Phosphoinositide 3-kinase/AKT Signaling Can Promote AIB1 Stability Independently of GSK3 Phosphorylation

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Abstract

The transcriptional coactivator AIB1 is an oncogene overexpressed in different types of tumors, including breast cancer. Although the subcellular compartimentalization of AIB1 seems to be intimately linked to abnormal proliferation, the molecular mechanisms that regulate its subcellular distribution are not well defined. Here, we report that the nuclear accumulation and half-life of AIB1 vary between cancer cell lines. Using these differences as an experimental model, our results reveal that alterations to the Akt signaling pathway and nuclear export determine the stability of AIB1 and nuclear content of this coactivator. Moreover, our results show that AIB1 is degraded in the nucleus by the proteasome in an ubiquitin-dependent manner. However, this process does not require phosphorylation by GSK3, thereby revealing an alternative mechanism for regulating the turnover of AIB1. We define a new region at the carboxy terminus of AIB1 that is required for proteasome-dependent transcriptional activation and is preceded by a PEST domain that is required for adequate protein turnover. Based on differences in Akt signaling and the subcellular distribution of AIB1 between different cell lines, our results suggest that dysregulation of nuclear shuttling and proteasomal degradation may modulate the oncogenic potential of AIB1. [Cancer Res 2008;68(13):5450–9]

Introduction

Pathways controlling signal transduction, protein turnover, and transcriptional regulation are frequently altered in human cancers. The phosphoinositide 3-kinase (PI3K)/Akt pathway controls fundamental aspects of metabolism and cell growth (1). As such, it also plays a pivotal role in the genesis of several diseases, including cancer (2). Akt is activated by multiple growth factors and hormones, but little is known about the regulation of endogenous Akt in tumor cells.

Protein turnover through the ubiquitin-proteasome pathway is also altered in cancers. Ubiquitination is a cellular mechanism that controls many physiologic processes, including protein half-life, transcriptional regulation, protein subcellular trafficking, recruitment of binding partners, and reversible allosteric conformations (3). The transcriptional coactivator AIB1 is a target of the proteasome and is degraded by the ubiquitin pathway, as well as by ubiquitin-independent mechanisms (4). AIB1 is overexpressed in ~60% of human cancers (5). However, the high levels of AIB1 can be explained by amplification of locus 20q in only ~5% of these tumors (6). Hence, other molecular mechanisms must modulate AIB1 levels via regulation of its transcription and/or protein stability. Two recent studies suggest that stability of AIB1 depends on its subcellular localization. However, the results from these studies are contradictory because one reports that AIB1 is degraded in the cytosol by the proteasome (7) whereas the other describes nuclear degradation by the proteasome (8). As a transcriptional regulator, AIB1 exerts its activity in the nucleus. Interestingly, nuclear staining for AIB1 is detected in the highly proliferative cell layer of the mammary gland (9). Furthermore, nuclear accumulation of AIB1 occurs during S phase of the cell cycle (10), suggesting a role for AIB1 during or after S phase, although it may also exert an important function during G_1 progression by coactivating E2F1 (11, 12) and promoting the transcription of cyclin D1 (13).

Several signaling pathways have been implicated in the nuclear shuttling of AIB1. Phosphorylation of AIB1 by Erk has been shown to promote its nuclear localization and interaction with nuclear receptors (14), as well as with p300 (15). IκB kinase (IKK), responsible for the degradation of IκB and subsequent activation of nuclear factor-κB, phosphorylates AIB1, permitting its translocation to the nucleus (16). This translocation is Ran-dependent and mediated by importin α3 (17). The SRC-interacting protein (SIP) sequesters AIB1 in the cytosol until phosphorylation by casein kinase II (CKII) releases AIB1 from this interaction (18). The PI3K/Akt pathway is also involved in the activation of IKK (19, 20), suggesting that PI3K/Akt pathway, when altered in cancer cells, could also modulate AIB1 nuclear shuttling and stability and, hence, its oncogenic potential. Interestingly, AIB1-deficient mice display altered regulation of insulin-like growth factor I (IGF-I) expression in specific tissues (21) whereas AIB1 overexpression in transgenic animals enhances IGF-I expression, thereby increasing Akt signaling (22). Furthermore, AIB1 is recruited to the target promoters of IRS-2 and IGF-I (23), suggesting a feedback loop between AIB1 stability/activity and IGF-I/Akt increased signaling. Importantly, the function of AIB1 in IGF-I–induced proliferation, signaling, cell survival, and gene expression seems to be independent of its role in estrogen receptor signaling (24). GSK3, another downstream target of Akt, phosphorylates AIB1, allowing the recruitment of the ubiquitin ligase SCF^{Fbw7a} (25).

With the present study, we provide evidence that nuclear localization explains the observed differences in the stability of AIB1 between certain cancer cell lines. Moreover, these differences in nuclear content of AIB1 reflect differences in the regulation of nuclear export rather than alterations to import molecules. Although nuclear localization is the major determinant of the half-life of AIB1, Akt activation counteracts the degradation of AIB1, thereby increasing its half-life. Additionally, our results show that estrogen receptor activity destabilizes AIB1, suggesting that multiple pathways may modulate the subcellular localization and

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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doi:10.1158/0008-5472.CAN-07-6433

stability of AIB1. We also identify two new domains, distinct from the known GSK3 phosphorylation site, that are required for ubiquitination and for proteasome-efficient transcriptional activation. Therefore, our results reveal a new mechanism for regulating the stability of the coactivator AIB1 and may provide unique insights into how subcellular localization of this molecule modulates various physiologic and pathologic processes, including the development of breast cancer.

Materials and Methods

Cell culture, transfections, and treatments. All cell lines were maintained in DMEM containing 10% fetal bovine serum (FBS). Transfections were performed with Lipofectamine LTX reagent (Invitrogen) according to company specifications. For estrogen depletion (−E2), MCF-7 cells were seeded in DMEM phenol red–free medium for 10 d and split when required to a final 80% confluence. The medium also included 10% charcoal and dextran-treated FBS (HyClone) to remove estrogens and glucocorticoids. Cells were treated with leptomycin B (10 ng/mL) and/or MG132 (20 μmol/L; Calbiochem) for 17 h. Epoxomicin treatment (20 μmol/L; Sigma) was also done for 17 h. E36-ts20 cells were grown at 32°C or heat shocked at 42°C for 30 min and then grown at 39°C overnight.

Paraffin embedding and immunostaining. Subconfluent growing cells were trypsinized, pelleted, and fixed with 10% formalin for 15 min at room temperature. After serial dehydration, cells were in paraffin by standard procedures. Sections of 5 μm thickness were processed for immunohistochemistry analysis after antigen retrieval.

