Ceramide-induced Apoptosis by Translocation, Phosphorylation, and Activation of Protein Kinase C in the Golgi Complex*

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Takatoshi Kajimoto‡, Yasuhito Shirai‡, Norio Sakai‡, Toshiyoshi Yamamoto‡, Hidenori Matsuzaki‡, Ushio Kikkawa, and Naoki Saito‡

From the Laboratories of Molecular Pharmacology and Biochemistry, Bioinformation Research Center, Kobe University, 1-1 Rokkodai-cho, Nada-ku, Kobe 657-8501, Japan, and the Department of Molecular and Pharmacological Neuroscience, Graduate School of Biomedical Sciences, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8553, Japan

Protein kinase C (PKC), a Ca²⁺/phospholipid-dependent protein kinase, is known as a key enzyme in various cellular responses, including apoptosis. However, the functional role of PKC in apoptosis has not been clarified. In this study, we focused on the involvement of PKCδ in ceramide-induced apoptosis in HeLa cells and examined the importance of spatiotemporal activation of the specific PKC subtype in apoptotic events. Ceramide-induced apoptosis was inhibited by the PKCδ-specific inhibitor rottlerin and also was blocked by knockdown of endogenous PKCδ expression using small interfering RNA. Ceramide induced the translocation of PKCδ to the Golgi complex and the concomitant activation of PKCδ via phosphorylation of Tyr³¹¹ and Tyr³³² in the hinge region of the enzyme. Unphosphorylatable PKCδ (mutants Y311F and Y332F) could translocate to the Golgi complex in response to ceramide, suggesting that tyrosine phosphorylation is not necessary for translocation. However, ceramide failed to activate PKCδ lacking the C1B domain, which did not translocate to the Golgi complex, but could be activated by tyrosine phosphorylation. These findings suggest that ceramide translocates PKCδ to the Golgi complex and that PKCδ is activated by tyrosine phosphorylation in the compartment. Furthermore, we utilized species-specific knockdown of PKCδ by small interfering RNA to study the significance of phosphorylation of Tyr³¹¹ and Tyr³³² in PKCδ for ceramide-induced apoptosis and found that phosphorylation of Tyr³¹¹ and Tyr³³² is indispensable for ceramide-induced apoptosis. We demonstrate here that the targeting mechanism of PKCδ, dual regulation of both its activation and translocation to the Golgi complex, is critical for the ceramide-induced apoptotic event.

The protein kinase C (PKC) family is a group of phospholipid-dependent serine/threonine protein kinases consisting of at least 10 subtypes that can be classified into three subgroups, classical, novel, and atypical (1–3). The classical PKCs (α, βI, βII, γ, and δ), which have a Ca²⁺-binding region (C2 region) and two cysteine-rich regions, are activated by Ca²⁺, phosphatidylserine and diacylglycerol, or phorbol esters (4). The novel PKCs (ε, η, ζ, and θ), which lack the C2 region, are activated by phosphatidylserine and diacylglycerol or phorbol esters without Ca²⁺ (5). The atypical PKCs (ζ and η), which lack the C2 region and have only one cysteine-rich region, are dependent on phosphatidylserine, but are not affected by diacylglycerol, phorbol esters, or Ca²⁺ (6).

There is increasing evidence showing that PKC is involved in apoptosis (7). Among multiple subtypes of PKC, PKCδ has been implicated as a pro-apoptotic kinase, mostly by acting as a target of caspase-3 (8), whereas PKCα and PKCδ inhibit apoptosis by phosphorylating Bcl-2 or by increasing the expression of Bcl-2, respectively (9, 10). Apoptotic stimuli such as DNA-damaging agents (11), Fas ligand (12), and etoposide (8, 13, 14) cause the cleavage of PKCδ and the accumulation of the constitutively active fragment in the nuclei, and then the catalytic domain of PKCδ is supposed to induce nuclear fragmentation and cell apoptosis (15). Recent studies have also demonstrated that the cleaved catalytic domain of PKCδ acts as a sphingosine-dependent kinase (16). In addition, it has been reported that PKCδ is essential for the apoptosis of keratinocytes and LNCaP cells in response to 12-phorbol 13-myristate 13-acetate (17, 18).

