CARM1 Regulates Estrogen-Stimulated Breast Cancer Growth through Up-regulation of E2F1

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Abstract

Estrogen receptor α (ERα) mediates breast cancer proliferation through transcriptional mechanisms involving the recruitment of specific coregulator complexes to the promoters of cell cycle genes. The coactivator-associated arginine methyltransferase CARM1 is a positive regulator of ERα-mediated transcriptional activation. Here, we show that CARM1 is essential for estrogen-induced cell cycle progression in the MCF-7 breast cancer cell line. CARM1 is specifically required for the estrogen-induced expression of the critical cell cycle transcriptional regulator E2F1 whereas estrogen stimulation of cyclin D1 is CARM1 independent. Upon estrogen stimulation, the E2F1 promoter is subject to CARM1-dependent dimethylation on histone H3 arginine 17 (H3R17me2) in a process that parallels the recruitment of ERα. Additionally, we find that the recruitment of CARM1 and subsequent histone arginine dimethylation are dependent on the presence of the oncogenic coactivator AIB1. Thus, CARM1 is a critical factor in the pathway of estrogen-stimulated breast cancer growth downstream of ERα and AIB1 and upstream of the cell cycle regulatory transcription factor E2F1. These studies identify CARM1 as a potential new target in the treatment of estrogen-dependent breast cancer. [Cancer Res 2008;68(1):301–6]
verified by dissociation curve analysis. See Supplementary Table S1 for a list of chromatin immunoprecipitation primers used in this study.

Real-time reverse transcription-PCR. RNA isolation and real-time reverse transcription-PCR (RT-PCR) were done as described in ref. 17. Expression was normalized to RPS28 and glyceraldehyde-3-phosphate dehydrogenase. See Supplementary Table S1 for a list of target primers used in this study.

Western blotting. Western blot assays were done as described in ref. 17.

Cell cycle distribution and growth assays. MCF-7 cell cycle distribution was analyzed 24 h after stimulation with 10 nmol/L E2 or vehicle using propidium iodide staining and flow cytometry. *, P < 0.05, versus siCONTROL-transfected cells treated with E2.

Data analysis. Data analysis was done using the Prism software. Statistical significance was determined using Student's t test comparison for unpaired data and was indicated as follows: *, P < 0.05; **, P < 0.01.

**Figure 1.** Effect of CARM1 silencing in MCF-7 cells on E2-induced cell cycle progression. A, siRNA-mediated silencing of CARM1 in MCF-7 cells. Two different CARM1-specific siRNAs or a control siRNA were transfected into hormone-depleted MCF-7 cells. Extracts were collected and Western blotting was done with anti-CARM1 antibodies. Anti-calnexin was used as a loading control. B, cell cycle analysis of CARM1-depleted MCF-7 cells. MCF-7 cells were transfected as described in A. Forty-eight hours later, cells were treated with 10 nmol/L E2 or ethanol vehicle for 24 h and harvested to analyze DNA content by propidium iodide staining and flow cytometry. *, P < 0.05, versus siCONTROL-transfected cells treated with E2. Columns, mean from one representative experiment done in triplicate; bars, SEM. **, P < 0.01, versus similarly treated siCONTROL-transfected cells.

**Figure 2.** CARM1 silencing affects E2-induced expression of E2F1. A to C, CARM1-specific or control siRNAs were transfected into hormone-depleted MCF-7 cells. Forty-eight hours after transfection, cells were treated with vehicle or 10 nmol/L E2 for the indicated time period, and RNA was isolated to measure the expression of TFF1 (A), E2F1 (B), and CCND1 (C) genes by real-time RT-PCR. Columns, mean of three independent replicates; bars, SD. Shown are results obtained from siCARM1-1 transfections; similar results were obtained from siCARM1-2 transfections. *, P < 0.05, versus siCONTROL-transfected cells. D, after 48 h of transfection with siCARM1 or control siRNA, MCF-7 cells were stimulated with 10 nmol/L E2 for 0, 24, and 48 h and whole-cell extracts from MCF-7 cells were analyzed by Western blot for E2F1.
Results

