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Advances in Brief

AIB1 Enhances Estrogen-dependent Induction of Cyclin D1 Expression[1]

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Abstract

***AIB1* was isolated as a gene amplified in breast cancer and encodes a protein that acts as a steroid receptor coactivator. The role of steroid receptor coactivators such as AIB1 in breast cancer development is not clear. It is possible that AIB1 cooperates with estrogen receptor α in regulating estrogen-dependent cell proliferation. Ectopic expression of the estrogen receptor α in different cell lines does not confer estrogen-induced proliferation. This inability of the estrogen receptor to drive proliferation has been recently correlated with a lack of estrogen-dependent cyclin D1 expression in cells engineered to express the estrogen receptor. In this study, we evaluated whether high levels of AIB1 enable the estrogen receptor to direct the transcription of cyclin D1. We show here that AIB1 and other steroid receptor coactivators can enhance the functional interaction of the estrogen receptor with the cyclin D1 promoter. Increases of AIB1 levels in breast cancer cells by amplification and/or overexpression may represent one way to confer estrogen-dependent mitogenic stimulation to breast cancer cells.**

Introduction

ER[4] expression and function are crucial for the development and maintenance of ER-positive breast carcinomas. For this reason, antiestrogens such as tamoxifen can prevent and control the proliferation of cells associated with this type of breast cancer. It is not clear, however, how the ligand-activated ER promotes cell proliferation. Normal MECs that express the ER within mammary tissue are usually nonproliferative (1, 2). In addition, stable ectopic expression of the ER in different cell lines does not mimic the estrogen-dependent proliferation observed in ER-positive, breast cancer-derived cells (3, 4). These observations suggest that within normal MECs, the ER is not functionally coupled with the cellular machinery that promotes cell proliferation.

The lack of an estrogen-driven mitogenic response has been correlated with the failure of estrogen to up-regulate cyclin D1 expression (5, 6). Cyclin D1 is amplified and overexpressed in breast cancer. This overexpression of cyclin D1 is already seen in early stages of breast cancer development (7–9), indicating that cyclin D1 up-regulation, possibly mediated by ligand-activated ER, may be one of the first steps in breast cancer progression. This highlights the importance of elucidating the mechanisms regulating cyclin D1 expression in breast cancers in general and, more specifically, in ER-positive breast tumors.

It is possible that the functional coupling of the ER and the *cyclin D1* gene is achieved in breast cancer cells through the actions of certain intermediary proteins. One such candidate protein is the product of the *AIB1* gene, which has been found to be amplified in three of four ER-positive breast cancer cell lines examined as well as in 5–10% of primary breast tumors (10, 11). These observations suggest a possible link between estrogen-driven mitogenicity and this steroid receptor coactivator. To elucidate the role of AIB1 in breast cancer pathogenesis, we evaluated the ability of AIB1 to regulate cyclin D1 expression in response to estrogen. Our data suggest that AIB1 cooperates with the ER to drive cyclin D1 transcription.

Materials and Methods

Cell Culture, Transfections, and Luciferase Assays. HaCaT-neo and HaCaT-ER cells were maintained in DMEM with 5% fetal bovine serum and antibiotics. For transfection, cells were seeded in DMEM without phenol red with 5% charcoal/dextran-stripped fetal bovine serum (CSS) at 100,000–200,000 cells/well in a 12-well dish. The next day, cells were transfected using Tfx-50 reagent (Promega, Madison, WI) for 1 h. Total DNA was made constant by adding pcDNA to the transfections as needed. Cells received either 3.3% or 5% CSS with or without 5 nM E_2 for 24 h before harvesting. In some experiments, antiestrogens were also added to estrogen-containing media. Transfection values were corrected for the efficiency of transfection using the dual-luciferase reporter assay system. For a better comparison between promoters, both cyclin D1 promoter sequences and a promoter containing estrogen-responsive element sequences (6) were subcloned into pGL3-luc (Promega, Madison, WI).

