We previously reported that apicidin, a novel histone deacetylase inhibitor, inhibited the proliferation of tumor cells via induction of p21WAF1/Cip1. In this study, we determined the molecular mechanisms by which apicidin induced the p21WAF1/Cip1 gene expression in HeLa cells. Apicidin induced p21WAF1/Cip1 mRNA independent of the de novo protein synthesis and activated the p21WAF1/Cip1 promoter via Sp1 site located at −82 and −77 relative to the transcription start site. This transcriptional activation appears to be mediated by protein kinase C (PKC), because calphostin C, a PKC inhibitor, significantly attenuated the activation of p21WAF1/Cip1 promoter via Sp1 sites, which was accompanied by a marked suppression of p21WAF1/Cip1 mRNA and protein expression induced by apicidin. Consistent with the transcriptional activation of p21WAF1/Cip1 promoter by apicidin, apicidin treatment led to the translocation of PKCe from cytosolic to particulate fraction, which was reversed by pretreatment with calphostin C, indicating the involvement of PKC in the transcriptional activation of p21WAF1/Cip1 via Sp1 sites by apicidin. However, the PKC-mediated transcriptional activation of p21WAF1/Cip1 by apicidin appears to be independent of the histone hyperacetylation, because apicidin-induced histone hyperacetylation was not affected by calphostin C. Furthermore, a PKC activator, phorbol 12,13-dibutyrate, alone induced the transcriptional activation of p21WAF1/Cip1 mRNA, and protein expression without induction of the histone hyperacetylation, suggesting that the transcriptional activation of p21WAF1/Cip1 by apicidin might have been mediated by a mechanism other than chromatin remodeling through the histone hyperacetylation. Taken together, these results suggest that the PKC signaling pathway plays a pivotal role in the transcriptional activation of the p21WAF1/Cip1 gene by apicidin.

Cell cycle progression is controlled by various cyclin-dependent kinases (Cdks), whose activation is carefully regulated at multiple levels including the induction and degradation of cyclin protein, Cdk phosphorylation by cyclin-activating kinase, and the induction of Cdk inhibitory proteins (1–5). The Cdk inhibitor p21WAF1/Cip1 was first cloned and characterized as a mediator of p53-induced growth arrest (6–8). p21WAF1/Cip1 can negatively regulate cell cycle progression from G1 to S phase by inhibiting the catalytic activity of Cdk (7–10). Although p21WAF1/Cip1 has been shown to be expressed in a p53-dependent fashion in response to DNA damage (11), its expression could also be up-regulated p53-independently by various stimuli, including transforming growth factor-β (TGF-β) (12), tamoxifen (13), progesterone (14), and nerve growth factor (15).

Recent accumulating evidence suggests that the p53-independent induction of p21WAF1/Cip1 by these stimuli could be influenced by signal transduction pathways that mediate cell growth, differentiation, and stress response. For example, it has been reported that the transcriptional activation of p21WAF1/Cip1 by TGF-β requires MEK pathway including Ras, Raf-1, and MEK, upstream regulators of mitogen-activated protein kinase, in human keratinocyte HaCaT cell (12). On the other hand, tamoxifen-induced p21WAF1/Cip1 expression has been shown to be mediated by protein kinase A (PKA) in H358 human lung cancer cells (13).

The induction of p21WAF1/Cip1 is one of the common phenomena observed after treatment with HDAC inhibitors such as trichostatin A (TSA), sodium butyrate (NaB), suberoylanilide hydroxamic acid (SAHA), oxamflatin, and MS-27–275 (16–20). The transcriptional activation of p21WAF1/Cip1 by these HDAC inhibitors has been proposed to be regulated by the chromatin remodeling resulting from the accumulation of acetylated histones H3 and H4 in p21WAF1/Cip1 promoter region. Moreover, recent studies have demonstrated that p21WAF1/Cip1 induction by HDAC inhibitors was mediated via Sp1 sites through Sp1 family transcription factors, Sp1 and/or Sp3 (17, 18, 21) as in case of TGF-β or nerve growth factor. However, there has been no study addressing the possible link between the transcriptional activation of the p21WAF1/Cip1 gene expression by HDAC inhibitor and signal transduction pathway.

