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Expression of p21$^{WAF1/Cip1}$ through Sp1 sites by histone deacetylase inhibitor apicidin requires PI 3-kinase–PKCε signaling pathway

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We previously reported that the activation of p21$^{WAF1/Cip1}$ transcription by histone deacetylase inhibitor apicidin was mediated through Sp1 sites and pointed to the possible participation of protein kinase C (PKC). In this study, we investigated the role and identity of the specific isoforms of PKC involved and identified phosphatidylinositol 3-kinase (PI 3-kinase) as an upstream effector in HeLa cells. Using an isoform-specific pharmacological inhibitor of PKC, a PKCε dominant-negative mutant, and antisense oligonucleotide to inhibit PKCε specifically, we found that among PKC isoforms, PKCε was required for the p21$^{WAF1/Cip1}$ expression by apicidin. In addition to PKCε, PI 3-kinase appeared to participate in the activation of p21$^{WAF1/Cip1}$ promoter by apicidin, since inactivation of PI 3-kinase either by transient expression of dominant-negative mutant of PI 3-kinase or its specific inhibitors, LY294002 and wortmannin, attenuated the activation of p21$^{WAF1/Cip1}$ promoter and p21$^{WAF1/Cip1}$ protein expression by apicidin. Furthermore, membrane translocation of PKCε in response to apicidin was blocked by the PI 3-kinase inhibitor, indicating the role of PI 3-kinase as an upstream molecule of PKCε in the p21$^{WAF1/Cip1}$ promoter activation by apicidin. However, the p21$^{WAF1/Cip1}$ expression by apicidin appeared to be independent of the histone hyperacetylation, since apicidin-induced histone hyperacetylation of p21$^{WAF1/Cip1}$ promoter region was not affected by inhibition of PI 3-kinase and PKC, suggesting that the chromatin remodeling through the histone hyperacetylation alone might not be sufficient for the expression of p21$^{WAF1/Cip1}$ by apicidin. Taken together, these results suggest that the PI 3-kinase–PKCε signaling pathway plays a pivotal role in the expression of the p21$^{WAF1/Cip1}$ by apicidin.
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Keywords: histone deacetylase inhibitor; apicidin; p21$^{WAF1/Cip1}$; PI 3-kinase; PKCε

Introduction

Histone acetylation plays a key role in transcriptional activation, whereas deacetylation of histones correlates with the transcriptional repression and silencing of genes (Grunstein, 1997). Some transcription factors bind histone acetyltransferases, such as CREB-binding protein and P/CAF to enhance nucleosomal relaxation and activation of transcription (Sternglanz, 1996; Wu, 1997), while other transcription factors bind histone deacetylases (HDACs) that stabilize nucleosomal structure and repress transcription (Grunstein, 1997; Wu, 1997). Accumulating evidence indicates that HDACs may be involved in cell-cycle regulation, differentiation, and human cancer through the regulation of gene transcription. Various HDAC inhibitors, such as sodium butyrate (Cousens *et al*., 1979), trapoxin (Kijima *et al*., 1993), trichostatin A (TSA) (Hoshikawa *et al*., 1994), depudecin (Kwon *et al*., 1998), FR901228 (Nakajima *et al*., 1998), oxamflatin (Kim *et al*., 1999), and MS-27-275 (Saito *et al*., 1999) promote accumulation of acetylated histones in the nucleus, arrest cell growth, and reverse neoplastic characteristics in cultured cells via the expression of a specific preprogrammed set of genes.

Cell-cycle progression is regulated by the sequential activation of cyclin-dependent kinases (Cdks), whose activation is carefully regulated at several levels, which include induction and degradation of cyclin protein, Cdk phosphorylation by cyclin-activating kinase, and induction of Cdk inhibitory proteins (Draetta, 1994; Hunter and Pines, 1994; King *et al*., 1994; Sherr, 1994; Sherr and Roberts, 1995). The Cdk inhibitor proteins, namely, p21$^{WAF1/Cip1}$, p27^{Kip1}, p57^{Kip2}, p16, p15, p18, and p19 can negatively regulate cell-cycle progression by inhibiting Cdk activity through the physical association with their target cyclin–Cdk complexes (Noda *et al*., 1994; Polyak *et al*., 1994; Halevy *et al*., 1995). Among them, p21$^{WAF1/Cip1}$ was cloned first and characterized as an inhibitor of cyclin E–Cdk2 complex kinase activity in p53-mediated cell-cycle arrest induced by DNA damage (El-Deiry *et al*., 1993; Harper *et al*., 1993; Dulic *et al*., 1994; El-Deiry *et al*., 1994). It was further shown that p21$^{WAF1/Cip1}$ could be transcriptionally upregulated by an

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accumulating list of physiological and pathological factors, such as tumor suppressors of the p53 family, differentiation factors, growth factors, cytokines, stress factors, and pharmacological agents (Kardassis *et al*., 1999). Some of these factors such as p53, vitamin D3, interferon γ, and other signals appear to act through different DNA motifs scattered in the region between -2300 and -210 base pairs upstream from the transcriptional initiation site of the p21 gene (El-Deiry *et al*., 1995; Chin *et al*., 1996; Liu *et al*., 1996). However, many factors such as TGF-β1, progesterone, phorbol esters, tamoxifen, and HDAC inhibitors act on the proximal region of the p21 promoter (base pairs -210 to $+1$), which contains several closely spaced G/C-rich motifs that serve as binding sites for members of the Sp1 family of transcription factors (Datto *et al*., 1995). Recent evidence suggests that the induction of $p21^{WAF1/Cip1}$ by these stimuli could be regulated by various signal transduction pathways that mediate cell growth, differentiation, and stress response (Hu *et al*., 1999; Lee *et al*., 2000; Mitsuuchi *et al*., 2000). Also, it has been demonstrated that $p21^{WAF1/Cip1}$ induction by HDAC inhibitors was mediated via Sp1 sites through Sp1 family transcription factors, Sp1 and/or Sp3 (Nakano *et al*., 1997; Sowa *et al*., 1999; Huang *et al*., 2000; Han *et al*., 2001). However, the detailed signal transduction pathways involved in $p21^{WAF1/Cip1}$ gene regulation by HDAC inhibitors still remain poorly understood.