Chromatin Immunoprecipitation. MCF-7 cells were grown in phenol red-free DMEM supplemented with 10% CSS for at least 3 days to a 95% confluence before E_2 or atRA was added. Cells were washed once with PBS and cross-linked with 1% formaldehyde at room temperature for 10 min. Then cells were rinsed twice with ice-cold PBS and collected into ice-cold PBS with protease inhibitors (Roche Molecular Biochemicals, Indianapolis, IN). Cells were pelleted and resuspended in lysis buffer [1% SDS, 5 mM EDTA, 50 mM Tris-HCl (pH 8.1), and 1× protease inhibitor cocktail] and sonicated three times at 15 s each at a maximal input (Fisher Sonic Dismembrantor; Model 300) before centrifugation for 10 min. Supernatants were collected and diluted in dilution buffer [1% Triton X-100, 2 mM EDTA, 150 mM NaCl, and 20 mM Tris-HCl (pH 8.1)] before immunoclearing with 2 μg of sheared salmon sperm DNA, 2 μl of preimmune serum, and protein A-Sepharose [45 μl of 50% slurry in 10 mM Tris-HCl (pH 8.1), and 1 mM EDTA] for 2 h at 4°C. Immunoprecipitation was performed for 6 h or overnight at 4°C with specific antibodies. For ER analyses, a mouse monoclonal (Ab-10) was used (NeoMarkers, Fremont, CA) at a 1:1500 dilution. After immunoprecipitation, 45 μl of protein A-Sepharose and 2 μg of salmon sperm DNA were added and the incubation was continued for an additional 1 h. Sepharose beads were washed sequentially for 10 min each in TSE I [0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.1), and 150 mM NaCl], TSE II [0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.1), and 500 mM NaCl], and buffer III [0.25 M LiCl, 1% NP40, 1% deoxycholate, 1 mM EDTA, and 10 mM Tris-HCl (pH 8.1)]. Then beads were washed three more times with TE buffer and extracted three times with 1% SDS and 0.1 M $NaHCO_3$. Elutes were pooled and heated at 65°C for 6 h or overnight to reverse the formaldehyde cross-linking. DNA fragments were purified with a DNA purification kit (QIAquick Spin Kit; Qiagen, Valencia, CA). For regular PCR, 1 μl of the 50-μl DNA extraction volume was used, and 21–25 cycles were allowed.

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[4] The abbreviations used are: ER, estrogen receptor α; MECs, mammary epithelial cells; ERE, estrogen receptor element; E_2,17β-estradiol; CSS, charcoal/dextran-stripped serum; ChIP, chromatin immunoprecipitation; atRA, all-*trans* retinoic acid.

Results

Role of AIB1 in Estrogen-dependent Cyclin D1 Transcription. To assess the role of AIB1 in estrogen-dependent cyclin D1 expression, we used human nontransformed epithelial cells of keratinocyte origin (HaCaT cells) engineered to stably express the estrogen receptor α (HaCaT-ER; Ref. 6). These cells express a functional ER, as gauged by its ability to efficiently regulate transcription of an ERE-containing promoter (ERE-luc) in an estrogen-dependent manner over a 10-fold range of induction as reported previously (6). However, estrogen treatment of these cells does not induce either cyclin D1 expression or cell proliferation.

We decided to use HaCaT-ER cells for our studies for several reasons. First, the proliferation of these cells is not affected significantly by estrogen. Secondly, these cells are nontransformed, and we wished to avoid the use of cancer cells that may contain altered signal transduction pathways. Thirdly, these cells express low levels of cyclin D1 in the absence of serum, allowing us to monitor changes in cyclin D1 expression more efficiently.

To determine whether levels of AIB1 expression may be important for estrogen-dependent cyclin D1 expression, we compared AIB1 expression by Western blot analysis in HaCaT-ER and MCF-7 cells. MCF-7 cells are known to overexpress AIB1 mRNA because of gene amplification (10). For this reason, we wanted to evaluate whether HaCaT-ER cells express lower levels of AIB1 than MCF-7 while expressing comparable levels of ER. As shown in Fig. 1, MCF-7 expressed at least 4-fold more AIB1 protein than did HaCaT-ER. However, the levels of ER expression were comparable in both cells.

