

transfected with FuGENE (Roche Diagnostics) for 16 to 24 h and then treated with hormones for 24 h. Cell extracts were prepared by using 100 μl of 1× passive lysis buffer (Promega Co.) and incubated at room temperature for 30 min on a rocker. Twenty microliters of the cell extract was assayed for firefly luciferase activity with the luciferase reporter assay kit (Promega Co.). Protein concentrations for each sample were determined using the Bradford protein assay. Luciferase values for each sample were normalized with their protein concentration.

Real-time reverse transcription-PCR. MCF-7 cells were transfected with AIB1 (3 μg) and ERα (0.5 μg) by electroporation (AMAXA kit V, program E-14) for 24 h. Cells were estrogen stripped and treated with 17β-estradiol (E2) (100 nM) for 3 h, and total RNA was harvested using RNA STAT (Tel-Test Inc.). One hundred fifty nanograms of RNA was used to perform real-time reverse transcription-PCR with the Platinum quantitative reverse transcriptase PCR ThermoScript one-step system (Invitrogen). Samples were reverse transcribed for 30 min at 56°C, followed by a denaturing step (3 min at 95°C) and 40 cycles (each cycle consisting of 15 seconds at 95°C and 1 min at 58°C). Fluorescence data were collected during the 58°C step (iCycler; Bio-Rad). pS2 (TFF-1 [trefoil factor 1]) probe and primers were purchased from Applied Biosystems (catalog no. Hs00170216_m1). The sequences of the beta-actin primers and probe were as follows: forward primer, 5' CCT GGC ACC CAG CAC AAT; reverse primer, 5' GCC GAT CCA CAC GGA GTA CT; probe, 5' 6-carboxyfluorescein-TCA AGA TCA TTG CTC CTC CTG AGC-Black Hole Quencher (IDT DNA Inc.).

Site-directed mutagenesis. The QuikChange XL II mutagenesis kit (Stratagene Co.) was used to introduce amino acid mutations in pCDNA3-AIB1-Δ3 and pCMV-3XFLAG-AIB1-Δ3. The following primers (IDT Inc.) were used for the mutagenesis reaction: for Y1357F, sense, 5' phosphate-CCG CAG GCT GCA TCC ATC TTC CAG TCC TCA GAA ATG AAG GG; antisense, 5' phosphate-CCC TTC ATT TGT GAG GAC TGG AAG ATG GAT GCA GCC TGC GG. The mutagenesis reaction was performed under the following conditions using the RoboCycler 40. The PCR mixture contained the following: 5 μl of 10× QuikChange reaction buffer; pCDNA3-AIB1-Δ3 (200 ng); sense primer (100 ng); antisense primer (100 ng); 1 μl of deoxynucleoside triphosphate mix; 3 μl Quik solution. The PCR mixture was brought up to a volume of 50 μl. PCR cycling conditions were as follows: step 1 was 2 min at 95°C; step 2 consisted of 25 cycles, with each cycle consisting of 1 min at 95°C, 1 min at 60°C, and 30 min at 68°C for 30 min; and step 3 was 7 min at 68°C. The DNA from the mutagenesis reaction was digested with 1 μl of DpnI restriction enzyme for 1 h at 37°C to digest template DNA. Four microliters of the digested reaction mixture was transformed into 45 μl of β-mercaptoethanol-treated *Escherichia coli* XL-10 gold competent cells. Plasmid DNA was prepared, and DNA sequencing was performed to confirm mutation.

Phospho-antibody production. A rabbit polyclonal antibody to phospho-Y1357 AIB1 was raised against the phosphorylated peptide NH₂-SlpYQSSEM KGWPSGNLC-COOH (pY is phosphorylated tyrosine) (Pacific Immunology Co.). Titers against the phosphorylated and nonphosphorylated peptides were confirmed by enzyme-linked immunosorbent assays. Phospho-specific antibodies were purified sequentially using nonphosphorylated and then phosphorylated peptide affinity columns.

IHC. AIB1/SRC-3^{-/-} (p/CIP^{-/-}) transgenic mice were previously described (59). FVB/N-TgN (mouse mammary tumor virus [MMTV]-HER2/neu) mice were purchased from Jackson Laboratory. Immunohistochemistry (IHC) analyses were performed on mammary gland 4 and tumor sections as previously described (50) using the phospho-Y1357 AIB1 rabbit polyclonal Ab. Briefly, tissues were fixed in 10% formalin and blocked in paraffin. Four-micrometer paraffin-embedded sections of mammary gland and tumor tissue were deparaffinized in xylene, rehydrated in alcohol, boiled for 10 min in citrate buffer (pH 6) (Zymed Laboratories) for antigen retrieval, and quenched with 3% hydrogen peroxide. The primary antibody was incubated overnight at 4°C. The phospho-Y1357 blocking peptide (Genscript) was prepared at four times the concentration of the phospho-Y1357 antibody. The peptide and antibody solutions were incubated together for 30 min at room temperature. The entire volume was added to the tissue section and incubated overnight at 4°C. Detection of rabbit primary antibodies were performed using the Dako Envision Plus horseradish peroxidase kit (Dako Cytomation). Bound antibody was visualized using diaminobenzidine substrate (Vector Laboratories). The slides were counter stained with hematoxylin (Polysciences, Inc.) for 30 s, dehydrated through an ascending concentration of ethanol, cleared in xylene, and mounted with Clearmount solution (Zymed Laboratories).

Protein modeling. (i) **Structure prediction.** Three-dimensional models of Y1357 were generated based on BLAST sequence alignment (1) with available crystal structures: 1SR9 (PDB annotation). Structure predictions for Y1357 were performed with the MODELLER 7v7 program (22).

