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Expression of Cyclin D3 Through Sp1 Sites by Histone Deacetylase Inhibitors is Mediated With Protein Kinase C-δ (PKC-δ) Signal Pathway

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Abstract The histone deacetylase (HDAC) inhibitors are an exciting new class of drugs that are targeted as anti-cancer agents. These compounds can induce growth arrest, apoptosis, and/or terminal differentiation in a variety of cancers. The inhibition of HDACs shifts toward hyper-acetylation, thereby driving transcriptional activation. In present study, HDAC inhibitor apicidin was used to elucidate the effect on expression of cell cycle related proteins and the molecular mechanism for transcriptional regulation of cyclin D3 in response to HDAC inhibitors in human colon cancer cells. We found that apicidin increases the transcriptional activity of cyclin D3 gene, which results in accumulation of cyclin D3 mRNA and protein. Apicidin-induced cyclin D3 expression is mediated by Sp1 sites within the cyclin D3 promoter. Apicidin-mediated cyclin D3 expression is attenuated by rottlerin, a specific protein kinase C-δ (PKC-δ) inhibitor, but not mitogen-activated protein kinases (MAPKs) inhibitors. Furthermore, suppression of PKC-δ expression by transfection with its siRNA prominently attenuated apicidin-induced cyclin D3 expression. These results indicate that the cyclin D3 induction caused by apicidin was associated with PKC-δ signaling pathway not MAPKs signaling pathways. Taken together, these results suggest that the activation of cyclin D3 transcription by HDAC inhibitor apicidin was mediated through Sp1 sites and pointed to the possible participation of PKC-δ· J. Cell. Biochem. 101: 987–995, 2007.

Key words: HDAC inhibitor; apicidin; TSA; cyclin D3; Sp1; PKC-δ

Reversible histone acetylation and deacetylation play major roles in transcriptional regulation. The turnover of histone acetylation is regulated by the opposing activities of histone acetyltransferases (HATs) and histone deacetylases (HDACs) [Gray and Teh, 2001; Gregory et al., 2001]. Since HDAC are involved in the control of gene expression, the inhibition of HDACs causes an accumulation of acetylated histones in the nucleus and selectively affects transcription of a small portion of the genes, including cell cycle regulators, such as cyclin-dependent kinase inhibitor, p21. Many accumulating reports showed that the inhibition of HDACs induces differentiation, growth arrest or apoptosis in a number of tumor cell lines in vivo and in vitro [Sambucetti et al., 1999; Marks et al., 2000; Marks et al., 2004].

Natural and synthetic compounds including sodium butyrate, apicidin, trichostatin A (TSA), and suberoylanilide hydroxamic acid (SAHA) possess the ability to inhibit HDAC [Riggs et al., 1977; Vigushin et al., 2001; Hong et al., 2003]. These compounds have also been known to inhibit cell proliferation by arresting the cell cycle and induce differentiation and apoptosis in tumor cell lines [Yoshida et al., 1990; Hong et al., 2003; Shao et al., 2004]. These biological effects are thought to be related to the transactivation of various genes by leading to the hyper-acetylation of core histones. Some HDAC inhibitors are being tested in phase I and II clinical trials in patients with cancer [Marks et al., 2000; McLaughlin and La Thangue, 2004]. It has been demonstrated that treatment

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with HDAC inhibitors such as apicidin and TSA upregulates the transcription of p21, cyclooxygenase-1 (COX-1), and cyclin E via histone hyper-acetylation [Han et al., 2001; Hou et al., 2002; Kim et al., 2006a]. In addition, Sp1 has been demonstrated to induce transcription of a large number of genes in response to HDAC inhibitors [Han et al., 2001; Yang et al., 2001; Kim et al., 2004]. In present study, we attempted to investigate the molecular mechanism for transcriptional regulation of cyclin D3 in response to apicidin in human colon cancer cells.

Recent accumulating evidence has suggested that the transcriptional activation by HDAC inhibitors requires a mechanism other than chromatin remodeling through histone hyperacetylation, which is associated with protein kinase signaling pathways including MAPKs, PI3K, Akt, and PKC pathways [Espinos and Weber, 1998; Mayo et al., 2003; Kim et al., 2003; Kim et al., 2006b; Minucci and Pelicci, 2006].

In this study, we showed that apicidin-induced Sp1-dependent cyclin D3 gene expression as well as apicidin-mediated cyclin D3 expression was associated with protein kinase C-δ (PKC-δ) signaling pathway.

MATERIALS AND METHODS

Cells and Materials

All reagents were purchased from Sigma–Aldrich unless otherwise stated. The human colon cancer cell line HCT116 were obtained from the American Type Culture Collection (Rockville, MD) and cultured in RPMI 1640 supplemented with 2 mM l-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% fetal calf serum. The cells were subcultured twice weekly and grown on 6-well plates at 1×10^6 cells per well, at 37 °C in fully humidified 5% CO_2 air. Anti-cyclin A, anti-p21, and anti-cyclin D3 were purchased from Santa Cruz Biotechnology. Anti-acetyl H3 and anti-acetyl H4 were purchased from Upstate Biotechnology (Lake Placid, NY).

