Cancer Genes and Genomics

Deregulated E2F and the AAA+ Coregulator ANCCA Drive Proto-Oncogene ACTR/AIB1 Overexpression in Breast Cancer

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Abstract

The proto-oncogene ACTR/AIB1, a coactivator for transcription factors such as the nuclear receptors and E2Fs, is frequently overexpressed in various cancers including breast cancers. However, the underlying mechanism is poorly understood. Here, we identified several functional, noncanonical E2F binding sites in the ACTR first exon and intron that are critical for ACTR gene activation. We also found that the newly identified AAA+ coregulator AAA+ nuclear coregulator cancer associated (ANCCA) is recruited to the ACTR promoter and directly controls ACTR expression in breast cancer cells. Importantly, immunohistochemistry analysis indicated that ACTR overexpression is highly correlated with the expression of E2F1 and ANCCA in a cohort of human primary and lymph node–metastasized breast cancer specimens. Along with previous findings from us and others that ACTR is involved in its own gene regulation, these results suggest that one major mechanism of ACTR overexpression in cancer is the concerted, aberrant function of the nuclear coregulators such as ANCCA and ACTR, and they point to therapeutic strategies that target the Rb-E2F axis and/or the coregulator ANCCA for ACTR-overexpressing cancers.

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Introduction

ACTR (also known as AIB1 or SRC-3) is a member of the p160 transcriptional coactivator family initially identified based on its ability to interact with hormone-bound nuclear receptors and enhance their transcriptional activation function (1-3). Since then, it has been shown to serve as a coactivator for other transcription factors including E2F1, activator protein-1, PEA3, ER81, and NF-kB (4-8). ACTR knockdown in various normal and malignant human cells blocked their ability to progress through the cell cycle, and gene disruption in mice impaired female reproductive function, mammary gland development, and growth (4, 9-13). These loss-of-function studies show that ACTR plays an important role in cell proliferation and normal physiologic processes.

ACTR has also been strongly implicated in tumorigenesis and cancer progression, including hormone-responsive and nonresponsive cancers (3, 14). Early studies reported that ACTR is overexpressed in 30% to 64% of breast tumors (1, 15). The high expression of ACTR significantly correlated with human epidermal growth factor receptor 2 positivity in breast tumors, and the incidence of having high levels of both was associated with poor response to tamoxifen therapy (15, 16). ACTR is also overexpressed in prostate, colorectal, gastric, and pancreatic cancers, and its overexpression often associates with poor prognosis (12, 17-20). In mouse models, targeted expression of ACTR resulted in frequent formation of malignant mammary tumors, whereas its inactivation impaired oncogene-induced tumorigenesis (8, 21-25). Furthermore, we found that ectopically overexpressed ACTR can transform human mammary epithelial cells (4). These studies suggest that overexpressed ACTR possesses oncogenic activities sufficient for malignant transformation and tumor formation.

Although the important role of ACTR in tumorigenesis is well established, relatively little is known about how ACTR becomes overexpressed in human cancers. Less than 10% of breast tumors in which ACTR is overexpressed are associated with gene amplification (1), indicating that transcriptional and/or posttranscriptional mechanisms are the main determinants for the aberrant levels of ACTR protein in various human cancers. Indeed, ACTR mRNA was found to be overexpressed in 30% to 40% of breast tumors compared with matched normal breast tissues (15). Interestingly, overexpression was observed in different stages of disease progression, suggesting that it is an early event in tumorigenesis and is sustained during metastasis. Several studies have revealed that steroid hormones and growth factors affect ACTR protein turnover, which in turn is regulated...
by proteosomal degradation in immortalized and cancer cell lines (26-29). In breast cancer cells, estrogen and transforming growth factor-β were shown to modulate ACTR gene expression primarily at the transcriptional level (30). However, the cis- and trans-acting factors responsible for the effect have not yet been determined. More recently, identification of the ACTR promoter led to the finding that the gene is directly regulated by the E2F transcription factor (10, 31). E2Fs, a transcription factor family that includes eight members, regulate the expression of genes involved in cell cycle progression and DNA synthesis (32-34). In response to mitogenic signaling, cyclin-dependent kinase complexes are activated and, in turn, phosphorylate retinoblastoma tumor suppressor family members, which include pRb, p107, and p130. They bind to and negate the transactivation function of E2F-1, E2F-2, and E2F-3a during quiescence. Their hyperphosphorylation releases E2Fs that are then able to mediate transcriptional activation of target genes with the recruitment of enzymatic complexes that facilitate chromatin restructuring. E2Fs possess a similar DNA-binding domain that recognizes the consensus sequence TTT(C/G)(C/G)CGC identified in the regulatory regions of classic E2F targets, including some of the cell cycle genes. Binding sequences that differ from the consensus have recently been found and shown to have a crucial role in the regulation of E2F target genes such as tumor suppressor p14(ARF) and apoptosis signal-regulating kinase 1 (ASK1), which are involved in checkpoint and apoptotic pathways, respectively (35-38). Furthermore, unbiased chromatin immunoprecipitation (ChiP)-chip data suggest that only a small percentage of E2F1-binding sites in the human genome consist of the consensus sequence (39).