Ceramide generated by transient hydrolysis of sphingomyelin has recently emerged as an intracellular lipid mediator implicated in various cellular responses, including programmed cell death (19–23). The regulation of PKC activity by ceramide has been reported, but the results are still controversial: ceramide has been shown to activate PKCα or to inhibit PKCδ in renal mesangial cells in vitro (24). It has also been reported that ceramide induces the translocation of PKCδ and PKCe from the membrane to the cytosol (25), of PKCα from the cytosol to the membrane (26), or of PKCδ from the cytosol to the mitochondria (27).

We have reported that ceramide-induced PKCδ-specific translocation to the Golgi complex is accompanied by PKCδ activation via its tyrosine phosphorylation in HeLa cells (28), although the functional significance of the Golgi-targeted PKCδ by ceramide is unknown. Furthermore, previous reports have demonstrated that various stimuli result in the tyrosine phosphorylation and activation of PKCδ: PKCδ is tyrosine-phosphorylated by 12-phorbol 13-myristate acetate (29), epidermal growth factor (30), pleiotropic growth factor (31), ligands for the IgE receptor (32), and H₂O₂ (33, 34). Tyrosine residues that are specifically phosphorylated by individual stimulation have been also determined. Tyrosines 52, 64, 155, and 187 of PKCδ are phosphorylated in response to 12-phorbol...
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Fig. 1. Effect of PKCδ on ceramide-induced apoptosis in HeLa cells. A, inhibition of ceramide-induced apoptosis in HeLa cells by rottlerin, a PKCδ-specific inhibitor. After pretreatment with 5 μM rottlerin for 1 h, HeLa cells were treated with 10 μM C2-ceramide (C2-Cer) or C2-dihydroceramide (DH-C2-Cer) for 24 h. Chromatin condensation of cell apoptosis was measured as described under “Experimental Procedures.” Bars = 20 μm. B, inhibition of ceramide-induced apoptosis in HeLa cells by siRNA from endogenous PKCδ. After knockdown of endogenous PKCδ using siRNA, HeLa cells were stimulated with 10 μM C2-ceramide. Cell apoptosis induced by C2-ceramide was also determined after 24 h by bisbenzimide (Hoechst 33258) staining. C, requirement of PKCδ for release of cytochrome c (Cyto c) from mitochondria. HeLa cells were transfected with siRNA from human PKCδ. After 2 days, the cells were treated with 10 μM C2-ceramide. 18 h after treatment, the cells were fixed, and cytchrome c was visualized by immunofluorescence as described under “Experimental Procedures.” Furthermore, the cells were stained with bisbenzimide to visualize the nuclei. Bars = 10 μm.

13-myristate acetate (29), platelet-derived growth factor (31), and etoposide (8), and H2O2 treatment results in the phosphorylation of tyrosines 311, 332, and 512 (33). It has also been reported that tyrosines 311 and 332 are phosphorylated by Src (34). However, the activation of PKCδ reported that tyrosines 311 and 332 are phosphorylated by Src (33). It has also been reported that tyrosines 311 and 332 are phosphorylated by Src family kinases (31, 34). However, the activation of PKCδ by tyrosine phosphorylation is not always accompanied by apoptosis (31, 35). In this study, we have determined the tyrosine of PKCδ that is phosphorylated by ceramide treatment and the significance of both the activation and translocation of PKCδ in ceramide-induced apoptosis.