Western Blotting

Cell lysates were prepared by suspending 1.5×10^6 cells in 100 μl lysis buffer (137 mM NaCl, 15 mM EGTA, 0.1 mM sodium orthovanadate, 15 mM MgCl2, 0.1% Triton X-100, 25 mM MOPS (4-morpholinepropane-sulfonic acid), 100 μM phenylmethyl- sulfonyl fluoride, and 20 μM leupeptin, adjusted to pH 7.2), disrupted by sonication, and extracted at 4 °C for 30 min. The proteins were electrotransferred to Immobilon-P membranes and detection of specific proteins was carried out with an ECL Western blotting kit according to the manufacturer's instructions.

RNA Isolation and Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Total cellular RNA was extracted from HCT116 cell lines using the TRI reagent (Molecular Research Center, Cincinnati, OH). Single-strand cDNA was synthesized from 2 μg of total RNA using Moloney–Murine leukemia virus (M–MLV) reverse transcriptase. The cDNA for cyclin A, cyclin D3, and actin was PCR amplified using the following specific primers: cyclin A (sense) 5′-CGTGGACTGGTTAGTTGA-3′ and (anti-sense) 5′-ATGGCAAATACTTGAGGT-3′; cyclin D3 (sense) 5′-CTGGCCATGAACTACC TGGA-3′ and (anti-sense) 5′-CCAGCAAATCATGTGCAA TC-3′; actin (sense) 5′-ACGACATGGAGAAGATCTGGCACC-3′ and actin (anti-sense) 5′-TCAGG CAGCTCATAGCTCTTCTC-3′. PCR amplification was carried out as follows: 1 × (94 °C, 3 min); 30 × (94 °C, 45 s; 57 °C, 45 s; and 72 °C, 1 min); and 1 × (72 °C, 10 min). PCR products were analyzed by agarose gel electrophoresis and visualized by ethidium bromide.

Cyclin D3 Promoter Construction

Chromosomal DNA was prepared from macrophage cell line Raw 264.7 cells using the DNAzol^{TM} reagent (Gibco-BRL). Mouse cyclin D3 promoter was amplified from chromosomal DNA with the following synthetic primers: 5′-TCATGTG GGTACCAGGGATC (-655 to -635, *Kpn*I, sense), 5′-CATTCCCTAGA GCTCTGAAA-3′ (−381 to −361, *Sac*I, sense), 5′-CTTGGTCAGAAGGTACCGAG-3′ (−224 to −204, *Kpn*I, sense), 5′-GTAGGAGCTCTCCACGGTTG-3′ (−100 to −80, *Sac*I, sense), 5′-TGTCAGGGTACCGGTGCGCG-3′ (−52 to −32, *Kpn*I, sense), and 5′-GAGCAA GCTTGCGGGTTAGCG (+76 to +90, *Hin*dIII, anti-sense). The PCR products were digested with *Kpn*I and *Hin*dIII and cloned upstream of the firefly luciferase gene of pGL2-basic (Promega). PCR products were confirmed by their size, as determined by electrophoresis and DNA sequencing. The cyclin D3 promoter plasmid was transfected into HCT116 cells using the

Lipofectamine reagent (Life Technologies) according to the manufacturer's instructions. To assess cyclin D3 promoter luciferase activity, cells were collected and disrupted by sonication in lysis buffer (25 mM Tris-phosphate, pH 7.8, 2 mM EDTA, 1% Triton X-100, and 10% glycerol). After centrifugation, aliquots of supernatants were tested for luciferase activity using the luciferase assay system (Promega), as specified by the manufacturer. Point mutations of the Sp1 binding sites to the cyclin D3 promoter were generated by a two-step PCR method using the following primers: Sp1-1 (5′-CTGCGGCCCC-GCCCCTTAGA-3′ to 5′- CTGCGGCAAA GT-TTCTTAGA-3′; Sp1-2 (5′-GCGAGGGGCGGG-GCGCCTGT-3′ to 5′- GCGAGAA CTTTGCG-CCT GT-3′); Sp1-3 (5′-GTGGCGGGCGGGCT-GGGGCT-3′ to 5′- GTGGCGAACTTGCT GGG-GCT -3′). Clones representing each point mutation were sequenced to ensure the accuracy of the PCR amplification procedure.