We and others previously found that ACTR is a coactivator of E2F1 and can activate its own expression through a positive feedback loop (10, 31). We have also recently identified the AAA+ ATPase AAA+ nuclear coregulator cancer associated (ANCCA) as a novel coactivator for E2Fs (40). AAA+ (ATPases associated with various cellular activities) proteins are an evolutionarily conserved family of enzymes that can alter conformations of proteins or complexes (41). ANCCA contains two AAA+ domains in the central region with the first one being crucial for its transcription coactivator function (40). ANCCA also possesses a bromodomain close to the COOH-terminus that likely recognizes specifically acetylated histones. We found that ANCCA is often overexpressed in breast and prostate cancer, and plays an important role for cancer cell proliferation through direct control of target genes such as cyclin D1, c-Myc, and E2F1 (14, 40, 42). We thus speculate that ANCCA may be involved in control of ACTR gene expression.

In this study, we determined that E2F activation of the ACTR gene involves direct binding to multiple noncanonical E2F binding sequences. We showed that deregulated E2F activity through adenovirus E1A is sufficient to upregulate the ACTR promoter. More importantly, we provide evidence that ANCCA is a key mediator of ACTR overexpression and that there is a strong association between the elevated levels of ACTR and the expression of E2F1 and ANCCA in human breast cancer specimens. Together with our previous demonstration that ACTR directly controls ANCCA expression (40), our studies suggest that coordinated deregulation of specific transcription coregulators such as ACTR and ANCCA constitutes a previously
unrecognized mechanism of promoting cell proliferation and tumorigenesis.

Materials and Methods

Recombinant Protein Purification and Electrophoretic Mobility Shift Assay

Glutathione S-transferase (GST) fusion protein expression was induced from recombinant pGEX construct in Escherichia coli cells and purified using glutathione agarose. Oligonucleotide probes (Supplementary Table S1) were radiolabeled using fill-in reaction with the Klenow fragment of DNA polymerase in the presence of 32P-labeled dCTP. For Fig. 2A, gel shift reaction mixtures included binding buffer [50 mmol/L KCl, 3 mmol/L MgCl2, 1 mmol/L DTT, 1 mmol/L EDTA, 1 μg dl-dC competitor DNA, 20 mmol/L Tris-HCl (pH 7.5), and 10% glycerol], 50 ng radiolabeled probe, and 1.5 μg GST/E2F1 in 15 μL volume. Reaction mixtures were incubated at room temperature for 30 min, followed by electrophoresis through 5% polyacrylamide gel (29 acrylamide/1 bisacrylamide) in 0.5X Tris-borate EDTA. Binding was quantitated with the PhosphorImager analysis. Briefly, the autoradiogram density of shifted E2F1-DNA complex was normalized as a fraction of total probe (shifted + free probe). Percentage binding was obtained by comparing the normalized complex density to that of a complex containing control radiolabeled probe for 20 min. At time zero, a 50-fold excess of unlabeled probe was added, and aliquots of the mixtures were harvested at the indicated times. Immunoblotting was done using affinity-purified, only-recognized antibodies that were produced as previously described (43) and included 7 μL of binding buffer [20 mmol/L HEPES (pH 7.9), 20% glycerol, 0.1 mol/L KCl, 0.2 mmol/L EDTA, and 0.5 mmol/L DTT], 1 μg of sheared salmon testes DNA, and 50 ng radiolabeled probe in a total volume of 25 μL for the competition experiment shown in Fig. 2A. GST/E2F1 was used for the experiments described (43) and included 7 μL of binding buffer [20 mmol/L HEPES (pH 7.9), 20% glycerol, 0.1 mol/L KCl, 0.2 mmol/L EDTA, and 0.5 mmol/L DTT], 1 μg of sheared salmon testes DNA, and 50 ng radiolabeled probe in a total volume of 25 μL. For the competition experiment shown in Fig. 2C, GST/E2F1/DP1 was incubated with radiolabeled probe for 20 min. At time zero, a 50-fold excess of unlabeled probe was added, and aliquots of the mixture were analyzed by gel shift assay after incubation for the indicated times. Conditions were adapted from Neuman et al. (43) and included 7 μL of binding buffer [20 mmol/L HEPES (pH 7.9), 20% glycerol, 0.1 mol/L KCl, 0.2 mmol/L EDTA, and 0.5 mmol/L DTT], 1 μg of sheared salmon testes DNA, and 50 ng radiolabeled probe in a total volume of 25 μL. For the competition experiment shown in Fig. 2C, GST/E2F1/DP1 was incubated with radiolabeled probe for 20 min. At time zero, a 50-fold excess of unlabeled probe was added, and aliquots of the mixture were analyzed by gel shift assay after incubation for the indicated times. The autoradiogram density of shifted E2F1-DNA complex was quantitated for analysis. GraphPad Prism 5 software was used to plot the curves, perform statistical analysis, and calculate the on- and off-rates for binding.