***In vivo* cell labeling.** Cells (2×10^6) were seeded on 6-cm Petri dish. Treatment with cyclosporin A 5 μmol/L (Sigma), a calcineurin inhibitor that arrests cells in G_0-G_1 phase of the cell cycle, was performed for 16 h before radioactive pulse and maintained with this inhibitor throughout the time

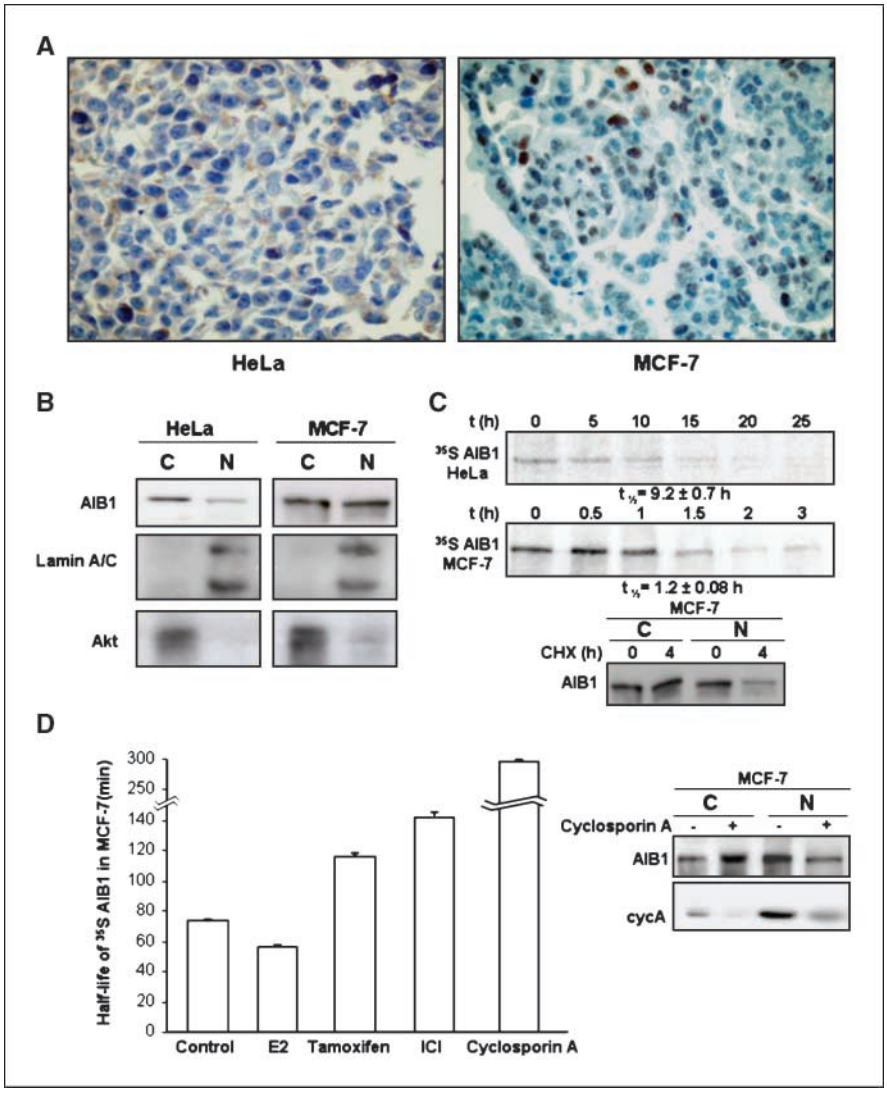

Figure 1. The subcellular distribution of AIB1 differs between cancer cell lines and correlates with its half-life. *A,* exponentially growing cells (80% confluence) were fixed and stained with anti-AIB1 antibodies. Sections were subsequently counterstained with hematoxilin. *B,* in parallel, cells were lysed and fractionated to obtain nuclear and cytoplasmic extracts that were analyzed by Western blotting with AIB1 antibodies. The Western blot is representative of five independent subcellular fractionations. *C,* a pulse-chase experiment was performed to determine AIB1 half-life. Subconfluent cultures were labeled with [^{35}S]methionine/cysteine and were lysed at the indicated times. Cleared lysates were immunoprecipitated with anti-AIB1 antibodies, resolved by SDS-PAGE, and radiolabeled AIB1 was quantified using a PhosphorImager. Images shown are representative of three independent experiments. Protein half-life ($t_{1/2}$) was measured by semilogarithmic linear regression. *Bottom,* Western blot for AIB1 of MCF-7 cells treated with 10 μg/mL cycloheximide (*CHX*) for the indicated times before the preparation of cytosol (*C*) and nuclear (*N*) fractions. *D,* AIB1 half-life in MCF-7 cells is also modulated by estrogen receptor activity. Estradiol or the estrogen receptor antagonists ICI and tamoxifen were added at time 0 (Materials and Methods). Cyclosporin A was added to subconfluent cultures 16 h before the pulse and chase experiment. Western blotting (*right*) confirmed cyclosporin A–mediated cell cycle arrest based on the loss of cyclin A expression which correlated with higher cytosolic AIB1 content.

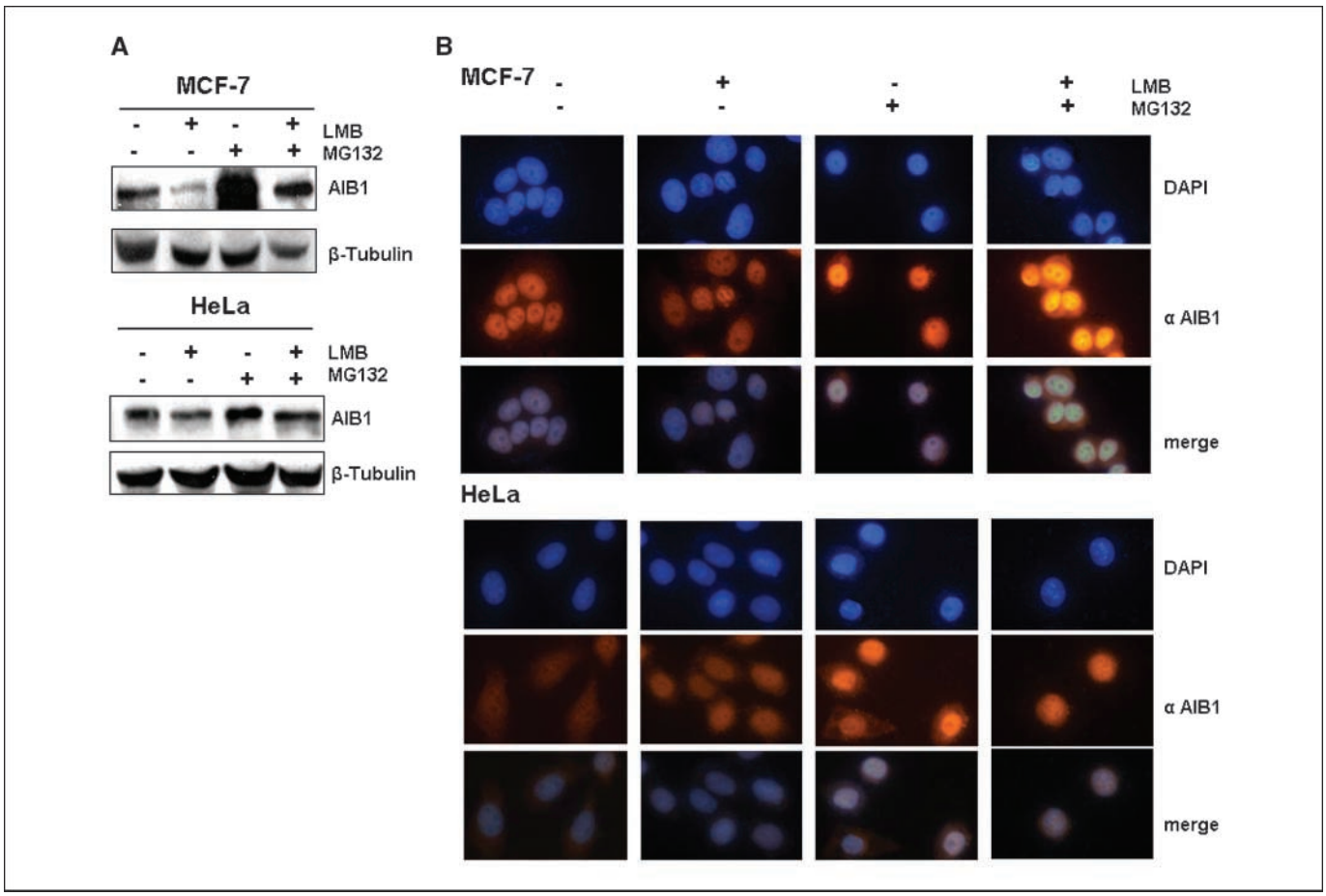

Figure 2. AIB1 turnover is mediated by the proteasome in the nucleus. *A,* subconfluent MCF-7 cells (*left*) and HeLa cells (*right*) were treated with MG132 (20 μmol/L), with leptomycin B (*LMB*; 10 ng/mL) or together as indicated for 17 h, and subsequently, lysates were subjected to Western blot analysis with AIB1 antibodies. Anti–β-tubulin was used to control the amount of protein loaded. *B,* cultures were treated exactly as described for *A* and stained with 4′,6-diamidino-2-phenylindole (*DAPI*; *blue in top*) and AIB1 antibodies (*bottom*).

