Ribozyme-targeting demonstrates that the nuclear receptor coactivator AIB1 is a rate limiting factor for estrogen-dependent growth of human MCF-7 breast cancer cells.

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Running Title: AIB1 is rate limiting for growth of MCF-7 cells in vivo
SUMMARY

Human breast tumorigenesis is promoted by the estrogen receptor pathway and nuclear receptor coactivators are thought to participate in this process. Here we studied whether one of these coactivators, AIB1 (amplified in breast cancer 1), was rate-limiting for hormone-dependent growth of human MCF-7 breast cancer cells. We developed MCF-7 breast cancer cell lines in which the expression of AIB1 can be modulated by regulatable ribozymes directed against AIB1 mRNA. We found that depletion of endogenous AIB1 levels reduced steroid hormone signaling via the ERα or PRβ on transiently transfected reporter templates. Downregulation of AIB1 levels in MCF-7 cells did not affect estrogen-stimulated cell cycle progression but reduced estrogen-mediated inhibition of apoptosis and cell growth. Finally, upon reduction of endogenous AIB1 expression, estrogen-dependent colony formation in soft agar and tumor growth of MCF-7 cells in nude mice was decreased. From these findings we conclude that, despite the presence of different ER coactivators in breast cancer cells, AIB1 exerts a rate-limiting role for hormone-dependent human breast tumor growth.
INTRODUCTION

Human breast tumorigenesis is promoted by enhanced activity of the estrogen receptor (ER) pathway. It has been shown that estrogens can directly cause proliferation of breast cancer cells (1) and that more than 70% of primary human breast cancers are ER positive. The activity of the ER is modulated by a recently discovered class of specific corepressors and coactivators, that inhibit or enhance the transcriptional activity of the ER as well as related nuclear hormone receptors (2-4). In the absence of ligand, some of the nuclear receptors are bound to corepressors such as SMRT and NCoR (5,6). After ligand binding, the corepressors are released and nuclear receptor coactivators are recruited. This leads to the enhancement of transcriptional activity of the nuclear receptor via interaction with chromatin remodelling complexes and members of the basal transcription machinery (2,3).

Some of the best characterized nuclear receptor coactivators belong to the p160/SRC (steroid receptor coactivator)-family. In humans this family consists of SRC-1 (7), TIF-2 (8) and AIB1 (9) (ACTR/RAC3/TRAM-1/SRC-3) (10-13). Special attention has been focused on the gene AIB1 (amplified in breast cancer 1), which is amplified in breast, ovarian, pancreatic, and gastric cancer (9,14,15). Amplification of the AIB1 gene was detected in 5-10% of primary breast tumors and AIB1 mRNA was found to be highly expressed in many breast tumor specimens (9,16-18). Furthermore, AIB1 amplification correlates with estrogen and progesterone receptor positivity of primary breast tumors as well as with tumor size (16,19). AIB1 binds directly to ER in vivo (20), and enhances in vitro the transcriptional activity of the estrogen receptor (9,10,13), as well as a
number of other nuclear receptors, including the progesterone, thyroid hormone, and retinoid acid receptor (10-12). In addition, it has been shown that AIB1 interacts with other transcription factors such as TEF (21) and NFκB (22) and that AIB1 inhibits p53-dependent transactivation (23). Interestingly, a recent study demonstrated that AIB1 overexpression is correlated with the absence of ER and PR but is positively correlated with the expression of p53 and HER2/neu, indicating that in a subset of breast tumors AIB1 might also be involved in signaling pathways other than for steroid hormones (24). p/CIP, the mouse homolog of AIB1, is required for CBP-dependent transcriptional activation induced by interferon-gamma and TPA (25). Disruption of p/CIP results in a pleiotrophic phenotype including reduced female reproductive function and blunted mammary gland development in mice as well as in the production of endogenous estrogen (26). In addition, p/CIP also seems to play a role for the expression of genes critical for somatic growth and in several growth hormone regulatory pathways (27). Taken together, these findings led to the hypothesis that human AIB1 contributes to the development of breast cancer, but evidence that AIB1 directly affects breast cancer growth and development is still lacking.