Transfections and Luciferase Gene Assays

Cells were plated onto 6-well plates at a density 5×10^5 cells/well and grown overnight. Cells were co-transfected with 2 μg of various plasmid constructs and 1 μg of the pCMV-β-galactosidase plasmid for 5 h by the Lipofectamine method. After transfection, cells were cultured in 10% FCS medium with vehicle (DMSO) or drugs for 24 h. Luciferase and β-galactosidase activities were assayed according to the manufacturer's protocol (Promega). Luciferase activity was normalized for β-galactosidase activity in cell lysate and expressed as an average of three independent experiments.

Small Interfering RNA Transfection

HCT116 cells were seeded at a density of 1×10^5 cells/well in six-well tissue culture plates the day before transfection to achieve 50–60% confluence. Transfections were done with 70 nmol/L of siRNA duplex using Lipofectamine Plus (Invitrogen) according to the instructions of the manufacturer. siRNA duplexes targeting PKC-δ was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Predesigned siRNA duplexes 5′-AAGACCCGCGCCGAGG-UGAAG-3′ for GFP were used as a negative control. Three independent PKC-δ silencing experiment were carried out to confirm the reproducibility of the findings.

RESULTS

Regulation of Cell Cycle Regulatory Proteins by Apicidin

Various classes of HDAC inhibitors have been shown to harbor potent anti-tumor activities in a range of tumor cells and animal models, indicating that these agents may have promising therapeutic values [McLaughlin and La Thangue, 2004; Minucci and Pelicci, 2006]. To

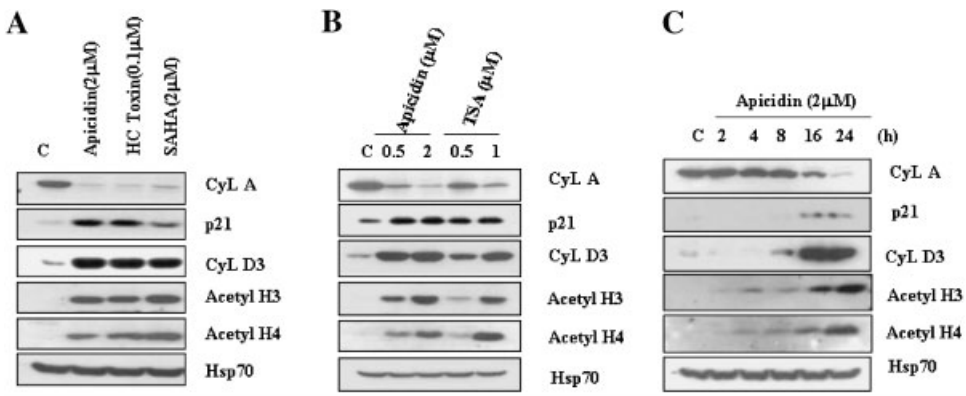

Fig. 1. Regulation of cell cycle regulatory proteins by HDAC inhibitors. **A**: HCT116 cells were treated with several of HDACs for 24 h and harvested. Equal amounts of cell lysates (40 μg) were subjected to electrophoresis and analyzed by Western blot for cyclin A, p21, cyclin D3, acetyl H3, acetyl H4, and HSP70. HSP70 used as control for the loading of protein level. **B**: The effect of two different concentrations of apicidin and TSA on expression levels of cell cycle regulatory proteins. HCT116 cells were treated with two different concentrations of apicidin or TSA for 24 h and harvested. The cells were lysed and the lysates were analyzed by Western blotting using anti-cyclin A, anti-p21, anti-cyclin D3, anti-acetyl H3, anti-acetyl H4, and anti-HSP70 antibodies. **C**: HCT116 cells were treated with 2 μM apicidin and harvested at the indicated times. The cells were lysed and the lysates were analyzed by Western blotting using anti-cyclin A, anti-p21, anti-cyclin D3, anti-acetyl H3, anti-acetyl H4, and anti-HSP70 antibodies.

investigate whether HDAC inhibitors had anti-proliferative effects in colon cancer cells, HCT116 cells were cultured vehicle alone or with HDAC inhibitors (2 μM apicidin, 0.1 μM HC-toxin, and 2 μM SAHA) for 24 h. As shown in Figure 1A, treatment of HCT116 cells with HDAC inhibitors induced accumulation of hyper-acetylated histone H3 and H4, which were accompanied by increased expression of p21 and decreased expression of cyclin A, suggesting that histone hyper-acetylation induced by HDAC inhibitors regulate specific genes that play important roles in cell cycle regulation. To further confirm the effects of HDAC inhibitors on expression levels of cell cycle regulatory proteins, we analyzed the effect of two different concentrations of apicidin and TSA on expression levels of cell cycle regulatory proteins. Similar expression patterns of cell cycle regulatory proteins were observed in HCT116 cells following treatment with apicidin or TSA (Fig. 1B). To investigate the relationship between expression levels of cell cycle regulatory proteins and histone acetylation, we carried out time kinetics studies. HCT116 cells were treated with 2 μM apicidin for indicated times. As shown in Fig. 1C, acetylation of histone H3 or H4 were induced at 2 h after apicidin treatment in HCT116 cells. The levels of cyclin A were progressively decreased from 16 to 24 h after apicidin treatment in HCT116 cells. However, treatment with apicidin significantly induced p21 and cyclin D3 at 16 h. These results suggest that acetylation of histone H3 and H4 preceded modulation of cell cycle regulatory proteins in HDAC inhibitors treated-cells.