**CARM1 is required for E2-induced proliferation in ERα-positive breast cancer cells.** CARM1 is thought to play an important role in hormone signaling through coactivation of nuclear receptor-mediated transcription and histone modification (18); however, this has been shown for a small number of target genes. Whereas both ERα and AIB1 have been shown to be involved in regulating cellular growth properties in both normal and breast cancer cells (19, 20), the mechanisms involving CARM1 in this process and its critical targets are not clearly defined. To explore the role of CARM1 in ERα-positive breast cancer cells, we examined the consequences of its suppression on the growth stimulatory effect of E2 using MCF-7 cells as a model. Expression of CARM1 was efficiently and specifically silenced in MCF-7 cells at the mRNA (data not shown) and protein levels (Fig. 1A) with either of two different CARM1 siRNA oligonucleotide duplexes. Upon silencing of CARM1, we observed that the E2-mediated stimulation of MCF-7 cell cycle progression was strongly reduced (Fig. 1B). Furthermore, we observe a decrease in the number of MCF-7 cells following growth stimulation with E2 when CARM1 was silenced compared with a control siRNA (Fig. 1C). We found a similar effect of CARM1 silencing on E2-induced proliferation of T47D cells, another model of ERα-positive breast cancer (Supplementary Fig. S1). These data point to a critical role for CARM1 in the proliferative response to estrogen in ERα-positive breast cancer cells.

**CARM1 controls breast cancer proliferation by regulating E2F1 expression.** CARM1 has been shown to regulate the transcription of a small number of genes in response to distinct stimuli (21–24). To address whether CARM1 regulates the cell cycle by modulating transcription of specific genes that are involved in this process, we examined the effect of CARM1 depletion on the expression of specific cell cycle genes. Previous results show that the key cell cycle regulator E2F1 and, subsequently, its downstream target genes are critical for hormone regulation of the proliferative program of ERα-positive breast cancer cells (25). We therefore investigated the effect of CARM1 silencing on the expression of E2F1. As a positive control, CARM1 silencing reduced expression of the TFF1 gene (Fig. 2A). Estrogen stimulation induced a 5- and 7-fold increase in E2F1 mRNA after 3 and 12 h, respectively, and this effect was very significantly reduced by CARM1 silencing (Fig. 2B). Interestingly, whereas CARM1 silencing impaired the transcription of E2F1, the estrogen-induced transcription of the CCND1 gene was unaffected by CARM1 silencing (Fig. 2C). Accordingly, we also observed that E2F1 protein expression levels were strongly reduced by CARM1 silencing (Fig. 2D). E2F1 expression was also diminished when CARM1 was silenced in another breast cancer cell model, the T47D cell line (Supplementary Fig. S2).

**CARM1 silencing blocks the E2 induction of E2F1-responsive genes.** Estrogen regulation of E2F1 is important for the regulation of several cell cycle–associated genes. Having shown that CARM1 is important for the estrogen regulation of E2F1, we examined whether CARM1 silencing resulted in reduced expression of the E2F1-responsive genes including CCNE1, CCNE2, CDC25A, and CCNA1. As predicted in E2-stimulated MCF-7 cells in which CARM1 was silenced, we observed a significant reduction in the levels of CCNE1 and CCNE2 mRNA, as well as CDC25A and CCNA1 mRNA, compared with the control (Fig. 3). Hence, these data support an important role for CARM1 in the regulation of E2F1 and E2F1-responsive genes.

**CARM1 directly mediates the E2-induced activation of the E2F1 promoter.** To examine the mechanism by which CARM1 is involved in the E2-mediated control of E2F1 transcription, we carried out chromatin immunoprecipitation assays. Previous studies have shown that a region up to −220 bp from the transcription start site is sufficient for full promoter activity (26, 27). This region contains two palindromic E2F-binding sites, several putative binding sites for SP1, a putative binding site for nuclear factor-κB, and two canonical CAAT boxes. The E2F1
promoter has neither a TATA motif nor an initiator element. We find that E2 enhances the recruitment of both ERα and E2F1 to the E2F1 promoter (Fig. 4A). In addition, we observe constitutive recruitment of SP1 to the E2F1 promoter independent of E2 stimulation (Fig. 4A). Because both AIB1 and CARM1 are thought to function in ERα- and E2F1-mediated transcriptional activation, we measured the enrichment of AIB1, CARM1, and H3R17me2 at the E2F1 promoter before and after E2 stimulation (Fig. 4B). Following E2 treatment, the coactivators AIB1 and CARM1, as well as the CARM1-catalyzed histone modification H3R17me2, are enriched at the E2F1 promoter (Fig. 4B). These results indicate that E2-mediated expression of E2F1 involves the recruitment of AIB1 and CARM1 to the E2F1 promoter and that this region of chromatin is subject to estrogen-dependent H3R17 dimethylation (H3R17me2). Interestingly, CARM1 was also recruited to the promoter of several E2F1 target genes (CCNE1, CCNE2, CDC25A, and CCNA1) following E2 stimulation (Supplementary Fig. S3). This suggests that in addition to the role of CARM1 in regulating E2F1 expression, it may play a second role in the regulation of E2F1 target genes. These results provide a direct link of CARM1 to cell cycle regulation through the transcriptional activation of E2F1 and E2F2 target genes.