Cell Lines, Reporter Constructs, Transfection, and Reporter Gene Assay

MCF-7, MCF10A, MCF12A, and HeLa cells were maintained as previously described (10). SKBR3 cells were maintained in McCoy’s 5A medium supplemented with 10% fetal bovine serum. Reporter gene assays were done by cotransfecting the cells using Fugene HD (Roche) with the indicated 0.6-kb ACTR promoter–linked pGL3 firefly luciferase reporter plasmid construct (see below), pCMX-β gal, and pcD-HCMV, pCMV-E2F1, pCMX-E1A, pSh-HCMV-ANCCA-HA, or pcD-HCMV-ACTR-HA as indicated. Cells were harvested 24 h posttransfection for luciferase and β-galactosidase activity using procedures previously described (4, 10). All transfections for reporter gene assays were done in triplicate, and luciferase values were normalized to β-galactosidase activity. Cells were harvested 24 h posttransfection for Western blot analysis as previously described (4).

The 0.6- and 1.6-kb ACTR promoter reporter constructs have been previously described (10). The 3′- and 5′-truncated and internal deletion constructs were obtained by PCR using the 1.6-kb construct as template and the primers indicated in Supplementary Tables S2 and S3. The PCR products were digested with HindIII and BamHI for Pro-A, Pro-B, and Pro-C or with HindIII and NcoI for Pro-D, Pro-E, Pro-F, and Pro-G, and were cloned into the pGL3 vector. The reporter constructs with point mutations were obtained using the QuikChange Mutagenesis kit (Stratagene). Putative E2F binding sequences were identified using the PROMO v.2.0 program for prediction of transcription factor binding sites (ALGGEN).

Small Interfering RNA, Western blot, Reverse Transcription-PCR and ChIP

SKBR3 cells were transfected with small interfering RNA (siRNA)–targeting ANCCA or a control sequence using Dharmafect (Dharmacon) as previously described (40), and then harvested at the indicated times. Immunoblotting with whole-cell lysates was done using the following specific antibodies: α-ACTR (BD Transduction Laboratories), α-E2F1 (KH95), α-actin (C4; Santa Cruz Biotechnology), and α-ANCCA antibody that was produced as previously described (40). Quantitative reverse transcription-PCR analysis of gene expression was done as previously described (4). ChIP was done as previously described (40). The ChIP DNA was purified using the Quick PCR purification kit (Qiagen) and was subjected to real-time PCR with ACTR promoter–specific primers as previously described (10).