Recently, we reported that apicidin, an HDAC inhibitor, inhibits proliferation of various tumor cells with a broad spectrum of antiproliferative activity against various cancer cell lines resulting from the induction of $p21^{WAF1/Cip1}$ (Han *et al*., 2000) and induces apoptosis through selective induction of Fas/Fas ligand, resulting in the release of cytochrome *c* from the mitochondria to the cytosol and subsequent activation of caspase-9 and caspase-3 in human acute promyelocytic leukemia cells (Kwon *et al*., 2002). In addition, it has been shown that the transcriptional activation of $p21^{WAF1/Cip1}$ by apicidin is dependent on protein kinase C (PKCs) as indicated by studies of the PKC inhibitor (Han *et al*., 2001). However, which of the PKC isoforms was involved in $p21^{WAF1/Cip1}$ gene expression by apicidin or upstream signaling events that leads to PKC activation are not yet clear.

Here, we have investigated the signaling pathway involved in apicidin induction of $p21^{WAF1/Cip1}$ expression and how this signaling pathway related to the hyperacetylation of histone in the region of $p21^{WAF1/Cip1}$ promoter. Our data suggest that the signaling events mediated by PKCε are required for the induction of $p21^{WAF1/Cip1}$ and that phosphatidylinositol 3-kinase (PI 3-kinase) has a role as an upstream effector of PKCε in this process. Furthermore, chromatin remodeling by histone hyperacetylation alone was not sufficient for the induction of $p21^{WAF1/Cip1}$ expression in response to apicidin. Taken together, these results suggest that PI 3-kinase–PKCε signal transduction pathway might be involved in the apicidin-induced transcriptional activation of the $p21^{WAF1/Cip1}$ gene expression via Sp1 sites.

Results

Involvement of Sp1 transcription factor in the expression of $p21^{WAF1/Cip1}$ by apicidin

We previously demonstrated that apicidin markedly upregulated transcription of $p21^{WAF1/Cip1}$ via Sp1 sites but without altering the DNA-binding affinities of Sp1 family transcription factors to DNA (Han *et al*., 2001). To determine whether or not apicidin's actions are dependent on Sp1 transcription factors themselves, we examined the effect of mithramycin, which is known to inhibit the binding of Sp1 family transcription factors to genes containing G + C-rich promoters (Blume *et al*., 1991). Mithramycin treatment significantly inhibited the expression of $p21^{WAF1/Cip1}$ protein by apicidin (Figure 1a). We also overexpressed a dominant-negative mutant (delta-Sp1), which contained a DNA-binding domain of human Sp1 that conferred sequence-specific DNA interactions, but did not contain a transactivation domain (Peterson and Thiel, 1996). As shown in Figure 1b, the apicidin induction of $p21^{WAF1/Cip1}$ was significantly attenuated by overexpression of delta-Sp1, compared to vector-transfected control cells. Collectively, these results suggested that $p21^{WAF1/Cip1}$ induction by apicidin was mediated through Sp1-binding sites by Sp1 transcription factor.

Requirement of PKCε signaling pathway for the expression of $p21^{WAF1/Cip1}$ by apicidin

It has been demonstrated that PKC signaling pathway is necessary for the induction of $p21^{WAF1/Cip1}$ (Han *et al*., 2001). To identify which of the various PKC isozymes were responsible for apicidin induction of $p21^{WAF1/Cip1}$, we examined the effects of specific PKC inhibitors.

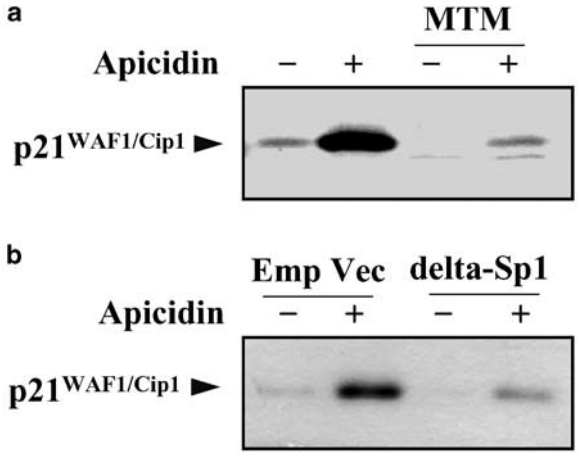

Figure 1 Induction of $p21^{WAF1/Cip1}$ by apicidin requires Sp1 transcription factor. (**a**) After pretreatment with 100-nM mithramycin (MTM), HeLa cells were treated with 1-μM apicidin for 24 h. Whole lysates (30 μg) of the HeLa cells were examined by 15% SDS–PAGE and analysed with immunoblotting using antibodies for $p21^{WAF1/Cip1}$. (**b**) HeLa cells were transiently transfected with the delta-Sp1 plasmid for 24 h, and incubated for an additional 24 h following treatment with 1-μM apicidin. The expression level of $p21^{WAF1/Cip1}$ was analysed with immunoblotting using a specific antibody for $p21^{WAF1/Cip1}$