Recently, we reported that apicidin, a HDAC inhibitor, inhibits proliferation of various tumor cells with a broad spec.

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** The abbreviations used are: Cdk, cyclin-dependent kinase; HDAC, histone deacetylase; PKC, protein kinase C; TSA, trichostatin A; NaB, sodium butyrate; SAHA, suberoylanilide hydroxamic acid; PDBu, phorbol 12,13-dibutyrate; ERK, extracellular-regulated protein kinase; PKA, protein kinase A; TGF, transforming growth factor; RT, reverse transcription; PCR, polymerase chain reaction.
In this study, we investigated the mechanism of apicidin-induced transcriptional activation of p21\textsuperscript{WAF1/Cip1} and the possible involvement of protein kinase signaling pathway in the p21\textsuperscript{WAF1/Cip1} gene expression. Apicidin induced p21\textsuperscript{WAF1/Cip1} gene expression independent of the de novo protein synthesis through Sp1–3 site located at −82 and −77 relative to the transcription start site of p21\textsuperscript{WAF1/Cip1}. Moreover, pretreatment with the PKC inhibitor, calphostin C, significantly attenuated the apicidin-induced activation of p21\textsuperscript{WAF1/Cip1} promoter, expression of p21\textsuperscript{WAF1/Cip1} mRNA and protein, and translocation of PKC\varepsilon from cytosolic to particulate fraction. Taken together, these results suggest that PKC signal transduction pathway is involved in the apicidin-induced transcriptional activation of the p21\textsuperscript{WAF1/Cip1} gene expression via Sp1 sites.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Human cervix cancer cell line HeLa was cultured in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (HyClone Laboratories, Logan, UT) and 1% penicillin/streptomycin (Life Technologies, Inc.).

**Reagents**—Apicidin, [cyclo(N-O-methyl-l-tryptophanyl-l-iso-leucinyl-l-pipeolicolyl-1-2-amino-8-oxodecanoyl)], was prepared from Fusarium sp. strain KCTC 16677 according to the method described previously (23). Cycloheximide, sodium butyrate, and trichostatin A were obtained from Sigma. DMSO, H-89, calphostin C, rottlerin, and FDBu were from Calbiochem (La Jolla, CA).

**RT-PCR**—Total RNA was extracted using TRIzol reagent (Life Technologies, Inc.). Integrity of RNA was checked by agarose gel electrophoresis and ethidium bromide staining. One microgram of RNA was used as a template for each RT-PCR using RNA PCR kit (Perkin Elmer). Primer sequences for p21WAF1/Cip1 and glyceraldehyde-3-phosphate dehydrogenase were described previously (19).

**Plasmids**—The human wild-type p21WAF1/Cip1 promoter-luciferase fusion plasmid, WWP-Luc, was a kind gift from Dr. Bert Vogelstein (Howard Hughes Medical Institute, Johns Hopkins University). The fusion plasmid, WWP-Luc, was a kind gift from Dr. Bert Vogelstein (Howard Hughes Medical Institute, Johns Hopkins University). The full-length human p21WAF1/Cip1 promoter-luciferase reporter construct, pWWP, is a series of mutant p21WAF1/Cip1 promoter constructs including pWP124, pWP101, pWP101-mSp1–3, pWP101-mSp1–4, pWP101-mSp1–5, and pWP101-mTATA were kind gifts from Dr. Yoshinari Sowa (Kyoto Prefectural University of Medicine). pWP-Del-Sna1 was generated by digesting pWWP with Sna1 and by religating.

**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 p21WAF1/Cip1 promoter analysis, the cells were transfected with 5 μg of p21WAF1/Cip1 promoter reporter plasmid DNA by using PrecFect transfection reagent (Promega, Madison, WI). Twenty-four h after the transfection, the medium was changed for medium with or without 1 μM apicidin, and cell lysates were collected for the luciferase assay 24 h later. The luciferase activities of the cell lysates were measured according to the manufacturer’s recommendations (Promega).