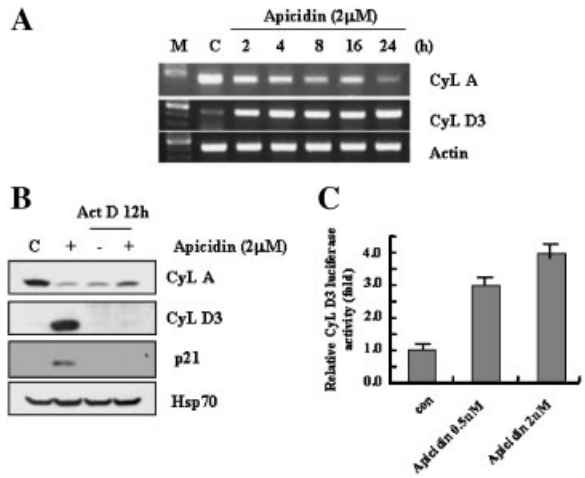

Fig. 2. Apicidin increases transcription of cyclin D3. **A**: The effect of apicidin on the cyclin D3 mRNA expression in HCT116 cells. HCT116 cells were treated with 2 μM apicidin and harvested at the indicated times. The total RNAs were isolated, and cyclin A, cyclin D3, and actin mRNA levels were analyzed by RT-PCR. A representative study is shown; two additional experiments yielded similar results. **B**: Induction of cyclin D3 by apicidin was inhibited by pretreatment with the RNA synthesis inhibitor, actinomycin D. HCT116 cell pretreated with actinomycin D were exposed to 2 μM apicidin for 12 h. The cells were lysed and the lysates were analyzed by Western blotting using anti-cyclin A, anti-p21, anti-cyclin D3, anti-p21, and anti-HSP70 antibodies. **C**: Apicidin induced the cyclin D3 promoter activity in a dose-dependent manner. HCT116 cells were transfected with cyclin D3 luciferase vector and luciferase activity was measured. Data represent the mean ± SD of at least three independent experiments.

Apicidin Increases Transcription of Cyclin D3

Recently, several reports have shown that induction of p21 and downregulation of cyclin A are associated with anti-proliferative activity of HDAC inhibitors [Siavoshian et al., 2000; Florenes et al., 2004]. Therefore, to examine the mechanism by which apicidin induces cyclin D3, we analyzed the effect of apicidin on the cyclin D3 expression in HCT116 cells. As shown in Figure 2A, apicidin treatment induced cyclin D3 mRNA in a time-dependent manner as determined by RT-PCR analysis. In addition, induction of cyclin D3 by apicidin was inhibited by pretreatment with the RNA synthesis inhibitor, actinomycin D (Fig. 2B). Since apicidin drastically induced the cyclin D3 mRNA expression, we investigated whether apicidin could stimulate the luciferase activity of the cyclin D3 promoter. As shown in Figure 2C, the luciferase activity was increased in a dose-dependent manner up to fourfolds by treatment with the indicated concentrations of apicidin.

Apicidin Activates the Cyclin D3 Promoter Through Sp1 Sites

To examine the mechanism by which apicidin activates the cyclin D3 promoter, we determined the regions of the cyclin D3 promoter responsible for the activation of cyclin D3 promoter by apicidin and TSA. A series of 5′-deletion constructs of the cyclin D3 promoter were transiently transfected into HCT116 cells, and luciferase activities following apicidin or TSA treatment were measured. Deletion from −655 to −100 (dF3) markedly reduced the apicidin or TSA-mediated activation as well as the basal promoter activity over the control in pGL2-cylD3, suggesting that these regions play an important role in both the basal and HDAC inhibitors-mediated activation of the cyclin D3