FIG. 1. Growth factor-induced tyrosine phosphorylation of AIB1. (A) IGF-1 induced tyrosine phosphorylation of AIB1 in MCF-7 breast cancer cells. Cells were serum starved for 24 h and treated with 50 ng/ml IGF-1 for 10 min (+) or not treated with IGF-1 (–). Whole-cell lysates were harvested and used for immunoprecipitation (IP) and Western blot (WB) analysis with anti-AIB1 and antiphosphotyrosine (PY) antibodies as indicated. The asterisk indicates the position of a phosphotyrosine-containing band with a molecular mass of 165 kDa, which was confirmed to be AIB1 by reprobing the blot with the AIB1 antibody. The panel represents noncontiguous lanes from the same WB. (B) EGF induced tyrosine phosphorylation of AIB1 in MDA-MB-231 breast cancer cells. Cells were stimulated for 10 min with 50 ng/ml of EGF (+) and then processed and analyzed as described above for panel A. IgG, immunoglobulin G. (C) A schematic of AIB1/SRC-3 protein showing conserved and functional domains, serine and threonine phosphorylation sites, and the region containing multiple methylation sites. Phosphorylation at the Y1357 residue was discovered utilizing mass spectrometry. SRC-3 amino acid numbering was used for consistency. bHLH, basic helix-loop-helix; PAS, Per-Arnt-Sim; EID, E2F1 interaction domain; RID, nuclear receptor interaction domain; CID, CBP/p300 interaction domain. (D) Comparisons of amino acids surrounding Y1357 in AIB1 with other members of the p160 family, SRC-1, TIF-2/SRC-2, and p/CIP, the mouse homologue of human AIB1. Conserved amino acids are highlighted.

(ii) **Energy minimization and molecular dynamics.** The predicted wild-type and phosphorylated structures were energy minimized using the consistent valence force field (CVFF) with default partial atomic charge available in Discover v3.0. Molecular dynamics simulations (300 ps) with distance-dependent dielectric constants were carried out using the SANDER module of the AMBER 7.0 suite programs (7) with PARM98 force field parameter (Accelrys Inc.).

Abl in vitro kinase assay. Recombinant c-Abl kinase (80 ng) (Invitrogen Co.) was incubated with glutathione *S*-transferase (GST)-AIB1 (1017 to 1420 amino acids [aa]) (kindly provided by Don Chen, University of Medicine and Dentistry of New Jersey-Robert Wood Johnson Medical School) purified from *E. coli* BL21 cell lysate. The reaction was performed for 30 min at 30°C in kinase buffer (50 mM Tris [pH 7.5], 10 mM MgCl₂, 0.01% NP-40, 1 mM dithiothreitol, 0.5 mM ATP). Phosphorylation was detected by Western blotting with pY1357 AIB1 polyclonal Ab.

Cell growth assays. Validated Abl small interfering RNAs (siRNAs) (exon 3, catalog no. 1346; exon 11, catalog no. 1431) were purchased from Ambion Co. and transfected as previously described (37). Hormone-stripped MCF-7 cells were plated in 1% charcoal-stripped calf serum and 10 nM ICI 182,780 (Tocris Biosciences) with or without 10 nM estrogen. Cell growth was measured by utilizing the WST-1 reagent (Roche Diagnostics) after 4 days.

Focus formation assays. AIB1/SRC-3^{-/-} mouse embryonic fibroblasts (MEFs) were kindly provided by Jianming Xu (Baylor College of Medicine). A total of 2 × 10⁶ SRC-3^{-/-} MEFs were transfected with 2 μg of H-ras V12 and either 4 μg of empty vector, AIB1-Δ3 (wild type), or AIB1-Δ3 Y1357F constructs using the

AMAXA MEF kit 2 (program A-23), plated in 100-mm dishes, and grown for 3 weeks with regular changes of the media. Plates were fixed with ice-cold methanol and stained with crystal violet (0.5% crystal violet, 25% methanol).

RESULTS

Tyrosine phosphorylation of AIB1 in breast cancer cell lines. We first investigated the change in overall tyrosine phosphorylation of AIB1 in MCF-7 breast cancer cells that had been treated with IGF-1. These cells were used because AIB1 is rate limiting for IGF-1 stimulation of their growth (37). AIB1 tyrosine phosphorylation was examined by immunoprecipitation of AIB1 from whole-cell extracts, and possible tyrosine phosphorylation of AIB1 was detected by Western blot analysis with an antiphosphotyrosine antibody (Fig. 1A). IGF-1 treatment increased by two- to threefold a phosphotyrosine-containing band with a molecular mass of 165 kDa, which was identified as AIB1 by reprobing the blot with the AIB1 antibody (Fig. 1A). We previously demonstrated that AIB1 is critical for EGF signal transduction in the MDA-MB-231 breast

cancer cell line (25). Therefore, we asked whether EGF treatment of this cell line would also increase tyrosine phosphorylated AIB1 levels. We observed a significant increase in the phosphotyrosine AIB1 levels after 10 min of EGF stimulation (Fig. 1B). The blot was stripped and reprobed with the AIB1 antibody to confirm that this phosphotyrosine band was AIB1. These results demonstrate that growth factor-induced tyrosine phosphorylation of AIB1 is not limited to a single breast cancer cell line and that AIB1 can be tyrosine phosphorylated by both IGF-1 and EGF signaling pathway kinases.

Mapping of a phosphorylated tyrosine residue (Y1357) in AIB1. To identify specific growth factor-induced tyrosine residues in AIB1, we employed the mass spectrometry technique, MALDI-TOF. AIB1 in total lysates from IGF-1- and EGF-treated MCF-7 cells was immunoprecipitated with an AIB1 MAb. Samples were run on a sodium dodecyl sulfate-polyacrylamide gel, a band corresponding to AIB1 was excised, and its protein sequence was confirmed by a nano liquid chromatography-tandem mass spectrometry technique before post-translational modification analysis was performed. After trypsin or Asp-N protease digestion, samples were analyzed by MALDI-TOF MS, and a phosphopeptide containing Y1357 was identified. In Fig. 1C, the location of Y1357 relative to previously identified serine/threonine phosphorylation sites is indicated (57). The major domains of AIB1 necessary for interaction with other transcriptional components are also indicated (3, 8, 13, 27, 31, 33, 49). The Y1357 site of SRC-3 is equivalent to ACTR Y1345, AIB1 Y1353, RAC3 Y1350, and TRAM-1 Y1357. The Y1357 site is located 67 aa proximal to the C terminus, juxtaposing a long polyglutamine tract (Fig. 1C) and is 264 aa distal to the C-terminal end of the CBP/p300 interaction domain. The Y1357 site and surrounding region has not been previously associated with any AIB1 functional domain. The Y1357 is also present in transcriptional intermediary factor 2 (TIF-2)/SRC-2 and in the mouse AIB1 homologue, p/CIP. Amino acids C terminal to the Y1357, notably Q and S residues, are also partially conserved in TIF-2/SRC-2 and p/CIP (Fig. 1D).

