Epidermal Growth Factor Receptor Tyrosine Phosphorylation and Signaling Controlled by a Nuclear Receptor Coactivator, Amplified in Breast Cancer 1

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Abstract

The steroid receptor coactivator amplified in breast cancer 1 (AIB1) as well as epidermal growth factor receptor (EGFR) family members are frequently overexpressed in epithelial tumors, and their expression is associated with poor prognosis. However, a direct role of AIB1 in EGF signaling has not been determined. To address this, we reduced endogenous AIB1 levels using RNA interference in lung, breast, and pancreatic cancer cell lines. We found that a knockdown of AIB1 levels resulted in a loss of the growth response of these cell lines to EGF. Further analysis revealed that the depletion of AIB1 reduced tyrosine phosphorylation of EGFR at multiple residues both at autophosphorylation and Src kinase phosphorylation sites. AIB1 knockdown did not affect tyrosine phosphorylation of the receptor tyrosine kinases, platelet-derived growth factor receptor and HER3, or overall tyrosine phosphorylation of cellular proteins. However, EGFR-dependent phosphorylation of HER2 was decreased. EGFR levels and membrane trafficking were not changed by AIB1 depletion, but there was less recruitment of Src homology 2 domain-containing proteins to the EGFR. This led to a substantial reduction in EGF-induced phosphorylation of signal transducers and activators of transcription 5 and c-Jun NH2-terminal kinase but no significant change in the activation of AKT. Vanadate treatment of cells revealed that the reduction in EGFR tyrosine phosphorylation is dependent in part on changes in cellular phosphatase activity. We propose that a portion of the oncogenic effect of AIB1 could be through control of EGFR and HER2 activity and subsequent modulation of cellular signaling pathways. [Cancer Res 2007; 67(15):7256–65]

Introduction

Amplified in breast cancer 1 (AIB1; also known as SRC-3/ACTR/NCOR3/RAC3/pCIP) and other members of the p160 steroid receptor coactivator (SRC) family, which includes SRC-1 and TIF2, enhance transcriptional activity of nuclear receptors (1) and other transcription factors, such as AP-1 (2) and E2F-1 (3). Knockout of p/CIP, the mouse homologue of AIB1, resulted in reduced somatic growth (4, 5) and impaired mammary gland development (5). AIB1 is the only SRC family member that has been found to be amplified and/or overexpressed in cancer and associated with cancer progression in human epithelial tumors (6–10). AIB1 transgenic mice exhibited preneoplastic changes and developed tumors in the mammary gland and in other organs (11, 12). In addition, AIB1 levels are limiting for both hormone (13) and insulin-like growth factor (IGF)-I signaling responses in mammary cancer cells in vitro (12, 14, 15) and for H-ras–induced mammary tumorigenesis in mice (15). These studies show that AIB1 can control tumorigenesis in hormone-dependent and hormone-independent epithelial tumors. However, a unifying mechanism of how AIB1 drives oncogenesis has not been elucidated.

Epidermal growth factor receptor (EGFR) and its ligands play an important role in many types of human cancer by promoting tumor growth, metastasis, and angiogenesis (16). Several studies suggest there may be a connection between AIB1 expression and EGFR/HER2 signaling. We previously reported that an isoform of AIB1 enhanced EGF-mediated gene transcription of an angiogenesis modulator, fibroblast growth factor-binding protein (FGF-BP; ref. 17). In breast cancer, AIB1 overexpression correlates with increased levels of EGFR and HER2 protein and resistance to tamoxifen therapy (18–20). In addition, amplification of AIB1 and EGFR genes has been associated with lymph node metastasis of oral squamous cell carcinoma (21). Moreover, EGFR is overexpressed in pancreatic cancer (22) and overexpression of AIB1 is highly correlated with advanced pancreatic cancer disease (8).

EGFR (HER1) is a member of the HER/ErbB family of transmembrane receptor tyrosine kinases, which also includes HER2/ErbB2, HER3/ErbB3, and HER4/ErbB4. EGFR is composed of an extracellular ligand-binding domain and a cytoplasmic COOH-terminal tyrosine kinase domain. Binding of ligands, including EGF and transforming growth factor-α, to the extracellular domain of EGFR results in the formation of homodimers and heterodimers with other HER/ErbB family members and the activation of tyrosine kinase activity. Receptor activation leads to autophosphorylation of tyrosine residues located within the cytoplasmatic COOH-terminal domain and recruitment to these sites of adaptor proteins that are involved in signal transduction to cytoplasmic effector proteins, including extracellular signal-regulated kinase (ERK) 1/2, phosphatidylinositol 3-kinase (PI3K), and signal transducers and activators of transcription (STAT; ref. 23).

In this study, we report that AIB1 protein levels are limiting for EGFR-mediated signaling in a variety of human cancer cell lines. We observed that AIB1 knockdown decreased EGFR tyrosine phosphorylation, signaling, and proliferation. There was no change in EGFR levels or membrane trafficking. Treatment of cells with vanadate revealed that the reduction in EGFR tyrosine phosphorylation is partially dependent on phosphatase activity. The attenuation of EGFR phosphorylation, resulting from a reduction in AIB1 levels, led to inhibition of EGF-induced HER2, STAT5,
c-Jun NH2-terminal kinase (JNK) phosphorylation but no significant change in activation of AKT. We propose that the oncogenic effects of AIB1 could be through control of EGFR activity and subsequent modulation of cellular signaling pathways.