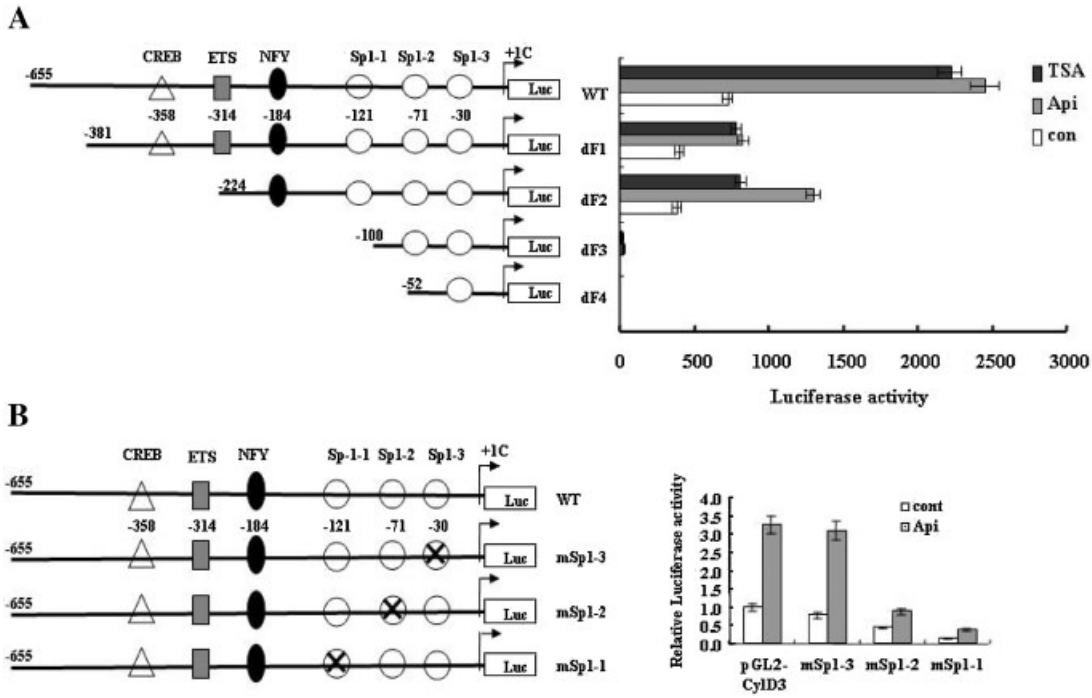

Fig. 3. Apicidin activates the cyclin D3 promoter through Sp1 sites. **A**: A schematic map of a series of 5′-deletion constructs of the cyclin D3 promoters and their luciferase activities following apicidin or TSA treatment. HCT116 cells were transfected with each cyclin D3 promoter vector and luciferase activities were measured. Data represent the mean ± SD of at least three independent experiments. **B**: A schematic map of a series of Sp1 point mutation constructs of the cyclin D3 promoter and their luciferase activities following apicidin treatment. The HCT116 cells were treated with apicidin and luciferase activities were measured. Nucleotides of the Sp1 consensus site in the promoter region were substituted as described in Materials and Methods. HCT116 cells were transfected with luciferase vectors in depicted in left panel. Data represent the mean ± SD of at least three independent experiments.

promoter. To further define the apicidin or TSA-responsive elements, we used pGL2-cyclD3 constructs with mutations in various Sp1 sites. Although mSp1-3 in which the Sp1-3 site was mutated, the basal activity of mSp1-3 and the activation of apicidin in mSp1-3 were similar to wild-type pGL2-cyclD3. When the Sp1-1 site was mutated (mSp1-1), the basal activity of mSp1-1 was reduced to 85% compared with that of pGL2-cyclD3 and the activation by apicidin in mSp1-1 was remarked decreased from 3.3- to 0.3-fold of pGL2-cyclD3. mSp1-2 (mutation of Sp1-2 site) revealed similar activation patterns in basal activity and activation of apicidin compared with that of mSp1-1, whereas luciferase activity of mSp1-2 is higher than that of mSp1-1. Taken together, these results demonstrate that the Sp1-1 site located between −131 and −121 relative to the transcription start site is the main apicidin-responsive element and that the Sp1-2 site is also partially involved in the activation.

To further confirm the functional role of Sp1 on cyclin D3 promoter activities, HCT116 cells were transfected with the Sp1 expression vector, and then luciferase activities were measured. Cyclin D3 promoter activities were significantly increased by Sp1 expression vector transfection in a dose-dependent manner (Fig. 4A). In subsequent experiments, HCT116 cells were transiently transfected with Sp1 reporter vector that contained Sp1 binding sites and Sp1 expression vector. Luciferase activity in the cells with the Sp1 construct is significantly increased by treatment with apicidin and TSA (Fig. 4B).

Mitogen-Activated Protein Kinases (MAPKs) are not Involved in the HDAC Inhibitors-Mediated Cyclin D3 Upregulation

We examined the role of MAPKs in the induction of cyclin D3 by apicidin or TSA to determine whether MAPKs signaling pathway is associated with HDAC inhibitors-mediated cyclin D3 upregulation. To test this possibility, well known specific kinase inhibitors, which have been usually employed as tools in analyzing specific signaling pathway, were used in this