IGF-1 and EGF induce Y1357 phosphorylation in breast cancer cells. To confirm that the phospho-Y1357 site discovered by mass spectrometry analysis was phosphorylated *in vivo*, a rabbit polyclonal antibody was generated against a peptide containing the phospho-Y1357 residue and affinity purified. AIB1 was immunoprecipitated from MCF-7 total lysate with this phospho-specific polyclonal Y1357 antibody and the AIB1 MAb was used for WB analysis (Fig. 2A). Phospho-Y1357 levels were significantly upregulated (two- to fivefold) after either IGF-1 or EGF treatment in all three cell lines examined (Fig. 2A), indicating that the phosphorylation of Y1357 was not limited to a single cell line or growth factor. A 10- to 30-min treatment with either IGF-1 or EGF resulted in peak phospho-Y1357 levels, without changing the total amount of AIB1 protein (Fig. 2A, input panels) (see Fig. S3 in the supplemental material). It was previously shown that estrogen (E2) treatments can cause an increase in serine/threonine phosphorylation of AIB1 (57). We examined whether estrogen induces phosphorylation of Y1357 in both ER α -positive (MCF-7) and ER α -negative (MDA-MB-231) breast cancer cell lines. We found that phospho-Y1357 levels increased by approximately twofold after estrogen treatment without chang-

ing total AIB1 levels in MCF-7 cells (Fig. 2B, top MCF-7 panel). However, we did not observe estrogen-induced phosphorylation at the Y1357 site in MDA-MB-231 cells (Fig. 2B, top MDA-MB-231 panel). Our results indicate that exposure to IGF-1, EGF, or estrogen, in ER α -positive cell lines, can cause increased phosphorylation at Y1357 without changing total AIB1 protein levels.

Since we observed a robust increase in phospho-Y1357 levels in breast cancer cells by growth factor or estrogen treatment, we asked whether phospho-Y1357 could be detected in mammary tumors. To investigate this possibility, we examined by IHC the levels of phospho-Y1357 in mammary tumors that develop in the mouse mammary tumor virus (MMTV)-driven HER2/neu (v-erb-b2 erythroblastic leukemia viral oncogene homolog 2) transgenic mouse model. This model is strongly dependent on HER/ErbB receptor family signaling for proliferation and metastasis (17). In these tumors, we observed a significantly higher percentage of positive nuclei stained with the phospho-Y1357 antibody than in healthy mammary epithelial cells, indicating that AIB1 (p/CIP) is highly and selectively phosphorylated at residue Y1357 in these tumors (Fig. 2C, compare tumor versus wild-type panels; results quantitated in the graph in the bottom right panel). The immunohistochemistry was specific for Y1357 AIB1, since no nuclei were visibly stained in mammary glands from SRC-3^{-/-} (p/CIP^{-/-}) mice with the phospho-Y1357 antibody (Fig. 2C, SRC-3^{-/-} panel). Prior incubation of the phospho-Y1357 antibody with a peptide containing the phosphorylated-Y1357 residue (blocking peptide) also resulted in no visible nuclei staining in both wild-type and tumor tissue sections (Fig. 2C, middle panel), further supporting the specificity of the phospho-Y1357 antibody.

Phosphorylation at Y1357 is necessary for AIB1's coactivator function. To help identify functions for phospho-Y1357, a phenylalanine mutant of Y1357 was generated (Y1357F), and its effect on AIB1's ability to function as a transcriptional coactivator was measured using several gene promoter reporters. Our analysis of the role of the Y1357 mutation in these experiments was performed in both full-length AIB1 and a naturally occurring ~130-kDa AIB1- Δ 3 isoform which differs from the full-length AIB1 by loss of the first 199 aa. We included the naturally occurring isoform AIB1- Δ 3 in addition to the full-length AIB1 in our experiments to define the effect of Y1357F because it has a significantly higher activity on a per mole basis than full-length AIB1 (42, 50). In addition, because of its lower molecular weight, the transfected AIB1- Δ 3 isoform can be detected in cell lines, such as COS-7 and HeLa cells, in which the endogenous full-length AIB1 is present at high levels (see Fig. S2 in the supplemental material). Compared to the wild type, the Y1357F mutant had ~50% coactivator activity on the estrogen-responsive promoter reporter in the context of both full-length AIB1 and AIB1- Δ 3 (Fig. 3A, left panel). The effect of the Y1357F mutant on AIB1's coactivation ability was also assessed by measuring estrogen-dependent induction of endogenous pS2 mRNA levels in MCF-7 cells. Transient transfection of wild-type AIB1 caused an increase in pS2 message, while no increase was observed with the Y1357 mutant in the presence of estrogen (Fig. 3A, right panel). We also compared the effect of the Y1357F mutant on another hormone-responsive promoter, progesterone-responsive MMTV, and again ob-