course of the experiment. The following day, cells were washed twice and labeled in DMEM without L-methionine and L-cysteine (Invitrogen 21013-024) plus 50 μCi of [^{35}S]methionine/cysteine (GE) for 2 h. Labeling media was then substituted with regular growing media or media containing either 10 nmol/L 17β-estradiol, 10 μmol/L 4-hydroxytamoxifen (Sigma), or 1 μmol/L ICI 182780 (AstraZeneca Pharmaceuticals). Cells were subsequently lysed at the indicated times. Lysates were immunoprecipitated with anti-AIB1 antibodies and run on a SDS-PAGE gel. Gel was fixed and incubated with enhancer solution (EN3HANCE, NEN) for 1 h before being dried and exposed on a PhosphorImager screen.

Antibodies. Antibodies used were as follows: monoclonal anti-AIB1 (BD); polyclonal anti-AIB1 (generated in our laboratory and used for immunoprecipitations); monoclonal anti-HA (Covance); monoclonal β-tubulin (D-10), polyclonal cyclin A (596), monoclonal Gal4–DNA-binding domain (Gal4-DBD; 510), and monoclonal Lamin A/C (636) from Santa Cruz Biotech; monoclonal β-actin (ac-15, Sigma); and monoclonal Akt and phosphorylated Akt (Ser^{473}) from Cell Signaling.

Real-time PCR. Isolated RNA (TRIzol, Invitrogen) from cells was digested with DNase followed by purification through QIAGEN columns (RNeasy kit). Subsequently, the RT reaction (Superscript, Invitrogen) was done with 1 μg of total RNA and oligo dT. Real-time PCR was performed with 10 ng of cDNA with 5AGCCAGGAGGTGGTGGAGAC3 and 5CTCGGCCTGTGCTCTTCTCA3 SIP, 5ATAAGGGGGATTTTGGCACTCA3 and 5CTCCAGCCCTCCA-CATTCTTCT3 importin α3, 5ACCACAGTCCATGCCATCAC3′ and 5′TCCACCACCCTGTTGCTGTA3′ GAPDH.

Immunoprecipitations and Western blotting. Subcellular fractionations were performed with a nuclear/cytosol fractionation kit (MBL, JM-K266) according to the manufacturer's instructions. Conventional lysates were done with commercial lysis buffer (Cell Signaling) supplemented with protease cocktail inhibitors (Roche), up to 150 mmol/L NaCl, 1 mmol/L AEBSF (Roche), and 1 mmol/L NaF. Lysis buffer was supplemented with 5 mmol/L *N*-ethylmaleimide (SIGMA) and 5 nmol/L ubiquitin aldehyde (Sta. Cruz Biotech) in ubiquitination experiments. Immunoprecipitations with AIB1 or HA antibodies were carried out overnight and immunocomplexes were washed four times before SDS-PAGE. Proteins were transferred to Immobilon-P membranes (Millipore) and blotted with the indicated antibodies.

Results

Differences in subcellular distribution and stability of AIB1 in various cancer cell lines. To study the mechanisms which regulate the subcellular distribution and degradation of AIB1, we used immunohistochemistry to compare the expression of AIB1 in the cervical cancer cell line HeLa with the breast cancer cell line MCF-7. Whereas the distribution of AIB1 was more cytosolic in HeLa cells, prominent nuclear staining was observed in MCF-7 cells (Fig. 1*A*). This difference in the distribution of AIB1 between HeLa and MCF-7 cells was further confirmed by Western blot analysis of subcellular fractions (Fig. 1*B*). Similar results have been previously reported by Western blotting (26). In other cancer cell lines (H1299, A549, BT-474), AIB1 was detected predominantly in the cytosolic fraction similar to HeLa or predominantly in the

nucleus as observed for the MCF-7 cell line (Supplementary Fig. S1*A* and data not shown), suggesting that nuclear accumulation of AIB1 may represent a fundamental biological difference between cell lines and, hence, may reflect alterations in the shuttling and/or turnover of AIB1. Given that recent studies have reported seemingly contradictory results as to whether AIB1 is degraded by the proteosome in the cytosol (7) or the nucleus (8), we sought to determine the degradation locus in our biological models. Because cytoplasmic content of AIB1 is higher in HeLa cells, a mechanism involving nuclear degradation would be consistent with a greater AIB1 stability in this cell line. Pulse-chase experiments revealed that the half-life of AIB1 was >9 hours in HeLa cells, whereas it was only slightly more than an hour in MCF-7 cells (Fig. 1*C*). Similar results were obtained with H1299 and A549 cells (Supplementary Fig. S1*B*). Furthermore, treatment with cycloheximide to prevent the appearance of newly synthesized protein diminished only the nuclear pool of AIB1 (Fig. 1*C, bottom*). When MCF-7 cells were arrested with the inhibitor cyclosporin A, cytosolic content of AIB1 and its half-life were increased (Fig. 1*D, right* and *graph*), demonstrating that subcellular distribution is a critical determinant of AIB1 half-life. Interestingly, the cellular levels of AIB1 were also influenced by estrogen receptor activity because treatment of MCF-7 cells with estradiol shortened the half-life of AIB1. Conversely, exposure to ER antagonists (ICI, tamoxifen) extended the stability of AIB1 (Fig. 1*D*), but these agents did not alter the subcellular compartmentalization of AIB1 (data not shown). Thus, the expression levels and activity of the ER represent an additional mechanism for modulating the half-life of AIB1. The significant differences in AIB1 turnover together with the cell line–specific subcellular distribution of AIB1 suggest that the nucleus may serve as the principal site for degradation of this coactivator.

Effects of inhibitors of nuclear export and the proteasome on the nuclear content of AIB1. To further explore the nuclear degradation of AIB1, we treated cells with leptomycin B, a specific inhibitor of the nuclear export factor CRM1. In both HeLa and MCF-7 cells, this inhibitor caused a notable decrease in AIB1 levels (Fig. 2*A, line 2*), presumably because AIB1 is trapped within the nucleus and, thus, is more accessible to mechanisms mediating its degradation. This effect was more striking in MCF-7 cells, probably due to the shorter half-life of AIB1 in this cell line. Immunofluorescence studies further confirmed the enhanced nuclear localization of AIB1 in both cell lines after inhibitor treatment (Fig. 2*B, line 2*). As expected, treatment of cells with the proteasome inhibitor MG132 greatly increased the levels of AIB1 as detected by Western blotting. However, when cells were coincubated with both inhibitors, MG132 potently prevented the leptomycin B–mediated reduction of AIB1 levels (Fig. 2*A* and *B, lane 4*), further demonstrating that nuclear accumulation of AIB1 coincides with degradation by the proteasome. Although leptomycin B reduced AIB1 levels in the presence of MG132 (Fig. 2*A, lanes 3* and *4*), this effect is unlikely to be at the level of stabilization but may rather reflect a decrease in protein translation as leptomycin blocks nuclear export of mRNAs. However, the degradation of AIB1 is presumably mediated by the proteasome because, otherwise, treatment with leptomycin alone or in combination with MG132 (*lanes 2* and *4*) would yield the same result. Taken all together, these results implicate a nuclear mechanism in the degradation of AIB1 by the proteasome and suggest that this may be avoided in HeLa cells by a higher rate of nuclear export of AIB1.