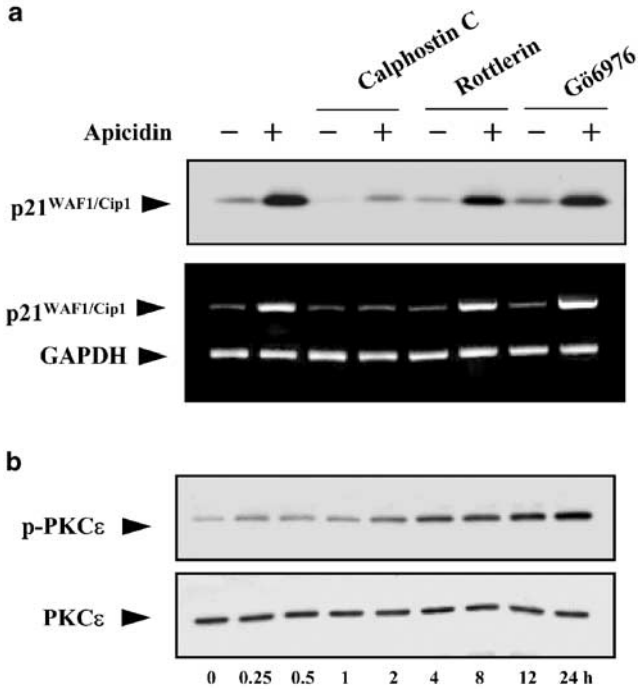

Figure 2 The induction of p21$^{WAF1/Cip1}$ by apicidin requires PKCε signaling pathway. (**a**) After pretreatment with PKC inhibitors for 1 h, HeLa cells were treated with 1-μM apicidin for 24 h. Lysates (30 μg) of the HeLa cells were examined by 15% SDS–PAGE and analysed with immunoblotting using antibodies for p21$^{WAF1/Cip1}$ (upper panel). Also, mRNA levels of p21$^{WAF1/Cip1}$ were analysed with RT–PCR as described under 'Materials and methods' (lower panel). (**b**) HeLa cells were treated with 1-μM apicidin for indicated times and the phoshorylation levels of PKCε were analysed using a specific antibody for phospho-PKCε

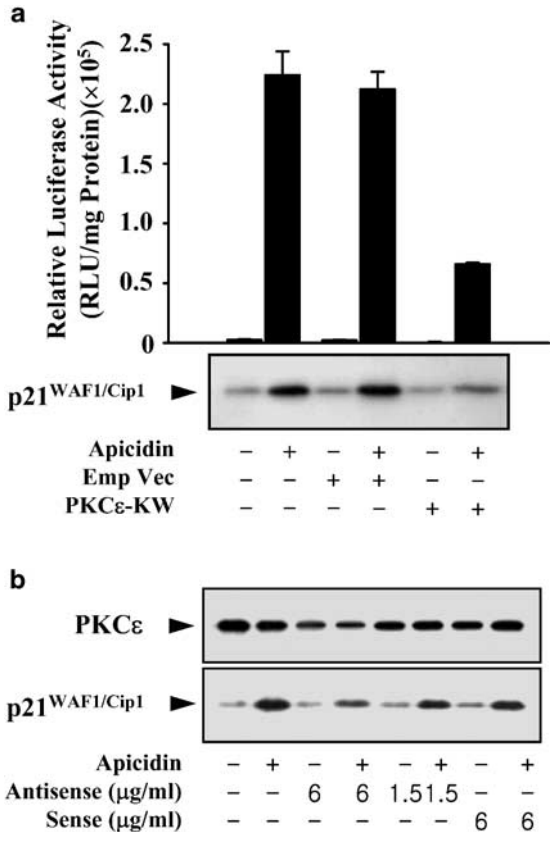

Figure 3 PKCε-mediated signaling pathways are required for the induction of p21$^{WAF1/Cip1}$ by apicidin. (**a**) HeLa cells were cotransfected with pWP101 reporter plasmid with or without PKCε-KW or an empty vector. After 24 h, the cells were incubated for an additional 24 h following treatment with 1-μM apicidin. The cells were harvested, and luciferase activity was determined and normalized to the protein content of each extract. In addition, the expression levels of p21$^{WAF1/Cip1}$ in the above extract were analysed using immunoblotting. (**b**) HeLa cells were transfected with either PKCε-specific antisense or sense oligonucleotides at the indicated concentrations. After 48 h, the cells were incubated for an additional 24 h following treatment with 1-μM apicidin, and the cellular levels of p21$^{WAF1/Cip1}$ and PKCε were measured using immunoblotting

Pretreatment of HeLa cells with calphostin C, a pan-specific inhibitor, significantly attenuated the induction of p21$^{WAF1/Cip1}$ by apicidin as well as the basal level of p21$^{WAF1/Cip1}$ expression (Figure 2a). However, the effects of rottlerin, a PKCδ-specific inhibitor, and Gö6976, an inhibitor of Ca^{2+}-dependent PKC α, β, and γ, on the expression of p21$^{WAF1/Cip1}$ by apicidin were less marked or not evident. Similarly, mRNA expression of p21$^{WAF1/Cip1}$ by apicidin was affected by calphostin C, but not by rottlerin or Gö6976. These and previous (Han *et al*., 2001) results suggest by default that PKCε rather than the other PKC isozymes played an important role in the induction of p21$^{WAF1/Cip1}$ by apicidin. Furthermore, phosphorylation of PKCε, which is a hallmark of PKCε activation, was significantly induced by apicidin treatment (Figure 2b), further indicating the participation of PKCε in the signaling pathway.

To obtain definitive evidence for the involvement of PKCε in the induction of p21$^{WAF1/Cip1}$, we examined the effect of a dominant-negative mutant of PKCε on the activation of the p21$^{WAF1/Cip1}$ promoter, pWP101, which contains the major apicidin-responsive region located at −82 and −77 relative to the transcription start site. As shown in Figure 3a, the overexpression of the dominant-negative mutant of PKCε significantly attenuated the activation of promoter activity by apicidin, compared to vector-transfected control cells. Consistent with this result, an increased level of p21$^{WAF1/Cip1}$ expression by apicidin was also reduced by the expression of the dominant-negative mutant of PKCε. To validate the fact that PKCε plays an essential role in the activation of p21$^{WAF1/Cip1}$ promoter and induction of p21$^{WAF1/Cip1}$ expression, we used an antisense oligonucleotide against PKCε. The endogenous levels of PKCε were measured by Western blot, following the transfection of HeLa cells with either antisense or sense oligonucleotides to PKCε for 48 h. Optimal inactivation of PKCε in HeLa cells was obtained with an antisense oligonucleotide at a concentration of 6 μg/ml, which resulted in a 65% reduction of total PKCε mass. The reduction of PKCε was accompanied by a 75% inhibition of the expression of p21$^{WAF1/Cip1}$ by apicidin (Figure 3b). The sense oligonucleotide did not significantly reduce the amount of PKCε in HeLa cells and the expression of p21$^{WAF1/Cip1}$ by apicidin (Figure 1b). Overall, these results indicate that PKCε mediates the expression of p21$^{WAF1/Cip1}$ in response to apicidin.