FIG. 2. In vitro and in vivo detection of phospho-Y1357 AIB1. (A) Phospho-Y1357 phosphorylation is observed in breast and lung cancer cell lines following growth factor stimulation using the phospho-Y1357 (p-Y1357) antibody. Cells were treated with 50 ng/ml of IGF or EGF for 10 min or not treated (-). Whole-cell lysates were harvested and used for IP/WB analysis with antibodies as indicated. (B) Estrogen (E2) induced phospho-Y1357 levels in MCF-7 (ER-positive) cells but not in MDA-231 (ER-negative) cells. Hormone-stripped cells were treated for 30 min with either ethanol (-) or E2 (10 nM) before whole-cell lysates were harvested for IP/WB analysis. Heavy (h) and light (l) chains of immunoglobulin G (IgG) are shown to the right of the gel for MCF-7 cells. (C) Increased phospho-Y1357 levels were observed in HER2/neu tumor tissue. Typical IHC staining patterns for phospho-Y1357 expression in paraffin-embedded mammary gland sections from female mice at 11 months (three HER2/neu mice with tumors) and from normal mammary gland 4 from mice at 6 months (three wild-type SRC-3 mice or two SRC-3^{-/-} mice). The phospho-Y1357 blocking peptide and phospho-Y1357 antibody were incubated together on each tissue section for 30 min. A total of 80 to 100 epithelial cells were counted per field. Ten fields were counted per genotype. The graph shows the quantitative results for wild-type and tumor panels. The values in the graph are means plus standard deviations (error bars). The values for phospho-Y1357-positive cells from wild-type mice and mice with tumors were significantly different ($P < 0.0022$) by the unpaired t test as indicated by the pair of asterisks.

served that the Y1357F mutation impaired the coactivating functions of AIB1 and AIB1-Δ3 (Fig. 3B).

The effect of the Y1357F mutant on steroid-independent coactivation was tested with multimerized NF-κB and AP-1

promoters. In the context of both AIB1 and AIB1-Δ3, the Y1357F mutant caused a ~40% reduction in the activity of the NF-κB promoter compared to the coactivating effect of wild-type AIB1 and AIB1-Δ3. The reduction in coactivator activity

FIG. 3. Functional role for phospho-Y1357 in steroid-dependent and -independent transcription. (A) (Left) Y1357F mutant coactivator effect on estrogen-stimulated transcription. AIB1 and AIB1-Δ3 constructs were cotransfected with ERα and estrogen-responsive promoter reporter (ERE) construct into hormone-stripped COS-7 cells. Cells were treated with ethanol (–) or 10 nM E2 (+) for 24 h and analyzed for reporter activity. Values for the ethanol- and E2-treated cells were significantly different by the unpaired *t* test as indicated by the following symbols: *, *P* < 0.03; #, *P* < 0.0012. (Right) ERα (0.5 μg) and AIB1 (3 μg) constructs were cotransfected into MCF-7 cells for 24 h and treated with E2 for 3 h. Total RNA was harvested and pS2 and beta-actin (b-actin) mRNA levels were measured using real-time quantitative reverse transcription PCR (qPCR). Values for the ethanol- and E2-treated cells were significantly different by the unpaired *t* test as indicated by the following symbols: *, *P* < 0.01; #, *P* < 0.001. (B) Y1357F mutant coactivator activity was measured on a progesterone-dependent promoter. PR-B expression plasmids were cotransfected with the MMTV reporter plasmids into hormone-stripped CHO cells. Cells were treated with either ethanol (–) or 10 nM R5020 (+) for 24 h and then analyzed for reporter activity. Cells from three mice were used for each treatment. Values for the ethanol- and R5020-treated cells were significantly different by two-way analysis of variance as indicated by the following symbols: *, *P* < 0.0012; #, *P* < 0.0007. (C and D) Y1357F mutant's coactivator effects on steroid-independent promoters. HeLa cells were cotransfected with AIB1 expression constructs as indicated and with either a multimerized NF-κB reporter construct (Stratagene Co.) (C) or a multimerized AP-1 reporter construct (Stratagene Co.) (D). Twenty-five nanograms of c-fos and c-jun expression vectors was also cotransfected with the AP-1 reporter. Twenty-four hours after transfection, extracts were prepared for reporter assays. Results are expressed as changes in the level of activation compared with empty vector-transfected cells. Values represent means plus standard deviations (error bars) for quadruplicate wells. Compared to the values for cells transfected with empty vector, the values were significantly different (*P* < 0.01) for the values for cells transfected with AIB1 Y1357F (*) and cells transfected with AIB1-Δ3 Y1357F (#) by the unpaired *t* test.

of the Y1357F mutant on an AP-1 promoter was also observed in the context of full-length AIB1 (Fig. 3D). In contrast, we observed an increase in activity of approximately threefold of the AIB1-Δ3 Y1357F mutant on the AP-1 promoter (Fig. 3C and D), suggesting a role for the N terminus of AIB1 in AP-1-mediated transcription. To investigate the surprising effect of the Y1357F mutation on AP-1-dependent expression

further, we analyzed its effect on a promoter fragment from the fibroblast growth factor-binding protein gene (19). The fibroblast growth factor-binding protein promoter is primarily AP-1 dependent and is coactivated by AIB1 in the presence of EGF (42). Although the Y1357F mutant activity was not significantly different than wild-type AIB1-Δ3 in its ability to coactivate this promoter (see Fig. S3 in the supplemental material),

FIG. 4. Phosphorylation of Y1357 modulates transcription cofactor interactions. Interaction of the AIB1 Y1357F mutant with transcription cofactors ERα (A1 and A2), p300-HA (B), and CARM1-HA (C). ERα, p300-HA, and CARM1-HA expression plasmids were separately cotransfected (+) with AIB1-Δ3-FLAG [AIB1 (FLAG)] constructs in 293T cells, and whole-cell lysates were prepared 24 h later for IP/WB analysis. The ratio of the amount of nonmutated AIB1-FLAG immunoprecipitated with the target protein (ERα, p300, or CARM1) was standardized to 1 and compared with the ratio of Y1357F FLAG immunoprecipitated with the target protein. These ratios are indicated below the panels. In panel A1, 293T cells were treated with ethanol (–) or 10 nM E2 (+) for 1 h before whole-cell lysates were prepared for analysis. The ERα input panel represents noncontiguous lanes of the same WB. In panel A2, empty vector (–), ERα, and AIB1 constructs were transfected separately into 293T cells and lysates were prepared. ERα- and AIB1-containing lysates were mixed and treated with either ethanol (–) or 100 nM E2 (+) before immunoprecipitation was performed. (D) Simulated effect of phosphorylated Y1357 on the local structure of AIB1. Amino acids with a white backbone were not phosphorylated, while amino acids with a green backbone were phosphorylated.

there was a trend toward increased activity even in the presence of a single AP-1 element in this promoter. The altered function of the Y1357F mutant's ability to coactivate both hormone- and growth factor-responsive promoters was not due to differences in exogenous AIB1 expressed protein levels (see Fig. S2 in the supplemental material). Overall, these functional data indicate that phosphorylation at Y1357 in AIB1 is important for both steroid-dependent and -independent transcriptional control, although the impact of Y1357 phosphorylation is highly dependent on the promoter context.