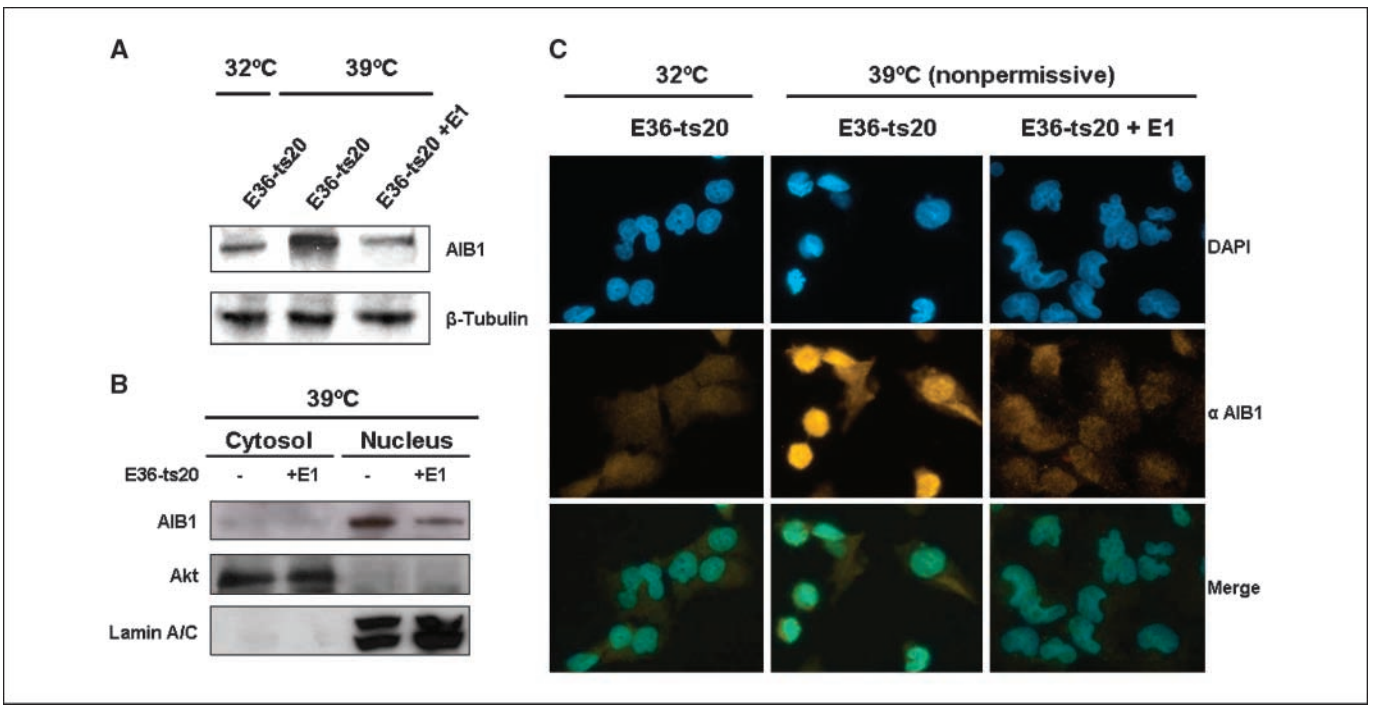

Figure 3. The ubiquitin-proteasome pathway degrades AIB1 in the nucleus of E36-ts20 cells. *A,* the thermosensitive cell line E36-ts20 was grown either at 32°C or at the nonpermissive temperature 39°C. The E1 ubiquitin ligase was transfected into these cells, and a stable clone was obtained (*E36-ts20 + E1*). Western blot analysis was performed to determine AIB1 levels, using anti–β-tubulin antibodies to control protein load. *B,* subcellular fractionation of E36-ts20 control cells (*−E1*) or transfected with E1 (*+E1*) and grown at the nonpermissive temperature 39°C reveals accumulation of AIB1 in the nucleus in the absence of E1 and degradation in the nucleus when E1 is expressed. Antibodies against Lamin A/C and Akt were used to assess cross-contamination of fractions. *C,* immunofluorescence with AIB1 antibodies and nuclear staining with DAPI confirms increasing intensity and accumulation in the nucleus of E36-ts20 cells when grown at the nonpermissive temperature.