Involvement of PI 3-kinase in the activation of p21WAF1/Cip1 promoter and expression by apicidin

Various protein kinase signaling pathways, including mitogen-activated protein kinase (MAPK), PKC, PI 3-kinase, and protein kinase A (PKA), were thought to be involved in the expression of p21WAF1/Cip1 in response to various stimuli (Espinos and Weber, 1998; Rivero and Adunyah, 1998; Besson and Yong, 2000; Mitsuuchi *et al*., 2000). However, in our previous study, we excluded the possibility of the involvement of extracellular-regulated protein kinase (ERK)1/2 and PKA signaling pathway in the expression of p21WAF1/Cip1 by apicidin (Han *et al*., 2001). Recent data suggest that PI 3-kinase could play a critical role in the expression of p21WAF1/Cip1 induced by cisplatin and paclitaxel (Mitsuuchi *et al*., 2000). These observations raised a possibility that PI 3-kinase might be involved in the expression of p21WAF1/Cip1 by apicidin. To test this possibility, we first examined the effect of PI 3-kinase inhibitors on the apicidin-induced activation and expression of the p21WAF1/Cip1 promoter activity. As seen in Figures 4a and 6 (lane 4), the activation of promoter activity and expression of p21WAF1/Cip1 by apicidin were largely blocked by the treatment of the cells with LY294002, but only partially inhibited by wortmannin. To confirm a possible role for PI 3-kinase, we used the catalytically inactive mutant of PI 3-kinase (PI3K p110α-KR), which is known to act as a dominant-negative mutant (Wymann *et al*., 1996). As expected, the overexpression of a dominant-negative mutant of PI 3-kinase significantly attenuated the activation of promoter activity as well as the protein expression of p21WAF1/Cip1 by apicidin (Figure 4b).

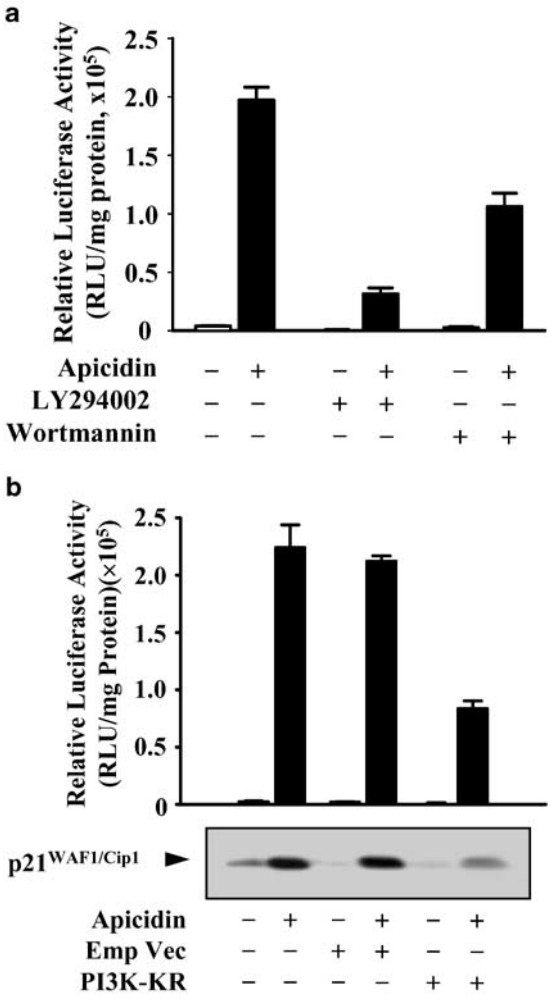

Figure 4 PI 3-kinase signaling is required for apicidin activation of the p21WAF1/Cip1 promoter activity and its expression. (**a**) HeLa cells were transfected with pWP101 reporter plasmid for 24 h. After pretreatment with either 25-μM LY294002 or 0.5-μM wortmannin for 1 h, the cells were incubated for an additional 24 h following treatment with 1-μM apicidin. The cells were harvested, and luciferase activity was determined and normalized to the protein content of each extract. (**b**) HeLa cells were cotransfected with pWP101 reporter plasmid with or without PI 3-kinase-KR or an empty vector. After 24 h, the cells were incubated for an additional 24 h following treatment with 1-μM apicidin. The cells were harvested, and luciferase activity was determined and normalized to the protein content of each extract. In addition, the expression levels of p21WAF1/Cip1 in the above extract were analysed using immunoblotting

Role of PI 3-kinase as an upstream effector of PKCε in apicidin-induced p21WAF1/Cip1 expression

To examine the relationship between PKCε and PI 3-kinase in the activation of p21WAF1/Cip1 expression, we examined the effects of PI 3-kinase inhibitors on the induction of p21WAF1/Cip1 expression in HeLa cells by direct stimulation of PKC. The PKC activator, phorbol-dibutyrate (PDBu), alone dramatically induced the expression of p21WAF1/Cip1 in HeLa cells. This induction was significantly inhibited by the PKC inhibitor, calphostin C, as expected, but not by PI 3-kinase inhibitors, LY294002 and wortmannin (Figure 5a) to indicate that PI 3-kinase acted in parallel with or upstream of PKC. To distinguish between these two possibilities, we next examined whether the membrane translocation of PKCε by apicidin, which is a hallmark of its activation, could be blocked by inhibiting PI 3-kinase activity. PKCε was translocated from the cytosol fraction to the particulate fraction by treatment with apicidin, as reported previously (Han *et al*., 2001). Apicidin-induced translocation of PKCε was, however, reversed by pretreatment of the cells with calphostin C and LY294002 (Figure 5b) to indicate that PKCε might play a role downstream of PI 3-kinase in the induction of p21WAF1/Cip1 expression by apicidin. Our results are consistent with the observations that PI 3-kinase activates novel and atypical PKCs in other systems (Toker *et al*., 1994) and that platelet-derived growth factor (PDGF) induced the translocation of PKCε from the cytosol to the membrane via the activation of PI 3-kinase in HepG2 cells (Yano *et al*., 1993).