In this study we investigated the function of AIB1 for breast cancer cell proliferation, apoptosis and tumor growth in mice. We selected the well-characterized human breast cancer cell line MCF-7 for our studies since it was shown earlier that these cells express high levels of AIB1 protein (13,18). In addition, AIB1 interacts with the endogenous estrogen receptor in these cells (20), enabling us to investigate the role of AIB1 for estrogen dependent growth. We now report that reduction of endogenous
AIB1 levels in MCF-7 cells by ribozyme-targeting reveals a significant role of this coactivator for estrogen-dependent growth and apoptosis as well as for tumor growth in mice.

EXPERIMENTAL PROCEDURES

Cell Culture- MCF-7 cells were cultivated in IMEM (Life Technologies) supplemented with 10% FCS (Life Technologies). MCF-7 cells which were stably transfected with ribozyme expression vectors were cultivated in IMEM supplemented with 10% FCS, 400 µg/ml G418 (Invitrogen) and 400 µg/ml zeocin for MCF-7/Rz12 (Invitrogen) or 1µg/ml puromycin for MCF-7/Rz23 (Sigma) in the presence or absence of 1µg/ml doxycycline (Sigma).

Ribozyme Expression Vectors and Generation of Stable Cell Lines- To generate the ribozyme constructs for transient transfections, synthetic sense and antisense oligonucleotides containing the catalytic center and flanking regions of the ribozymes as well as AIB1 homologous regions were annealed and ligated into the HindIII/NotI sites of pRc/CMV (Invitrogen). The sequences for the upper strand oligonucleotide were 5’-AGCTTGAATCGATACTGATGAGTCCGTTAGGACGAAACTG GGGTTGC-3’ for ribozyme 12 and 5’-AGCTTAGAACTACCTGATGAGTCCGTTAGGA CGAAACACCTGAAGC-3’ for ribozyme 23. Ribozyme expressing cell lines were obtained by co-transfection of MCF-7 cells, which stably express the tetR-VP16 transactivator protein (28), with ribozyme expression vectors which had been
constructed by insertion of the synthetic ribozymes (see above) into the tetracycline regulatable vector pTET (29), and pSV2 NEO (Clontech) (MCF-7/Rz12) or pBabePuro (30) (MCF-7/Rz23). The cells were selected in IMEM/10% FCS supplemented with G418 (400 µg/ml), zeocin (400 µg/ml) or puromycin (1 µg/ml) in the presence of doxycycline (1 µg/ml) for 4-6 weeks. Individual clonal cell lines were obtained by selection following transfection of the cells.

RNA Preparation and Northern Blot Analysis- For the preparation of cytoplasmic RNA 70-80% confluent cells were lysed in ice cold lysis buffer (0.2 M Tris-HCl pH 8.0, 140 mM NaCl, 2 mM MgCl₂, 0.5% NP-40). After incubation for 4 min on ice the mix was centrifuged at 12,000 rpm at 4°C. Cytoplasmic RNA was obtained by extraction with STE-buffer (5 mM Tris-HCl pH 8.5, 2 mM EDTA, 0.2% SDS) and phenol/chloroform/isoamylalcohol (25:24:1 v/v). Northern blot analysis was performed with 15 µg of cytoplasmic RNA using a radiolabeled 0.75 kb EcoRI fragment from AIB1/ACTR for hybridization (10). AIB1 transcript levels were quantified by phosphorimager (Molecular Dynamics). All AIB1 levels were corrected with GAPDH for loading differences.