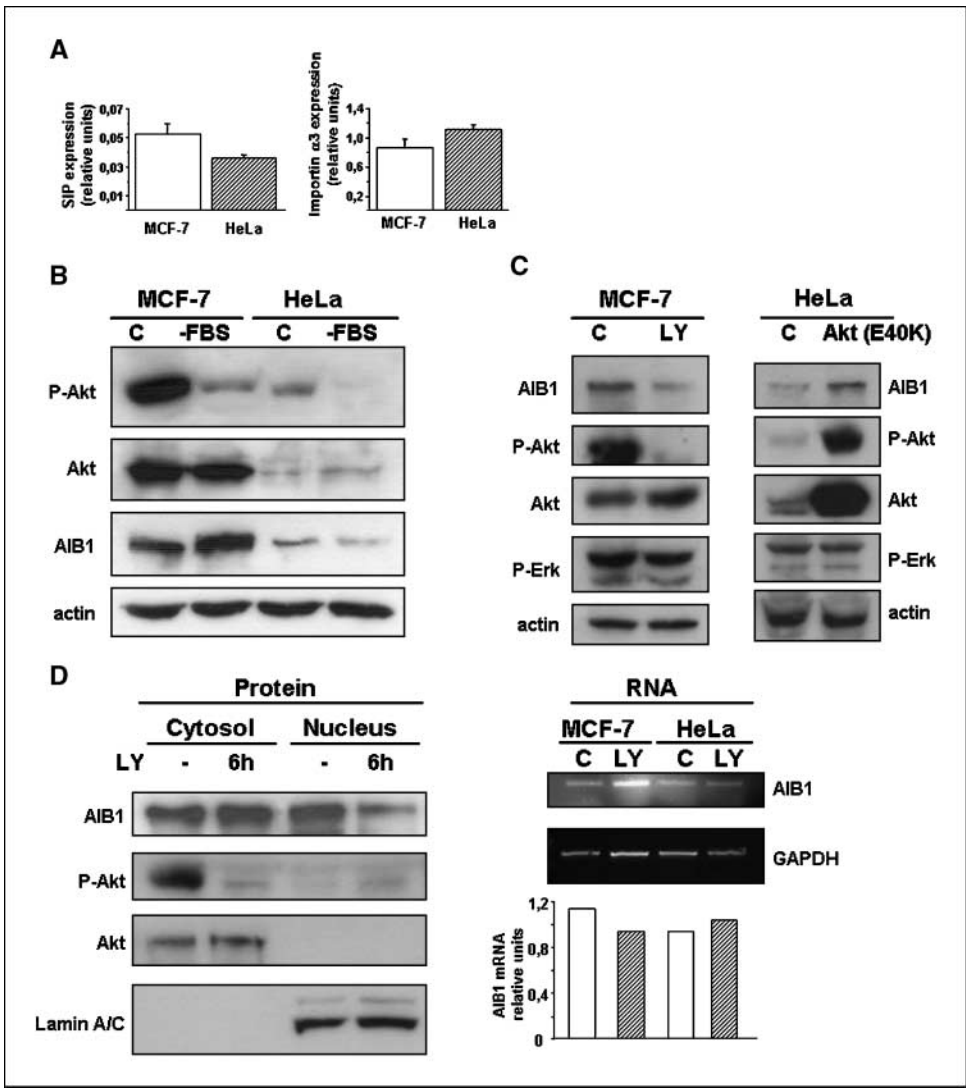

Figure 4. Differences in Akt signaling correlate with AIB1 stability. *A,* RNA was isolated from subconfluent MCF-7 and HeLa cells and used to analyze the expression of SIP (*left graph*) and importin α3 (*right graph*) by real-time PCR. *B,* subconfluent cultures were maintained under normal conditions (*C*) or deprived of serum overnight (−*FBS*). Lysates were prepared and analyzed by Western blotting with the indicated antibodies. *C,* proliferating MCF-7 cells were treated with 50 μmol/L LY294002 for 6 h (*LY*) or with vehicle alone (*C*). Cell lysates were subjected to Western blotting analysis using the indicated antibodies. HeLa cells were transfected with empty vector (*C*) or with pCMV HA-Akt(E40K), a soluble activated form of Akt. Two days posttransfection, cells were lysed and subjected to Western blot analysis with the indicated antibodies. *D,* subcellular fractions were prepared from MCF-7 cells treated with LY for the indicated times and analyzed by Western blot to detect AIB1 levels (*left*). LY efficiently blocked Akt activation as determined by analysis with phosphorylated Akt antibodies. Total Akt and Lamin A/C were used as cytoplasmic and nuclear markers. In parallel, transcription of endogenous AIB1 was assessed by semiquantitative RT-PCR (*right*) in untreated (*C*) and 50 μmol/L LY294002 (*LY*)–treated cells. Intensity of the bands was quantified using Quantity One software (Bio-Rad) and normalized to GAPDH levels as a house-keeping gene (*bottom columns*).

Proteasomal degradation of AIB1 in the nucleus requires ubiquitination. Some proportion of AIB1 proteasomal turnover occurs in an energy and ubiquitin-independent manner (4). Therefore, we sought to define the role of ubiquitination in the observed nuclear degradation of AIB1 by the proteasome pathway. In the thermosensitive cell line E36 (E36-ts20), the ubiquitin-activating enzyme E_1 is inactive when cells are grown at 39°C, thus representing a tool for studying the absence of protein ubiquitination. At the nonpermissive temperature (39°C), nuclear content of AIB1 was higher in E36-ts20 cells (Fig. 3*A, line 2*) than when cells were cultured at the permissive temperature of 32°C (Fig. 3*A, line 1*). Moreover, when cells were transfected with functional E1 (*E36-ts20 + E1*) and grown at 39°C, levels of AIB1 were similar to those observed at the permissive temperature of 32°C (Fig. 3*A, line 3*), an important control to show that the observed alterations in AIB1 levels are related directly to the ubiquitination process and not to some indirect effect of temperature in this thermosensitive line. In the absence of ubiquitination, AIB1 accumulated in the nucleus (Fig. 3*B* and *C*). These results strongly suggest that degradation of AIB1 mainly occurs through the ubiquitin-proteasome pathway and that this requires ubiquitination within the nucleus.

Akt signaling favors AIB1 stability. We next focused on investigating the mechanisms that may differentially regulate the stability and shuttling of AIB1 in MCF-7 versus HeLa cells. A recent report has shown that the SIP recruits and retains SRC proteins in the cytoplasm (18). We reasoned that differences in the expression levels of SIP might explain the differences in AIB1-nuclear content observed between these two cell lines. However, no significant differences were detected when SIP levels were analyzed by reverse transcription–PCR (RT-PCR) in both HeLa and MCF-7 cells (Fig. 4*A, left graph*). Importin α3 has also been implicated in AIB1 nuclear transport (8). We also assessed levels of importin α3 mRNA but did not observe significant differences between these two cell lines (Fig. 4*A, right graph*).

AIB1 is phosphorylated by the IKK complex in response to tumor necrosis factor α, resulting in enhanced transcriptional activity (16). IKK is also a target of Akt, and thus, we evaluated whether alterations to Akt signaling might explain differences in AIB1 stability and transcriptional activity between MCF-7 and HeLa cells. As would be expected, serum depletion (−FBS) in HeLa cells reduced the phosphorylation of Akt (Fig. 4*B*). This reduction coincided with a decrease of AIB1 levels. Surprisingly, serum

withdrawal in MCF-7 cells produced no such effect on Akt phosphorylation; the levels of phosphorylated Akt were still significantly higher than in HeLa cells after serum withdrawal (Fig. 4*B*), suggesting that Akt may be more active in the MCF-7 cell line. Furthermore, depletion of estrogen in cultures of MCF-7 cells reduced proliferation and cyclin A expression (Supplementary Fig. S2*A*). However, even under these growth-restricting conditions, the levels of phosphorylated Akt remained much higher than those observed in nonproliferating HeLa cells. It is important to note that both the levels of total Akt, as well as phosphorylated Akt, were higher in MCF-7 than in HeLa cells, and the expression of AIB1 was moderately, but reproducibly, increased by either serum or estrogen deprivation in the MCF-7 line (Fig. 4*B* and Supplementary S2*A*). In contrast, serum withdrawal reduced AIB1 expression in HeLa cells, coincident with a reduction in Akt phosphorylation levels and proliferative markers (Fig. 4*B* and Supplementary S2*A*). Similar results were also observed with A549 and H1299 cells (Supplementary Fig. S2*B*). These observations show that, in the absence of proliferation, AIB1 levels were slightly increased in MCF-7 cells. Moreover, these studies reveal substantial differences in Akt signaling between HeLa and MCF-7 cells and suggest that this pathway may be involved in the regulation of AIB1 stability/activity.

To further test the hypothesis that Akt modulates AIB1 degradation, we used LY294002, a potent and specific PI3K inhibitor, to block Akt activation. Treatment of MCF-7 cells with LY294002 abolished phosphorylation of Akt and produced a reduction of AIB1 levels (Fig. 4*D, left*). LY294002 treatment did not alter AIB1 RNA levels (Fig. 4*D, right*). Subcellular fractionation revealed that the LY294002 treatment induced a reduction of AIB1 levels in the nucleus, but not in the cytosol (Fig. 4*D, right lanes in the left panels*). Conversely, ectopic expression of a soluble, constitutively active form of Akt (E40K) in HeLa cells increased AIB1 levels (Fig. 4*C, right*). These results support a role of Akt in AIB1 stabilization. Importantly, treatment with LY294002 did not alter Erk phosphorylation (Fig. 4*C, left*) suggesting that the observed Akt effects on AIB1 stability are independent of Erk activation (14, 15).