**Subcellular Fractionation**—HeLa cells were partitioned into soluble (cytosolic) and particulate fractions. HeLa cells were lysed in lysis buffer (50 mM Tris-HCl, pH 7.5, 120 mM NaCl, 20 mM NaF, 1 mM EDTA, 5 mM EGTA, 15 mM sodium PPI, 30 mM p-nitrophenyl phosphate, 1 mM benzamidine, and 0.1 mM phenylmethylsulfonyl fluoride) and homogenized for 10 s at 6,000 rpm. The cytosolic and particulate fractions were separated by ultracentrifugation at 100,000 × g for 45 min at 4 °C. Supernatant was collected and formed the cytosolic fraction. The pellet was resuspended in lysis buffer containing 1% Triton X-100, incubated on ice for 30 min, cleared by centrifugation for 10 min at 10,000 × g at 4 °C, and formed the particulate fraction.

**Immunoblotting**—HeLa cell lysates were boiled in Laemmli sample buffer for 3 min, and 30 μg of each total protein were subjected to SDS-polyacrylamide gel electrophoresis on 15% slab gels for the analysis of p21WAF1/Cip1 and β-tubulin and 10% slab gels for the analysis of PKC isoforms. For immunodetection of acetylated histone H3, histone fractions were isolated by the established techniques (22) and subjected to 10–20% Tricine SDS-polyacrylamide gel electrophoresis. The proteins were transferred to polyvinylidene difluoride membranes, and the membranes were blocked for 30 min in Tris-buffered saline containing 0.1% Tween 20 and 5% (w/v) dry skim milk powder and incubated overnight with anti-p21WAF1/Cip1 (Santa Cruz Biotechnologies, Inc.), β-tubulin (BD PharMingen, Inc.), acetylated histone H3, H4 (Upstate Biotechnology Inc.), PKC isoform (α, β, δ, ε, η, μ, ζ, η, γ and δ; Santa Cruz Biotechnologies, Inc.), and PKCl (Upstate Biotechnology Inc.) antisera. The membranes were then washed with Tris-buffered saline containing 0.1% Tween 20 and incubated for 1 h with an anti-rabbit or an anti-mouse secondary antibody. Bound antibodies were detected with the enhanced amplified alkaline phosphatase immunoblot system (Bio-Rad).

**RESULTS**

Apicidin Increases p21WAF1/Cip1 mRNA and Protein Levels and Activates the p21WAF1/Cip1 Promoter—Previously, we demonstrated that p21WAF1/Cip1, which controls the cell cycle, was markedly up-regulated by apicidin in HeLa, human cervix cancer cell lines (22). To examine the mechanism by which apicidin induces p21WAF1/Cip1, we first analyzed the effect of apicidin on the p21WAF1/Cip1 expression in HeLa cells. As shown in Fig. 1 (A and B), apicidin treatment induced p21WAF1/Cip1 mRNA in a
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Fig. 2. Activation of the p21\textsuperscript{WAF1/Cip1} promoter through the Sp1 sites by apicidin. A, 5 deletion analysis of the p21\textsuperscript{WAF1/Cip1} promoter. The indicated plasmids, pWWP, pWP124, pWP101, and pWPdel-SmaI, were transiently transfected into HeLa cells, and luciferase activities were analyzed after treatment with 1 \mu M apicidin for 24 h in comparison with full-size promoter, pWWP. Fold induction by apicidin is also calculated and indicated. The data are shown as the means (bars, S.E.) (n = 3). B, mutation analysis of the p21\textsuperscript{WAF1/Cip1} promoter. Four different mutants, shown on the left, are identical to the wild-type pWP101 except for the mutations in bold letters. These constructs were transiently transfected into HeLa cells, and apicidin-induced luciferase activities were analyzed. Relative luciferase activity is shown as raw light units (RLU) in cell lysates/1 mg of cell protein. The mean of net RLU of untreated control of each plasmid is as follows. pWP101, 28,180; pWP101-mtSp1\textsuperscript{-3}, 25,405; pWP101-mtSp1\textsuperscript{-4}, 10,810; pWP101-mtSp1\textsuperscript{-5,6}, 177; pWP101-mtTATA, 456; and background, 150. Fold induction by apicidin was also calculated and indicated. The data are shown as the means (bars, S.E.) (n = 3).