Western Blot Analysis- For Western blot analysis, 70-80% confluent cells were washed with PBS, harvested with a cell scraper, washed once with PBS and twice with wash buffer (10 mM HEPES pH 7.8, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT, protease inhibitor cocktail (Complete™; Boehringer Mannheim)). The cell pellet was
resuspended in lysis buffer (20 mM HEPES pH 7.8, 1.5 mM MgCl$_2$, 420 mM NaCl, 25% (v/v) glycerol, 0.2 mM EDTA, 0.5 mM DTT, protease inhibitor cocktail, 0.1% NP-40) and incubated for 10 min on ice. The suspension was centrifuged at 10,000 x g at 4°C for 5 min. 40 µg of supernatant protein was electrophoresed on a 4-20% SDS polyacrylamide gel. After electrophoresis, the proteins were transferred to a nitrocellulose membrane and the membrane was incubated for 1 h at room temperature with 5% nonfat milk in PBST (PBS, 0.05% Tween 20) followed by washing three times for 15 min each with PBST. The membrane was incubated for 1 h at room temperature with primary anti-AIB1 antibody (Transduction Laboratories), washed as described above, and incubated for 1 h with a secondary antibody-peroxidase conjugate (10,000-fold dilution in PBST). After washing, the membranes were incubated for 1 min with ECL detection solution (Amersham) and then exposed to film. Bands were quantitated using densitometry.

**Transient Transfections**- In order to measure transcriptional activation from an estrogen responsive reporter (pERE-Luc), MCF-7 cells were plated at 60-70% confluence in IMEM/10% FCS in 6-well plates (5 x 10$^5$ cells/well) 24 h prior to transfection. The cells were transfected with 1 µg of pERE-Luc harboring three copies of the *Xenopus* vitellogenin A$_2$ estrogen response element driving a luciferase reporter (pERE-Luc) (31), 0.1 ng pRL-CMV Renilla luciferase reporter and 3 µg of the AIB1 ribozyme expression vectors or control vector in 8 µl LipofectAMINE™ (Gibco-BRL). After 5 h, the transfection mix was removed and the cells were cultivated for 72 hrs in IMEM/10% FCS in the presence of 10 nM ICI 182,780 ± 100nM 17ß-estradiol. In order
to test progesterone activity, MCF-7 cells were plated in IMEM/1% charcoal stripped fetal calf serum, transfected with 1 µg of a plasmid harboring the MMTV promoter (pMMTVLuc), 20 ng pPRß, 0.1 ng pRL-CMV Renilla luciferase reporter and 3 µg of the AIB1 ribozyme expression vectors or control vector and cells were treated for 72 h with 1 nM R5020 or vehicle. Transfections with MCF-7 cell lines which stably expressed the AIB1 ribozymes, were performed in the presence or absence of 1 µg/ml doxycycline and carried out for 24 h instead of 72 h. Cells were washed twice with PBS and resuspended in lysis buffer (0.1 M potassium phosphate buffer pH 7.8, 0.1% Triton X-100, 100 mM DTT). After centrifugation, the luciferase assay and the correction for transfection efficiency was performed with 10 µl of supernatant as described previously (32).

**Proliferation Assays** - 24 hrs before treatment cells were plated in IMEM/10% FCS in 96-well plates (1,500-3,000 cells/well). The cells were then treated with IMEM/10% FCS containing 10 nM ICI 182,780 ±100nM 17ß-estradiol and cultivated ± 1 µg/ml doxycycline for up to six days. Cell numbers were determined by a colorimetric assay, based on the cleavage of the tetrazolium salt Wst-1 in viable cells, according to the protocol of the manufacturer (Boehringer Mannheim).

**Cell Cycle Analysis** - Cells were serum-starved in the presence of 10 nM ICI 182,780 for 48 h, then treated for 24 h with 10 nM ICI 182,780 in the absence or presence of 100nM 17ß-estradiol and harvested. Cell cycle analysis was performed by the Vindelov staining method as described (33). In short, 2 x 10^6 cells were
resuspended in 100 µl of 40mM citrate/DMSO buffer. After addition of trypsin inhibitor and ribonuclease A for 10 min, the cells were stained with propidium iodide and cell cycle analysis was performed by flow cytometry.