Immunohistochemistry and Statistical Analysis

Tissue microarray sections (BR1004, U.S. Biomax, Inc.) were dewaxed and rehydrated. Antigen retrieving was done using a microwave oven, with 0.01 mol/L sodium citrate buffer (pH 6.0). After nonspecific antibody binding was blocked, sections were incubated with affinity-purified, anti-ANCCA antibody (at 1:300 for 30 min, or anti-E2F1 (KH95, at 1:100; Santa Cruz), or anti-AIB1 (monoclonal, at 1:100; BD) for 90 min. After incubation with biotin-conjugated secondary antibodies, the sections were dewaxed and rehydrated. Antigen retrieving was done using the Olympus IX81 inverted fluorescence microscope system. The tissue section immunoreactivity to each antibody was scored negative if <10% of the epithelial cells displayed any staining, and immunoreactivity was scored positive if >10% of the epithelial cells displayed staining.
FIGURE 2. Noncanonical E2F binding sites located in the first exon and intron of the ACTR gene are important for E2F1/DP1 binding and transactivation. A, top, schematic showing the position of the EMSA oligonucleotide probes as short lines labeled in lower case letters corresponding to 20- to 40-bp sequence segments of the ACTR promoter region. Middle, GST-E2F1 binding to ACTR probes was expressed as percentage of binding relative to control adenovirus E2 probe with consensus E2F binding sites (WT Ad E2; see Materials and Methods). Ad E2 probe containing mutated binding sites was used as negative control (Mut Ad E2). Average results from three experiments are shown in graph. Bottom, the E2F1-DNA complexes from a representative EMSA experiment are shown. B and C, DP1 enhances E2F1 binding to ACTR promoter by increasing the on-rate. B, GST-E2F1, either alone or with GST-DP1, was incubated with wild-type (WT) Ad E2 probe or ACTR probe h. The percentage of maximum binding observed for each protein-DNA complex was plotted against incubation time. Points, mean of two independent experiments. The rate of E2F1 binding was significantly increased by the presence of DP1 for both Ad E2 and ACTR probes (indicated by * and † next to the curves, respectively; P < 0.05 by paired samples t test). The statistically significant difference in rate of E2F1 binding to Ad E2 and ACTR probes (indicated by ‡ next to the two lower curves) was eliminated in the presence of DP1 (no significant difference between the two upper curves, P = 0.0668). An autoradiogram of shifted complexes from a representative experiment is shown above the graph. On-rate for E2F1/DP1 binding to Ad E2 = 3.95 × 10^4 M⁻¹ min⁻¹, to ACTR = 4.64 × 10^5 M⁻¹ min⁻¹ (C) Gel shift reactions were set up and analyzed as in B except that the recombinant proteins were prebound to radiolabeled probes before competition with unlabeled probes. No statistical difference between the plots of binding versus time was observed for Ad E2 and ACTR probes as determined by paired samples t test (P = 0.0644). Off-rate for E2F1/DP1 binding to Ad E2 = 0.3302 min⁻¹, to ACTR = 0.2212 min⁻¹. D, reporter gene assay was done and analyzed as described in Fig. 1 using ACTR promoter fragment D (Pro-D) containing internal deletions (left) or point mutations (right) in the sequences corresponding to the EMSA probes indicated in A. Sequences within the CCGCC motif of putative E2F binding sites were selected for point mutation on the same reporter construct (Supplementary Table S1). Average relative luciferase values from two independent experiments are shown. *, P < 0.05 for fold increase in transactivation by E2F1 compared with wild-type Pro-D.
with moderate to high intensity. The association between the different protein staining was analyzed using the Pearson’s $\chi^2$ test.

For reporter gene assays, independent sample $t$ test was used for statistical analysis.

Results

Identification of Functional E2F-Binding Sites in ACTR Promoter

We previously isolated a 0.6-kb genomic DNA fragment encompassing the first exon of ACTR, and showed that this promoter-containing region associates with and is transactivated by E2F1 (10). To identify the critical sequence(s) responsible for conferring responsiveness to E2F stimulation, we first performed luciferase reporter gene assay using truncated ACTR promoter fragments. As shown in Fig. 1, deletion from 3′ of the 0.6-kb promoter sequence identified a 150-bp region between +65 and +215 being critical for mediating E2F stimulation, as promoter fragment Pro-A retained most of the activity observed in the 0.6-kb promoter, whereas Pro-B and Pro-C displayed only marginal E2F responsiveness. This assessment was verified by results obtained from the 5′-truncated promoters, in which Pro-D and Pro-E, which contain the −30 to +215 nucleotide region, largely retain the E2F-stimulated activity, whereas Pro-F and Pro-G with further deletions from the 3′ end showed little response to E2F. Together, these results indicate that the sequence between −30 and +215 contains element(s) critical for E2F-mediated transcriptional response of ACTR promoter.