EXPERIMENTAL PROCEDURES

Materials—n-erythro-C2-ceramide and n-erythro-dihydro-C2-ceramide (36) were purchased from BIOMOL Research Labs Inc. (Plymouth Meeting, PA). PP2 (AG1879; 4-amino-5-(chlorophenyl)-7-(butyl)pyrazolo[3,4-d]pyrimidine), herbimycin A, rottlerin (37), and Hoechst 33258 (bisbenzimide) were purchased from Sigma. Vanadate (Na3V04) was purchased from Wako Pure Chemical Industries (Osaka, Japan). Anti-PKCδ polyclonal antibody was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Anti-phosphotyrosine antibody (clone 4G10) was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Anti-active caspase-3 antibody was purchased from Promega (Madison, WI). Peroxidase-conjugated goat anti-mouse or anti-rabbit IgG and Alexa 546-labeled goat anti-mouse IgG were purchased from Amer sham Biosciences. Calf thymus histone H1 was purchased from Roche Applied Science (Basel, Switzerland). Ac-DMDQ-CHO (Ac-Asp-Met-Gln-Asp-H (aldehyde)) was purchased from the Peptide Institute, Inc. (Osaka).

Construction of Plasmids Encoding the PKCs—FLAG epitope-tagged expression plasmids of rat PKCδ were constructed as described (33, 34). The constructs encoding green fluorescent protein (GFP)-conjugated rat PKCδ were previously described (35). GFP was fused to the C terminus of PKCδ. The cDNA encoding the C1B domain (amino acids 231–260) deletion mutant of PKCδ was generated by PCR using BS391 (rat PKCδ-GFP in pTB701) (35) as the template. The primers were synthesized with an AvaiI site at both the 3′ terminus (amino acid 230) and the 5′ terminus (amino acid 281) to maintain the amino acids of the joint region. The PCR product were digested with AvaiI/ EcoRI or AvaiI/BamHI and then subcloned into the EcoRI/BglII sites of BS340 (GFP in pTB701) (35).

Expression of PKCδ Protein in HeLa Cells—HeLa cells were purchased from the Riken Cell Bank (Tsukuba, Japan) and cultured in minimal essential medium containing 100 units/ml penicillin and 100 μg/ml streptomycin with 10% fetal bovine serum. For lipofection, cDNAs (~5.5 μg) were transfected into 5 × 10⁶ HeLa cells using FuGENE 6 (Roche, Indianapolis, IN) according to the manufacturer’s standard protocol.

Observation of PKCδ-GFP Translocation—HeLa cells expressing PKCδ-GFP fusion proteins were spread onto glass-bottomed culture dishes (Mattek Corp., Ashland, MA) and cultured for at least 16 h before observation. The culture medium was replaced with normal HEPES buffer composed of 135 mM NaCl, 5.4 mM KCl, 1 mM MgCl2, 1.8 mM CaCl2, 5 mM HEPES, and 10 mM glucose (pH 7.3). The fluorescence of GFP was monitored under a Zeiss LSM 510 confocal laser scanning fluorescence microscope at 488 nm excitation with a 505/550-nm band-pass filter. All experiments were performed at 37 °C.

Data Analysis of PKCδ-GFP Translocation—After treatment with C2-ceramide, the time course of translocation was recorded as a time series of 30 images at 1-min intervals for each experiment. Fluorescence in the Golgi complex, cytoplasm, or nucleoplasm was measured using Zeiss LSM 510 software. Because the PKCδ distribution in the nucleoplasm scarcely changed, the fluorescence intensity in the nucleoplasm was used as the fluorescence intensity in the cytoplasm or Golgi complex divided by the fluorescence intensity in the nucleoplasm in a 5–10-μm² region of interest. For each time point, the Golgi or cytoplasmic fluorescence was calculated in at least five different regions of interest. These values were averaged and plotted to generate a time course of translocation.