Histone acetylation-independent inhibition of p21WAF1/Cip1 expression by the PI 3-kinase inhibitors

To gain an insight into the molecular mechanisms underlying the effects of apicidin on the expression of

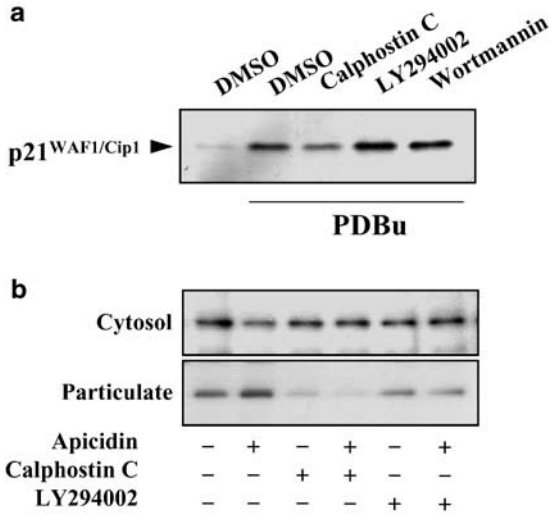

Figure 5 PI 3-kinase plays a role as an upstream effector molecule of PKCε in apicidin induction of p21$^{WAF1/Cip1}$. (**a**) HeLa cells were pretreated with or without 0.5-μM calphostin C, 0.5-μM wortmannin, or 25-μM LY294002 for 1 h and then incubated in the presence or absence of 50-nm PDBu for 24 h. The cellular level of p21$^{WAF1/Cip1}$ was determined by immunoblot analysis as described in 'Materials and methods'. (**b**) After pretreatment with either 0.5-μM calphostin C or 25-μM LY294002 for 1 h, HeLa cells were treated with 1-μM apicidin. The cells were lysed and fractionated into cytosolic and particulate fractions as described in 'Materials and methods'. The amounts of PKCε translocated in particulate fraction were investigated by immunoblot analysis with PKCε-specific antibody

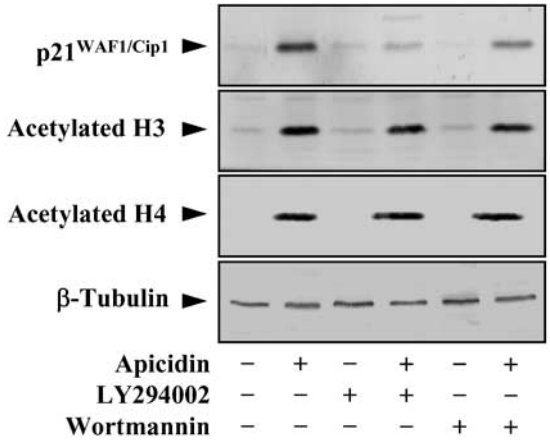

Figure 6 PI 3-kinase inhibitors attenuate the expression of p21$^{WAF1/Cip1}$ by apicidin in a histone acetylation-independent manner. HeLa cells were pretreated with or without either 25-μM LY294002 or 0.5-μM wortmannin for 1 h, and then incubated in the presence or absence of 1-μM apicidin for 24 h. The cellular levels of p21$^{WAF1/Cip1}$ and acetylated H3 and H4 histones were investigated by immunoblot analysis, as described in 'Materials and methods'

p21$^{WAF1/Cip1}$, we investigated the relationship between global histone acetylation and PI 3-kinase–PKCε signaling pathway in the apicidin induction of p21$^{WAF1/Cip1}$ expression by use of Western blot with specific antibodies against acetylated histone H3 and H4. Apicidin treatment led to the hyperacetylation of histone H3 and H4, which was accompanied by the induction of p21$^{WAF1/Cip1}$ (Figure 6). Surprisingly, PI 3-kinase inhibitors decreased the induction of p21$^{WAF1/Cip1}$ by apicidin without any alteration of hyperacetylation of histones H3 and H4, indicating that p21$^{WAF1/Cip1}$ induction by apicidin might be independent of chromatin remodeling through histone hyperacetylation. To confirm this observation, we examined the effect of apicidin on the acetylation of histone H3 associated with the p21$^{WAF1/Cip1}$ gene promoter using chromatin immunoprecipitation assay. Two primer sets were used: primer I, 282 bp, primer II, 255 bp as previously described (Richon *et al*., 2000) (Figure 7a). Acetylated histone H3 associated with the p21$^{WAF1/Cip1}$ gene promoter was undetectable in the absence of apicidin. Apicidin treatment caused a transient increase in acetylation levels of histone H3 in the p21$^{WAF1/Cip1}$ promoter region corresponding to primer set II, having a maximum effect at 6 h, but such a treatment did not induce the hyperacetylation of histone H3 in the p21$^{WAF1/Cip1}$ promoter region corresponding to primer set I (Figure 7b). These patterns were consistent with those of other report (Richon *et al*., 2000). Treatment of the cells with the PKC inhibitor or PI 3-kinase inhibitor