Materials and Methods

Cell culture. A549, MDA-MB-231, SK-BR-3, U-87, and PANC-1 cells were obtained from Georgetown University. COLO 357PL cells were obtained from Dr. Jessup (Georgetown University, Washington, DC; ref. 8). All media were from Invitrogen and supplemented with 1% fetal bovine serum. H1975 cells were obtained from the American Type Culture Collection and cultured in RPMI 1640. PAN-C-1 and COLO 357PL cells were cultured in Iscove’s MEM (IMEM) and all other cells were cultured in DMEM.

Antibodies and reagents. Antibodies against the following proteins were used: AIB1, c-Stat, SHP-1, PTP-1B, and Grb2 (BD Transduction); EGFR (1005); phosphorylated EGFR (phospho-EGFR; Tyr845, Tyr927, Tyr1045, Tyr1068, Tyr1173, and Ser1068/Ser1072), phosphorylated AKT (phospho-AKT; Ser473), phosphorylated HER2 (phospho-HER2; Tyr1221/Tyr1222), phosphorylated HER3 (phospho-HER3; Tyr1289), phosphorylated platelet-derived growth factor receptor β (phospho-PDGFRβ); Tyr575, phosphorylated ERK1/2 (phospho-ERK1/2; Thr202/Tyr204), phosphorylated JNK1/2 (phospho-JNK; Thr183/Tyr185), HER2, ERK1/2, AKT, JNK1/2, and STAT5 (Cell Signaling); and FGFR-BP and FGFR (NeoMarkers) were added to 500 μg of anti-HER2(N12) and g of anti-EGFR antibody (NeoMarkers) were added to 500 μg of anti-HER2(N12) and g of anti-EGFR antibody (NeoMarkers). All other chemicals were obtained from Sigma.

Gene silencing with small interfering RNA. AIB1 small interfering RNA (siRNA) oligonucleotides targeting different regions of AIB1 mRNA were synthesized by Qiagen. The AIB1(1) siRNA target sequence is 5′-AAAGACTCCCTTATAGCGCCTCT-3′ (14), and the AIB1(2) siRNA target sequence is 5′-GGTGAATCGAGACGGAAAC-3′ (1). The control siRNA used in the experiments is a scrambled sequence (5′-AAUUCCGAAGCGUUGACG-GUdTdT-3′). For siRNA transfection, 60 nmol/L of siRNAs were diluted in Opti-MEM with LipofectAMINE 2000 (Invitrogen) according to the manufacturer’s instructions. For short-hairpin RNA infection, see Supplementary Materials and Methods.

Real-time quantitative PCR. See Supplementary Materials and Methods.

Western blotting and immunoprecipitation. The Western blot protocol was followed as in ref. 14. Band density was quantified by densitometry using Adobe Photoshop 7.0 software and normalized to actin levels. Phospho-specific blots were normalized to the corresponding total protein. For EGFR and HER2 immunoprecipitation, either 1 μg of anti-EGFR (528), antibody (Santa Cruz Biotechnology) or 4 μg of anti-HER2 (N12) antibody (NeoMarkers) were added to 500 μg of lysate with Gammalbind G-Sepharose beads and incubated at 4°C for 16 h while rotating. The immunoprecipitates were washed thrice with lysis buffer and resolved by SDS-PAGE on 4% to 12% Tris-glycine gels.

Flow cytometry analysis of EGFR surface expression. Cells (1.0 × 10⁶) were resuspended in 100 μL PBS containing 0.4 μg/mL of anti-EGFR (528) antibody and incubated on ice for 1 h. The cells were then washed thrice with PBS and resuspended in 100 μL PBS containing 0.4 μg/mL of anti-mouse IgG conjugated with Cy2 (Invitrogen). The cells were incubated in the dark for 30 min and then washed thrice with PBS. As a control for background labeling, cells were incubated with only secondary anti-mouse IgG. The cells were resuspended in 400 μL PBS and analyzed by a BD FACScan flow cytometer.

Cell proliferation assay. Cells were transfected with siRNA for 24 h and seeded into each well of 96-well plates. After the cells attached, the medium was changed to serum-free medium for overnight and then serum-free medium/0.5% bovine serum albumin was added to the cells in the presence or absence of EGF (50 ng/mL). The number of cells was counted after 72 h by the WST-1 colorimetric assay (Roche) at 450 nm.

Soft agar colony-forming assay. PAN-C-1 cells were transfected with siRNA for 24 h. (1.0 × 10⁶ per dish) were resuspended in 0.35% agar and then seeded on top of a 1 mL solidified 0.6% agar layer in 35-mm dishes with IMEM or EGF (100 ng/mL) added to the top layer. Colonies >80 μm in diameter were counted after 14 days.