Phosphorylation of Y1357 alters AIB1 interaction with transcription cofactors. Since the phosphorylation status of Y1357 affected AIB1's coactivating ability on steroid- and NF-κB-dependent promoters, we postulated that phosphorylation can affect functional interactions between AIB1 and other proteins assembled in transcription complexes formed in response to steroid hormones and growth factor signals. We first examined interactions with the estrogen receptor ERα. In IP assays, we found ~50% less interaction between the Y1357F-FLAG mutant and ERα (Fig. 4A1). However, when AIB1 and ERα were

cotransfected together, we consistently observed a slight reduction in total ER α levels when cotransfected with Y1357F mutant. To determine whether the interaction between ER α and Y1357F was reduced due to an alteration in their binding affinity and not due to a reduction in total ER α available for interaction, we transfected the FLAG-tagged AIB1 and ER α constructs separately into 293T cells and mixed the lysates in the presence or absence of estrogen and then performed the FLAG IP followed by Western blotting for ER α (Fig. 4A2). Total expression of levels of AIB1 and ER α were also evaluated in the input lysates. With equal expression of ER α and AIB1, we observed a marked decrease in the affinity of Y1357F mutant for ER α compared to wild-type AIB1 (Fig. 4A2).

The interaction of AIB1 with CBP/p300, a histone acetyltransferase, is also a critical interaction for coactivation (8). HA-tagged p300 was cotransfected with FLAG-tagged AIB1 or Y1357F mutant construct, and coimmunoprecipitations were performed with the anti-FLAG antibody. Again, the Y1357F mutant interacted ~50% less than AIB1 in this assay for binding to p300 (Fig. 4B). We also noticed that Y1357 is close to a CARM1 methylation and interaction site on AIB1 (Fig. 1C). Unlike CBP/p300, engagement of the CARM1 cofactor has been demonstrated to inhibit transcription complex formation and to have a repressive effect on gene transcription (33). In contrast to the interaction results with p300 and ER α , we observed slightly increased amounts of CARM1 binding to the Y1357F mutant (Fig. 4C) compared to nonmutated AIB1. These results suggest that phosphorylation of this residue may play a minor role in stabilizing the interaction of AIB1 with CARM1. Overall, these data support the role of Y1357 phosphorylation in controlling the interaction between AIB1 and cofactors, such as ER α , p300, and CARM1, that ultimately alter its transcriptional activity.

Since mutation of Y1357 altered interactions with ER α and p300, we investigated whether Y1357 phosphorylation caused discernible differences in AIB1's structure that could explain changes in cofactor binding. Protein structure predictions were made with the MODELLER 7v7 program and 300-ps molecular dynamics simulations of the region surrounding Y1357 and phospho-Y1357 were carried out using distance-dependent dielectric constants (Fig. 4D). Upon phosphorylation, both phospho-Y1357 and nearby residues S1350, S1355, I1356, and E1361 (amino acids with a green backbone) move away from one another to avoid steric hindrance with the added, charged phosphate group, illustrating possible structural and functional roles for both Y1357 and phospho-Y1357. Therefore, phosphorylation at Y1357 could cause local structural alterations that increase the stability of AIB1's interactions with transcription machinery components, such as p300 and ER α , while dephosphorylation could maintain the stability of CARM1 binding, at the expense of p300 and ER α binding.

AIB1 Y1357 is phosphorylated by the Abl kinase pathway. Since we determined that phosphorylation at Y1357 had a functional role for AIB1's ability to coactivate by promoting the formation of transcription cofactor complexes, we wanted to determine the tyrosine kinase that was responsible for Y1357 phosphorylation. To narrow down the possible tyrosine kinases that could phosphorylate AIB1, the amino acid sequences around Y1357 were analyzed using Scansite 2.0 software program to determine whether the sequences formed a

consensus substrate for a particular tyrosine kinase (36). The Scansite program predicted that Y1357 and surrounding residues in AIB1 was a possible Abl tyrosine kinase substrate based on the presence of isoleucine at position -1 to Y residue which was also found in other substrates of Abl kinase, such as Dok (60), and Cas (44) (Fig. 5A). A general consensus for Abl kinase phosphorylation substrate has been derived from six known substrates (4, 10, 12, 44, 60, 63) (Fig. 5A). Interestingly, the isoleucine at position -1 was given a higher selectivity value than the proline at position +3 in a study that originally characterized Abl's substrate sequence specificity (47). However, it appears from the comparison in Fig. 5A that the proline at position +3 is a common feature of many known Abl substrates. To determine whether AIB1 was indeed phosphorylated by Abl kinase, we first performed an *in vitro* kinase assay to determine whether a GST fragment containing the Y1357 residue could be phosphorylated by recombinant Abl kinase. We found that a GST-AIB1 fragment from 1017 to 1420 aa was readily phosphorylated at Y1357 by exogenous Abl kinase, as detected by the phospho-Y1357 antibody (Fig. 5B). To confirm that Abl kinase could phosphorylate AIB1 in whole cells, we overexpressed Abl kinase using an Abl-AU5-tagged construct and cotransfected it with an AIB1-FLAG construct into 293T cells. We immunoprecipitated AIB1 with either a FLAG or phospho-Y1357 antibody and detected phosphorylated AIB1 by Western blotting. Since CrkL is an Abl/Bcr-Abl substrate (11), phospho-CrkL (Y207) levels were measured (Fig. 5C, input panels) to ensure that the transfected Abl kinase was functional. Consistent with the *in vitro* kinase assay, we detected a large amount of Y1357 phosphorylation only in the presence of transfected active Abl kinase (Fig. 5C, IP: FLAG panels).