Co-detection of Tyrosine Phosphorylation and PKCδ-GFP Translocation by Ceramide—HeLa cells were fixed before or after ceramide treatment with a fixative containing 4% paraformaldehyde and 0.2% picric acid in 0.01 M phosphate-buffered saline (pH 7.4) for 30 min. The cells were then incubated with anti-phosphotyrosine monoclonal antibody (diluted 1:200) and with Alexa 546-labeled goat anti-mouse IgG (diluted 1:1000) for 30 min. The fluorescence of GFP and Alexa 546 was observed under the confocal laser scanning fluorescent microscope.
**Fig. 2. Effect of caspase-3 on ceramide-induced apoptosis.** A, effect of Ac-DMQD-CHO on apoptosis in HeLa cells induced by C2-ceramide. After pretreatment with the caspase-3-specific inhibitor Ac-DMQD-CHO (100 \mu M) for 1 h, HeLa cells were treated with 10 \mu M C2-ceramide (C2-Cer). Cell apoptosis induced by C2-ceramide was also determined after 24 h by bisbenzimide staining. B, immunoblot analysis of active caspase-3 in HeLa cells. After treatment with 10 \mu M C2-ceramide for 24 h or with 50 \mu M etoposide for 24 h, total cell lysates from HeLa cells expressing wild-type PKC6-GFP were subjected to SDS-PAGE and Western blot analysis. The membrane was probed with anti-active caspase-3 antibody.

**Immunoprecipitation**—HeLa cells expressing various types of PKC6 were harvested with 1 ml of homogenate buffer (250 mM sucrose, 10 mM EGTA, 2 mM EDTA, 20 mM Tris-HCl, 200 \mu g/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 10 mM NaF, and 1 mM Na3VO4 (pH 7.4)) and centrifuged at 2000 \times g. The cells were resuspended in 300 \mu l of buffer composed of 10 mM Tris-HCl, 1% Nonidet P-40, 0.15 M NaCl, 1 mM EDTA, 10 \mu g/ml aprotinin, 10 mM NaF, and 1 mM Na3VO4 (pH 7.8) containing 1% Triton X-100 and sonicated (output 5, 50% duty, 10 times at 4 °C; UD-210, Tomy Seiko Co. Ltd., Tokyo, Japan), and the supernatant was used after centrifugation at 19,000 \times g for 15 min. For immunoprecipitation, the supernatant of the total fraction was incubated with anti-FLAG monoclonal antibody or anti-GFP polyclonal antibody (diluted 1:10,000) using an enhanced chemiluminescence detection kit (Amer sham Biosciences).

**Kinase Analysis**—PKC6 activity was assayed by measuring the incorporation of 32P from [gamma-32P]ATP into substrate as described (34, 35). In brief, the kinase activities of FLAG-PKC6 or PKC6-GFP fusion proteins immunoprecipitated from HE-La cells after stimulation with C2-ceramide or H2O2 (10 \mu l of suspended pellet) were measured with calf thymus histone H1 as the substrate without any PKC activators such as phosphatidylserine or diolein. The reaction mixture contained 20 mM Tris-HCl (pH 7.5), 10 mM MgCl2, 20 \mu M ATP, 15–50 kBq of [gamma-32P]ATP, and 200 \mu g/ml histone H1. The incubation was carried out for 10 min at 30 °C.
ing RNA (siRNA) transfection. For expression of exogenous PKCs, cDNA was transfected after 24 h of siRNA transfection.

Measurement of Cell Apoptosis—After various treatments in serum-free minimal essential medium for 24 h, HeLa cells were collected and fixed with 4% paraformaldehyde and 0.2% picric acid for 15 min. The cells were then stained with 1 ml of bisbenzimide (16 μg/ml) in phosphate-buffered saline, and the number of cells showing apoptotic chromatin changes in their nuclei was counted under a fluorescence microscope. Cells with two or more condensed chromatin fragments were considered to be apoptotic. For detection of cytochrome c release from mitochondria, the cells were incubated with anti-cytochrome c antibody and with Alexa 488-labeled goat anti-mouse IgG (Amersham Biosciences).

