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Histone deacetylase inhibitor apicidin induces cyclin E expression through Sp1 sites

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Abstract

We show that a histone deacetylase (HDAC) inhibitor apicidin increases the transcriptional activity of cyclin E gene, which results in accumulation of cyclin E mRNA and protein in a time- and dose-dependent manner. Interestingly, apicidin induction of cyclin E gene is found to be mediated by Sp1- rather than E2F-binding sites in the cyclin E promoter, as evidenced by the fact that specific inhibition of Sp1 leads to a decrease in apicidin activation of cyclin E promoter activity and protein expression, but mutation of E2F-binding sites of cyclin E promoter region fails to inhibit the ability of apicidin to activate cyclin E transcription. In addition, this transcriptional activation of cyclin E by apicidin is associated with histone hyperacetylation of cyclin E promoter region containing Sp1-binding sites. Our results demonstrate that regulation of histone modification by an HDAC inhibitor apicidin contributes to induction of cyclin E expression and this effect is Sp1-dependent.

Keywords: Histone deacetylase; Apicidin; Cyclin E; Sp1; E2F

Cyclin-dependent kinases (CDKs) play a pivotal role in controlling progression of the cell cycle [1]. CDKs form complexes with their regulatory subunits, cyclins, to function as kinases of specific proteins at different phases of the cell cycle. Current evidences suggest that the S-phase promoting function of cyclin D and cyclin E is mediated by the kinase activities of cyclin-CDK complexes to phosphorylate Rb protein (pRb), leading to releasing E2F from an inactive pRb-E2F complex [2,3]. When the inactivation of pRb occurs via its phosphorylation and E2F is subsequently activated, E2F upregulates the expression of target genes including cyclin E gene required for the G_1/S transition of the cell cycle. The increase in cyclin E expression results in an activation of cyclin E/CDK2 complex, leading to further phosphorylation of pRb [4].

Chromatin remodeling from reversible acetylation of core histones has been suggested to be a critical component of transcriptional regulation [5]. The turnover of histone acetylation is regulated by the opposing activities of histone acetyltransferases (HATs) and histone deacetylases (HDACs) [6,7]. The inhibition of HDACs causes an accumulation of acetylated histones in the nucleus and subsequent activation of transcription of target genes. Indeed, it has repeatedly been demonstrated that treatment with HDAC inhibitors such as apicidin and trichostatin A upregulates the transcription of $p21^{WAF1/Cip1}$, cyclooxygenase-1, and telomerase reverse transcriptase via histone hyperacetylation [8–11]. In addition, these transcriptions

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require Sp1 transcription factor which plays a key role in the activation of a large number of genes containing upstream 'GC Box' promoter elements [12,13].

The transcription of cyclin E has been known to be E2F-dependent, but recently, several investigators have demonstrated that Sp1 synergistically acts with E2F to induce transcription of cyclin E and DHFR genes through physical interactions of Sp1 with E2F, suggesting the possible involvement of Sp1 transcription factor in cyclin E expression [14–16]. Furthermore, Sp1 can transactivate or interact with several G_1/S regulators, resulting in G_1/S transition and S phase progression in the cell cycle [17]. However, it has not been addressed whether Sp1 alone induces cyclin E expression without cooperation of E2F. In this study, we attempted to investigate the molecular mechanism for transcriptional regulation of cyclin E in response to HDAC inhibitor apicidin. Our results show that apicidin induces Sp1-dependent cyclin E gene expression, as demonstrated by use of Sp1 family inhibitor mithramycin, dominant negative delta-Sp1, and site-directed Sp1 mutants, which are associated with hyperacetylation of histones in cyclin E promoter region containing Sp1-binding sites.

Materials and methods

Cell culture and reagents. Human cervix cancer cell line HeLa was cultured in Dulbecco's modified Eagle's medium (Life Technologies, Gaithersburg, MD), supplemented with 10% fetal bovine serum (HyClone Laboratories, Logan, UT), and 1% penicillin/streptomycin (Life Technologies). Apicidin, (cyclo(*N*-*O*-methyl-L-tryptophanyl-L-isoleucinyl-D-pipecolinyl-L-2-amino-8- oxodecanoyl)), was prepared from *Fusarium* species Strain KCTC 16677, according to the method previously described [18]. Trichostatin A and mithramycin were obtained from Sigma (St. Louis, MO).