We then evaluated whether ectopically expressed AIB1 could enhance the ability of the stably expressed ER in HaCaT cells to activate a promoter containing ERE sequences. In parallel, we assayed vector-transfected cells (termed "HaCaT-neo") as control cells. Confirming previously reported observations (6), the stably expressed ER in HaCaT-ER cells is capable of up-regulating ERE-luc in an estrogen-dependent manner (Fig. 2*A*). Introduction of an AIB1 expression construct into these cells by transient cotransfection with the reporter gene led to an additional 2-fold enhancement of estrogen-dependent ERE-luciferase expression (from 12-fold in the absence of additional AIB1 to almost to 24-fold estrogen-dependent transcription in the presence of transfected AIB1). In contrast, HaCaT-neo cells lacking ER did not show enhanced estrogen-dependent transcription even at high doses of AIB1 (Fig. 2*A*). Hence, the ER stably expressed in HaCaT-ER cells was functional and allowed estrogen-dependent transcription responsive to changes in AIB1 levels. Moreover, the ability of AIB1 to enhance estrogen-dependent transcription was dependent on the presence of ER, as evidenced by the lack of this effect in HaCaT-neo cells.

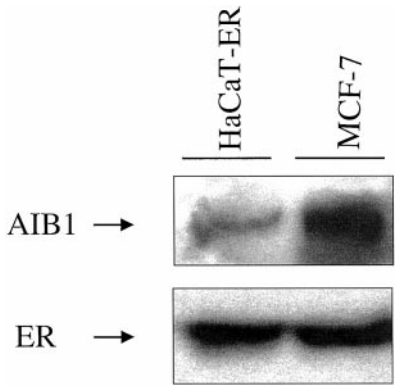

Fig. 1. Western blot analysis of AIB1 (*top*) and ER α (*bottom*) expression. Similar amounts of cell extracts obtained from asynchronously growing HaCaT-ER and MCF-7 cells were run on an 8% SDS-PAGE and subjected to Western blot analysis. After determining AIB1 expression using a rabbit polyclonal antibody, the membrane was reprobed for ER expression as described previously (6).

Cyclin D1 has been proposed to be an important effector of the mitogenic functioning of the ER. Thus, in MCF-7 and T47D human breast carcinoma cells, both of which are ER-positive, the addition of antiestrogen results in rapid down-regulation of cyclin D1 mRNA (12, 13). Conversely, in MCF-7 cells the addition of estrogen results in rapid induction of *cyclin D1* gene expression, the production of cyclin D1 protein, and a resulting entrance of the cell into the active growth cycle, including a progression through its G_1 phase (14–17). This has suggested that the cyclin D1 promoter is a proximal target of ER transcriptional activation.

The cyclin D1 promoter is not, however, a classic ER target, because its sequences do not contain an ERE. Responding to this, some have proposed that the observed estrogen-dependent cyclin D1 expression is mediated by non-ERE sequences in the cyclin D1 promoter that are recognized by AP-1 family members (18, 19). Independent of this, others have shown that the AIB1 coactivator can enhance transcription mediated by AP-1 factors (20). It has remained unclear, however, whether AIB1 can enhance estrogen-dependent activation mediated through this class of transcription factors. We explored this possibility by monitoring the changes of estrogen-dependent transcription using the cyclin D1 gene promoter as an element in an expression reporter construct.

As shown in Fig. 2*B*, a cyclin D1-promoter construct driving luciferase expression was poorly induced by estrogen in both HaCaT-neo and HaCaT-ER cells in the absence of added AIB1. However, increases in the levels of ectopically expressed AIB1 enhanced estrogen-dependent cyclin D1-luciferase expression, specifically in HaCaT-ER cells. The results of eight independent experiments indicate that in the absence of AIB1, the induction of the cyclin D1 promoter by estrogen in HaCaT-ER cells was 1.34 ± 0.22 (average $\pm$ SE), and that in the presence of high levels of AIB1, the induction was 2.14 ± 0.25 (average $\pm$ SE). These results suggest that AIB1 levels can enhance the ability of the ER to interact productively with the cyclin D1 promoter in an estrogen-dependent manner. A comparison of the effect of AIB1 in ERE-luc and cyclin D1-luc showed that the percentage of AIB1-dependent enhancement with both promoters was equivalent (Fig. 2*C*), indicating a similar responsiveness of both types of promoter sequences to increased AIB1 levels. This A1B1-dependent enhancement of estrogen-mediated transcription was not observed in control cells lacking ER (Fig. 2*C*), stressing the important contribution of the ER to this induction.