CARM1 recruitment and E2-mediated histone modification at the E2F1 promoter are dependent on AIB1. It is thought that CARM1 is targeted to ERα-regulated genes via an interaction with SRC/p160 members including AIB1 (28). To test the requirement of AIB1 for CARM1 recruitment and histone arginine dimethylation at the E2F1 promoter, we silenced AIB1 in MCF-7 cells using AIB1-specific siRNAs and carried out CARM1 and H3R17me2 chromatin immunoprecipitation assays. Following efficient silencing of AIB1, we detect a significantly reduced recruitment of CARM1 to the E2F1 promoter compared with cells transfected with control siRNA (Fig. 5A) both in the absence of hormone and, more significantly, after E2 stimulation. Importantly, the reduction of AIB1 had no measurable effect on the levels of CARM1 protein (Fig. 5A, right). Additionally, silencing AIB1 reduced the levels of H3R17me2 at the E2F1 promoter. The silencing of AIB1 affects histone methylation to a similar degree as when CARM1 is silenced (Fig. 5B). Thus, AIB1 functions in the recruitment of CARM1 and the subsequent H3R17 dimethylation during transcriptional activation of E2F1 and may in part explain the oncogenic functions of AIB1.

Discussion

Estrogens elicit proliferative responses in breast cancer cells through ERα-mediated transcriptional mechanisms (29). CARM1 has been implicated in the positive regulation of ERα-mediated gene activation in response to estrogen signaling (28). Thus, a presumed but not fully tested model for CARM1 action is that it functions in the control of cell proliferation through the activation of key cell cycle genes. In the current study, we describe a specific role for CARM1 in regulating the cell cycle by inducing the expression of E2F1 in MCF-7 cells following E2 treatment.

We show that CARM1 is required for the E2-mediated activation of E2F1 and for the induction of E2F1 target genes, including CDC25A, CCNA1, CCNE1, and CCNE2. These data are in agreement with a recent report showing that CARM1 functions in E2F1-mediated transactivation through AIB1 to positively regulate the expression of CCNE1 on cell cycle entry (22). Transfection of siRNA oligonucleotide duplexes into MCF-7 cells reduced the mRNA and protein levels of CARM1 by >70%. Reduced levels of CARM1 were sufficient to affect the E2-mediated activation of the TFF1 gene, suggesting that CARM1 was functionally silenced. Significantly, CARM1 silencing does not affect the E2-stimulation of CCND1, showing that CARM1 does not function in the entire E2 response but rather has an important role in targeting a subset of ERα targets including E2F1. The observed gene-specific effect of CARM1 is consistent with the reported promoter-specific role for CARM1 in the activation of specific nuclear factor κB–dependent genes (23).

At the E2-responsive TFF1 promoter, CARM1 recruitment was shown to follow ERα recruitment. Thus, CARM1 is thought to be recruited by ERα to this promoter (15, 30). However, it is not known whether the recruitment of CARM1 occurs at additional E2-responsive sites including nonclassical ERα target sites. Here, we show that CARM1 is recruited to the E2F1 promoter, a nonclassical ERα site (6). Our chromatin immunoprecipitation data confirm the recruitment of ERα to the promoter of E2F1 and show that E2 induces the recruitment of both AIB1 and CARM1. CARM1 targeting to the E2F1 promoter coincides with enhanced levels of H3R17 dimethylation. Importantly, H3R17me2 at this site is undetectable in CARM1-depleted cells, signifying that CARM1 is the main or sole enzyme responsible for the dimethylation