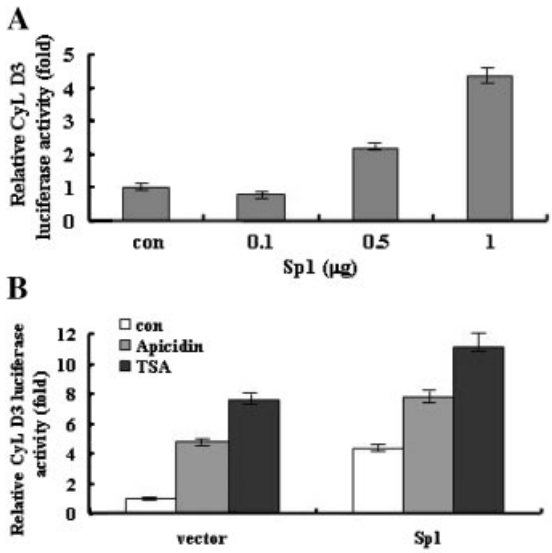

Fig. 4. Sp1 activates cyclin D3 promoter and apicidin induces the cyclin D3 promoter activity through Sp1. **A**: The functional role of Sp1 on cyclin D3 promoter activities. HCT116 cells were transfected with the indicated concentration of Sp1 expression vectors and cyclin D3 promoter vector, and then luciferase activities were measured. Data represent the mean ± SD of at least three independent experiments. **B**: HCT116 cells were transfected with the Sp1 expression vectors (0.5 μg) and cyclin D3 promoter vector (0.5 μg) and treated with 2 μM apicidin and 1 μM TSA, and then luciferase activities were measured. Data represent the mean ± SD of at least three independent experiments.

study. As shown in Figure 5, pretreatment with MAPKs inhibitors [PD98059 (a MEK inhibitor), SB203580 (a p38 MAP kinase inhibitor), SP600125 (a JNK inhibitor)] did not affect the upregulation of cyclin D3, downregulation of cyclin A and acetylation of histone H4. These

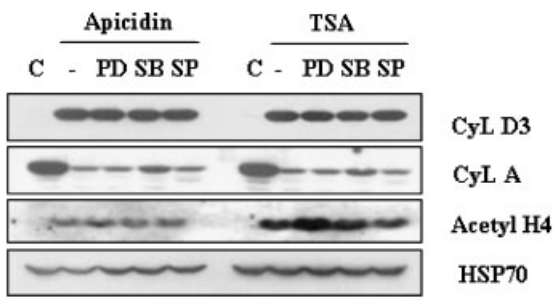

Fig. 5. MAPKs are not involved in the HDAC inhibitors-mediated cyclin D3 upregulation. HCT116 cells pretreated with MAPKs inhibitors such as PD98059, SB203580, and SP600125 were cultured with the presence of 2 μM apicidin and 1 μM TSA for 24 h. The cells were lysed and the lysates were analyzed by Western blotting using anti-cyclin A, anti-cyclin D3, anti-acetyl H4, and anti-HSP70 antibodies.

results suggest that MAPKs signaling pathway did not involve in apicidin-mediated cyclin D3 upregulation.

Apicidin Mediated Cyclin D3 Expression is Associated With Protein Kinase C-δ (PKC-δ) Signaling Pathway

To determine whether PKC activation was involved in the signal transduction pathway leading expression caused apicidin, the PKC inhibitors (rottlerin, staurosporine, and Go 6976) were used. The pretreatment of cells for 1 h with rottlerin, a PKC-δ-specific inhibitor, attenuated the apicidin-induced cyclin D3, and p21 expressions (Fig. 6A). However, the effects of sturosporine (a pan-specific inhibitor) and Go6976 (an inhibitor of Ca^{2+}-dependent PKC-α and -β) on the expression of cyclin D3 by apicidin were less marked (Fig. 6A). As shown in Figure 6A, rottlerin was more effective than staurospirine for cyclin D3 downregulation. In order to further confirm the functional role of PKC-δ on apicidin-mediated cyclin D3 expression, we examined the effect on the cyclin D3 promoter activity and protein expression by treatment with rottlerin. Pretreatment with rottlerin markedly suppressed the promoter activity and protein expression of cyclin D3 activated by apicidin (Fig. 6B,C). To further investigate PKC involvement in induction of cyclin D3 expression by apicidin, we determined the effect of expressing either PKC-δ or dominant-negative PKC-δ (dN-PKC-δ) constructs on the induction of cyclin D3 promoter activity by apicidin. As shown in Fig. 6D, transfection of dN-PKC-δ-δ completely blocked promoter activity of cyclin D3 activated by apicidin. We further examined whether downregulation of PKC-δ utilizing its siRNA could inhibit the effect of apicidin on cyclin D3 expression. As shown in Figure 6E, transfection of HCT116 cells with siRNA against PKC-δ resulted in a suppression of cyclin D3 expression compared with cells transfected with control siRNA in apicidin-treated cells.