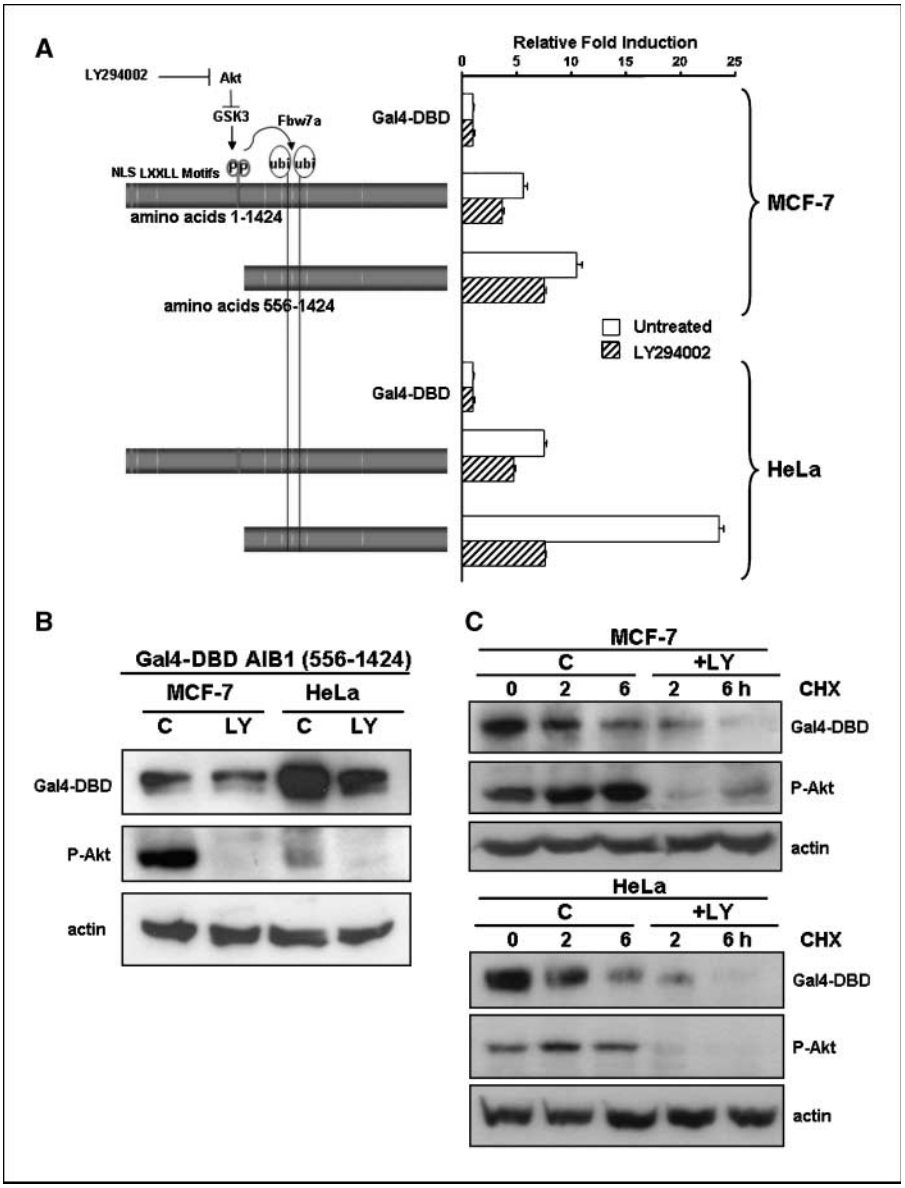

Figure 5. Akt signaling stabilizes AIB1 independently of GSK3. *A,* MCF-7 and HeLa cells were transiently transfected with the indicated AIB1 fragments fused to the Gal4-DBD together with a luciferase reporter. Two days after the transfection, cells were left untreated (*open bars*) or treated (*hatched bars*) with 50 μmol/L LY294002 at 6 h. Subsequently, cells were lysed and luciferase activity was measured and normalized to luciferase values obtained with the Gal4-DBD empty vector alone. Gal4 DBD fused in frame to the VP16 activation domain was also used as a control (*GD-VP16*). In contrast to AIB1 fragments, VP16 activity increased upon LY294002 treatment: MCF-7 (GD-VP16 = 564 ± 22; GD-VP16 + LY = 2466 ± 57); HeLa (GD-VP16 = 319 ± 6; GD-VP16 = 2565 ± 47). *NLS,* nuclear localization signal; *LXXLL Motifs,* interaction with transcription factors and other coactivators. *B,* in parallel, transfected cells were analyzed by Western blot analysis for expression of Gal4-DBD AIB1 (556-1424), revealing that treatment with LY294002 (*LY*), results in reduced protein levels. *C,* loss of stability of ectopically expressed Gal4-DBD AIB1 (556-1424) protein by inhibition of PI3K. HeLa and MCF-7 cells were treated with 10 μg/mL of cycloheximide (*CHX*) 2 d posttransfection. Treatment was performed for the indicated times in the absence (*C*) or presence (+*LY*) of PI3K inhibitor. Without *de novo* protein synthesis, Gal4-DBD hybrid was more unstable in the presence of LY294002.