Statistical analysis. One-way ANOVA was the statistical method used to compare control siRNA versus AIB1 siRNA for each respective treatment group. The columns in the histograms represent the mean ± SD of triplicate values from independent experiments.

Results

EGF-induced proliferation, anchorage-independent growth, and gene expression require AIB1 in epithelial tumor cell lines. Initially, we determined if reducing AIB1 protein levels, by using AIB1 siRNA, would affect EGF-induced phenotypic changes in a diversity of EGFR-expressing cancer cell lines. A reduction of AIB1 protein levels in MDA-MB-231 breast cancer, A549 lung cancer, and COLO 357PL and PANC-1 pancreatic cancer cell lines (Fig. 1A) with AIB1 siRNA resulted in a significant inhibition of EGF-induced proliferation in comparison with control siRNA-treated cells (Fig. 1A). Similar results were observed with a second AIB1 siRNA in COLO 357PL and PANC-1 cells (Supplementary Fig. S1A). These experiments suggest that AIB1 levels are limiting for EGF-induced proliferation of human epithelial cancer cell lines.

A hallmark of tumorigenesis in vitro is the ability of cells to grow in anchorage-independent conditions in soft agar. We observed that PANC-1 cells responded most effectively to EGF stimulation for anchorage-independent growth. Therefore, we investigated the role of AIB1 in EGF-induced anchorage-independent growth in this cell line. A reduction of AIB1 protein levels with AIB1 siRNA (Fig. 1B, inset) resulted in a significant reduction in EGF-stimulated anchorage-independent growth relative to control siRNA–treated cells (Fig. 1B, left and right).

We next determined if the expression of EGF target genes, FGF-BP and cyclin D1, was affected by treatment of cells with AIB1 siRNA. We have previously shown that FGF-BP gene expression is up-regulated by EGF stimulation (24). From the cancer cell lines used in the proliferation assay, we found that the induction of FGF-BP gene expression was most pronounced in COLO 357PL pancreatic cancer cells (data not shown). Treatment of COLO 357PL cells with AIB1 siRNA significantly reduced AIB1 protein levels (Fig. 1C, inset), which abrogated the induction of FGF-BP mRNA levels by EGF (Fig. 1C). Cyclin D1 is an EGR-regulated gene in H1975 non–small cell lung cancer cells that harbor activating mutations in EGFR (25). Cyclin D1 expression is also regulated by EGFR in A549 lung cancer cells because treatment with AG1478, an EGFR-specific tyrosine kinase inhibitor, reduced cyclin D1 protein levels (Supplementary Fig. S1B). Our results show that cyclin D1 protein levels were significantly decreased by AIB1 siRNA treatment of H1975 and A549 cells (Fig. 1D, left and right), thus suggesting that AIB1 regulates EGFR-dependent cyclin D1 expression. Therefore, in a variety of cancer cell lines, AIB1 regulates EGF-induced proliferation and gene expression.

EGF-dependent activation of signal transduction pathways requires AIB1. Previous data have shown that AIB1 regulates the gene expression of molecules in the IGF-1 receptor (IGF-IR)/insulin receptor substrate-1/PI3K/AKT pathway in breast (14) and prostate cancer cell lines (26) as well as in AIB1-induced mammary tumorigenesis in mouse models (12). Because EGF can activate the PI3K/AKT pathway through EGFR recruitment of the GAB1 adaptor molecule, we determined whether the antiproliferative effect of AIB1 knockdown in the cancer cell lines could be mediated through inhibition of PI3K/AKT activation. However, a reduction in AIB1 levels with AIB1 siRNA did not alter the EGF-induced increase in AKT phosphorylation in MDA-MB-231, A549, PAN-C-1, and COLO 357PL cells (Fig. 2A, left, middle, and...
right; data not shown). We also observed that there was no change in EGF-induced Gab1 tyrosine phosphorylation (Supplementary Fig. S2A). We next examined whether the effect of AIB1 siRNA on EGF-induced proliferation could be mediated by changes in ERK signaling. In AIB1 siRNA-treated cells, we observed a small decrease in EGF-induced ERK phosphorylation in MDA-MB-231 and A549 cells and no change in PANC-1 and COLO 357PL cells (Fig. 2A, left, middle, and right; data not shown).