Abl has the ability to phosphorylate and bind directly to its substrate targets, such as c-Jun (4) and Cas (44). We therefore determined whether Abl has the ability to complex with AIB1 and whether this binding was affected by the phospho-Y1357 residue. We cotransfected Abl with either AIB1 or the AIB1 Y1357F mutant into 293T cells and examined their interaction with Abl kinase by coimmunoprecipitation and WB analysis. Abl interacted strongly with AIB1, and ~50% of this binding was lost between Abl and the Y1357F mutant (Fig. 5D, IP: FLAG, WB: AU5 gel, lane 3). This result indicated that phosphorylation of the Y1357 residue increased the affinity for Abl kinase but was not absolutely required for the AIB1 interaction with Abl kinase. Like other nonreceptor tyrosine kinases, Abl mainly exists intracellularly in an inactive form and becomes activated by either external signals, such as growth factor stimulation or cell adhesion, or as a response to DNA damage (as reviewed in reference 39). Conversely, the Abl kinase inhibitor imatinib (Gleevec; STI-571) binds to the ATP binding pocket when Abl is in its inactive conformation (45). To determine whether the activation of Abl kinase was necessary for the interaction with AIB1, 293T cells were pretreated with imatinib 1 h prior to harvesting the cells for IP analysis. Inhibition of Abl kinase activity eliminated phosphorylation at Y1357 and completely prevented the interaction between Abl and AIB1 (Fig. 5D, IP: FLAG, WB: AU5 gel, lane 4). This result strongly suggests that AIB1 can interact only with the active form of Abl kinase. The inhibition of Abl kinase activity by imatinib was confirmed by measuring phospho-CrkL levels (Fig. 5D,

FIG. 5. Abl kinase directly phosphorylates phospho-Y1357 and binds to AIB1. (A) AIB1 Y1357 contains a partial Abl kinase recognition site. Amino acids immediately surrounding the AIB1 Y1357 residue were compared to an Abl kinase consensus sequence peptide and known Abl kinase substrates. Amino acids that are identically positioned are highlighted. (B) Abl kinase phosphorylates an AIB1 GST fragment in vitro. An in vitro kinase assay was performed with purified GST-AIB1 protein (aa 1017 to 1420) and recombinant Abl kinase. (C) Expression of constitutively active Abl phosphorylates AIB1 at Y1357. Abl-AU5 was cotransfected with AIB1- Δ 3-FLAG constructs in 293T cells. Phosphorylated

input, lane 4). Since we observed that estrogen could increase the phosphorylation of the Y1357 site (Fig. 2B) and that conversely, mutation of the Y1357 site diminished ER α interaction with AIB1 interaction (Fig. 4A), we were also interested to determine how increasing Abl kinase activity would affect the ER α -AIB1 complex formation. To accomplish this, we transfected 293T cells with a combination of ER α , Abl-AU5, and AIB1-FLAG expression constructs and determined by immunoprecipitation and Western blot analysis the amount of ER α -AIB1 complex formation in the presence or absence of added estrogen. As expected, IP of ER α brings down AIB1, and this interaction is increased in the presence of 10 nM estrogen (Fig. 5E, lane 5). Some interaction with ER α and AIB1 was observed in the absence of estrogen. Due to the high expression of transfected ER α , residual estrogens in the charcoal-stripped serum media was enough to cause some ER α -AIB1 complex formation (Fig. 5E, lanes 2 and 3). Interestingly, when Abl kinase is active, a significant increase in the amount of complex between ER α and AIB1 occurs (Fig. 5E, lane 6). Consistent with the idea that Abl phosphorylates AIB1, we also observed a significant upward mobility shift in the immunoprecipitated AIB1 in the lanes where Abl kinase is overexpressed (Fig. 5E, lanes 3 and 6). These data suggest that phosphorylation of AIB1 by Abl kinase facilitates the interaction with ER α and this is considerably enhanced in the presence of estrogen. To confirm that Abl kinase phosphorylates AIB1 in a breast cancer cell line, we used an siRNA directed against endogenous Abl kinase to determine whether reducing Abl kinase levels/activities resulted in a corresponding decrease in phospho-Y1357 levels. As shown in Fig. 2A, EGF treatment in MDA-231 cells resulted in an increase in phospho-Y1357 levels. When Abl kinase activity was reduced in MDA-231 cells with siRNA transfection, phospho-Y1357 levels were reduced dramatically (Fig. 5F). Total levels of Abl were difficult to detect in MDA-231 cells; therefore, phospho-CrkL activation was used as a surrogate marker for Abl siRNA knockdown. We observed a 20 to 40% decrease in phospho-CrkL levels when transfected with the Abl siRNA (Fig. 5F). These data clearly indicate that the Y1357 site on AIB1 is a substrate for Abl kinase in breast cancer cells.