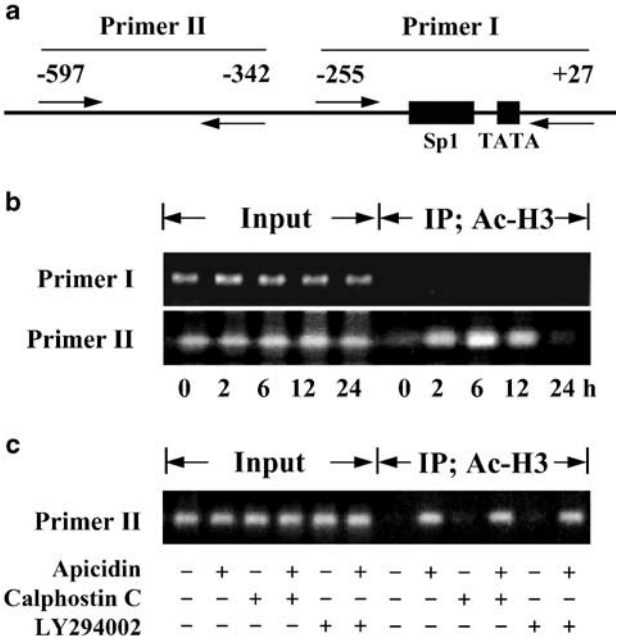

Figure 7 Inhibition of PKC and PI 3-kinase does not alter the apicidin-induced hyperacetylation of nucleosome on the p21$^{WAF1/Cip1}$ promoter region. (**a**) Schematic representation of the human p21$^{WAF1/Cip1}$ gene. Sp1 sites are located at −82 and −69, and amplified with primer I. (**b**) Soluble chromatin was immunoprecipitated with antiacetylated histone H3 antibodies from HeLa cells cultured with or without 1-μM apicidin for the indicated times. PCR primer for the promoter region of the p21$^{WAF1/Cip1}$ gene was used to amplify the DNA isolated from the immunoprecipitated chromatin. (**c**) HeLa cells were pretreated with or without either 0.5-μM calphostin C or 25-μM LY294002 for 1 h, and further incubated in the presence or absence of 1-μM apicidin for 6 h. Soluble chromatin was immunoprecipitated with antiacetylated histone H3 antibodies from the above HeLa cells. The PCR primer for the promoter region of the p21$^{WAF1/Cip1}$ gene was used to amplify the DNA isolated from the immunoprecipitated chromatin

did not alter the apicidin-induced hyperacetylation of histone H3 in the p21WAF1/Cip1 promoter region (Figure 7c) in contrast to the inhibitory effects of these inhibitors on apicidin induction of p21WAF1/Cip1 protein expression as noted earlier. These results strongly suggest that chromatin remodeling by histone hyperacetylation itself is not sufficient for the induction of p21WAF1/Cip1 expression in response to apicidin, and other signaling mediators such as PI 3-kinase and PKCε are required for its expression.

Discussion

Among the genes that are directly transcriptionally upregulated in cells treated with HDAC inhibitors is the Cdk inhibitor p21WAF1/Cip1, which plays an important role in the arrest of cell growth through inhibition of cyclin/Cdk complexes. It has been proposed that p21WAF1/Cip1 could be one of the better candidates for gene-regulating chemotherapy or chemoprevention, because it is mutated less frequently than p53 gene in human common cancers (Hollstein *et al*., 1991; Chedid *et al*., 1994; Li *et al*., 1995) and its induction by HDAC inhibitors is mediated by a p53-independent pathway (Nakano *et al*., 1997; Sowa *et al*., 1999; Huang *et al*., 2000; Han *et al*., 2001). It is, therefore, important to understand the induction mechanism of p21WAF1/Cip1 by HDAC inhibitors in chemotherapy or chemoprevention.

We recently reported that apicidin, an HDAC inhibitor, activated p21WAF1/Cip1 transcription through Sp1 sites and that the transcriptional activation of p21WAF1/Cip1 by apicidin required a signaling pathway mediated by PKCs (Han *et al*., 2001), although the precise mechanism of action of apicidin is not yet clear. This study demonstrates that the signaling events mediated by PI 3-kinase and PKCε are indispensable for the transcriptional activation of p21WAF1/Cip1 by apicidin and that chromatin remodeling through the histone hyperacetylation alone might not be sufficient for the expression of p21WAF1/Cip1 by apicidin.

Recent evidence has suggested that the p21WAF1/Cip1 transcriptional activation by HDAC inhibitors is due to its ability to induce histone hyperacetylation in its promoter region, followed by transcriptional activation by facilitating the binding of transcription factors to nucleosomal DNA (Richon *et al*., 2000). In this study, however, the transcriptional activation and expression of p21WAF1/Cip1 gene by apicidin were sensitive to specific inhibitors of PKCε and PI 3-kinase, although apicidin induction of neither global histone hyperacetylation nor hyperacetylation of histone H3 in the p21WAF1/Cip1 promoter region was affected by these inhibitors. 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 its interaction with other proteins, or modification of proteins interacting directly or indirectly with Sp1/3. These possibilities are consistent with the observations that various protein kinase signaling pathways, including MAPK, PKC, PI 3-kinase, and PKA, are involved in the expression of p21WAF1/Cip1 in response to various stimuli (Espinos and Weber, 1998; Rivero and Adunyah, 1998; Besson and Yong, 2000; Mitsuuchi *et al*., 2000). 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 in human keratinocyte HaCaT cell (Hu *et al*., 1999) and that tamoxifen-induced p21WAF1/Cip1 expression was mediated by PKA in H358 human lung cancer cells (Lee *et al*., 2000). In addition, the p53-dependent induction of p21WAF1/Cip1 by cisplatin and paclitaxel has been shown to be mediated by PI 3-kinase/AKT signal transduction pathway (Mitsuuchi *et al*., 2000). Our studies demonstrated that apicidin activates PI 3-kinase–PKCε signaling pathway, which was responsible for the transcriptional activation and expression of p21WAF1/Cip1 through Sp1 sites. LY294002, a specific inhibitor for PI 3-kinase, markedly inhibited the apicidin-induced transcriptional activation of the p21WAF1/Cip1 promoter, pWP101, which contains the major apicidin-responsive region located at −82 and −77 relative to the transcription start site (Figure 4). The inhibition of transcriptional activity by apicidin was accompanied by a decrease in the expression level of p21WAF1/Cip1 (Figure 6) to indicate an essential role of PI 3-kinase in apicidin induction of p21WAF1/Cip1. The involvement of the PI 3-kinase signaling pathway was further confirmed by transient transfection of dominant-negative mutant of PI 3-kinase. When PI 3-kinase signaling pathway was impaired by LY294002, apicidin-induced translocation of PKCε from the cytosol to the membrane was almost completely inhibited, suggesting that PKCε might play a role of a downstream effector of PI 3-kinase in the induction of p21WAF1/Cip1 expression by apicidin.