To rule out the possibility that the increase of cyclin D1-luc expression was nonspecific, we characterized the impact of high levels of AIB1 on another cell cycle gene by using the cyclin E promoter in a reporter construct (21). Cyclin E promoter activity was not induced by estrogen, nor was it affected by high levels of AIB1 (Fig. 2*D*). In a similar way, the pGL3-luc vector lacking promoter sequences did not show any changes in expression by either estrogen or AIB1 levels. Therefore, these studies argue against a nonspecific effect of AIB1 on the cyclin D1 promoter. Instead, they support a role of AIB1 overexpression in facilitating estrogen-dependent cyclin D1 expression. It is not clear from these results whether the enhancement of cyclin D1 transcription occurs through the ER acting at the cyclin D1 promoter, because the cyclin D1 promoter lacks ERE sequences. Therefore, we wanted to determine whether the ER was being recruited to the cyclin D1 promoter after estrogen addition.

Estrogen-dependent Recruitment of ER to Cyclin D1 Promoter in MCF-7 Cells. If cyclin D1 promoter activity were regulated directly by the ER in tumor cells, this would suggest that the ER is physically located at the site of the cyclin D1 promoter in these cells.

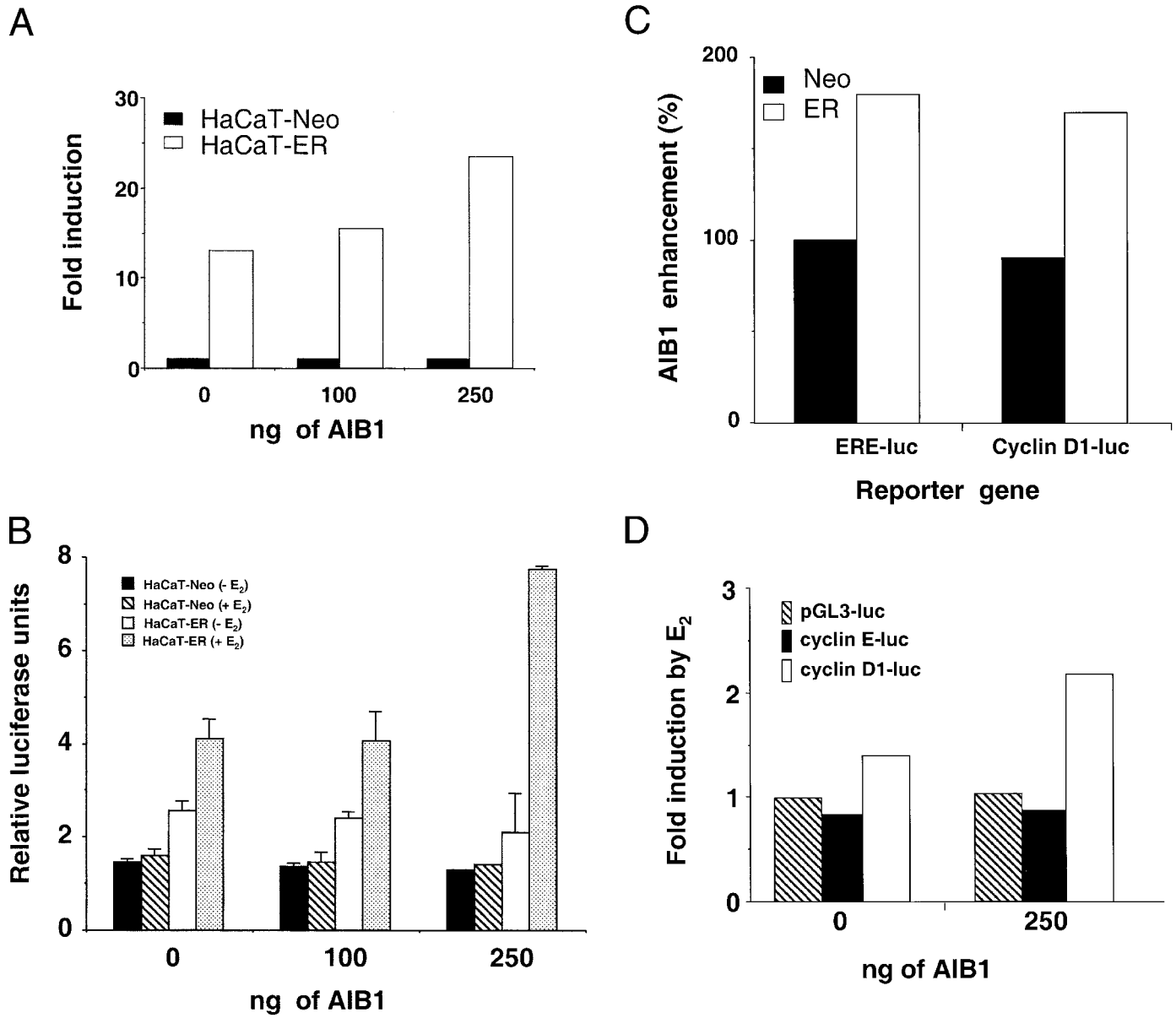

Fig. 2. Responsiveness of cyclin D1 promoter to AIB1 levels. *A*, ERE-luc responsiveness. HaCaT-neo and HaCaT-ER cells were transfected with ERE-luciferase to evaluate the effect of the indicated doses of pcAIB1 in estrogen-dependent transcription. Data represents fold induction over control (no added E_2). *B*, cyclin D1-luc responsiveness. HaCaT-neo cells and HaCaT-ER cells were transfected with cyclin D1-luc. Relative levels of luciferase units corrected by cotransfection with tk-Renila-luciferase are plotted as observed in the absence ($-E_2$) or presence ($+E_2$) of 5 nM E_2 for 24 h. Values represent the average ± SE for a representative experiment. *C*, comparison of ERE-luc and cyclin D1-luc responsiveness to AIB1. AIB1 enhancement of estrogen-dependent transcription of each promoter was calculated as the ratio between induction by estrogen after transfection of 250 ng of AIB1 and in the absence of transfected AIB1. The values observed in the absence of additional AIB1 were taken as 100%. *D*, specificity of the effect of AIB1. HaCaT-ER cells were transfected with pGL3-luc vector lacking any promoter activity, cyclin E-luc, and cyclin D1-luc. Estrogen-dependent expression was determined at different doses of AIB1.