Abl activity and phospho-Y1357 site contribute to AIB1's function as a critical coactivator and role in tumorigenesis. To assess the effect of Abl on AIB1 coactivator activity in MCF-7 cells, we inhibited endogenous Abl in MCF-7 cells with imatinib. MCF-7 cells carry the AIB1 gene amplification and therefore express very large amounts of AIB1 protein. Imatinib inhibited both basal and exogenous AIB1 coactivation of a MMTV promoter reporter in the presence of R5020 (Fig. 6A). AIB1 is rate limiting for estrogen-induced growth of MCF-7

cells (28). Imatinib or Abl siRNA treatment significantly reduced MCF-7 cell growth after 4 days of estrogen treatment (Fig. 6B). These findings demonstrate that Abl activity is necessary for AIB1's coactivation of hormone-dependent gene promoters and, ultimately, necessary for hormone-dependent growth of breast cancer cells. To directly assess the Y1357 site's contribution to AIB1-dependent tumorigenesis, focus formation assays were performed with transiently transfected H-ras V12 and the Y1357F mutant constructs in AIB1/SRC-3^{-/-} MEFs. AIB1 has been shown to reduce the incidence and latency of breast tumors in the MMTV v-Ha-ras mammary tumorigenesis mouse model (24). Wild-type AIB1 alone or the Y1357F mutant did not induce focus formation (data not shown), while H-ras V12 alone did result in the formation of a limited number of foci. Wild-type AIB1 plus H-ras V12 produced an increased number of foci, while the Y1357F mutant plus H-ras V12 produced fewer foci (Fig. 6C, chart). These data demonstrate that the Y1357 site directly contributes to AIB1's role in an oncogene-dependent transformation assay. We propose a molecular model (Fig. 6D) in which activated Abl binds to and phosphorylates AIB1 at Y1357. Phosphorylated-Y1357 AIB1 leads to a conformational alteration that stabilizes AIB1's interaction with cofactors, such as ER α and p300, while simultaneously resulting in a less stable interaction with CARM1. Phosphorylation at Y1357 is required for AIB1's ability to mediate steroid receptor-dependent gene transcription as well as its ability to contribute to breast cancer tumorigenesis.

DISCUSSION

This is the first study, to our knowledge, that describes the tyrosine phosphorylation of a steroid receptor coactivator. Although AIB1 tyrosine phosphorylation is initiated by membrane tyrosine kinases, it appears to be eventually mediated by Abl, a nonreceptor tyrosine kinase. Our results are consistent with a model outlined in Fig. 6D whereby Abl kinase is activated by an extracellular signal and in its activated form creates a complex with AIB1. AIB1 is then rapidly phosphorylated by Abl at tyrosine Y1357, thereby changing its local conformation and increasing its affinity for p300 and steroid receptors and decreasing its affinity for the repressor CARM1. At promoters that harbor estrogen, progesterone, or NF- κ B response elements, this leads to an overall increase in transcription. At other promoter elements, such as AP-1 sites, the tyrosine phosphorylation of AIB1 seems to be less important in formation of the transcription complex and may normally even repress transcription. This suggests that other AIB1 cofactor interactions may play a rate-limiting role in this promoter context. Inter-

CrkL (P-CrkL) (Y207) levels were detected to determine Abl activation. (D) Interaction between Abl and AIB1 is partially mediated by Y1357 and is fully dependent on Abl kinase activity. Abl-AU5 and AIB1- Δ 3-FLAG (AIB1 or Y1357F) constructs were used as described above for panel C. Transfected 293T cells were pretreated for 4 h with either dimethyl sulfoxide (–) or 10 μ M imatinib prior to collection of lysates and IP. (E) Abl forms a complex with ER α and AIB1 in the presence (+) of estrogen. 293T cells were transfected (+) with Abl-AU5, ER α , and AIB1- Δ 3-FLAG for 24 h and treated with either ethanol (lanes 1 to 3) or 10 nM E2 before whole-cell lysates were harvested. Lysates were immunoprecipitated with ER α followed by Western blot analysis for FLAG or ER α . (F) Reduction of Abl results in a decrease in endogenous AIB1 Y1357 phosphorylation in MDA-231 cells. MDA-231 cells were transfected with Abl (exon 11) siRNA for 48 h, serum starved, and treated with vehicle (–) or EGF (+) for 10 min. Phosphorylated CrkL (P-CrkL) levels were used to assess reduction in Abl activity. Activated CrkL levels were quantitated as ratios of the control siRNA-untreated lane. IgG, immunoglobulin G.

FIG. 6. Abl activity is necessary for AIB1's role in hormone-induced promoter coactivation and proliferation of breast cancer cells. (A) Inhibition of Abl kinase activity by imatinib reduces AIB1's ability to coactivate progesterone-dependent gene promoter activity. MCF-7 cells were transfected with MMTV reporter, PR-B, and AIB1 vectors for 24 h, pretreated 1 h with 10 μ M imatinib, and then treated with 10 nM R5020 with 10 μ M imatinib for an additional 24 h before reporter analysis. Each experiment was performed in triplicate. Results are expressed as changes in the activation of cells transfected with an empty vector. Values that were significantly different ($P < 0.002$) by two-way analysis of variance are indicated by the bracket and pair of asterisks. (B) Inhibition of Abl kinase significantly reduces E2-induced cell growth of MCF-7 breast cancer cells. For imatinib growth assays, hormone-stripped MCF-7 cells were pretreated with 10 μ M imatinib for 1 h and then treated with ethanol or 10 nM E2 with imatinib for 4 days. For Abl siRNA growth assays, hormone-stripped MCF-7 cells were transfected with scrambled (control [con.]) siRNA or with abl.3 or abl.11 siRNAs (specific for exon 3 or 11) for 24 h. Cells were treated with ethanol (–) or 10 nM E2 for 4 days. Each experiment was performed in triplicate. Values that were significantly different by two-way analysis of variance are indicated by brackets and the following symbols: *, $P < 0.001$; **, $P < 0.0002$. In panels A and B, values are means plus standard deviations (error bars). (C) Y1357F mutant demonstrates reduced H-ras V12-dependent focus formation in AIB1/SRC-3^{-/-} MEFs. AIB1/SRC-3^{-/-} MEFs was transfected with H-ras V12 and empty vector, AIB1- Δ 3 (wild type [WT]) or AIB1- Δ 3 Y1357F (Y1357F) constructs. After 3 weeks, focus formation was assessed after staining with crystal violet. Three independent experiments were performed. (D) A proposed model for the role of Abl tyrosine phosphorylation of AIB1 in steroid receptor signaling. Activated Abl binds to and phosphorylates AIB1 at Y1357. Phospho-Y1357 AIB1 stabilizes its interaction with cofactors, such as ER α and p300, while simultaneously resulting in a less stable interaction with CARM1. P, phosphate group; E, estrogen; NR, nuclear receptor.