Because apicidin drastically induced the p21\textsuperscript{WAF1/Cip1} mRNA expression, we then investigated whether apicidin could stimulate the activity of the p21\textsuperscript{WAF1/Cip1} gene promoter in cells transiently transfected with 2.4-kilobase wild-type p21\textsuperscript{WAF1/Cip1} promoter-luciferase fusion plasmid, WWP-Luc. As shown in Fig. 1C, the luciferase activity was increased in a dose-dependent manner up to a 5.5-fold by treatment with 2 \mu M apicidin. Together, these results demonstrate that apicidin induces the expression of p21\textsuperscript{WAF1/Cip1} protein and mRNA and activates its promoter activity in a dose-dependent manner.

Apicidin Activates the p21\textsuperscript{WAF1/Cip1} Promoter through Sp1 Sites—To delineate the mechanism by which apicidin activates the p21\textsuperscript{WAF1/Cip1} promoter, we first determined the regions of the p21\textsuperscript{WAF1/Cip1} promoter responsible for the activation of p21\textsuperscript{WAF1/Cip1} promoter by apicidin. A series of 5 deletion constructs of the p21\textsuperscript{WAF1/Cip1} promoter were transiently transfected into HeLa cells, and luciferase activities following apicidin treatment were measured relative to the full-size p21\textsuperscript{WAF1/Cip1} promoter (pWWP). The pWP124 plasmid lacking the two p53 binding sites was strongly activated by apicidin up to a 9.8-fold, a level comparable with that of the full-size promoter pWWP (Fig. 2A), indicating that apicidin activates the p21\textsuperscript{WAF1/Cip1} promoter in a p53-independent pathway. This observation well correlates with our previous report that the expression of p21\textsuperscript{WAF1/Cip1} is induced by a p53-independent fashion in HeLa cells (22). To determine whether the Sp1 binding sites are involved in the transcriptional activation by apicidin, a series of 5 deletion plasmids of pWP124 harboring six Sp1 binding sites, pWP101 lacking Sp1–1 and Sp1–2, and pWPdel-SmaI, lacking Sp1–1 to Sp1–4 sites, were used and assayed for luciferase activity in the presence or absence of apicidin. Deletion of Sp1–1 and Sp1–2 sites did not significantly affect the apicidin-mediated activation of the promoter, because apicidin still activated pWP101 by about 9.5-fold, a level similar to that of activation by the full-size promoter (pWWP) (Fig. 2A). On the other hand, deletion of Sp1–3 and Sp1–4 sites from positions –101 to –60 (pWPdel-SmaI) markedly reduced the apicidin-mediated activation as well as the basal promoter activity over the control in pWP101, suggesting that Sp1–3 and Sp1–4 sites play an important role in both the basal and apicidin-mediated activation of the p21\textsuperscript{WAF1/Cip1} promoter. To further define the apicidin-responsive elements, we used pWP101 constructs with mutations in various Sp1 sites or TATA element. Although the basal activity of pWP101-mtSp1–1 in which the Sp1–3 site is the main responsive element in the TATA element) did not activate the promoter at all, the basal and apicidin-mediated activation of the p21\textsuperscript{WAF1/Cip1} promoter activity decreased to 25% of pWP101, the activation by apicidin in pWP101-mtSp1–3 was markedly decreased from 9.5- to 1.2-fold of pWP101 activation (Fig. 2B). When the Sp1–4 site was mutated (pWP101-mtSp1–4), the basal activity of pWP101-mtSp1–4 was reduced to 42% compared with that of pWP101, and the activation by apicidin decreased to 25% of pWP101. These results indicate that both Sp1–3 and Sp1–4 site are important, but the Sp1–3 site is the main responsive element in apicidin-induced activation. On the other hand, pWP101-mtSp1–5,6 (mutated in Sp1–5,6) and pWP101-mtTATA (mutated in the TATA element) did not activate the promoter at all, because the basal promoter activity of pWP101-mtSp1–5,6 and pWP101-mtTATA was reduced to the background levels, suggesting that Sp1–5,6 and TATA sites were indispensable for the p21\textsuperscript{WAF1/Cip1} basal promoter activity. Taken together, these
results demonstrate that the Sp1–3 site located between −82 and −77 relative to the transcription start site is the main apicidin-responsive element and that the Sp1–4 site is also partially involved in the activation.