Alternatively, if the ER were not present at the cyclin D1 promoter, it would indicate that estrogen-dependent enhancement of cyclin D1 transcription occurs in an indirect manner. To examine the possible physical association of the ER with the cyclin D1 promoter, we carried out ChIP assays to determine whether the ER associates with the cyclin D1 promoter in an estrogen-dependent manner. To do so, we used MCF-7 cells, because they display estrogen-dependent cyclin D1 expression. Estrogen-deprived MCF-7 cells were treated with 17β-estradiol for 45 min before harvesting for ChIP analysis using ER-α antibodies. The estrogen-responsive region of the cyclin D1 promoter has been mapped previously to a fragment starting at -966 from the transcription start site (18). For this reason, we analyzed whether ChIP of the ER would selectively bring down this region of the promoter in response to estrogen treatment of MCF-7 cells.

Fig. 3*A* shows the results of the ChIP analysis using this region of the promoter. Although the input of DNA was similar in the presence or absence of E_2 (Fig. 3*A*, *left Lanes of inset*), after ChIP analysis, only the sample treated with E_2 showed a significant recovery of the promoter region of the cyclin D1. These data imply that the ER was recruited to the cyclin D1 promoter after estrogen addition to MCF-7 breast cancer cells. This effect was specific, because the ER is not recruited to another promoter such as β-actin (Fig. 3*B*) or to a region further upstream in the *cyclin D1* gene (Fig. 3*C*). To show that this effect is attributable to the direct action of estrogen, we treated MCF-7 cells with atRA, another molecule capable of recruiting steroid receptor coactivators to selected promoters. This treatment did not show an enhanced recovery of the proximal region of the cyclin D1 promoter after a similar ChIP analysis (Fig. 3*D*). Therefore, these results sug-