DISCUSSION

Many structurally divergent classes of HDAC inhibitors have been identified that induce cell-cycle arrest, terminal differentiation, and apoptosis in various cancer cell lines [McLaughlin and La Thangue, 2004; Minucci and Pelicci, 2006]. Inhibition of HDAC activity by HDAC

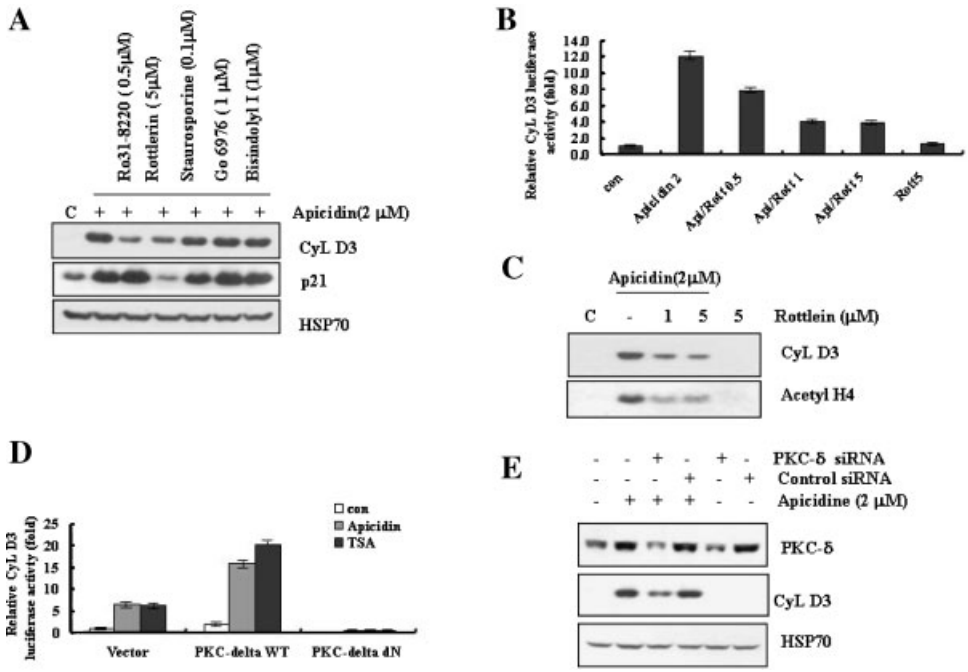

Fig. 6. Apicidin-mediated cyclin D3 expression is associated with PKC-δ signaling pathway. **A**: PKC activation was involved in the signal transduction pathway leading cyclin D3 expression caused by apicidin. HCT116 cells were pretreated with PKC inhibitors (rottlerin, staurosporine, and Go 6976) and exposed to 2 μM apicidin for 24 h. The cells were lysed and the lysates were analyzed by Western blotting using anti-cyclin D3, anti-p21, and anti-HSP70 antibodies. **B**: The effect on the cyclin D3 promoter activity by treatment with rottlerin. HCT116 cells were pretreated with the indicated concentration of PKC-δ specific inhibitors, rottlerin, and exposed to 2 μM apicidin for 24 h. Cell extracts were harvested and luciferase activities were measured as described in Materials and Methods. Data represent the mean ± SD of at least three independent experiments. **C**: Rottlerin markedly suppressed protein expression of cyclin D3 activated by apicidin treatment in a dose-dependent manner. HCT116 cells were pretreated with the indicated concentration of rottlerin and exposed to 2 μM apicidin for 24 h. Cell extracts were harvested and Western blotting was performed as described in Materials and Methods. **D**: The effect of expressing either PKC-δ or dN-PKC-δ constructs on the induction of cyclin D3 promoter activity by apicidin. HCT116 cells were transfected with empty vector, wild-type PKC-δ and PKC-δ dominant negative (PKC-δ–dN) and cyclin D3 promoter vector, and treated with 2 μM apicidin and 1 μM TSA for 24 h. Cell extracts were harvested and luciferase activities were measured as described in Materials and Methods. Data represent the mean ± SD of at least three independent experiments. **E**: The effect of PKC-δ siRNA on the induction of cyclin D3 expression by apicidin. HCT116 cells were transfected with PKC-δ siRNA and control siRNA, and treated with 2 μM apicidin for 24 h. Cell extracts were harvested and Western blotting was performed as described in Materials and Methods.

inhibitors results in the accumulation of acetylated histone proteins and subsequent activation of transcription of target genes. Activation of transcription results from a reduction in histone-DNA interactions and loosening of chromatin structure due to HAT acetylation of adjacent histones.