estingly, it has been shown that AP-1-mediated transcription is impacted by serine and threonine phosphorylation of AIB1 (57). Furthermore, it has been postulated that phosphorylation at a particular residue of AIB1 may be a driving event, enabling subsequent posttranslational modifications (55). It would of interest to determine whether Y1357 is a primary permissive phosphorylation or a secondary occurrence after other post-

translational modifications including as yet uncharacterized additional tyrosine, serine, and threonine phosphorylation sites in AIB1. Tyrosine phosphorylation is usually a consequence of rapid activation of growth factor receptor tyrosine kinases and cytoplasmic protein tyrosine kinases upon ligand stimulation. Therefore, it may be more likely that tyrosine phosphorylation of AIB1 is an early rate-limiting modification which influences

phosphorylation or posttranslational modifications at other sites.

The phosphorylation of AIB1 by Abl kinase was a somewhat surprising result, especially as the Abl kinase consensus surrounding the Y1357 residue is not highly conserved. The role of Abl kinase in oncogenesis is complex. The oncogenic forms for Abl, v-Abl and Bcr-Abl, have been extensively studied and well described; however, the normal cellular functions of Abl are still being characterized (39, 52). Unlike Src tyrosine kinase, Abl has been found to have both a cytoplasmic and nuclear function, and it has profoundly different functions depending on its subcellular localization. Cytoplasmic Abl is associated with cell growth, motility, migration, and adhesion, while nuclear Abl is associated with apoptosis (15, 52). Similar to Abl kinases, AIB1 is also both a cytoplasmic and nuclear protein, albeit the full-length protein appears to be predominantly nuclear (29, 41). The mechanisms which alter the localization of AIB1 have been a topic of intense focus, as it may be important in regulating posttranslational modifications and protein stability of AIB1 (2, 26, 62). It would be of interest to determine whether the Abl-AIB1 interaction and phosphorylation occurs in a specific subcellular compartment, what other modifications precede or follow Y1357 phosphorylation, and the resulting functional consequences.

Our results strongly suggest that phosphorylation of and interaction with AIB1 by Abl kinase play a role in either Abl- or AIB1-mediated oncogenesis. As stated above, Abl can have different roles in oncogenesis depending on its subcellular localization and also the level of its activated expression. Similarly, AIB1 can be oncogenic when overexpressed in mammary epithelium and other epithelial tissue (50, 51, 61). Conversely, AIB1/SRC-3^{-/-} transgenic mice develop lymphomas as they age (9), suggesting that in this context AIB1 may normally suppress oncogenesis. It would be of interest to determine whether different functional interactions between Abl and AIB1 in the hematopoietic system compared with epithelial cells alter the role of AIB1 in oncogenesis. It may be possible that an epithelial tissue growth factor and steroid receptor pathways activate Abl and thus AIB1. However, in the hematopoietic system, a different paradigm may operate between Abl and AIB1, possibly in a different subcellular compartment. These are intriguing questions for further study.

Abl is activated by platelet-derived growth factor (PDGF) and EGF (40), but whether IGF-1 or insulin is a possible activator of Abl kinase seems to be somewhat cell line dependent and is still not fully understood (14, 46, 48). Regardless of the extracellular activator of Abl kinase, we postulate that other intracellular Abl-activated proteins (Fig. 6D) will be a necessary part of the Abl-AIB1 complex. Abl usually exists in an inhibited state in which either Abl keeps both its kinase domain and Src homology 2 (SH2)/SH3 domain tightly bound to itself (18, 35) or by binding to inhibitory proteins, such as ABI-1 (39). Activation of Abl kinase, perhaps due to phosphorylation (6, 34), results in exposure of the N-terminal myristoyl group and exposure of the SH2/SH3 domains to bind to phosphorylated proteins. It has been postulated that Abl substrates are initially phosphorylated by basal kinase activity of Abl, which initiates a positive-feedback loop by activating SH2 domain-dependent activation of Abl and finally results in the recruitment of its substrate (18). Discovering the components

of the AIB1-Abl kinase complex, especially a SH2/SH3 domain-containing protein that also binds to AIB1, may add further levels of complexity to the regulation of AIB1 function.

A possible clinical application of this study is the utilization of the phospho-specific antibody to detect phosphorylated AIB1 at Y1357 as a marker for activated Abl kinase in tumors and possible responsiveness to Abl kinase inhibitors, such as imatinib. At the writing of this article, seven clinical trials were ongoing to study the beneficial effects of using imatinib in conjunction with other therapies to treat metastatic breast cancer. One of the inclusion criteria of these trials is the presence of molecular markers, c-kit and PDGF receptor (PDGFR) beta. Autocrine PDGF/PDGFR signaling has been shown to promote metastasis in MMTV-Neu transgenic mice, and imatinib treatment was shown to reduce metastasis (21). This finding is interesting, since we also observed an increase in activated phospho-Y1357 AIB1 in HER2/neu tumors (Fig. 2C), thus suggesting that AIB1 may be downstream of PDGFR signaling. It will be interesting to determine in patient samples the levels of tyrosine-phosphorylated AIB1 and whether this is predictive of outcome in therapies directed at reducing growth factor and/or Abl kinase signaling. Since Abl kinase promotes complex formation between ER α and AIB1, as well as reducing NF κ B-mediated transcription, imatinib may have an inhibitory effect on mammary tumor growth in both steroid-dependent and -independent settings in breast cancer. Finally, due to the successful use of imatinib in the treatment of multiple human leukemias and the emergence of imatinib resistance in patients, a large number of drugs that target Abl, PDGFR beta, and Src are in the pipeline for drug development and testing. These inhibitors may also be applicable in the treatment of breast cancer, especially those that have high levels of phospho-Y1357 AIB1.

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