Mitogen-activated Protein Kinase (ERK1/2) and PKA Are Not Involved in the Activation of p21WAF1/Cip1 Promoter via Sp1–3 Site by Apicidin—Recent accumulating evidence suggests that the activation of p21WAF1/Cip1 promoter in response to various stimuli could be regulated by protein kinases, including PKC (25) and PKA (26). Furthermore, HDAC inhibitors such as NaB and trapoxin have been demonstrated to be able to activate protein kinases including PKC (25) and PKA (26). Furthermore, HDAC inhibitors might be mediated by a protein kinase signaling pathway. To test this possibility, different types of well known specific kinase inhibitors, which have been usually employed as tools in analyzing specific signaling pathway, were used in this study. We first examined the role of ERK1/2 in the activation of p21WAF1/Cip1 promoter by apicidin. As demonstrated in Fig. 3A, pretreatment with PD98059 that blocks MEK1 did not attenuate the apicidin-induced luciferase activity as monitored by a transient transfection of pWP101 as a reporter plasmid, suggesting that ERK1/2 was not involved in the apicidin-induced transcriptional activation of p21WAF1/Cip1 promoter via Sp1 sites. We then investigated the role of PKA in the induction of p21WAF1/Cip1 promoter activity. Pretreatment of a selective inhibitor of PKA, H-89, did not affect the activation of p21WAF1/Cip1 promoter via Sp1 sites by apicidin (Fig. 3A). Taken together, these results suggest that the ERK1/2 and PKA signaling pathway might not be involved in the activation of p21WAF1/Cip1 promoter via Sp1–3 sites by apicidin.

Activation of p21WAF1/Cip1 Promoter via Sp1–3 Site by Apicidin Is Mediated by Protein Kinase C—Recently, the possible link between p21WAF1/Cip1 induction by HDAC inhibitors and PKC activation has been evoked by NaB. NaB can stimulate the PKC activation during erythroid differentiation (27), and PKC activator alone has also been shown to induce p21WAF1/Cip1 expression (28, 29). Thus, we examined whether PKC could be a mediator in the induction of p21WAF1/Cip1 by apicidin. Pretreatment with selective PKC inhibitors calphostin C and rottlerin markedly suppressed the promoter activity of p21WAF1/Cip1 activated by apicidin (Fig. 3B). Moreover, treatment with a PKC activator, PDBu, alone activated the p21WAF1/Cip1 promoter activity up to about 8-fold in the absence of apicidin in HeLa cells. Co-treatment of PDBu with apicidin further elevated the luciferase activity but did not have a synergistic effect on apicidin-induced p21WAF1/Cip1 promoter activity (Fig. 3B). To confirm the ability to activate p21WAF1/Cip1 promoter activity through Sp1–3 site by PDBu, we used pWP101 mutants, pWP101-mSp1–3, pWP101-mSp1–4, and pWP101-mSp1–5,6. Consistent with the mutant analysis demonstrated in Fig. 2B, PDBu did not induce the luciferase activity in the cells transfected with mutants, suggesting that PKC may regulate the activity of p21WAF1/Cip1 promoter through Sp1–3 site (Fig. 3C).

To test whether the ability of PKC to up-regulate p21WAF1/Cip1 promoter activity by apicidin is due to the structural property of apicidin, we examined the effect on the p21WAF1/Cip1 promoter activity by other HDAC inhibitors, TSA and NaB, that are structurally unrelated to apicidin. Using pWP101 as a reporter plasmid, we measured the luciferase activity in cells treated with TSA or NaB in the presence or absence of calphostin C. The increased promoter activities by TSA and NaB were also completely inhibited by pretreatment with calphostin C, comparable with the inhibitory effect of calphostin C on apicidin-induced promoter activities (Fig. 3D). These results suggest that the PKC signal transduction pathway might be contributed to the p21WAF1/Cip1 transcriptional activation through Sp1 sites by apicidin and that other HDAC inhibitors might also adopt PKC as an indispensable mediator in the induction of p21WAF1/Cip1 gene expression.