Plasmids. The full-length wild-type human cyclin E promoter sequences from −363 to +1007 (p10-4 Luc), the cyclin E promoter sequences with three mutated E2F sites at −354, −292, and −282 (pMUT I + II + III Luc), the wild-type cyclin E promoter sequences from −207 to +77 (pE(−207)Luc), and the cyclin E promoter sequences with two mutated E2F sites at −16 and +7 (pME(−207)Luc) were kind gifts from Dr. E. Aubrey Thompson (Mayo Clinic Jacksonville). The delta-Sp1 plasmid, dominant-negative mutant, was kindly supplied by Dr. Gerald Thiel (Department of Medical Biochemistry and Molecular Biochemistry, University of the Saarland Medical Center). Site-directed mutagenesis of the cyclin E promoter sequences from −195 to −15, as shown in Fig. 4A, was performed by the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA).

Transfection and luciferase assay. HeLa cells were plated into 6-well plates at a density of 1×10^5/well and incubated for 24 h. For cyclin E promoter analysis, the cells were transfected with 2 μg/well of cyclin E promoter reporter plasmid DNA using ProFection transfection reagent (Promega, Madison, WI). Following transfection for 24 h, the medium was freshly changed with or without 1 μM apicidin,and cell lysates were prepared for the luciferase assay 24 h later. The luciferase activities were measured according to the manufacturer's recommendations(Promega, Madison, WI) and normalized for the amount of the protein in cell lysates.

RT-PCR. Total RNA was extracted using Easy-Blue™ reagent (iNtRON Biotechnology, Sungnam, Gyeonggi). Integrity of RNA was checked by agarose gel electrophoresis and ethidium bromide staining. One microgram of RNA was used as a template for each reverse transcriptase (RT)-mediated PCR (RT-PCR) using RNA PCR kit (Takara Bio, Otsu, Shiga). Primer sequences for cyclin E and GAPDH were described previously [19].

Immunoblotting. HeLa cell lysates were prepared, clarified, and subjected to immunoblot analysis as described previously [11].

Chromatin immunoprecipitation assay. Chromatin immunoprecipitation (ChIP) assays were performed using the acetyl-histone H3 immunoprecipitation assay kit (UpstateBiotechnology, Lake Placid, NY), according to the manufacturer's instructions.Briefly, chromatin from 1×10^6 HeLa cells sheared by a sonicator was precleared with salmon sperm DNA-saturated protein G–Sepharose, and then was immunoprecipitated with anti-acetylated histone H3 antibodies. Samples were analyzed by PCR using Ex *Taq* polymerase (Takara Bio Inc, Otsu, Shiga).

Results and discussion

Since HDAC inhibitors such as trapoxin or oxamflatin have been shown to induce cyclin E expression [20,21], we first examined the effect of apicidin on the protein expression of cyclin E in HeLa cells. As shown in Figs. 1A and B, apicidin induced expression of cyclin E protein in a time- and dose-dependent manner. We next analyzed the changes of cyclin E mRNA levels in response to apicidin treatment. Apicidin resulted in a time-dependent induction of cyclin E mRNA: apicidin-induced cyclin E mRNA levels were first observed at early time points between 1 and 2 h, slightly decreased about around 8–12 h, and reached maximum by 24 h end time point of this experiment (Fig. 1C). To further understand molecular mechanisms by which apicidin induces cyclin E mRNA and protein expression, we investigated whether apicidin could stimulate the transcriptional activity of cyclin E, using HeLa cells transfected with the wild-type cyclin E promoter luciferase fusion plasmid (p10-4 Luc). Apicidin dramatically increased the expression of cyclin E reporter gene in a dose-dependent manner, and this effect was consistent with the other HDAC inhibitor trichostatin A (Fig. 1D). In addition, treatment of HeLa cells with a protein synthesis inhibitor, cycloheximide (10 μM), did not alter apicidin-induced cyclin E mRNA levels, indicating that cyclin E mRNA expression seems to be regulated directly through transcriptional mechanism (Fig. 1E). Collectively, these findings demonstrate that apicidin induces the expression of cyclin E mRNA and protein through transcriptional activation.