Figure 1. AIB1 regulates EGF-induced proliferation and gene expression in epithelial cancer cell lines. A, MDA-MB-231, A549, COLO 357PL, and PANC-1 cells were transfected with either control siRNA (black columns) or AIB1(1) siRNA (white columns) for 24 h, seeded into 96-well plates at a density of 2,500 per well (MDA-MB-231 and A549 cells) or 1,000 per well (COLO 357PL and PANC-1 cells), and then serum starved for 24 h followed by EGF (50 ng/mL) treatment for 72 h. Cell proliferation was measured using the WST-1 assay. Insets, level of AIB1 protein expression by Western blot analysis after 5 d of siRNA treatment. Columns, mean of triplicate values from four independent experiments; bars, SD. **, P < 0.001; one-way ANOVA, relative to control siRNA results for each respective treatment group. B, PANC-1 cells were transfected with either control siRNA (black columns) or AIB1(1) siRNA (white columns) for 24 h and resuspended in IMEM/0.35% agar in the presence or absence of EGF (50 ng/mL). Cell colonies were grown for 14 d and subsequently counted. Insets, AIB1 protein levels were measured by Western blot analysis after siRNA treatment. Right, quantitation of the colonies. Columns, mean of four replicate values from two independent experiments; bars, SD. *, P < 0.05, one-way ANOVA. C, COLO 357PL cells were transfected with either control siRNA (white columns) or AIB1(1) siRNA (black columns) for 24 h, serum starved for 24 h, and then treated with EGF (50 ng/mL) for 1 h. FGF-BP mRNA levels were analyzed by real-time quantitative PCR and normalized to glyceraldehyde-3-phosphate dehydrogenase mRNA levels. The data are represented as fold induction of EGF-stimulated cells. Insets, AIB1 protein levels were measured by Western blot analysis after siRNA treatment. D, H1975 (left) or A549 (right) cells were transfected with AIB1(1) siRNA for 48 h. Total cyclin D1 protein levels were detected by Western blot analysis.
pathway (28). Because it has been shown that EGFR-dependent activation of STAT5 is required for the proliferation of breast, head and neck, and lung cancer cells (29–31), we tested whether a knockdown of AIB1 would affect EGF-induced phosphorylation of STAT5. Treatment of MDA-MB-231, A549, and PANC-1 cells with AIB1 siRNA resulted in a 60% decrease in EGF-induced STAT5 phosphorylation (Fig. 2B, left, middle, and right). Similar results were observed in A549 cells with a second AIB1 siRNA (Supplementary Fig. S2B).

JNK is activated by both apoptotic stimuli and growth factors and to have dual roles in both apoptosis and proliferation (32). A549 cells were previously shown to require JNK for proliferation in

**Figure 2.** Reduction of AIB1 levels modulates the activation of EGF-induced signaling pathways. A to C, MDA-MB-231, A549, PANC-1, and COLO 357PL cells were transfected with either control or AIB1 siRNA for 24 h, serum starved for 24 h, and stimulated with EGF (50 ng/mL) for 10 min. A, phospho-AKT (p-AKT) and ERK1/2 (p-ERK1/2) and total levels of AKT and ERK1/2 were detected by Western blot analysis. B, phosphorylated STAT5 (p-STAT5) and total STAT5 levels were detected by Western blot analysis. C, phospho-JNK (p-JNK) and total JNK levels were detected by Western blot analysis. D, PANC-1 cells were seeded at a density of 1,000 per well in 96-well plates and serum starved for 24 h. The cells were pretreated with 0.2, 2, and 20 μmol/L of SP600125 for 45 min followed by EGF (50 ng/mL) treatment for 72 h. Cell proliferation was quantified using the WST-1 assay. Columns, mean of triplicate values from two independent experiments; bars, SD. *, P < 0.05, one-way ANOVA; **, P < 0.001, one-way ANOVA.
response to EGF (33). Therefore, we tested whether AIB1 knockdown would affect EGF-induced JNK phosphorylation. We observed that EGF was a strong stimulus of JNK phosphorylation in A549, PANC-1, and COLO 357PL cells (Fig. 2C, left, middle, and right), but not in MDA-MB-231 cells (data not shown), and reducing AIB1 expression decreased the levels of phospho-JNK by >50% with no change in total JNK levels (Fig. 2C, left, middle, and right). Similar results were observed with a second siRNA in A549, PANC-1, and COLO 357PL cells (Supplementary Fig. S2C). JNK is phosphorylated as a result of upstream signaling cascades, which involves the activation of MKK4 (32). We observed that treatment of PANC-1 cells with AIB1 siRNA resulted in decreased EGF-induced MKK4 phosphorylation (Supplementary Fig. S2D).

The data thus far indicate that AIB1 is required for EGF-induced proliferation of a panel of epithelial cells and that a major portion of this effect could be mediated through activation of STAT5 and JNK rather than through the ERK or PISK/akt pathway. A549 cells were previously shown to require activation of JNK and not ERK for proliferation (33). To confirm the role of JNK in EGF-stimulated proliferation of PANC-1 cells, we treated the cells with SP600125, a chemical inhibitor of JNK (34), which resulted in a dose-dependent decrease in proliferation (Fig. 2D). Therefore, inhibition of JNK in these cells, either through a drug or indirectly through reduction of AIB1 levels, significantly inhibits EGF-induced proliferation.