**Apoptosis assay-** Cells (1 x 10⁶) were serum-starved in the presence of 10 nM ICI 182,780 for 72 h, then treated for 48 h with 10 nM ICI 182,780 in the absence or presence of 100nM 17ß-estradiol and harvested. After washing, the cells were resuspended in 100 µl of propidium iodide-annexinV-FITC dual staining solution according to the protocol of the manufacturer (Trevigen) and incubated in the dark for 15 min at room temperature. 400 µl of 1x binding buffer was added to the cell suspension and cells were analyzed by flow cytometry within 1 h.

**Soft-agar colony formation assays-** MCF-7/Rz23-9 cells suspended in 0.35% agar (20,000 cells/dish) were layered on top of 1 ml of solidified agar (0.6%) in a 35 mm dish in the presence or absence of 10 nM ICI 182,780 ± 100 nM 17ß-estradiol. IMEM growth media with a final concentration of 10% fetal bovine serum was included in both layers ±1 µg/ml doxycycline. After 7 to 9 days incubation, colonies with a diameter of ≥80 µm were counted with an image analyzer (Omnicon). Experiments were carried out in triplicate.

**Tumor growth in nude mice-** Twenty million tumor cells (MCF7/Rz23-9 cells) suspended in 0.2 ml PBS were injected subcutaneously into the flanks of athymic female nude mice. One day before injection the mice received one estrogen pellet
(0.72 mg/pellet 17ß-estradiol; Innovative Research of America) and were fed with either a doxycycline containing diet (200 mg/kg doxycycline; Bioserv) or normal food throughout the study. Tumor growth was followed for 2 month by measuring the tumor area every two to three days.

RESULTS

Ribozyme targeting of endogenous AIB1 in MCF-7 cells- For reduction of AIB1 levels in MCF-7 cells we designed five different hammerhead ribozymes directed against different regions of AIB1 mRNA. Four ribozymes were directed against the translated region of the AIB1 mRNA and one against the 3’-untranslated region (Fig.1A). We first screened for ribozyme activity by transiently transfecting MCF-7 cells with different ribozyme expression vectors. Based on previous observations that AIB1 increases nuclear receptor-mediated transcription in transient transfection assays (9-13) we predicted that downregulation of AIB1 should decrease transcriptional activation by the ER and we used this as a read-out for our initial assays. We transiently transfected the empty vector or each of the ribozyme expression vectors under the control of the CMV promoter together with a luciferase reporter harboring an estrogen responsive promoter (pERE-Luc). After 72 h of hormone induction, we found that two of our ribozyme expression vectors, pCMVRz-12 and pCMVRz-23, reduced estrogen-mediated activation of pERE-Luc by 20% and 25% respectively (Fig. 1B) indicating ribozyme activity. Other ribozyme expression vectors in the same vector backbone had no effect on the estrogen response (data not shown) indicating that the
reduction of ER activity was due to the inserted ribozyme in the Rz 12 and 23 constructs. To test whether the ribozyme effect was only on estrogen-mediated transcription or also affected transcriptional activation mediated by other hormones, we transiently transfected MCF-7 cells with a progesterone responsive reporter (pMMTVLuc) together with the most effective ribozyme pCMVRz-23. Consistent with the effect on ER signalling we found a 35% reduction of the progesterone mediated induction of transcription (Fig. 1C) indicating that endogenous AIB1 is involved in at least two hormone mediated signaling pathways in MCF-7 cells and that these ribozymes could effectively regulate endogenous AIB1 levels in MCF-7 cells.