Cloning of the cyclin E promoter identified several putative E2F-binding sites within the promoter sequences from −363 to +1007, and the E2F-binding sites have been thought to regulate cyclin E transcription [2,22,23]. To assess whether apicidin stimulates cyclin E promoter through E2F-binding sites, we utilized two sets of cyclin E promoter constructs. As shown in Fig. 2A, the first set (p10-4 Luc, pMut I + II + III Luc) contains the full-length wild-type human cyclin E promoter sequences from −363 to +1007 (p10-4 Luc) or the cyclin E promoter sequences (−363 to +1007) with three mutated E2F sites at −354, −292, and −282 (pMut I + II + III). The second set of constructs (pE(−207)Luc and pME(−207)Luc) contains

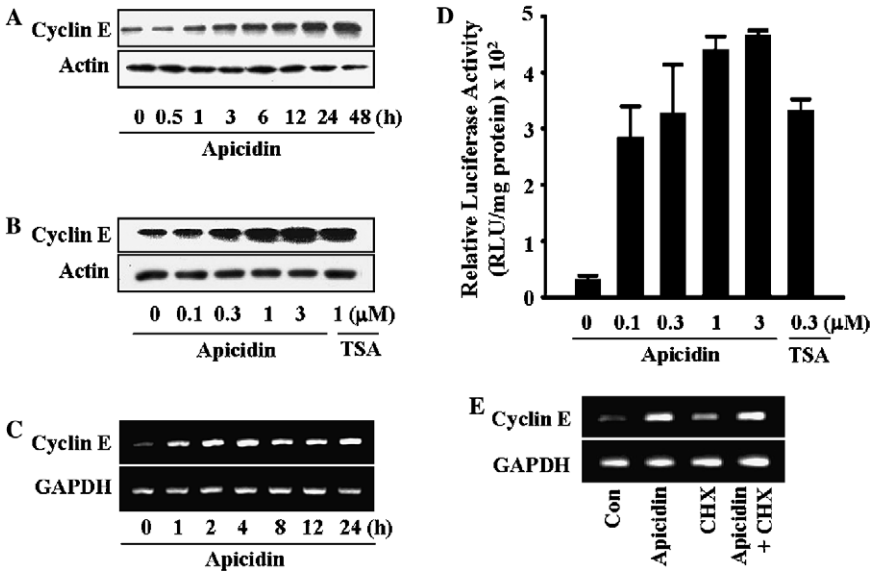

Fig. 1. Effect of apicidin on cyclin E protein expression, mRNA levels, and promoter activity in HeLa cells. (A) HeLa cells were treated with 1 μM apicidin for indicated times, and equal amounts of cell lysates (40 μg of total proteins) were subjected to immunoblot analysis using anti-cyclin E antibodies as described in Materials and methods. (B) HeLa cells were exposed to 0.1, 0.3, 1, and 3 μM apicidin, or 1 μM trichostatin A (TSA) for 24 h. (C) Cyclin E mRNA levels from total RNA (1 μg) were analyzed by RT-PCR as described in Materials and methods. (D) HeLa cells were transiently transfected with 1 μg of the cyclin E promoter constructs for 24 h and further incubated for 24 h with various concentrations of apicidin (0.1, 0.3, 1, and 3 μM) or TSA (0.3 μM). Luciferase activity was determined and normalized to the protein content of each extract as described in Materials and methods. Results from independent transfections are shown as relative luciferase unit (RLU) per mg protein. (E) Following pretreatment of HeLa cells with cycloheximide (CHX, 10 μM) for 1 h, the cells were further incubated for additional 24 h with or without 1 μM apicidin.