AIB1 regulates EGF phosphorylation of multiple tyrosine residues in cancer cells. Because we observed a reduction in EGF-mediated signaling in AIB1 siRNA–treated cells, we determined if AIB1 knockdown affected EGF levels or its autoposphorylation. Ligand-bound EGF results in activation of tyrosine kinase activity and phosphorylation of multiple intracellular tyrosine residues. As a result of AIB1 knockdown in MDA-MB-231 breast cancer cells, we observed no change in EGF levels (Fig. 3A). However, we observed a significant decrease in overall EGF-induced tyrosine phosphorylation of EGFR as detected with a phosphotyrosine antibody (Fig. 3A). Therefore, we examined the effect of AIB1 knockdown on EGF-induced phosphorylation of individual EGFR tyrosine residues. We assessed the phosphorylation levels of EGFR autophosphorylation sites (Y992, Y1045, Y1068, and Y1173) and a Src kinase phosphorylation site (Y845). Treatment of MDA-MB-231 cells with either of the AIB1 siRNAs (AIB1(1) or AIB1(2)) resulted in decreased EGF-induced phosphorylation of EGFR on individual tyrosine residues with no change in the total EGFR protein level (Fig. 3B). Therefore, AIB1 controls the phosphorylation of multiple EGF tyrosine residues, thus indicating an effect on EGF tyrosine kinase activity or increased phosphatase activity. For further experiments, the phospho-specific EGF antibody against Tyr1068 (phospho-EGFR<sup>1068</sup>) was used to assess EGF tyrosine phosphorylation. Similar results were also observed with other phospho-EGFR antibodies. We then tested whether AIB1 knockdown affected EGF-induced EGFR tyrosine phosphorylation in other EGF-responsive cancer cell lines used in this study. As a result of AIB1 siRNA treatment, EGF-induced phospho-EGFR<sup>1068</sup> levels decreased 57% in A549 lung cancer cells (Fig. 3C, left) and 86% in PANC-1 pancreatic cancer cells (Fig. 3C, right). In H1975 non–small cell lung cancer cells, which harbor activating mutations in EGF, we also observed that reduction of AIB1 with siRNA reduced phospho-EGFR<sup>1068</sup> levels without altering EGF levels (Fig. 3D). To verify that the effect of AIB1 siRNA treatment on EGF phosphorylation was unrelated to the siRNA method of gene silencing, we tested the ability of lentiviral-delivered AIB1 short hairpin RNAs (shRNA) to reduce AIB1 expression. We observed that AIB1-703 shRNA was the most effective at reducing AIB1 protein levels in A549 cells (Supplementary Fig. S3A). In A549 cells transduced with AIB1-703 shRNA, there was a 62% decrease in EGF-induced levels of phospho-EGFR<sup>1068</sup> compared with cells transduced with control shRNA (Supplementary Fig. S3B).

It was possible that reduced AIB1 expression did not actually reduce the absolute levels of EGFR tyrosine phosphorylation but altered the onset of receptor phosphorylation. To examine this, we assessed the effect of AIB1 knockdown on the kinetics of EGF-induced phosphorylation of EGFR<sup>1068</sup> in MDA-MB-231 breast cancer cells. During a time course of EGF stimulation up to 120 min, there was reduced phospho-EGFR<sup>1068</sup> levels at the peak of EGF stimulation (10 min) and at all other time points in cells treated with AIB1 siRNA (Fig. 4A, left and right). Thus, lowering the cellular levels of AIB1 reduces the absolute levels of tyrosine phosphorylation of EGFR.

The cellular response to EGF stimulation is dependent on the recruitment of Src homology 2 (SH2) domain–containing signaling molecules, such as Shc, c-Cbl, Grb2, phospholipase Cγ, and p85, to tyrosine-phosphorylated residues located within the cytoplasmic region of EGFR. We expected that reduced levels of tyrosine phosphorylation would result in decreased recruitment of EGF-interacting proteins. From a representative experiment, we observed that a knockdown of AIB1 in MDA-MB-231 cells resulted in a 55% decrease in EGF-induced receptor tyrosine phosphorylation, along with a 40% decrease in Shc recruitment, 20% for Grb2, and 68% for c-Cbl (Fig. 4B).

Cbl is recruited to EGFR in response to EGF stimulation and targets EGFR for degradation in the lysosomal compartment (35). Altered membrane localization or internalization of EGFR could affect EGFR activity as a result of increased or decreased receptor availability for EGF binding. We speculated that a decrease in c-Cbl recruitment to EGFR, as a result of a reduction in AIB1 (Fig. 4B), could alter the trafficking of the EGFR and this in turn would affect the autophosphorylation of the receptor. Thus, we tested the possibility that knockdown of AIB1 in MDA-MB-231 cells decreased the amount of EGFR localized at the membrane and/or increased EGF-induced internalization. However, we did not observe any difference in the nonstimulated levels of cell surface EGFR or EGF-stimulated EGFR internalization as a result of AIB1 knockdown (Fig. 4C). This suggests that AIB1 does not regulate the internalization or degradation of the EGFR.