To further examine the effect of PKC inhibitor on the induc-
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FIG. 4. Suppression of apicidin-induced p21WAF1/Cip1 expression by treatment with PKC inhibitor independent of histone acetylation in vivo. A, HeLa cells were pretreated with or without 5 μM calphostin C for 1 h and further incubated in the presence or absence of 1 μM apicidin for 24 h. In some experiments, the cells were treated with PDBu (50 nM) with or without 1 μM apicidin for 24 h. The induction of p21WAF1/Cip1 and acetylated H3, H4 histone was investigated by immunoblot analysis as described under “Experimental Procedures.” To verify equal loading of the amount of protein in each lane, the β-tubulin expression level was also examined. B, HeLa cells were treated with cycloheximide (CHX, 10 μM) for 1 h were treated with PDBu (50 nM) for 24 h. After treatment, the cells were analyzed for p21WAF1/Cip1 mRNA expression by RT-PCR analysis as described under “Experimental Procedures.” Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used to normalize the amount of loaded RNA. DMSO, dimethyl sulfoxide.

FIG. 5. Translocation of PKCs from cytosolic to particulate fraction by apicidin in HeLa cells. A, whole cell lysates (50 μg of protein) were separated on 10% SDS-polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membrane, and detected with using isoform-specific PKC antibodies. B, after pretreatment with calphostin C (5 μM) for 1 h, HeLa cells were treated with 0.1% Me2SO alone, 1 μM apicidin, or 50 nM PDBu. The cells were lysed and fractionated into cytosolic (C) and particulate (P) fractions as described under “Experimental Procedures.” Thirty micrograms of protein was loaded in each well.

DISCUSSION

Among the genes that are directly transcriptionally up-regulated in cells treated with HDAC inhibitors is the Cdk inhibitor p21WAF1/Cip1. It binds and inhibits cyclin/Cdk complexes, resulting in tumor cell cycle arrest. It has been proposed that p21WAF1/Cip1 could be one of the better candidates for gene-regulating chemotherapy or chemoprevention, because it is less frequently mutated than p53 gene in human common cancers (31–33) and its induction by HDAC inhibitors is mediated by a p53-independent pathway (17, 18, 21). It is therefore important to understand the induction mechanism of p21WAF1/Cip1 by HDAC inhibitors in chemotherapy or chemoprevention.

This study demonstrates that apicidin induced the p21WAF1/Cip1 gene independent of the de novo protein synthesis by a transcriptional activation of p21WAF1/Cip1 promoter through Sp1–3 site. Consistent with our results, other HDAC inhibitors, such as NaB, TSA, and SAHA have been shown to activate p21WAF1/Cip1 promoter through Sp1 sites (17, 18, 21). Furthermore, the responsive element for HDAC inhibitors is also involved in the transcriptional activation of p21WAF1/Cip1 promoter by various stimuli, including TGF-β (12), tamoxifen (13), the PKC activator phorbol ester (34), the phosphatase 1 and 2A inhibitor okadaic acid (34), the geranylgeranyltansferase I inhibitor GGTI-298 (35), progesterone (14), and nerve growth factor (15), indicating the essential role of Sp1 sites in the transcriptional activation of p21WAF1/Cip1 gene. However, the Sp1 family transcription factors, Sp1 or Sp3, appear to be differentially involved in the activation of p21WAF1/Cip1 promoter via Sp1 sites depending on different kinds of inducers and cellular systems as previously reported (18, 35, 36). Apicidin-induced transcriptional activation of p21WAF1/Cip1 through Sp1 sites does not appear to be attributed to the increase in DNA binding activity of Sp1/3, because apicidin treatment did not disrupt the interaction between Sp1/3 and...
Sp1–3 element (data not shown), which is consistent with previous observations from other well-known HDAC inhibitors such as NaB (17), TSA (21), and SAHA (18).

Recently, accumulating evidence has suggested that the p21^{WAF1/Cip1} transcriptional activation by HDAC inhibitors is triggered by the histone hyperacetylation in its promoter region, followed by transcriptional activation by facilitating the binding of transcription factors to nucleosomal DNA (16). In this study, however, the transiently transfected reporter plasmids harboring the apicidin-responsive elements of p21^{WAF1/Cip1} promoter were still responsive to the apicidin-mediated stimulation in p21^{WAF1/Cip1} gene activation, although they did not appear to adopt a complete chromatin structure. This observation raised the possibility that the transcriptional activation by apicidin could be mediated by mechanisms other than alteration of chromatin structure, such as post-translational modification of Sp1/3 by a protein kinase signaling pathway, alteration of PKC signaling pathway would thus be of great interest.