To study the influence of AIB1 on the proliferation of MCF-7 cells we stably transfected MCF-7 cells with expression vectors for Rz-12 and Rz-23. For these experiments we placed these ribozymes under the control of tetracycline regulated expression vectors (pTETRz-12 and pTETRz-23 respectively; tet-off system). This system allowed us to specifically regulate AIB1-ribozyme expression (and hence endogenous AIB1 levels) by tetracycline or doxycycline withdrawal in isogenic cells and thus to avoid effects based on clonal selection. We transfected MCF-7 cells that stably expressed the tetR-VP16 transactivator with the ribozyme expression vectors and selected individual clones. To test the efficacy of the transfected ribozymes, we performed Northern and Western blot analysis of various clones that were obtained after 4-6 weeks of cultivation in selection media. We detected a 5-15% reduction of AIB1 mRNA and 25-40% of protein levels in clone MCF-7/Rz12-9 and a 30-50% reduction of mRNA and over 50% of protein for clone MCF-7/Rz23-9 (Fig. 2A and 2B). These data demonstrate
that we established two clonal MCF-7 cell lines containing regulatable ribozymes in which we can specifically downregulate AIB1 levels.

*Influence of endogenous AIB1 on steroid hormone-dependent transcriptional activation*- We tested whether downregulation of endogenous AIB1 levels had any functional consequences for hormone signaling in these cells as suggested from the transient transfection assays (Fig. 1). Indeed, cotransfection of the PR-sensitive pMMTVLuc and PR-β into the cell line MCF-7/Rz23-9 showed a strong reduction of PR-β activity induced with the synthetic progesterone R5020 in the cells which expressed the AIB1 ribozyme (Fig. 3A). As a negative control we used MCF-7 cells stably transfected with an empty vector (Fig. 3B). From this we concluded that in MCF-7 cells containing a tetracycline-regulated AIB1 ribozyme, reduction of AIB1 levels correlated with a reduction of progesterone-mediated transcriptional activation indicating a rate-limiting role for AIB1 in hormone signaling *in vivo*.

*AIB1 Function in MCF-7 Breast Cancer Cell Growth*- Based on the reduced ability of the ER and PR to activate the expression of a hormone responsive reporter gene after downregulation of endogenous AIB1 protein levels (see Fig. 1 and Fig. 2) we hypothesized that AIB1 could be a rate-limiting factor for estrogen-dependent growth in MCF-7 cells. To test this hypothesis, we first performed cell growth assays. As Fig. 4 demonstrates, when we downregulated AIB1 in MCF-7/Rz23-9 cells, 17β-estradiol induced growth was reduced by 50%. A similar effect was observed with the clonal cell line MCF-7/Rz12-9 (data not shown). Since estrogens contribute to cell
cycle progression (34,35) and inhibition of apoptosis in MCF-7 cells (36), we analyzed whether lowered estrogen-mediated growth after AIB1 downregulation might have resulted from a reduced ability of these cells to progress through the cell cycle or whether this effect might have been based on their altered susceptibility towards apoptosis. When we tested cell cycle progression of MCF-7/Rz23-9 cells after estrogen-induction, we detected no significant difference in cell cycle progression dependent on the AIB1 level of these cells (Fig. 5A and B). However, when we challenged MCF-7/Rz23-9 cells by serum-starvation, the ability of estrogen to inhibit apoptosis of these cells was strongly reduced in cells in which AIB1 levels were downregulated (Fig. 6A and B). We conclude from these data, that AIB1 is essential for estrogen-dependent growth of MCF-7 cells and that reduced growth caused by downregulation of AIB1 is at least partially due to the reduced ability of estrogen to inhibit apoptosis.