**AIB1 knockdown decreases EGFR tyrosine phosphorylation by a phosphatase-dependent mechanism.** We next determined whether AIB1 knockdown causes a decrease in total cellular tyrosine phosphorylation levels or whether the effect was specific to EGFR. As indicated in Fig. 5A (left, arrow), an antiphosphotyrosine immunoblot showed that EGF stimulation resulted in a dramatic increase in the intensity of a band above 150 kDa (Fig. 5A, lanes 2 and 4), corresponding to the molecular weight of EGFR of ~180 kDa, which is decreased ~40% in cells treated with AIB1 siRNA (Fig. 5A, lane 4). However, as shown in the longer exposure (Fig. 5A, right), the intensity of a band around the 150 kDa marker (Fig. 5A, arrow) is greater in the cells treated with AIB1 siRNA (Fig. 5A, lane 3) compared with control siRNA (Fig. 5A, lane 1). In addition, AIB1 knockdown has no detectable effect on the level of EGF-induced tyrosine phosphorylation of other proteins, such as indicated by the arrows below the 100 kDa marker in lanes 2 and 4 (Fig. 5A, right). Therefore, the effect of AIB1 knockdown did not cause a general decrease in tyrosine phosphorylation levels of all proteins, but the effect is specific for EGFR.
Following this result, we hypothesized that reduced AIB1 expression may either directly or indirectly regulate the expression or activity of a phosphatase that has specificity for tyrosine-phosphorylated residues on EGFR. It was previously reported that EGFR tyrosine phosphorylation is regulated by the tyrosine phosphatases SHP-1 and PTP-1B (36, 37). The protein levels of SHP-1 and PTP-1B were not increased in MDA-MB-231 cells treated with AIB1 siRNA (Fig. 5B). However, this result does not rule out the possibility that AIB1 knockdown results in increased expression or activity of other tyrosine phosphatases.

To further assess the role of tyrosine phosphatase activity due to a reduction in AIB1, we pretreated MDA-MB-231 cells with vanadate, a broad specificity tyrosine phosphatase inhibitor. We rationalized that if a specific tyrosine phosphatase was activated following AIB1 knockdown, then a general inhibition of tyrosine phosphatase activity with vanadate should diminish the AIB1 knockdown effect. This in turn would lead to a higher level of EGF-induced phosphorylation compared with cells treated with control siRNA. As expected, vanadate treatment increased phospho-EGFRY1068 levels more than EGF alone in both the presence and absence of AIB1 siRNA (Fig. 5C, left). However, the magnitude of these changes is reproducibly different; vanadate treatment of control siRNA–treated cells caused a smaller increase in phospho-EGFRY1068 levels than vanadate treatment of AIB1 siRNA–treated cells (1.9-fold versus 2.8-fold; Fig. 5C, right). This result suggests that a significant portion of the AIB1 knockdown effect on EGFR tyrosine phosphorylation results from the induction or activation of a tyrosine phosphatase that targets EGFR.

AIB1 knockdown does not affect ligand-induced phosphorylation of HER3 and PDGFRβ but reduces EGF-induced HER2 phosphorylation. We tested whether AIB1 knockdown affected the tyrosine phosphorylation of other EGFR family members, including HER2 and HER3, and an unrelated receptor tyrosine kinase, PDGFRβ. In MDA-MB-231, A549, PANC-1, and COLO 357PL...
cells, we did not observe activation of HER3 or PDGFRβ with their respective ligands, heregulin-β and PDGF-BB; therefore, we tested other EGFR-positive cancer cell lines. Heregulin-β binding to HER3 causes dimerization with HER2 and subsequent autophosphorylation of cytoplasmic tyrosine residues. We used SK-BR-3 HER2-overexpressing breast cancer cells, which also express EGFR and HER3, to test whether AIB1 knockdown would affect EGF-induced and heregulin-β–induced tyrosine phosphorylation of EGFR and HER3, respectively. As a result of AIB1 siRNA treatment, we observed 49% less EGF-induced phospho-EGFR Y1068 levels (Fig. 6A) but no decrease in the levels of heregulin-β–induced phospho-HER3 Y1289 (Fig. 6A). HER2 is constitutively activated in SK-BR-3 cells through HER2 homodimerization but not through heterodimerization with EGFR. Therefore, we assessed whether HER2 tyrosine phosphorylation was affected as a result of AIB1 knockdown. However, there was no change in phospho-HER2 Y1221 levels in SK-BR-3 cells treated with AIB1 siRNA (Fig. 6A). Because EGF stimulation of EGFR can cause heterodimerization with HER2, we tested A549 cells, which have lower levels of HER2 expression than SK-BR-3 cells, whether EGF-induced HER2 tyrosine phosphorylation was affected by AIB1 knockdown. As a result of AIB1 siRNA treatment, there was a 64% decrease in EGF-induced tyrosine phosphorylation of HER2 (Fig. 6B). Finally, we tested U-87 glioblastoma cells, which express both EGFR and PDGFRβ, to determine whether AIB1 knockdown affected both EGF-induced and PDGF-BB–induced phosphorylation of EGFR and PDGFRβ, respectively. As a result of AIB1 knockdown, we observed an 80% decrease in EGF-induced phospho-EGFR Y1068 levels (Fig. 6C) but no decrease in levels of PDGF-BB–induced phospho-PDGFRβ Y751 (Fig. 6C). Therefore, a reduction in AIB1 protein levels decreases EGF-stimulated tyrosine phosphorylation of EGFR and HER2 but not of other ligand-inducible receptor tyrosine kinases tested.