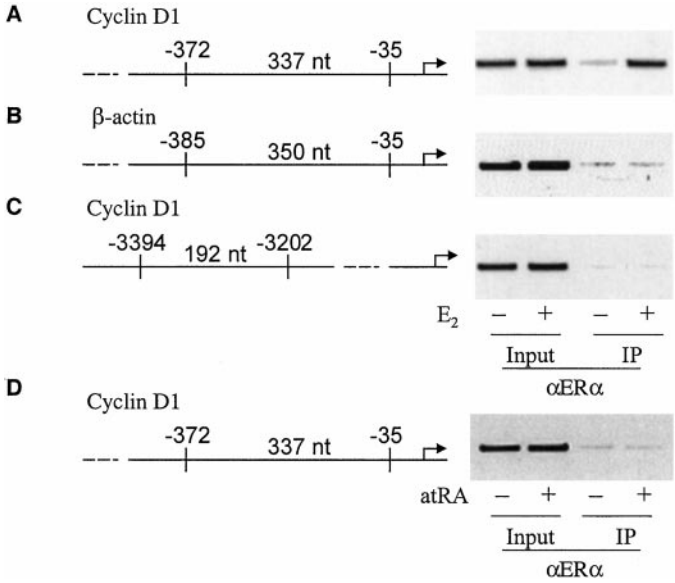

Fig. 3. Estrogen induces the occupancy of cyclin D1 promoter by ER. *A*, MCF-7 cells were grown in the absence of estrogen for 3 days and treated with 100 nM of E_2 (*E_2*) for 45 min. The occupancy of ER on the cyclin D1 gene promoter was measured by ChIP experiments with antibodies against ER α. *B*, E_2 does not induce the occupancy of ER on β-actin gene promoter in the MCF-7 cells. *C*, E_2 does not induce the occupancy of ER on the distal region of cyclin D1 gene promoter. *D*, atRA (*atRA*) does not induce the occupancy of ER on the cyclin D1 gene promoter.

gest that estrogen alone can target the ER to the cyclin D1 promoter in breast cancer cells that show estrogen-dependent cyclin D1 expression and argue for a direct action of the ER at the cyclin D1 promoter.

Role of Other Steroid Receptor Coactivators and Antiestrogens in Estrogen-dependent Cyclin D1 Expression. It is possible that other steroid receptor coactivators may enhance estrogen-dependent cyclin D1 expression despite the fact that, to date, only AIB1 has been found amplified in breast cancer. Accordingly, we tested whether other members of the steroid receptor coactivator family or other coactivators could mediate a similar activation of the cyclin D1 promoter in response to estrogen. Transient transfection of HaCaT-ER cells with vectors expressing either the steroid receptor coactivators GRIP or SRC-1 showed that both coactivators have the potential of enhancing cyclin D1 transcription in response to estrogen comparable with that of AIB1 (Fig. 4*A*). In contrast, the more general coactivator CBP did not enhance estrogen-dependent cyclin D1 expression (Fig. 4*A*). These observations argue for a special role of steroid receptor coactivators in linking ER signaling to the cell cycle machinery.

It is also possible that these steroid receptor coactivators may be enhancing transcription in an indirect way. Therefore, to ensure that the enhancement of cyclin D1 transcription is dependent on ER function, we tested whether antiestrogens could block the effect of AIB1 on cyclin D1 transcription. The addition of either partial antiestrogens, such as 4-hydroxytamoxifen and tamoxifen, or the pure antiestrogen ICI 164,384 completely abolished the effect of estrogen and AIB1 on cyclin D1 transcription (Fig. 4*B*). Our results indicate, therefore, that the ability of steroid receptor coactivators to enhance cyclin D1 transcription is dependent on ER function, which is known to be antagonized by these and a variety of other antiestrogens.

Discussion

In these studies, we have characterized the role of steroid receptor coactivators in regulating the ability of the ER to direct transcription of the cyclin D1 promoter. Our goal was to determine whether the steroid receptor coactivator AIB1, whose gene is found amplified in many breast cancers (11), and/or other steroid receptor coactivators, confer estrogen-dependent cyclin D1 transcription. Our previous results had indicated that ER expression and function were not sufficient for estrogen-dependent cyclin D1 expression and mitogenesis (6). For this reason, we were interested in finding specific molecules that cooperate with the ER in mediating estrogen-dependent cyclin D1 expression. The finding that AIB1 is amplified and overexpressed in ER-positive breast tumors (11) indicated that this steroid receptor coactivator may be involved in estrogen-dependent proliferation in breast tumors. We therefore evaluated the effect of overexpression of AIB1 on estrogen-dependent cyclin D1 expression.