Our previous ChIP analysis showed that endogenous E2Fs associate with the promoter region of ACTR gene centered around −0.3 to +0.5 kb (10). To examine whether the −30 to +215 region and/or other regions in the 0.6-kb ACTR promoter harbor E2F binding sites, we analyzed by electrophoresis mobility shift assay (EMSA) a panel of oligonucleotide probes for their potential E2F binding activity. Multiple putative noncanonical E2F binding sites (5′-CCGCC/G-3′) were identified based on software prediction analysis of sequence within the minimal E2F-responsive region (see Fig. 2A schematic and Supplementary Table S1). EMSA was used with recombinant E2F1 and radiolabeled probes consisting of short nonoverlapping genomic DNA sequences that span the region. As seen in Fig. 2A, E2F1 binding clustered within exon 1 (probes d and e), as well as a small region in intron 1 that is downstream of exon 1 (probe h). The binding assay was DNA sequence specific because binding of E2F1 was detected with the wild-type but not the mutant form of the well-characterized E2F binding site from the adenovirus E2 gene. Furthermore, ACTR promoter probes u, a, b, f, and j, which do not contain any predicted E2F binding sequence, did not bind E2F1. Interestingly, similar to the adenovirus E2 probe, the addition of heterodimerization partner DP1 significantly enhanced E2F1 binding to the ACTR probe h by increasing the binding on-rate (Fig. 2B).

Differences between the probes for adenovirus E2 and ACTR sequences in their on- or off-rates of E2F1 binding were not significant in the presence of DP1, indicating similar binding affinity (Fig. 2B and C).

Because sequences in probes d, e, and h bound most strongly to E2F1, we focused on them for further analysis. To determine whether the probe sequences correspond to functioning E2F binding sites, reporter gene assay was done using the ACTR promoter fragment D reporter construct containing internal deletions of probe sequence d, e, h, or i. When sequence d was deleted, E2F1 transactivation was not markedly reduced, indicating that E2F binding to d is not critical for conferring E2F responsiveness in the promoter context (Fig. 2D). Deletion of sequences e or h, however, more strongly reduced E2F induction of the promoter. When both sequences e and h were deleted together, a synergistic inhibitory effect was observed (∼1.7-fold decrease for e, 2.1-fold decrease for h, but ∼10-fold decrease for e+h). As expected, deletion of sequence i, which did not show significant E2F1 binding, did not have a significant effect on induction.

Upon further examination of sequences e and h, we found that they each contain two putative E2F binding sites (Supplementary Table S1). To determine if these E2F binding sites are functional, the core GC nucleotides of each putative binding sequence in e and h were mutated to effect disrupt E2F binding. Indeed, G to T and C to A mutations in either e or h significantly reduced E2F1 induction of the promoter (from 27-fold to ∼12-fold). Changing the GCs in e and h further eliminated the E2F induction of the promoter (from 12-fold to 7-fold; Fig. 2D). Together, these results show that the ACTR noncoding region (up to 150 nucleotides downstream of the transcription start site) contains multiple functional E2F binding sites and that these sites seem to function together to mediate E2F activation of the ACTR promoter.

ACTR Gene Expression Is Associated with ACTR Promoter Activity that Responds to E2F1 Activation

E2F1 is tightly regulated in vivo by pocket proteins that bind and sequester the transcription factor to prevent the activation of its target genes in the absence of appropriate growth signals. During transformation, oncogenes disrupt the interaction between E2F and pocket proteins, leading to constitutively active free E2F species. To determine whether this mode of endogenous E2F deregulation is sufficient to upregulate the ACTR promoter, we introduced the commonly used E1A oncogene into a nontransformed mammary epithelial cell line. Transfection of E1A in MCF10A cells significantly increased ACTR promoter activity by 3- to 4-fold in a dose-dependent manner (Fig. 3A, left), suggesting that deregulation of endogenous E2F activity can drive ACTR promoter activity. Although transfection of E2F1 itself more dramatically increased ACTR promoter activation (Fig. 3A, right), E1A similarly upregulated endogenous ACTR protein level (Fig. 3B). Because endogenous E2F1 levels were only moderately
affected by E1A, we conclude that the increase in ACTR gene expression is due, at least in part, to the enhanced activation of E2F1.