**Discussion**

This is the first report showing that AIB1 is a limiting factor for EGF-induced EGFR tyrosine phosphorylation, signaling, and

**Figure 4.** AIB1 knockdown alters the kinetics of EGF-induced EGFR tyrosine phosphorylation but not its internalization. **A,** MDA-MB-231 cells were transfected with either control or AIB1(1) siRNA for 24 h, serum starved for 24 h, and stimulated with EGF (50 ng/mL) from 5 to 120 min. Total and phospho-EGFR Y1068 levels were detected by Western blot analysis. Right, densitometry values of phospho-EGFR Y1068 normalized to total EGFR levels. Data are representative of two experiments. **B,** MDA-MB-231 cells were transfected with either control or AIB1(1) siRNA for 24 h, serum starved for 24 h, and stimulated with EGF (50 ng/mL) for 10 min. EGFR was immunoprecipitated from whole-cell lysate and the relative amount of SH2-containing proteins interacting with EGFR was detected by Western blot analysis. Data are representative of two experiments. **C,** MDA-MB-231 cells were transfected with either control or AIB1(1) siRNA for 24 h, serum starved for 24 h, and stimulated with EGF (10 ng/mL) for 30, 60, and 120 min. Membrane levels of EGFR were measured by antibody labeling and flow cytometry analysis. Inset, AIB1 protein levels were detected by Western blot analysis after 48 h of siRNA treatment. Data are representative of two experiments.
proliferation in a variety of different epithelial cancer cell lines. We observed a decrease in EGF-induced EGFR phosphorylation at multiple tyrosine residues, including autophosphorylation sites (Y1068, Y992, and Y1173) and a Src kinase phosphorylation site (Y845), suggesting that AIB1 regulates the overall levels of EGFR tyrosine phosphorylation. From our experiments, this effect was limited to EGF-induced tyrosine phosphorylation of both EGFR and HER2. A knockdown in AIB1 levels did not affect HER2 tyrosine phosphorylation, resulting from HER2 overexpression, or ligand-induced tyrosine phosphorylation of HER3 or PDGFRβ.

A plausible explanation for decreased EGF-induced receptor tyrosine phosphorylation could be that a knockdown in AIB1 levels results in an increase in expression and/or activity of a specific EGFR-associated tyrosine phosphatase. Our data suggest that a portion of the AIB1 knockdown effect on EGFR phosphorylation is through a cellular tyrosine phosphatase. However, because we did not observe a complete reversal of the AIB1 knockdown effect with vanadate treatment, it is possible that vanadate treatment did not sufficiently inhibit tyrosine phosphatase activity or other mechanisms of control of EGFR phosphorylation also play a role. Other possible explanations for reduced EGF-induced EGFR tyrosine phosphorylation may be due to decreased levels of EGFR at the cell membrane as a result of altered receptor trafficking, decreased high-affinity ligand-binding sites (38), or increased phosphorylation of threonine and serine residues (39, 40). In the present study, knockdown of AIB1 in MDA-MB-231 cells did not change the level of EGFR on the cell surface or the kinetics of EGFR internalization in response to EGF. It has been reported that phosphorylation of EGFR on Ser1068/Ser1076 decreases EGF-induced EGFR tyrosine phosphorylation (39). Therefore, we measured the phosphorylation of EGFR Ser1068/1076 with a phospho-specific antibody. However, there was no increase in the phosphorylation of these serine residues as

Figure 5. AIB1 knockdown specifically decreases EGFR tyrosine phosphorylation by a phosphatase-dependent mechanism. A, MDA-MB-231 cells were transfected with either control or AIB1(1) siRNA for 24 h, serum starved for 24 h, and stimulated with EGF (50 ng/mL) for 10 min. Total tyrosine phosphorylation levels were detected from whole-cell lysates by Western blot (WB) analysis after a short (left) and longer autoradiograph exposure (right). Arrows, tyrosine-phosphorylated protein bands. B, MDA-MB-231 cells were transfected with either control or AIB1(1) siRNA for 48 h. The level of protein expression of tyrosine phosphatases was detected by Western blot analysis. C, to determine if knockdown of AIB1 levels affects tyrosine phosphatase activity, a broad specificity tyrosine phosphatase inhibitor vanadate was used to block tyrosine phosphatase activity. MDA-MB-231 cells were transfected with either control or AIB1(1) siRNA, serum starved for 24 h, pretreated for 2 h with 1 mmol/L vanadate, and stimulated with EGF (50 ng/mL) for 10 min. Left, phospho-EGFR Ser1068 levels were detected by Western blot analysis; right, densitometry values of phospho-EGFR Ser1068 normalized to total EGFR levels. Data are representative of two experiments.
a result of AIB1 knockdown (data not shown). Whatever the mechanism involved as a result of AIB1 knockdown, it is reasonable to conclude that, because we did not observe a general decrease in the cellular levels of tyrosine phosphorylation or of other EGF-independent receptor tyrosine kinases, the effect of AIB1 knockdown is relatively specific for the EGFR.