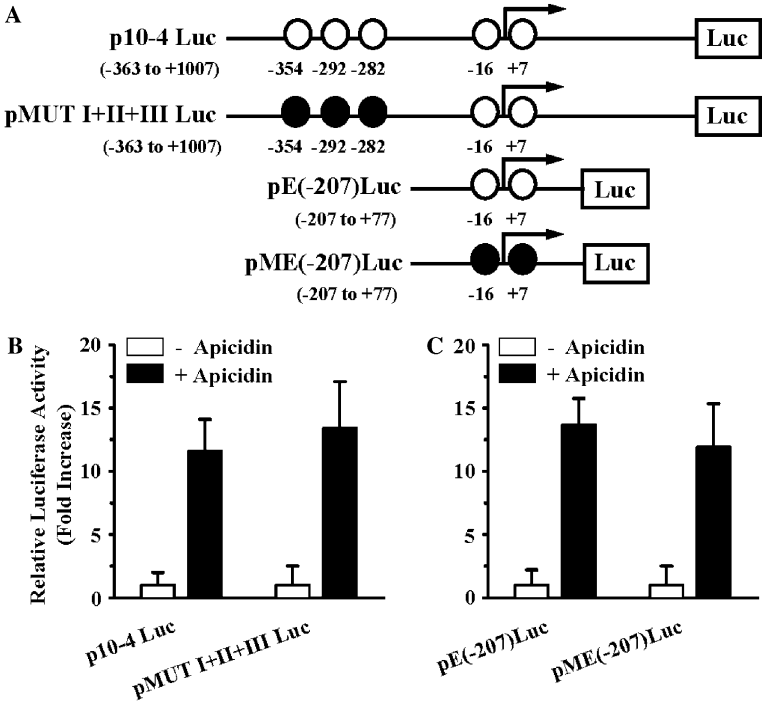

Fig. 2. Apicidin activates the cyclin E promoter independent of E2F-binding sites. (A) Wild-type E2F sites (○) and mutated E2F sites (●) of the cyclin E promoter constructs are represented. (B,C) HeLa cells were transfected with 2 μg p10-4 Luc, pMUT I + II + III (B), pE(−207)Luc or pME(−207)Luc (C) for 24 h, and the cells were further incubated for 24 h with or without 1 μM apicidin. Luciferase activity is expressed as fold increase relative to the untreated control. Data shown are the results from at least three independent experiments.

either the wild-type cyclin E promoter sequences from −207 to +77 or the cyclin E promoter sequences with two mutated E2F sites at −16 and +7. We transiently transfected these reporters into HeLa cells and examined the roles of these E2F sites in mediating apicidin-induced transcriptional activation of cyclin E. Any mutation of

the E2F sites in these reporter systems did not inhibit apicidin activation of the cyclin E (Figs. 2B and C). Furthermore, no significant changes in apicidin-induced activation of cyclin E in pE(−207)Luc were observed, compared with that in p10-4 Luc. However, the observation that apicidin activates cyclin E promoter independent of E2F was to some extent predictable. The cell cycle regulation of E2F-dependent transcription is mediated by phosphorylation of pocket protein, pRb. In G_0 and early G_1, pRb is hypo-phosphorylated and only this form can interact with E2Fs, leading to blocking the ability of E2F to activate transcription. We have previously demonstrated that apicidin arrests cell cycle at G_1 phase and decreases pRb phosphorylation [24]. Thus, E2F-mediated gene activation is expected to be blocked by hypo-phosphorylated pRb. Collectively, these findings indicate that apicidin-induced cyclin E activation might be mediated through E2F-independent mechanisms and does not require the promoter sequences from −363 to −207 and from +77 to +1007.