In our present study, HDAC inhibitors induced the accumulation of hyper-acetylated histone H3, H4 and upregulation of cyclin D3 and cdk/cyclin inhibitor p21, and downregulation of cyclin A expression. These data suggested that histone hyper-acetylation induced by HDAC inhibitors was responsible for antiproliferative activity through selective regulation of genes that play important roles in cell cycle regulation. Interestingly, we found that expression of cyclin D3 protein was induced by HDAC inhibitors treatment. Apicidin induced activation of the luciferase activity of the cyclin D3 as well as increase of cyclin D3 mRNA expression. Recent study demonstrated that apicidin-induced cyclin E activation might be mediated through Sp1-dependent mechanisms [Kim et al., 2006a], suggesting the possibility that apicidin-induced cyclin D3 accumulation could be mediated by Sp1 sites within cyclin D3 promoter region. We identified three putative Sp1-binding sites within the promoter sequences from −655 to +90. Using a combination of deletion and site direct mutation to luciferase report assay for analysis of the importance of each Sp1 binding site, we demonstrated that the Sp1-1 site located between

−131 and −121 relative to the transcription start site is the main apicidin-responsive element and that the Sp1-2 site is also partially involved in the activation (Fig. 3B). This observation was further supported by ectopic expression of Sp1. Taken together, the transcriptional activation of cyclin D3 following apicidin treatment might be mediated through the specific Sp1 sites within cyclin D3 promoter region.

Recent studies have shown that the biological effects of HDAC inhibitors require the transcriptional activation by HDAC inhibitors as well as several protein signaling pathways, such as ERK1/2, PI3K, Akt, and PKC signaling pathways [Espinos and Weber, 1998; Kim et al., 2003, Kim et al., 2006b; Mayo et al., 2003]. These studies led us to hypothesize that the increase of cyclin D3 expression induced by apicidin exposure might be linked with other protein kinase signaling pathways. First, we investigated whether MAPKs signaling pathway was required for induction of cyclin D3 in response to apicidin treatment. Pretreatment with selective MAPKs inhibitors did not affect apicidin-mediated cyclin D3 induction. This result indicated that MAPKs signaling pathways did not involved in apicidin-induced cyclin D3 regulation in HCT116 cells. We next investigated the role and identity of the specific isoforms of PKC involved in cyclin D3 induction in apicidin-treated HCT116 cells. Using an isoform-specific pharmacological inhibitor of PKC such as rottlerin, staurosporine and Go 6976, we found that rottlerin, a PKC-δ-specific inhibitor, attenuated the apicidin-induced cyclin D3 expression (Fig. 6A). Furthermore, we found that transfection of PKC-δ dominant negative mutant construct significantly inhibited the promoter activity of cyclin D3 activated by apicidin treatment as well as transfection of PKC-δ siRNA attenuated cyclin D3 expression increased by apicidin treatment. Collectively, these results suggested that apicidin-mediated cyclin D3 expression is associated with PKC-δ signaling pathway. Interestingly, we found that rottlerin treatment lessened the acetylation of histone 4 induced by apicidin, suggesting that the acetylation of histone 4 caused by apicidin was associated with PKC-δ signaling pathway. Recent studies have shown that several nuclear factors, called coactivators such as CREB (cAMP response element binding protein)-binding protein (CBP) and p300/CBP associated factor (P/CAF), have HAT activities that transfer an acetyl base to the lysine residue on histone [Ogryzko et al., 1996; Mizzen and Allis, 1998; Struhl, 1998]. The HAT activity of CBP and p300 is upregulated through phosphorylation by ERK, cdk2, or protein kinase A (PKA) [Kalkhoven, 2004]. Based on these reports, we assumed that apicidin treatment induces the activation of PKC-δ that might increase the HAT activity of coactivators. In contrast to our result, apicidin treatment induced the transcriptional activation of p21WAF1/Cip1 via activation of PKC-ε and histone acetylation that is independent of PKC-ε activation in human cervical cancer cells [Han et al., 2001]. In addition, Yuan et al. (2002) reported that PKC-δ-mediated phosphorylation inhibits p300 intrinsic HAT activity of CBP. Taken together, PKC signaling pathways are maybe involved in regulating the acetylation of cellular proteins such as histone and nonhistone proteins as a critical modulator in HDAC inhibitors-treated cells.

However, recent studies reported that butyrate and TSA stimulated the protein expression of cyclin D3 without affecting its mRNA expression in human intestinal epithelial cells [Siavoshian et al., 2000] and that TSA treatment led to induction in cyclin D3 that was due to increased protein stability [Florenes et al., 2004]. In contrast to these results, we found that apicidin treatment induced cyclin D3 accumulation, which was associated with apicidin-induced cyclin D3 transcription in HCT116 cells. Although, there is a discrepancy of regulation mechanism of cyclin D3 expression in HDAC inhibitors-treated cells; we assumed that cyclin D3 induction might be linked to the growth inhibitory or apoptotic effects of HDAC inhibitors.

In conclusion, a HDAC inhibitor apicidin increases the transcriptional activity of cyclin D3 gene, which results in accumulation of cyclin D3 mRNA and protein. In addition, Sp1-dependent cyclin D3 gene expression might be mediated with PKC-δ signal pathway in apicidin-treated cells.

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