ANCCA, a Novel E2F Coactivator, Is Required for ACTR Overexpression in Breast Cancer Cells

We recently showed that the AAA+ ATPase ANCCA plays an important role in the proliferation of many types of cancer cells including estrogen receptor (ER)-negative breast cancers. We found that ANCCA directly interacts with members of the E2F family and is a bona fide E2F coactivator that mediates the expression of key cell cycle genes.3 Because ACTR is a direct target of E2F, we decided to test the hypothesis that ANCCA is an important regulator of ACTR expression in breast cancer cells. To this end, ER-negative SKBR3 cells were transfected with siRNA targeting ANCCA. As shown in Fig. 4A, ANCCA protein levels were significantly knocked down in ANCCA-siRNA-transfected cells compared with control-siRNA-transfected cells. Although ANCCA depletion did not affect E2F1 expression under this condition, it markedly decreased ACTR expression at both mRNA and protein levels (Supplementary Fig. S1; Fig. 4A and B). To determine whether ANCCA can stimulate endogenous ACTR expression, ANCCA was ectopically overexpressed in the nonmalignant human mammary epithelial cell line MCF12A through transient transfection. As expected for an E2F target gene, ACTR expression was strongly increased in cells ectopically overexpressing E2F1 (Fig. 4C, lane 2). Strikingly, ectopic ANCCA expression resulted in significantly increased ACTR expression (Fig. 4C, lane 3). Together, these results suggest that ANCCA plays a critical role in maintaining high levels of ACTR expression in breast cancer cells.

ANCCA, Together with E2F and ACTR, Stimulates ACTR Gene Expression

To examine whether ANCCA mediates ACTR expression through its function as an E2F coactivator, we first determined whether ANCCA stimulates ACTR promoter transactivation using reporter gene analysis. Although ANCCA did not significantly elevate E2F1-stimulated transactivation, simultaneous expression of ANCCA and ACTR synergistically increased the E2F1-mediated induction of ACTR promoter (Fig. 5A; only about 20% or 30% increase by ACTR or ANCCA respectively if comparing lane 5 and 6 with lane 2, but over 200% increase by ACTR plus ANCCA if comparing lane 7 with lane 2). Conversely, siRNA-mediated knockdown of endogenous ANCCA in breast cancer cells significantly reduced the E2F1 transactivation of the ACTR promoter (Supplementary Fig. S2). These results suggest that ANCCA and ACTR may act together as E2F1 coactivators to mediate transcriptional up-regulation of ACTR gene expression. Indeed, we have previously shown that ANCCA directly associates with ACTR (40). Pull-down assay with recombinant proteins showed that ANCCA specifically and directly interacts with E2F1 and, to a lesser extent, its heterodimerization partner DP1 (Supplementary Fig. S3).

We next examined using ChIP whether ANCCA is recruited with E2Fs to the ACTR promoter. Proliferating SKBR3 cells, which express high levels of ANCCA (Fig. 4A), not unpublished data, were used in a ChIP-reChIP experiment. Precipitation using antibodies against E2F1 and E2F3, but not IgG control, recovered genomic DNA corresponding to the ACTR promoter (Fig. 5B, left), in accord with previous studies showing occupancy of the region by activator E2Fs (10). Remarkably, in the same experiment, ANCCA was also readily detected on the promoter. In the second-round immunoprecipitation using ANCCA antibody, we can clearly show the presence of ANCCA protein in ACTR promoter chromatin complexes eluted from the first ChIP using E2F1 or E2F3 antibodies, but not normal IgG (Fig. 5B, right). The E2F and ANCCA binding are specific for the ACTR promoter, as the antibodies did not precipitate sequence upstream of the 0.6-kb region (~3.6 kb) in either the first- or second-round immunoprecipitation. Along with reporter gene analysis, these results strongly suggest that ANCCA is directly involved in the E2F regulation of the ACTR promoter.
ACTR Overexpression Correlates with High Levels of E2F1 and ANCCA in Human Breast Cancer