Sp1 has been demonstrated to induce transcription of various genes in response to HDAC inhibitors [8,11,25]. Our observation that the activation of cyclin E transcription by apicidin was found to be E2F-independent led us to examine the involvement of Sp1 sites in cyclin E expression. Therefore, we examined whether Sp1 mediates apicidin activation of cyclin E in HeLa cells transfected with either pE(−207)Luc or pME(−207)Luc, using mithramycin, which interferes with the binding of Sp1 family transcription factors to GC-rich promoter [26]. Mithramycin pretreatment dramatically inhibited apicidin-induced luciferase activities in both pE(−207) Luc- and pME(−207)Luc-transfected cells (Figs. 3A and B). In addition, mithramycin pretreatment also abolished the expression of cyclin E protein induced by apicidin (Fig. 3C), strongly suggesting that apicidin activation of cyclin E is dependent on Sp1 sites. This observation was further supported by the fact that apicidin did not induce cyclin E expression in HeLa cells in which delta-Sp1, a dominant-negative mutant to inhibit endogenous Sp1 activity, was transfected (Fig. 3D). We next tried to determine which Sp1 sites mediate apicidin activation of cyclin E, using site-directed mutants of seven Sp1-binding sites within pE(−207)Luc sequences between −194 and −28 (Fig. 4A). Following transient transfection, HeLa cells were exposed to 1 μM apicidin for 24 h and analyzed for luciferase activity. Both Sp1-6 and Sp1-7 site mutants (denoted as pE-mt-Sp1-6 and pE-mt-Sp1-7, respectively) dramatically inhibited apicidin activation of cyclin E, compared with that in pE(−207)Luc (Fig. 4B). Taken together, these findings demonstrate that apicidin-induced cyclin E transcription is mediated through Sp1-6 and/or Sp1-7 sites.

Covalent modification of the N-terminal tails of histones is an important mechanism in regulating chromatin structure and function. Pairs of opposing enzymes, such as HATs and HDACs, regulate the balance of histone acetylation [5–7,27]. The inhibition of HDACs shifts this equilibrium toward hyper-acetylation, thereby driving transcriptional activation. To investigate whether the

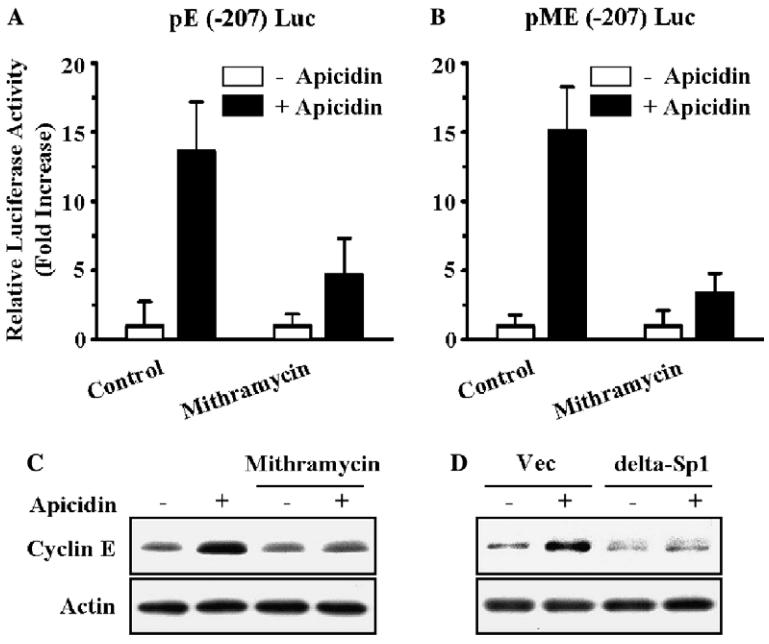

Fig. 3. Apicidin-induced activation of cyclin E requires Sp1-binding sites. (A,B) After transfection of HeLa cells with 1 μg pE(−207)Luc (A) or pME(−207) Luc (B) for 24 h, the cells were pretreated with 200 nM mithramycin and incubated for additional 24 h with or without 1 μM apicidin. Luciferase activity is expressed as fold increase relative to the untreated control. (C) HeLa cells were pretreated with 200 nM mithramycin for 1 h and further incubated for 24 h with or without 1 μM apicidin. (D) Delta-Sp1-transfected HeLa cells were treated with apicidin for 24 h. The cell lysates were immunoblotted with anti-cyclin E antibodies.