In a separate measure of effects on tumor cell growth, we tested whether anchorage-independent soft agar colony formation of these cells in response to estrogen would also be affected. In the presence of the anti-estrogen ICI 182,780 MCF-7/Rz23-9 cells do not form colonies (Fig. 7A and 7C). However, when these cells are treated with 17β-estradiol, a striking difference between the AIB1-reduced cells (Rz on) and control cells (Rz off) became apparent. Reduction of AIB1 reduced the ability of these cells to form colonies in response to estrogen (Fig. 7D vs. 7B and 7E) indicating a rate-limiting role for AIB1 in estrogen-stimulated anchorage-independent growth of human breast cancer cells.
Subcutaneous Growth of MCF-7 Tumor Cells in Nude Mice- The previous results raised the question whether downregulation of AIB1 in MCF-7 cells also limits their growth potential in an environment exposed to physiological stimuli from stromal tissue. While MCF-7 cells cultured in vitro are exposed to a limited number of autocrine and paracrine growth factors we wanted to determine whether factors supplied by the host may compensate for lower AIB1 levels in order to stimulate MCF-7 cell growth. We injected MCF-7/Rz23-9 cells subcutaneously into nude mice and followed tumor growth (Fig. 8). Animals in which ribozyme expression was prevented by feeding them a doxycycline containing diet developed a significantly higher number of tumors relative to controls (6 of 10 vs. 1 of 8 respectively; p<0.05) (Fig. 8 A) which was also reflected in a larger average tumor size relative to controls (Fig. 8 B). This indicates that neither host factors nor other nuclear receptor cofactors present in MCF-7 cells can compensate for reduction of the nuclear receptor coactivator AIB1 during estrogen dependent growth in vivo.

DISCUSSION

The discovery of specific coactivators and corepressors that modulate the transcriptional activity of the ER and the identification of ER cofactors that are amplified and overexpressed in breast tumors led to the hypothesis that some of these cofactors contribute directly to the development of breast cancer. Some of the best-characterized nuclear receptor coactivators to date include CBP/p300 (37,38) and
members of the p160/SRC-family including SRC-1, (7), TIF-2 (8) and AIB1 (9) (ACTR/RAC3/TRAM-1/SRC-3) (10-13). Cofactors that are amplified and overexpressed in breast tumors include PBP, ACS2, SRA (39-41) and AIB1. Despite many similarities of these cofactors, which have been shown to bind to the same nuclear receptors and enhance the transcriptional activity of the same receptors in vitro, it is hard to predict their function in vivo. For example, CBP and p300 both enhance retinoic acid-mediated transcription in vitro (37,42), but they have distinct functions in vivo during retinoic acid-induced differentiation of carcinoma F9 cells (43). In addition, functional redundancy of nuclear receptor cofactors might compensate for the loss or for reduced levels of one of these cofactors as exemplified by a study which showed that SRC-1 potentiates PPARα activity in vitro, still was not essential for PPARα-regulated gene expression in vivo (44). It is therefore imperative to identify the function of these coactivators directly in a cellular context. So far, despite several reports (9,16-19,39-41,45) demonstrating differential expression patterns of some of these coactivators in breast tumors and defining their interaction with various signaling molecules in vitro, the functional role for these cofactors in normal mammary gland development and for breast tumor development is unclear.

In this study we demonstrate for the first time that ribozyme-targeting of the nuclear cofactor AIB1 reduces estrogen-dependent proliferation and neoplastic growth of human MCF-7 breast cancer cells. Based on these data, we propose that AIB1 overexpression provides a selective advantage for tumor growth in mammary epithelium. This hypothesis is in concordance with the finding that AIB1 amplification in breast tumors correlates with ER and PR positivity and tumor size, as shown in a
study based on 1,157 human breast tumors (16). In addition, it has been shown that endogenously expressed human ERα and AIB1 interact in MCF-7 cells (20) supporting the idea that AIB1 could be a rate limiting factor for estrogen-mediated growth in breast tumor cells. Our results would also suggest that human AIB1 and its mouse homolog p/CIP might have similar functions in the development of mammary epithelium since deletion of the p/CIP gene showed blunted mammary gland development in the mouse (26). Furthermore, a recent study showed that Taiman, the Drosophila homolog of AIB1, contributes to steroid hormone-mediated motility of Drosophila border cells but interestingly has no effect on the proliferation of these cells (46). These findings raise the question whether there are fundamental differences in the function of these closely related coactivators based on species or tissue context, or whether some of these effects might be compensated by functional redundancy of nuclear receptor coactivators.