To address the disease relevance of the ANCCA-E2F mechanism in ACTR overexpression, we examined by immunohistochemistry the expression of ACTR, ANCCA, and E2F1 in a cohort of 96 human breast cancer specimens on a tissue microarray, with half from primary invasive tumor tissues and the other half being matched, lymph node metastatic tumor tissues. Consistent with the findings from previous studies (15, 44, 45), we found that ACTR is overexpressed in 35% of primary tumors and in 44% metastatic tumors, and that E2F1 is overexpressed in 52% of the primary tumors and in 38% metastatic tumors (Table 1; Fig. 6). Interestingly, ACTR overexpression was significantly correlated to the expression of E2F1 in the primary tumors ($P < 0.002$) and in the metastatic tumors ($P < 0.0004$). When ANCCA expression was analyzed, a very strong association between ANCCA and ACTR was also found in the primary and metastatic tumors ($P < 0.00001$). These results provide the clinical evidence that ACTR overexpression in breast cancer is likely resulted from the deregulated expression in breast cancer is likely resulted from the deregulated expression in breast cancer is likely resulted from the deregulated expression in breast cancer is likely resulted from the deregulated expression in breast cancer is likely resulted from the deregulated expression in breast cancer is likely resulted from the deregulated expression in breast cancer.

Discussion

The mechanism underlying the deregulated expression primarily at transcript level of the oncogene ACTR in multiple types of cancers is thus far poorly understood. Our study here provides several important insights into the molecular mechanisms involved in the transcriptional regulation of oncogene ACTR. Through rather comprehensive analysis of ACTR promoter, we have identified several sequences important for E2F-mediated regulation. We also provide evidence that ACTR promoter activation requires direct E2F binding. Activation of endogenous E2Fs through disruption of the pRb-E2F regulatory axis is sufficient to upregulate the ACTR promoter. Furthermore, we
show that the recently identified E2F coactivator ANCCA has an important function in controlling ACTR expression in breast cancer cells. More importantly, we found that ACTR overexpression is correlated strongly with high levels of E2F1 and the newly identified coregulator ANCCA in human breast carcinomas. ACTR, ANCCA, and E2F1 have all been shown to be involved in regulating cell proliferation through their control of key cell cycle genes. We propose

**FIGURE 5.** A, HeLa cells were cotransfected with pGL3-0.6kb ACTR promoter reporter and expression constructs for E2F1, ACTR, and/or ANCCA as indicated. Columns, mean of normalized luciferase values from triplicate samples; bars, SEM. *, *P < 0.05 for coactivation of E2F1 by ACTR or ANCCA alone compared with E2F1 transactivation with both ACTR and ANCCA. B, ChIP assay was done using asynchronously proliferating SKBR3 cells with ANCCA-, E2F1-, or E2F3-specific antibodies, or with IgG-nonspecific control (left). Half of the chromatin complexes precipitated using E2F antibodies or IgG were then subject to a second precipitation with ANCCA antibody (right). Protein occupancy was analyzed by qPCR analysis of ChIP or input DNA from three independent experiments done in triplicate using ACTR promoter or upstream primers amplifying the region indicated in schematic. PCR data from ChIP DNA was normalized to that of input and calculated as percent of input.

**Table 1.** The number of tumors stained positive and negative for ACTR, E2F1 and ANCCA, and their association

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<th>Primary tumors</th>
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<tr>
<td></td>
<td>ACTR negative</td>
<td>ACTR positive</td>
<td>P</td>
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<tr>
<td>No. of tumors</td>
<td>29</td>
<td>19</td>
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<tr>
<td>E2F1</td>
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<tr>
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<td>24</td>
<td>7</td>
<td>&lt;0.002</td>
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<tr>
<td>Positive</td>
<td>5</td>
<td>12</td>
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<tr>
<td>ANCCA</td>
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<tr>
<td>Negative</td>
<td>23</td>
<td>1</td>
<td>&lt;0.00001</td>
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<td>Positive</td>
<td>6</td>
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Abbreviation: LN, lymph node.
that ANCCA and activator E2Fs, particularly E2F1, cooperate to influence ACTR expression levels. Thus, deregulation of ACTR expression as seen in cancer cells may result from factors that aberrantly alter the expression and/or activity of E2F1 or of ANCCA.

Previous studies show that ACTR is a direct target of E2F1 (10, 31). In our further study of the mechanism for E2F control of ACTR gene expression, we identified several functional E2F binding sites, located within the first exon and the 5′ end of the first intron of ACTR gene. The functional sites were identified based on the results from different in vitro and in vivo analyses using series of wild-type and mutant probes and promoter constructs. Recently, based on sequence conservation between human and mouse, two putative E2F binding sites distinct from the ones found in this study were identified but later found not to be crucial for E2F occupancy of the ACTR/SRC-3 promoter (31). Intriguingly, a Sp1 binding site downstream of the putative E2F sites was found to be important for the E2F occupancy at and transactivation of the ACTR promoter. Although our results support the notion that E2Fs bind directly to the sites we identified to mediate stimulation of ACTR promoter, we cannot rule out the possibility that Sp1 contributes to the E2F occupancy at these sites. Alternatively, E2Fs may occupy distinct subsets of binding sites in the ACTR promoter region through both direct binding and through concerted binding with other transcription factors such as Sp1 that are also involved in control of ACTR promoter activity. The latter mode of facilitated E2F genomic association has been proposed to explain the widespread genomic localization of sites occupied by E2Fs (39) and showed experimentally on specific E2F targets (46-48).