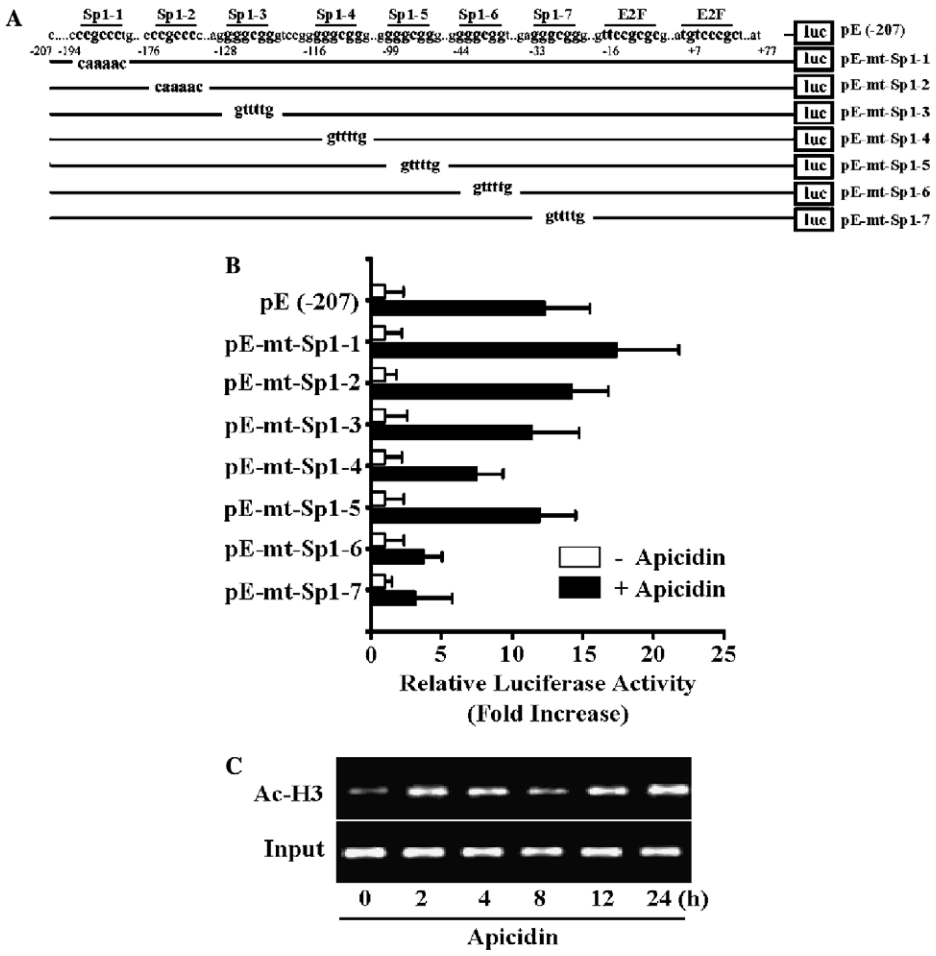

Fig. 4. Apicidin-induced hyperacetylation at the specific Sp1-binding sites within cyclin E promoter is associated with transcriptional activation of cyclin E. (A) Seven Sp1-binding sites and two E2F-binding sites of the wild-type pE(−207)Luc are underlined. (B) Seven site-directed Sp1 mutants were transiently transfected into HeLa cells, and luciferase activities were analyzed. (C) Chromatin was immunoprecipitated with anti-acetylated histone H3 antibodies from HeLa cells treated with 1 μM apicidin for indicated times. PCR was performed as described in Materials and methods.

acetylation of histones was associated with apicidin-induced cyclin E transcription, we next examined the acetylation status of histone H3 in apicidin-responsive region (sequences from −69 to +46) of cyclin E promoter, using chromatin immunoprecipitation (ChIP) assay. Following apicidin treatment, histone H3 acetylation at cyclin E promoter including Sp1 sites (Sp1–6 and Sp1–7) increased in 2 h, slightly decreased at 8 h, and peaked at 24 h (Fig. 4C), whereas the acetylation level of coding region (sequences from +492 to +665) within cyclin E promoter was not significantly affected (data not shown). These data are similar to previous findings on apicidin induction of cyclin E mRNA levels (Fig. 1C), suggesting that the HDAC inhibitor apicidin-induced hyperacetylation around the specific Sp1 sites within cyclin E promoter region is responsible for the transcriptional activation of cyclin E.

In conclusion, our studies provide evidence that an HDAC inhibitor apicidin increases hyperacetylation of histones at cyclin E promoter region containing specific Sp1 sites, resulting in induction of cyclin E expression through Sp1-dependent and E2F-independent mechanism.

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