To clarify the possible link between acetylation of nonhistone proteins and the protein kinase signaling pathway, we examined these possibilities demonstrated that PKC inhibition completely suppressed not only the promoter activity but also the mRNA and protein expression of p21^{WAF1/Cip1} induced by apicidin, suggesting that PKC might be involved in the transcriptional activation of p21^{WAF1/Cip1} through Sp1 sites by apicidin. Indeed, apicidin could translocate only PKCε among the PKC isoforms expressed in HeLa cells from cytosolic to particulate fraction, indicating a selective activation of PKCε by apicidin, as previously reported with other HDAC inhibitor in human K562 erythroleukemia cells (27). Furthermore, apicidin-induced translocation of PKCε from the cytosolic to particulate fraction was reversed by pretreatment with calphostin C, which was accompanied by an inhibition of p21^{WAF1/Cip1} mRNA and protein expression as well as its promoter activity by pretreatment with calphostin C. However, little is known about how apicidin activates PKCε through translocation from the cytosol to the membrane. We could not also rule out the possibility that apicidin induced translocation of PKCε from the cytosol to the nucleus as previously observed with NaB (27).

Our results could be further supported by other observations that the induction of p21^{WAF1/Cip1} was up-regulated by PKC activator phorbol ester (25) and that the induction of p21^{WAF1/Cip1} gene could be regulated by the Sp1 and 3 phosphorylation in response to geranylgeranylation transferase I inhibitor GTI-298 (35). Although well-known HDAC inhibitors including apicidin have been reported to induce selective changes in the expression of few genes such as p21^{WAF1/Cip1} (16–20, 22), gelsolin (19, 22), Fas/FasL (37), or TGF-β type II receptor (38), whether apicidin generally up-regulates all genes with Sp1 binding sites remains to be unraveled. This identification would be important to elucidate the role of PKC in regulation of whole new categories of genes.

The ability of PKC to up-regulate p21^{WAF1/Cip1} promoter activity by apicidin appears not to be due to the structural property of apicidin, because the transcriptional activation of p21^{WAF1/Cip1} promoter by other HDAC inhibitors, TSA and NaB, that are structurally unrelated to apicidin, was also completely suppressed by pretreatment with calphostin C, indicating that HDAC may be still the main target of apicidin. However, the transcriptional activation of p21^{WAF1/Cip1} by apicidin might be mediated by hyperacetylation of histone proteins rather than chromatin remodeling through histone hyperacetylation, because calphostin C could block p21^{WAF1/Cip1} expression independent of histone acetylation by apicidin. This view may be supported by the fact that the function of the nonhistone protein such as E2F transcription factor may be regulated by reversible acetylation (39). To clarify the possible link between acetylation of nonhistone proteins and the protein kinase signaling pathway would thus be of great interest.

In summary, we demonstrated that PKC might be an important mediator of apicidin-induced transcriptional activation of p21^{WAF1/Cip1} promoter. Apicidin induced the p21^{WAF1/Cip1} gene independent of the de novo protein synthesis through Sp1–3 site on p21^{WAF1/Cip1} promoter. In addition, PKCε may be responsible for the transcriptional activation of p21^{WAF1/Cip1} through Sp1 sites, because the inhibition of PKCε translocation from cytosolic to particulate fraction by pretreatment with calphostin C significantly attenuated the activation of p21^{WAF1/Cip1} promoter as well as the expression of p21^{WAF1/Cip1} mRNA and protein by apicidin. To our knowledge, the results we present here are the first study elucidating the possible link between the transcriptional activation of p21^{WAF1/Cip1} by apicidin and the PKC signaling pathway. Taken together, our results provide a new insight into the role of PKC in the transcriptional regulation of p21^{WAF1/Cip1} expression through Sp1 sites by HDAC inhibitors.

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Activation of p21^{WAF1/Cip1} Transcription by Apicidin

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