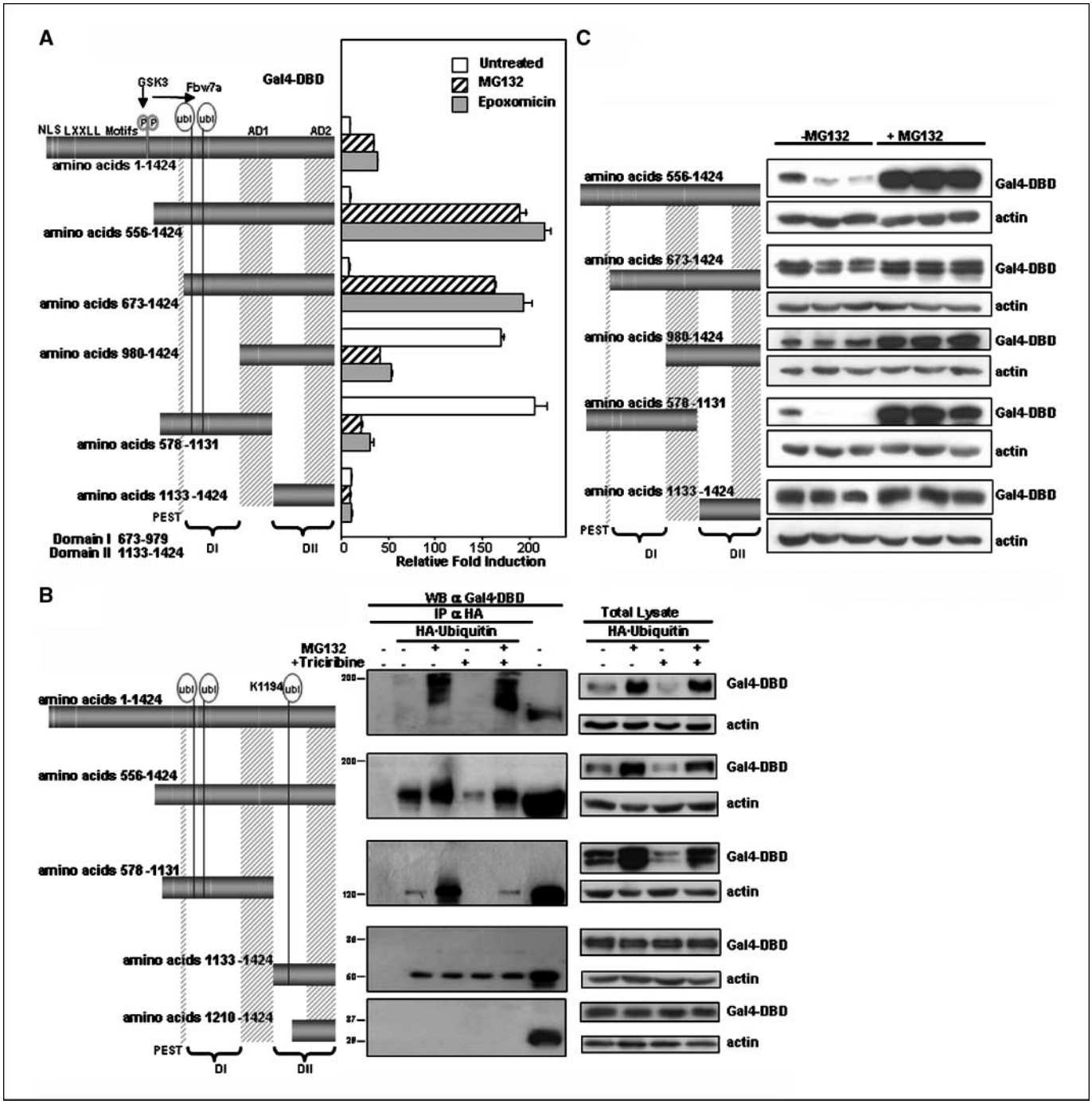

Figure 6. Two distinct domains at the carboxy terminus of AIB1 mediate sensitivity to proteasome inhibitors and binding to ubiquitin. *A,* the indicated fragments of AIB1 were fused to the Gal4-DBD and transiently transfected into COS cells together with a luciferase reporter under the Gal4-responsive element. Cells were left untreated or were exposed to the proteasome inhibitors epoxomycin or MG132 (20 μmol/L each). *AD,* activation domain. *B,* Gal4-DBD constructs were transiently cotransfected with HA-tagged ubiquitin. Two days later, COS cells were treated with the proteasome inhibitor MG132 and/or with the Akt inhibitor Triciribine (40 μmol/L) for 6 h, as indicated. Cells were lysed and analyzed by immunoprecipitation with anti-HA antibodies, followed by Western blotting with Gal4-DBD antibodies (*right*). The Western blots (*extreme right*) present levels of Gal4-DBD expression and acting as a loading control. *C,* the indicated fragments were fused to Gal4-DBD and were transiently expressed in COS cells. Two days posttransfection, cells were treated with cycloheximide (*CHX*) and cultures were lysed at the indicated times. Gal4-DBD antibodies were used to detect the levels of hybrid protein. In parallel, cells were also treated with MG132 to inhibit proteasomal degradation.

Regulation of AIB1 activity and stability by Akt occurs independently of GSK3. Two residues in the amino terminus of AIB1 (K17 and R18) are essential for its nuclear localization (8). To evaluate the role of Akt in modulating AIB1 activity independently of nuclear shuttling, we generated two fragments of AIB1 fused to the Gal4-DBD harboring a nuclear localization signal. One construct contained full-length AIB1 (amino acids 1–1424) and the other one lacked the known GSK3 phosphorylation site (amino acids 556–1424). Each construct was cotransfected with the luciferase gene under the Gal4-responsive element. As expected,

treatment with LY294002 inhibited AIB1 transcriptional activity in both MCF-7 and HeLa cells (Fig. 5*A*). Surprisingly, the activity of the fragment lacking the phosphorylation site for GSK3 was also regulated by Akt signaling pathway (see scheme and results in Fig. 5*A*), suggesting the existence of a regulatory mechanism distinct from the one previously reported for GSK3 (25). To further correlate transcriptional activity with protein levels, we analyzed ectopic expression of AIB1 fragment (556-1424) by Western blotting. As shown in Fig. 5*B*, this fragment lacking the phosphorylation site for GSK3 was less stable when PI3K/Akt was inhibited by LY294002. Furthermore, protein stability, as determined by cycloheximide treatment to prevent *de novo* protein synthesis, was significantly diminished in both cell lines when PI3K signaling was inhibited (Fig. 5*C*). Similar results were also obtained using the Akt specific inhibitor Triciribine (Supplementary Fig. S3). These results suggest that PI3K/Akt signaling is important for conferring stability of AIB1 protein.

Two domains in the carboxy terminus of AIB1 are concomitantly required for efficient proteasome-dependent transcriptional activation. To investigate the potential role of GSK3 phosphorylation in AIB1-mediated transcription, we generated various fragments of AIB1 fused to the Gal4-DBD. Individual constructs were cotransfected into COS cells together with the luciferase gene under the Gal4-responsive element. Treatment with the inhibitor MG132 or epoxomicin, a more specific inhibitor of the proteasome, potently enhanced the transcriptional activity of those fragments containing the carboxyl-half of AIB1 (Fig. 6*A*). Importantly, these fragments did not contain S505 and S509, which participate in GSK3/Fbw7α-mediated ubiquitination (25), suggesting that other ubiquitin ligase may also be involved in AIB1 ubiquitination. However, the DI domain (amino acids 673–979; Fig. 6*A*) containing the two lysines reported to be ubiquitinated by Fbw7α was required, but not sufficient, to mediate the sensitivity to proteasomal inhibitors. Another domain, DII (1133–1424), together with DI, was necessary for the effects induced by MG132 or epoxomicin. Emerging evidences suggest a role for the ubiquitin-proteasome system in the transcriptional activation of genes (27, 28). These results suggest that the DI and DII domains of AIB1 are concomitantly required for efficient proteasome-mediated transcriptional activation. A potential caveat to the interpretation of these experiments is the possibility that some of these ectopically expressed fusion proteins were not localized to the nucleus. However, the fact that they display potent transcriptional activity is consistent with nuclear localization. Furthermore, nuclear staining of these domains of AIB1 was verified by immunofluorescence (Supplementary Fig. S4). Thus, these observations strongly suggest that the carboxy terminus of AIB1 contains the structural information needed for the proper regulation of AIB1-mediated transcription by the proteasome.

The DI and DII domains are both ubiquitinated independently of GSK3 phosphorylation and are differentially stabilized by Akt. GSK3 couples AIB1 phosphorylation to ubiquitination by the E3 ubiquitin ligase $SCF^{Fbw7\alpha}$. To further study the role of Akt in the stabilization of AIB1, we analyzed ubiquitination of the AIB1 fragments devoid of the GSK3 phosphorylation site in response to the Akt inhibitor Triciribine. Treatment with this inhibitor potently reduced ubiquitination levels in all DI-containing fragments, but not in the DII fragment alone (Fig. 6*B*; compare lane 2 without inhibitor to lane 4 with Triciribine). This reduction in ubiquitination levels was due to increased degradation since inhibition of the proteasome restored, at least in part, ubiquitination of the DI-containing fragments (Fig. 6*B, lane 5* treated with both inhibitors). Moreover, treatment with Triciribine reduced the levels of these mutant proteins as detected in total cell lysates (Fig. 6*B, extreme right*). These results reveal a role for Akt in the stabilization of fragment DI. Additionally, we show that the ubiquitination of DII, in contrast to DI, is rather stable (Fig. 6*B* and *C*). Sequence analysis of AIB1 revealed a third potential ubiquitination site in lysine 1194, located precisely within the DII domain. A shorter version of DII lacking K1194 was not ubiquitinated (Fig. 6*B*). It is not clear whether the absence of a ladder reflects increased susceptibility of these fragments to deubiquitinases in contrast to the full-length AIB1 or whether, alternatively, polyubiquitination may require other structural information present only in the full-length molecule.

The PEST-containing domain preceding the DI and DII domains is required to destabilize AIB1 protein. To further study the role of DI and DII domains in AIB1 turnover, we performed a time course experiment wherein cycloheximide was added to the cultures to prevent *de novo* synthesis of proteins. AIB1 contains a PEST domain (647–672) typically involved in protein degradation; however, deletion of this domain had no effect on the activity of AIB1 (Fig. 6*A*). Analysis of protein stability revealed that only those fragments containing the PEST region were unstable in the absence of proteasome inhibitor (Fig. 6*C*). However, treatment with MG132 potently induced the accumulation of these highly unstable fragments (Fig. 6*C*, fragments 556–1424 and 578–1131). These findings reveal a previously unrecognized role for the PEST domain of AIB1 in mediating the proteasomal degradation of this coactivator. In contrast, fragment DII, although ubiquitinated, was stable (Fig. 6*B* and *C*), thus implicating the DII domain in proteasome-mediated transcriptional activation but not in degradation.