The data presented here indicate that increased levels of AIB1 can enhance estrogen-dependent transcription of the cyclin D1 promoter. Recently, it has been shown that the addition of estrogen to MCF-7 breast cancer cells promotes the interaction between AIB1 and ER (22). The fact that ER is recruited to the cyclin D1 promoter by estrogen suggests that AIB1 may be enhancing estrogen-dependent transcription of cyclin D1 by interacting with ER at the promoter. Although the enhancement of estrogen-dependent cyclin D1 transcrip-

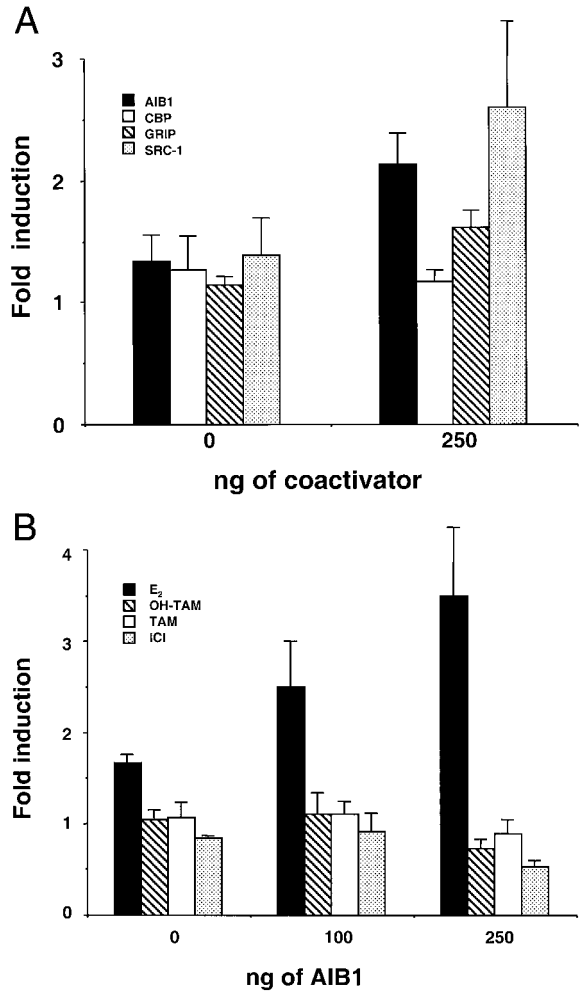

Fig. 4. Effect of coactivators and antiestrogens in estrogen-dependent expression of the cyclin D1 promoter. *A*, HaCaT-ER cells were transfected with cyclin D1-luciferase and the indicated coactivators at the doses shown. Values represent the average of three independent experiments except for AIB1, in which eight independent experiments were averaged. *B*, HaCaT-ER cells were transfected with cyclin D1-luciferase in the presence or absence of AIB1 and treated with 3.3% CSS containing media ± 5 nM E_2 (*E_2*). Some of the cells receiving E_2 also had received 1 μM 4-hydroxytamoxifen (*OH-TAM*), 1 μM tamoxifen (*TAM*), or 1 μM ICI 164,384 (*ICI*). After correcting for transfection efficiency, the luciferase values were divided by the value obtained in the presence of 3.3% CSS to determine the fold induction by each treatment. The results represent the average of four independent experiments.

tion by AIB1 is <2-fold, this level of induction is commonly seen when analyzing enhancement by steroid-receptor coactivators and has been shown with AIB1 using artificially constructed promoters (10).

The ability of estrogen to modulate cyclin D1 expression may confer direct estrogen-stimulated mitogenesis on mammary epithelial cells. We have not determined whether transient overexpression of AIB1 allows estrogen-dependent proliferation. However, in support of the idea that AIB1 and other steroid receptor coactivators can enhance estrogen-dependent proliferation, recent studies have characterized the effects of ectopic expression of steroid receptor coactivators in MCF-7 cells which, in the absence of ectopically expressed coactivators, already show substantial estrogen-stimulated proliferation. MCF-7 cells overexpressing SRC-1 had a greater proliferative response to estrogen than did those lacking ectopically expressed SRC-1 (23). In addition, the steroid receptor coactivator GRIP has also been shown to enhance cell cycle progression in MCF-7 cells (24).

Several studies have indicated the importance of estrogen-dependent cyclin D1 expression for the proliferation of MCF-7 cells (15–18). Amplification of AIB1 during breast cancer development may confer a selective advantage to ER-positive mammary epithelial cells by establishing a functional linkage of ER and the transcriptional machinery necessary to drive cyclin D1 expression. The lack of amplification of other steroid receptor coactivators such as SRC-1 in ER-positive breast cancers might be related to the absence of this protein in normal ER-positive mammary epithelial cells (25). Moreover, the recent observation that ER complexes with AIB1, but not with SRC-1, after estrogen treatment of MCF-7 cells argues for a specific role of AIB1 in mediating estrogen-dependent transcription and cell proliferation in breast cancer cells (22).

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