The specific involvement of PKCε among PKC isozymes in the induction of p21WAF1/Cip1 by apicidin was confirmed using specific inhibitors for each PKC isozyme (Figure 2a), antisense oligonucleotide to PKCε (Figure 3b), and a dominant-negative mutant of PKCε (Figure 3a) This notion was further supported by other observations that PKCε signaling pathway was necessary for the induction of p21WAF1/Cip1 expression by PMA in an NIH3T3 cell (Petrovics *et al*., 2002), and that another HDAC inhibitor, sodium butyrate, could activate PKCε and induce translocation from cytosolic to particulate fraction (Rivero and Adunyah, 1998). The possible involvement of PKCδ in the induction of p21WAF1/Cip1 by apicidin was suggested based on the effects of rottlerin, a PKCδ-specific inhibitor, on the promoter activity of p21WAF1/Cip1 (Han *et al*., 2001), but this promoter activity was determined using reporter gene assay, an indirect method for the determination of transcriptional activation of genes. However, we have ruled out this possibility by determining that endogenous mRNA and protein level of p21WAF1/Cip1 were unaffected by rottlerin pretreatment (Figure 2a). Consistent with our observation that PI 3-kinase played a role as an upstream effector of PKC in apicidin

induction of p21WAF1/Cip1, PI 3-kinase has been demonstrated to be essential for PKCε activation by PDGF and other growth factors (Moriya *et al*., 1996). Furthermore, recent evidence suggests that PKCε signaling pathway could be mediated by PDK-1. PKCε has been shown to be phosphorylated by PDK-1 *in vitro* and in cells (Le Good *et al*., 1998). PDK-1 phosphorylates PKCε at the activation-loop residue Thr566 and Ser729, leading to its activation (Parekh *et al*., 1999; Ziegler *et al*., 1999; Cenni *et al*., 2002). Although PDK-1 has been reported to be constitutively activated in cells, it is hypothesized that upon stimulation of cells by growth factors, PDK-1 is recruited to the membrane due to binding of phosphatidylinositol-3,4,5-triphosphate to its PH domain (Anderson *et al*., 1998). The above observations suggest that PKCε activation by apicidin could be regulated by PI 3-kinase signaling pathway as an upstream signaling molecule. This possibility is supported by earlier observations that PI 3-kinase plays a critical role in the expression of p21WAF1/Cip1 induced by cisplatin and paclitaxel (Mitsuuchi *et al*., 2000) and that PDGF treatment leads to the translocation of PKCε via the activation of PI 3-kinase in HepG2 cells (Toker *et al*., 1994).

It has been reported that p21WAF1/Cip1 expression by HDAC inhibitors could be mediated by Sp1 family transcription factors through an Sp1-binding site. p21WAF1/Cip1 transcription could be activated by Sp3, but not Sp1, transcription factor through Sp1 site in TSA-treated cells (Sowa *et al*., 1999), while in suberoylanilide hydroxamic acid-treated cells, its transcription could be activated by both Sp1 and Sp3 transcription factors (Huang *et al*., 2000). Our present data suggest that p21WAF1/Cip1 transcriptional activation by apicidin might be mediated by Sp1 transcription factor through Sp1-binding sites, since specific inhibition of Sp1 by its dominant-negative mutant could significantly abrogate the expression of p21WAF1/Cip1 in response to apicidin. However, it is possible that the dominant-negative mutant of Sp1 inhibited the DNA binding of both Sp1 and Sp3, because the DNA-binding domains of Sp1 and Sp3 are highly related (Suske, 1999), thus complicating the interpretation of our results. Therefore, we could not rule out the possible involvement of the Sp3 transcription factor in apicidin-induced transcription of p21WAF1/Cip1.

There are several possible mechanisms by which the Sp1 transcription factor could be activated in response to apicidin. Like many other transcription factors, the transcriptional activity of Sp1 could be regulated by post-translational modification, including phosphorylation (Bouwman and Philipsen, 2002). The Sp1 transcription factor might be directly or indirectly phosphorylated by apicidin-activated PI 3-kinase–PKCε signaling pathway. In support of this assumption, treatment of Hep3B cells and SL2 cells with HDAC inhibitor TSA caused the phosphorylation of Sp1, leading to the activation of its transcriptional activity (Choi *et al*., 2002). We also do not exclude the possibility that the transcriptional activation of p21WAF1/Cip1 by apicidin might be mediated by hyperacetylation of Sp1 and/or Sp3, because Sp3 can be acetylated *in vivo* (Braun *et al*., 2001). Also, other nonhistone proteins such as the p53 or E2F transcription factor are thought to be regulated by reversible acetylation (Martinez-Balbas *et al*., 2000; Prives and Manley, 2001). Clarification of the possible link between acetylation of nonhistone proteins and the protein kinase signaling pathway would thus be of great interest. In addition, since transcriptional activity of Sp1 could be regulated by interaction with other transcriptional coactivators or corepressors (Bouwman and Philipsen, 2002), Sp1-mediated transcriptional activation of p21WAF1/Cip1 by apicidin might be due to modifications of proteins interacting directly or indirectly with Sp1 and/or Sp3. At least, however, Sp1-dependent transcriptional activation of p21WAF1/Cip1 by apicidin does not appear to be attributed to the alteration of DNA-binding activity of Sp1, because apicidin treatment did not disrupt the interaction between Sp1 and its DNA-binding element and this binding affinity was not altered by inhibiting PKC and PI3K signaling pathways (data not shown).