Interestingly, the repetitive motif in the E2F binding sequences found in ACTR promoter is 5′-CCGCC-3′ (Supplementary Table S1), which is significantly deviated from the consensus sequence TTT(C/G)(C/G)CGC. This noncanonical sequence was previously identified as an E2F1 binding site that occurs as a variable number of tandem repeats polymorphism in the regulatory region of the SMYD3 histone methyltransferase gene (37). Notably, SMYD3 gene expression was found to be elevated in colorectal, liver, and breast cancers, and the allele containing three tandem repeats of the binding motif correlated with increased risk of these cancers compared with the allele with only two repeats. In support of our notion that the CCGCC sequence constitutes a functional E2F binding site in the ACTR promoter, point mutations changing G or C to A or T in the individual motifs were sufficient to attenuate E2F1-stimulated transactivation of ACTR promoter. Mutating the GCs in both E2F binding regions almost completely abrogated the E2F induction, suggesting that the CCGCC motifs in ACTR promoter, like the ones in SMYD3 promoter, cooperate in mediating E2F occupancy.

We found that ectopic introduction of E1A oncprotein significantly enhanced basal ACTR promoter transactivation in nontransformed mammary epithelial cells, suggesting that deregulated endogenous E2F activity through inactivation of pocket proteins can promote ACTR overexpression. This idea is supported by the finding that the E2F-targeted $p14^{ARF}$ tumor suppressor gene is induced in normal fibroblasts only in response to aberrant E2F activity stemming from E1A inactivation of Rb or ectopic E2F1 expression (36). Interestingly, similar to what we observed for ACTR promoter, transactivation of $p14^{ARF}$ under these aberrant conditions also involves nonconsensus E2F binding sequences. In light of our previous findings that the novel E2F coactivator ANCCA is a direct target of ACTR (40), we speculated that ANCCA might also be involved in control of E2F activation of ACTR expression. Indeed, we found that ANCCA is recruited to the ACTR promoter, likely as a complex with activator E2Fs. Reporter gene analyses indicated that ANCCA, along with ACTR, synergistically enhances E2F-induced transactivation of the ACTR promoter. Whether this finding reflects a singular E2F1 coactivator complex formed by ANCCA and ACTR has not yet been determined and will require further analysis. Nonetheless, these results raise the intriguing possibility that aberrant expression or activation of coregulator

![FIGURE 6. ACTR overexpression correlates strongly with E2F1 and ANCCA in breast cancers. Sections from tissue microarrays were processed for immunohistochemistry analysis with specific antibodies as indicated for ACTR, E2F1, and ANCCA. Examples of immunohistochemistry staining of primary tumors were shown either in low magnification (left) or with the areas indicated by dashed frames displayed in high magnification (right).](image-url)
ANCCA, as might be observed in cancer cells, can lead to the transcriptional overactivation of ACTR promoter leading to its overexpression.

E2F1 is well known for its paradoxical function as oncoprotein and tumor suppressor in different cancers. Nevertheless, its overexpression in multiple cancers including breast cancer and lung cancer has been strongly linked to cancer progression (45, 49–53). E2F control of gene expression likely involves multiple distinct chromatin modifying coregulators (54). Among the E2F coregulators reported thus far, ACTR is unique in that it possesses a potent oncogenic activity when overexpressed. Our finding that E2F1 overexpression strongly correlates with elevated levels of oncoprotein ACTR not only provides the clinical evidence for E2F-mediated deregulation of ACTR but also suggests that the E2F-ACTR–positive feedback loop may underlie their aberrant expression and function in cancers. Future studies to further investigate the interdependency between E2F1, ACTR, and possibly ANCCA in tumorigenesis should shed light on how distinct gene expression programs are altered by the specific E2F-coregulator complexes that directly contribute to cancer development.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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