An unexpected observation was that reduction of AIB1 levels did not alter AKT activation in any of the diverse cell types studied. In breast and prostate cancer cell lines, we and others have reported that reducing AIB1 significantly reduced IGF-I–induced phospho-AKT levels (12, 14, 26). Overexpression of AIB1 resulted in activation of the PI3K/AKT pathway (12), and it has been suggested that AIB1 drives tumorigenesis primarily through this pathway by modulation of the expression levels of IGF-I signaling molecules (41). However, our data suggest that AIB1 also regulates signal transduction pathways through different mechanisms of control, which may depend on the specific oncogenic pathways that are driving tumorigenesis. In the case of EGFR signaling, GAB1 is the adaptor molecule that relays the signal from EGFR to PI3K/AKT. Because we did not observe any affect on EGF-induced GAB1 phosphorylation as a result of AIB1 knockdown (Supplementary Fig. S2A), this suggests that the reduced EGFR phosphorylation levels are still sufficient to recruit GAB1. For ERK signaling, Grb2 and Shc are adaptor molecules that relay the signal from EGFR to Ras/mitogen-activated protein kinase/ERK kinase/ERK. Less Grb2 and Shc were recruited to EGFR after EGFSimulation in MDA-MB-231 cells, which resulted in a decrease in ERK phosphorylation. It should be noted that MDA-MB-231, A549, PANC-1, and COLO 357PL cells harbor mutated Ras, which may affect EGFR signaling through this pathway.

The reduction in AIB1 protein levels resulted in a dramatic decrease in the EGF-dependent activation of both STAT5 and JNK in multiple tumor types. STAT5 is important for the proliferation of breast, head and neck, and lung cancer cells (30, 31, 42) and may play a role in the EGF-dependent proliferation in the cell lines we studied. Moreover, our results with AIB1 siRNA treatment or with a JNK inhibitor show that activation of the JNK signaling pathway by EGF is a major contributor for the proliferation of PANC-1 pancreatic cancer cells. In addition, it was previously shown that EGF-stimulated proliferation of A549 lung cancer cells requires JNK and not ERK (33). Consistent with a role for JNK in EGF-induced proliferation, several reports provide evidence that JNK mediates pro-proliferative phenotypes in cancer (33, 43, 44) and a recent report indicates that mutations in the JNK pathway are associated with human cancer (45).

Several questions arise from our data. It will be interesting to determine if the cellular mechanism for the worse clinical prognosis seen in breast cancer patients with high HER2 and high AIB1 expression is related to the effect of AIB1 on EGFR/HER2 signaling activity. Studies have shown that EGFR signaling is important for HER2 signaling. HER2-mediated tumorigenesis has been reported to require EGFR signaling (46). Another study showed that Iressa, an EGFR tyrosine kinase inhibitor, is able to inhibit the growth of HER2-overexpressing cancer cells and increased apoptosis of cells treated with Herceptin, an antibody that targets HER2 (47). Possibly increased AIB1 expression in breast cancer cells will enhance EGFR signaling through HER2 activation and make cells more resistant to Herceptin treatment. An additional possibility is that the reduction of EGFR phosphorylation by AIB1 knockdown will affect IGF-IR signaling. Several laboratories have shown that, in some cellular contexts, IGF-I induction of proliferation requires EGFR (48, 49). Interestingly, activation of EGFR has been shown to regulate the expression of IGF-I signaling molecules through the JNK pathway (50). Therefore, it is possible that the effects of AIB1 on IGF-I signaling and the reports of AIB1 as an oncogene (12) are mediated in some contexts through EGFR signaling. The EGF pathway has not been a focus of AIB1 studies thus far, but our data indicate that it most likely plays a significant role in cancer induced by AIB1. In conclusion, our observation that AIB1 affects the signaling capacity of EGFR in

Figure 6. AIB1 knockdown does not affect the ligand-induced phosphorylation of HER3 and PDGFRβ. A, SK-BR-3 breast cancer cells were treated with either control or AIB1102 siRNA for 24 h, serum starved for 24 h, and stimulated with either EGF (50 ng/mL) or heregulin-β (10 ng/mL) for 10 min. Phospho-EGFRY1068, phospho-HER2Y1221, and phospho-HER3Y1289 levels were detected by Western blot analysis. B, A549 cells were treated as in (A) and stimulated with EGF. Total levels of immunoprecipitated HER2 and HER2 tyrosine phosphorylation were assessed by Western blot analysis with anti-HER2 and antiphosphotyrosine, respectively. C, U-87 glioblastoma cells were treated as in (A), except that cells were stimulated with PDGF-BB (40 ng/mL) for 10 min. Phospho-EGFRY1068 and phospho-PDGFRβY751 levels were detected by Western blot analysis.
cancer cells suggests that AIB1 could play an important role in EGFR-mediated oncogenic processes.

Acknowledgments
Received 3/19/2007; revised 5/2/2007; accepted 5/23/2007.

References

Grant support: National Cancer Institute grants CA11677 and CA108441 (A.B. Reiter) and Department of Defense Predoctoral grants W81XWH0510250 (T. Lahusen) and W81XWH0610350 (M. Fershteh). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
We thank the Gordon Family Cancer Foundation for their support and Tom Matteson for manuscript editing.
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