Figure 4. E2-mediated E2F1 expression corresponds with arginine methylation of nucleosomal histones located at the E2F1 promoter. A, chromatin immunoprecipitation analysis of the human E2F1 promoter. Chromatin immunoprecipitation was done with primers specific for the proximal portion of the E2F1 promoter with antibodies against Sp1, E2F1, and ERα. Formaldehyde cross-linked chromatin samples prepared from vehicle- and E2-treated MCF-7 cells were used for chromatin immunoprecipitation analyses with the indicated antibodies and analyzed by real-time PCR for the presence of the E2F1 promoter fragment as diagramed. Columns, mean of three independent replicates; bars, SD. B, chromatin immunoprecipitation analysis of the E2F1 promoter with antibodies against AIB1, CARM1, and H3R17me2.
of histone H3 at arginine 17. We also observe a major defect in CARM1 recruitment to the E2F1 promoter. Specific siRNAs targeting AIB1 or control siRNAs were transfected into MCF-7 cells 48 h before E2 stimulation. CARM1 chromatin immunoprecipitation (ChIP) analyses for the E2F1 promoter were done as in Fig. 4. Western blot analysis of AIB1 and CARM1 was done with AIB1- and CARM1-specific antibodies. Calnexin was used as a loading control. *, P < 0.05, versus siCONTROL-transfected cells treated with E2. B, depletion of CARM1 or AIB1 results in decreased levels of H3R17me2 at the promoter of E2F1. siRNAs targeting a nonspecific control, CARM1, or AIB1 were transfected into MCF-7 cells and H3R17me2 chromatin immunoprecipitation was done. *, P < 0.05, versus siCONTROL-transfected cells treated with E2.

Methylation of histones by PRMTs is increasingly being acknowledged as an important aspect for the dynamic regulation of gene expression. For example, CARM1/PRMT4 and PRMT1, two type I PRMTs, have been proposed to play a role in gene activation and were shown to catalyze monomethylation and asymmetrical N\(^{\omega}\), N\(^{\omega}\)-arginine dimethylation on histone H3R2, H3R17, H3R26, and H4R3, respectively (31). In contrast, PRMT5, a type II PRMT, was shown to catalyze histone H4 monomethylation and symmetrical N\(^{\omega}\), N\(^{\omega}\)-arginine dimethylation, a modification that coincides with gene repression (32, 33). Whereas it is recognized that specific PRMT enzymes are important transcriptional coregulators, it is unclear how these enzymes and their associated histone modifications function to coordinately regulate gene expression in vivo. In this report, we reveal that the arginine methyltransferase CARM1 functions in breast cancer proliferation through specific gene activation.

Aberrant expression of CARM1 has been linked to human prostate and breast cancers (21, 22, 34). Consistent with a study showing that CARM1 \(^{-/-}\) murine embryonic fibroblasts display an altered capacity to enter the cell cycle upon serum stimulation (22), we show that CARM1 depletion also adversely affects breast cancer cell proliferation when stimulated with estrogen. Our finding that CARM1 is required for E2F1 expression, but not CCND1 expression, following estrogen simulation suggests that the pathway of estrogen-stimulated growth involves the direct transcriptional effect of ER\(\alpha\), AIB1, and CARM1 on the E2F1 promoter. In this model (Fig. 6), the ability of estrogen acting through ER\(\alpha\) and AIB1 to activate CCND1 (35) and subsequently inactivate Rb is independent of CARM1, although the ability of estrogen to

Figure 5. Depletion of AIB1 inhibits CARM1 recruitment and E2F1 promoter methylation. A, siRNA-mediated depletion of AIB1 affects CARM1 recruitment to the E2F1 promoter. Specific siRNAs targeting AIB1 or control siRNAs were transfected into MCF-7 cells 48 h before E2 stimulation. CARM1 chromatin immunoprecipitation (ChIP) analyses for the E2F1 promoter were done as in Fig. 4. Western blot analysis of AIB1 and CARM1 was done with AIB1- and CARM1-specific antibodies. Calnexin was used as a loading control. *, P < 0.05, versus siCONTROL-transfected cells treated with E2. B, depletion of CARM1 or AIB1 results in decreased levels of H3R17me2 at the promoter of E2F1. siRNAs targeting a nonspecific control, CARM1, or AIB1 were transfected into MCF-7 cells and H3R17me2 chromatin immunoprecipitation was done. *, P < 0.05, versus siCONTROL-transfected cells treated with E2.

Figure 6. Model of E2-mediated breast cancer cell proliferation.
stimulate the cell cycle is dependent on CARM1 activation of E2F1 and several of its downstream targets. Taken together, these results suggest that CARM1 may represent an important target for therapeutic intervention in hormone-dependent breast cancer.

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References


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