Previous studies of SRC-1 demonstrate that it is a coactivator of ER (7), however, its role in breast cancer seems less pronounced and our studies suggest that it is unable to compensate for a loss of AIB1 function. Consistent with this, gene disruption studies of SRC-1 in mice showed only relatively subtle defects in the development of estrogen-dependent tissues which might be due to the compensatory upregulation of the related coactivator TIF-2 but not of AIB1 (47). In addition, the highest levels of SRC-1 were found in normal human breast tissue compared to lower levels in breast tumors (45) and in contrast to AIB1, SRC-1 expression did not correlate with ER status of these tumors (45). Furthermore SRC-1 does not colocalize with the ER in rat
mammary epithelium (48) nor did it interact with the endogenous ER in MCF-7 cells (20). Overall our data and these previous observations indicate a lesser role for SRC-1 in human breast tumorigenesis.

The gene targets of AIB1 in breast cancer are currently not known but may involve its interactions with a number of transcription factors including p53 (23), CBP (10,12), CARM1 (49), NFκB (22) and TEF (21) as well as with several members of the nuclear receptor family. It is an interesting possibility that many of these interactions might not only be relevant for breast tumorigenesis but also for a variety of other cancers such as ovarian, pancreatic and gastric cancer in which AIB1 is also amplified or overexpressed (9,14,15). Ribozyme-targeting of AIB1 will be a valuable tool to explore this.
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FIG. 1. Transiently transfected AIB1 ribozymes reduce estrogen- and progesterone-mediated transcriptional activation. A, ribozyme target sites in the mRNA of AIB1. The position of the helix-loop-helix domain (bHLH), the Per-Arnt-Sim domains A and B (PAS-A and PAS-B), a poly-Q rich region, various nuclear receptor interacting domains (LXXLL motif) as well as AIB1 translation start and stop site are indicated. The target sites of the tested ribozymes are indicated. B, MCF-7 cells were transfected with an estrogen responsive luciferase reporter (pERE-Luc), and empty vector or the pCMV-Rz 12 or Rz 23 expression vectors. The fold induction by estradiol of control (empty vector) transfected cells was set at 100%. C, MCF-7 cells were transfected with a progesterone responsive reporter (pMMTVLuc) and pCMVRz23 and the effect of R5020 induction relative to that of cells transfected with an empty vector (control) is shown. The data are means +/- SEM from two (B) or three (C) independent experiments done in triplicate. *P<0.05 versus values from control transfected cells (Student’s t test). Details under Experimental Procedures.
FIG. 2. **Reduction of endogenous AIB1 levels in MCF-7 cells.** *A,* Northern blot analysis of AIB1 from MCF-7 cells stably transfected with AIB1 ribozymes. The top panel shows a representative Northern blot from the clonal cell lines MCF-7/Rz12-9 and MCF-7/Rz23-9. Cells were cultivated in the presence (Rz off) or absence (Rz on) of doxycycline. RNA loading was corrected for by GAPDH mRNA levels. Quantitation, shown in the lower panel, was done with a phosphoimager. The AIB1 mRNA levels for doxycycline treated cells were set arbitrarily as 100% for each cell line. *B,* Western blot analysis of AIB1 from cells which were cultivated in the presence (Rz off) or absence (Rz on) of doxycycline. The top panel shows a representative Western blot. Quantitation, as shown in the lower panel was done by densitometry, whereby the AIB1 levels for doxycycline treated cells were arbitrarily set as 100%.