It has been suggested that the action of HDAC inhibitors on gene expression is nonselective, because currently available HDAC inhibitors have a broad spectrum of inhibitory effects on almost all HDACs, and HDACs also act as general transcription corepressors. However, only about 2% of the expressed genes in cells cultured with TSA changed their expression twofold or more compared with untreated control cells, thus indicating that the action of HDAC inhibitors on gene expression was highly selective (Van Lint *et al*., 1996, unpublished data). Therefore, our data suggest a signaling pathway, such as PI 3-kinase and PKCε, as a possible molecular basis of the gene selectivity of HDAC inhibitors. Taken together, our data strongly suggest that the PI 3-kinase–PKCε signaling pathway is responsible for the transcriptional activation p21WAF1/Cip1 through Sp1 sites. This finding reflects our understanding of the increasing complexity of how HDACs may work and how HDAC-dependent effects may be determined by nonhistone protein interactions. These data also confirm the notion that chromatin remodeling by hyperacetylation of histone in the p21WAF1/Cip1 promoter region alone may not be sufficient for the expression of p21WAF1/Cip1 by apicidin.

Materials and methods

Cell culture

The human cervix cancer cell line HeLa was cultured in Dulbecco's modified Eagle's medium (Invitrogen), supplemented with 10% fetal bovine serum (HyClone Laboratories, Logan, UT, USA), and 1% penicillin/streptomycin (Invitrogen).

Reagents

Apicidin, cyclo(*N*-*O*-methyl-L-tryptophanyl-L-isoleucinyl-D-pipecolinyl-L-2-amino-8-oxodecanoyl)], was prepared from

Fusarium sp. Strain KCTC 16677 according to a previously described method (Park *et al*., 1999). Calphostin C, rottlerin, LY294002, wortmannin, and PDBu were obtained from Calbiochem (La Jolla, CA, USA). Mithramycin and Gö6976 were obtained from BioMol (PA, USA).

Plasmids and transfection

The pWP101 reporter gene, which contains the major apicidin-responsive response Sp1-3 site located at −82 and −77 relative to the transcription start site, was provided by Dr Yoshihiro Sowa (Kyoto Prefectural University of Medicine). The pEBG-Sp1 plasmid, dominant-negative mutant, was kindly supplied by G Thiel (Medical Biochemistry and Molecular Biochemistry, University of the Saarland Medical Center) and kinase dead mutants of PKCε (PKCε-KW) and PI 3-kinase (PI3K p110α-KR) were gifts from A Toker (Boston Biomedical Research Inst., Harvard Medical School), and M Wymann (Institute of Biochemistry, University of Fribourg, Switzerland), respectively. HeLa cells were plated into six-well plates at a density of 1×10^5 per well and incubated for 24 h. For $p21^{WAF1/Cip1}$ promoter analysis, cells were transfected with 1 μg per well of pWP101 reporter plasmid DNA and/or mammalian expression plasmids by using ProFection transfection reagent (Promega, Madison, WI, USA). At 24 h after the transfection, the medium was changed to a 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, Madison, WI, USA).

Treatment of cells with oligonucleotides

Antisense oligonucleotides complementary to the translation initiation region of mRNA specific for human PKCε, that is, 5′-GGCTGGTACCATCACAAG-3′, were used, whereas the sense oligonucleotide, 5′-CCGACCATGGTAGTGTTC-3′, was taken as a control. Oligonucleotides were dissolved in sterile deionized water to a final concentration of 1 mM, aliquoted, and stored at −20°C until use. HeLa cells were transfected with each oligonucleotide using LipofectAMINE (Life Technologies, Inc.). Oligonucleotides (6 μg), transfection reagent (21 μl), and OPTI-MEM (1 ml) were mixed and added to wells of confluent cell monolayers according to the manufacturer's instructions. The oligonucleotide incubation medium was replaced every 12 h for 48 h.

Subcellular fractionation

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 6000 rpm. The lysates were fractionated into cytosolic and particulate fractions by ultracentrifugation at 100 000 *g* for 45 min at 4°C. 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 designated as the particulate fraction.

Immunoblotting

HeLa cell lysates were boiled in a Laemmli sample buffer for 3 min, and 30 μg of each total protein was subjected to SDS–polyacrylamide gel electrophoresis (PAGE) on 15% slab gels for the analysis of $p21^{WAF1/Cip1}$, and 10% slab gels for the analysis of PKC isoforms. For immunodetection of acetylated histone H3/H4, histone fractions were isolated by established techniques and subjected to SDS–PAGE on 15% slab gels (Han *et al*., 2000). Proteins were transferred onto polyvinylidene difluoride membranes, blocked for 30 min in TBS containing 0.1% Tween-20 and 5% (w/v) dry skim milk powder, and incubated overnight with primary antibodies. The membranes were then washed with TBS-T and incubated for 1 h with an anti-rabbit or an anti-mouse secondary antibody. Bound antibodies were visualized with the Enhanced Chemiluminescence detection kit (Amersham Life Sciences, IL, USA).

RT–PCR

Total RNA was extracted using TRI_{ZOL} reagent (Invitrogen). The integrity of RNA was verified by agarose gel electrophoresis and ethidium bromide staining. In total, 1 mg of RNA was used as a template for each reverse transcriptase (RT)-mediated PCR (RT–PCR) using an RNA PCR kit (Perkin-Elmer). Primer sequences for $p21^{WAF1/Cip1}$ and GAPDH were described previously (Han *et al*., 2001).

Chromatin immunoprecipitation

Analysis was performed by means of a kit protocol (Upstate Biotechnology). Chromatin from sonicated 1×10^6 HeLa cells was precleared with salmon-sperm DNA-saturated protein G sepharose and was precipitated using acetylated histone H3 antibody. Samples were analysed by PCR using ExTaq polymerase (Takara, Japan) and primers to amplify the p21 promoter region.

Abbreviations

HDAC, histone deacetylase; PKC, protein kinase C; Cdk, cyclin-dependent kinase; TSA, trichostatin A; NaB, sodium butyrate; SAHA, suberoylanilide hydroxamic acid; PDBu, phorbol-dibutyrate; ERK, extracellular-regulated protein kinase; PKA, protein kinase A; MAPK, mitogen-activated protein kinase.

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