Discussion

In the present study, we report that nuclear content of AIB1 varies between cancer cell lines and these differences in subcellular distribution correlate with striking differences in the half-life of this oncogenic molecule. Additionally, we show that sustained phosphorylation of Akt profoundly influences AIB1 stability and transcriptional activity, independently of the known mechanism involving GSK3 phosphorylation (25). As a transcriptional coactivator, AIB1 exerts its major function in the nucleus; here, we confirm that the majority of AIB1 is degraded by the proteasome also in the nucleus. Interestingly, in the quiescent noncancerous epithelial cells of mammary ducts, AIB1 is localized to the cytoplasm whereas very strong nuclear staining has been observed in proliferating cells of the same structures (10). Our data suggest that overexpression of AIB1 alone might not be sufficient to trigger neoplastic changes but that alterations to other pathways (i.e., Akt signaling, nuclear shuttling, and proteasomal degradation) are required to produce the tumors associated with AIB1. Consistent with this idea, tumor development in transgenic mice overexpressing AIB1 is not observed until at least at 8 months of age, which is much later than the effects of other oncogenes that have been overexpressed in animal models (22). One possible explanation for this stepwise development of cancer is that aging or environmental factors produce other alterations that function in synergy with the overexpression of AIB1. The PI3K/Akt pathway is frequently altered in cancers, and hence, it may play a pivotal role in AIB1-mediated oncogenesis.

We did not detect differences in the expression of SIP and importin α3, two molecules directly implicated in AIB1 nuclear transport, between MCF-7 and HeLa cells. However, we cannot exclude the possibility that cell line–specific differences may exist for regulating the activation level of these molecules. Although we have focused on the observed differences in Akt activation, CKII may also play a role in the modulation of AIB1 degradation, given that Akt and ERα are substrates of CKII (18, 29). Phosphorylation of SIP by CKII in its PEST domain releases AIB1 from the interaction in the cytosol (18). However, CKII activity alone cannot explain the differences we observe in activity and stabilization of AIB1 fragments lacking the NH_2 terminal region that mediates the interaction with SIP (Fig. 6).

Our results show that the half-life of AIB1 is shorter in MCF-7 than in HeLa cells. By blocking nuclear export with LMB and inhibiting the proteasome with MG132 (Fig. 2), our studies reveal that turnover of AIB1 occurs in the nucleus in both cell lines and suggest that nuclear export of AIB1 is more active in HeLa cells because higher levels of this coactivator were observed in the cytoplasm of this cell line in the absence of inhibitors. This cytosolic localization may explain the longer half-life of AIB1 which we observed in HeLa cells, although other alterations, such as ERα expression may also contribute to AIB1 stability (Fig. 1*D*; ref. 30). MCF-7 cells display unusually high levels of activated Akt. Serum withdrawal is much less effective at reducing phosphorylated Akt levels in MCF-7 cells compared with the effects on HeLa cells. Furthermore, prolonged estrogen depletion can effectively cause growth arrest in MCF-7 cells without significantly reducing phosphorylated Akt levels. This sustained activation of Akt is most likely due to the high levels of total Akt expressed by MCF-7 cells compared with HeLa cells (Fig. 4*B*). Overexpression of AIB1 itself may also play a role in these observations because its ablation in mice reduces the expression of upstream signaling molecules, such as IGF-IR and IRS proteins, which in turn diminish the activation of Akt upon IGF stimulation (24). However, other mechanisms specific to MCF-7 cells may account for the observed dysregulation of Akt because the cell line A549 also overexpresses AIB1 but Akt activation is comparatively normal (Supplementary Fig. S1*A*). Our data (fragment 556–1424; Figs. 5 and 6*D*) suggest the existence of an additional or alternative mechanism for regulating the proteasomal degradation of AIB1, which is distinct from that involving GSK3 phosphorylation and targeting to Fbw7α (25).

We have identified two new domains that are concomitantly required for proteasome-dependent transcriptional activation of AIB1 independently of the GSK3 phosphorylation site. Both DI and DII domains are subject to ubiquitination (Fig. 6*B*). We also cotransfected fragments DI and DII to test whether the combined presence of these two independent proteins could restore transcriptional efficiency to the proteasome. However, as independent fragments, these domains were not able to reconstitute sensitivity as was observed for the clone expressing both domains in tandem (data not shown), perhaps owing to a loss of structure or adequate orientation. One possible explanation is that efficient proteasome-mediated transcriptional activation requires the presence of all three ubiquitination sites.

In contrast to the PEST-DI domains, DII ubiquitination is not required for the degradation of AIB1 by the proteasome (Fig. 6*B* and *C*; ref. 25). Furthermore, we found that only PEST-DI domains are stabilized by Akt. One plausible explanation is that Akt inhibits DI ubiquitination as has been described for other molecules, such as MDM2, XIAP, and cyclin D2 (31–33). A recent publication documents the stabilization of AIB1 by PKCζ (34). PKCζ is a known substrate of PI3K/PDK1 signaling pathway (35). Our data are distinct but complementary to this new report, as they provide an alternative mechanism mediated by Akt, another effector of PDK1, whereby AIB1 is stabilized through its PEST-DI domains.

Our results suggest that the oncogenic potential of AIB1 is likely to be modulated by various pathways that function to determine its subcellular localization and thereby its susceptibility to proteasome degradation. One of our present aims is to identify mutations in human AIB1 that render it insensitive to degradation by the proteasome. These mutations are of most interest for the role they may play in modulating the oncogenic potential of overexpressed AIB1. On the one hand, certain genetic and/or environmental signals are required to trigger overexpression of AIB1. One mechanism, which has been associated with overexpression of AIB1, is chromosomal amplification, but this has been observed in only ~5% of the human tumors bearing high levels of AIB1. Thus, other mechanisms must modulate the cellular levels of AIB1; for example, mutations in the degradation domains (PEST-DI) or in the domains required for proteasome-mediated coactivation (DI and DII) would be expected to block degradation or full activation of AIB1 and, thereby, altering its oncogenic potential. Additionally, the participation of AIB1 in abnormal cellular growth may involve alterations to its subcellular localization, which directly or indirectly affect its transcriptional activity in the nucleus. The Akt pathway is quite likely to be involved in these processes because our results clearly link sustained phosphorylation of Akt with increased stability/activity of AIB1 in cancer cells. Thus, when increased expression of AIB1 coincides with another molecular anomaly that promotes its activation/stabilization, this coactivator may function as a potent oncogene by promoting cell proliferation and tumor development.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

Received 11/30/2007; revised 5/1/2008; accepted 5/1/2008.

Grant support: Grant SAF2006-12470 (Ministerio de Educación, Ciencia, Spain), FIS grant PI030818 (Instituto de Salud Carlos III, Spain), and Conselleria d'Educació predoctoral fellowship from Generalitat Valenciana (M. Ferrero).

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We thank M. Piechaczyk for the cells E36-ts20 and E36-ts20 + E1, P.K. Vogt (Scripps Research Institute) for the activated form of Akt, P. Puigserver for Gal4-DBD-VP16, R. Farras and E. Knecht for constant advice and help, and D. Burks for the editing of this article.

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Phosphoinositide 3-kinase/AKT Signaling Can Promote AIB1 Stability Independently of GSK3 Phosphorylation

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Cancer Res 2008;68:5450-5459.