FIG. 3. **Stable A1B1-ribozyme expression reduces R5020-mediated transcriptional activation.** A, MCF-7/Rz23-9 cells were transfected with a progesterone responsive luciferase reporter (pMMTVLuc) and a PR-ß expression vector and treated ± 1 nM R5020 for 24 h ± doxycycline. The induction of luciferase activity by R5020 relative to control (+ doxycycline = Rz off) is shown. Mean +/- SEM from four independent experiments done in triplicate is shown. *P<0.05 versus values from doxycycline treated cells (Student’s t test). B, MCF-7/600 control cells, which contained no ribozyme expression vector, were transfected, treated with 1 nM R5020 in the presence or absence of doxycycline and analyzed as described in (A).
FIG. 4. Estrogen-dependent growth of MCF-7 cells is reduced in AIB1 ribozyme expressing cells. Estrogen-stimulated cell proliferation in MCF-7/Rz23-9 cells was measured by a colorimetric assay. The OD for cells cultivated in the presence of doxycycline (Rz off) was set as 100%. Mean +/- SEM from three independent experiments done in triplicate is shown. *P<0.05 versus values from doxycycline treated cells (Student’s t test).
FIG. 5. **Cell cycle progression of MCF-7 cells is not affected by AIB1 levels.**  
**A,** cell cycle analysis profile of MCF-7/Rz23-9 cells in the presence (Rz off) or absence (Rz on) of doxycycline. Cells were serum-starved for 48 h, then treated for 24 h with 10 nM ICI 182,780 in the absence (left panels) or presence (right panels) of 100nM 17β-estradiol. **B,** mean of the percentage of cells in S-phase +/- SEM from three independent experiments done in triplicate is shown.
FIG. 6. **Downregulation of AIB1 levels reduces estrogen-mediated inhibition of apoptosis.** A, shows the FACS analysis of MCF-7/Rz23-9 cells kept in the presence (Rz off) or absence (Rz on) of doxycycline. The cells were stained with fluorescein isothiocyanate (FITC)-conjugated annexinV and propidium iodide (PI) and analyzed by flow cytometry. Cells were serum-starved for 72 h, then treated for 48 h with 10 nM ICI 182,780 in the absence (left panels) or presence (right panels) of 100nM 17ß-estradiol. Cells in the early stages of apoptosis were used for quantitation (shown in B) stain for annexin V and are shown in the **lower right quadrant.** B, mean of estrogen-mediated inhibition of apoptosis +/- SEM from one representative experiment done in duplicate. *P<0.05 versus values from doxycycline treated (Rz off) cells (Student’s t test).
FIG. 7. Anchorage-independent growth of MCF-7 cells can be inhibited by AIB1 ribozyme targeting. A-D, MCF-7/Rz23-9 cells (A and B: with doxycycline (Rz off); C and D: no doxycycline (Rz on)) were cultivated in soft agar in the absence (A and C) or presence of estrogen (B and D). Representative images (A-D) of colony formation and the mean +/- SEM from one representative experiment done in triplicate (E) are shown. *P<0.05 versus values from doxycycline treated (Rz off) cells (Student's t test).
FIG. 8. **Tumor growth of MCF-7/Rz23-9 cells in athymic nude mice.** *A and B,* MCF-7/Rz23-9 cells were injected subcutaneously into the flanks of athymic nude mice at 2 x 10^7 cells per injection site and two sites per animal (n = 5 animals in the presence of doxycycline (AIB1 ribozyme blocked); n = 4 animals without doxycycline (AIB1 ribozyme active)). *A,* shows the tumor incidence two month after injection from animals fed with doxycycline containing diet (Rz off) and from the animals fed with normal diet (Rz on) (*p-value < 0.05  for Chi-square test). *B,* the tumor area was measured every two to three days (closed circles: Rz off; open squares: Rz on) and mean tumor size is shown for two month following the injection date.
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Ribozyme-targeting demonstrates that the nuclear receptor coactivator AIB1 is a rate limiting factor for estrogen-dependent growth of human MCF-7 breast cancer cells
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