RESULTS

Role of PKCδ in the Apoptotic Effect of Ceramide—We first investigated the morphological changes in HeLa cells in response to Cδ-ceramide, a membrane-permeable analog of ceramide. As shown in Fig. 1A, ≈80% of the ceramide-treated HeLa cells, but not the Cδ-dihydroceramide (an inactive form of Cδ-ceramide)-treated cells, showed an altered nuclear structure with condensed chromatin fragments upon bisbenzimide staining), indicating the specificity of the apoptotic action of Cδ-ceramide. Possible involvement of PKCδ in ceramide-induced apoptosis was examined using the PKCδ-specific inhibitor rottlerin and knockdown of PKCδ by siRNA. Rottlerin significantly inhibited the apoptotic effect of Cδ-ceramide, reducing the number of apoptotic cells to 23.5% (Fig. 1A) without affecting the basal level of cell apoptosis. Knockdown of endogenous PKCδ in HeLa cells using siRNA from human PKCδ (38) significantly inhibited the apoptotic response to Cδ-ceramide in HeLa cells (Fig. 1B). Furthermore, the release of cytochrome c from mitochondria (27) by Cδ-ceramide treatment was also blocked by knockdown of endogenous PKCδ (Fig. 1C). These findings strongly suggest that activation of PKCδ is indispensable for ceramide-induced apoptosis.

PKCδ can be cleaved by a caspase-dependent process during apoptosis (8, 39). We therefore examined the ability of the cell-permeable caspase-3-specific inhibitor Ac-DMQD-CHO (100 μM) to block the apoptosis induced by Cδ-ceramide in HeLa cells. As shown in Fig. 2A, pretreatment of the cells for 1 h with Ac-DMQD-CHO failed to inhibit the apoptotic effect of Cδ-ceramide. In addition, we examined whether active caspase-3 is expressed in Cδ-ceramide-treated HeLa cells. Using Western blot analysis, we found that the active form of caspase-3 was expressed in the etoposide-treated cells, but not in the Cδ-ceramide-treated cells (Fig. 2B).

PKCδ Tyrosine Phosphorylation Sites in Ceramide-treated Cells—To determine the phosphorylation sites responsible for ceramide-induced PKCδ activation, we mutated four tyrosine residues (Y187, Y311, Y332, and Y512) in PKCδ to phenylalanine, as these tyrosine residues have been identified as the phosphorylation sites responsible for PKCδ activation in response to various stimuli, such as etoposide, platelet-derived growth factor, and H2O2 (8, 31, 33). The Y187F and Y512F mutants showed significant phosphorylation by ceramide (as much as wild-type PKCδ), whereas mutation of Y311F or Y332F evidently reduced the ceramide-induced phosphorylation, and phosphorylation was undetectable in the double mutant Y311F/Y332F (Fig. 3A). Antibodies against phosphopeptides corresponding to each phosphorylation site (Y311F or Y332F) demonstrated that both Y311F and Y332F were phosphorylated after ceramide treatment (Fig. 3B). Ceramide-induced tyrosine phosphorylation of PKCδ was blocked by herbimycin A and PP2 inhibitors of Src family protein-tyrosine kinases (Fig. 3C).

To study the correlation between phosphorylation of Tyr311 and Tyr332 and the kinase activity of PKCδ, we measured the kinase activities of immunoprecipitated PKCδ and its mutants in HeLa cells with and without ceramide treatment. As shown in Fig. 4, Cδ-ceramide increased by 1.5-fold the kinase activities of wild-type PKCδ and the Y187F and Y512F mutants, but the kinase activities of the Y311F, Y332F, and Y311F/Y332F mutants were not altered. As each single mutation of Tyr311 or Tyr332 abolished the ceramide-induced activation, simultaneous phosphorylation of Tyr311 and Tyr332 may be essential for PKCδ activation.

Tyrosine Phosphorylation and Activation of PKCδ in the Golgi Complex—We have demonstrated the ceramide-induced activation of PKCδ concomitant with its translocation to the
Golgi complex (28). To determine whether the tyrosine phosphorylation of PKCδ is required for translocation of PKCδ to the Golgi complex, we investigated the ceramide-induced translocation of GFP-tagged PKCδ, Y311F, Y332F, and Y311F/Y332F in HeLa cells. Similar to wild-type PKCδ, the unphosphorylatable mutants also translocated to the Golgi complex in response to ceramide (Fig. 5). We concluded that the tyrosine phosphorylation and activation of PKCδ are not necessary for the translocation of PKCδ by ceramide stimulation.

As tyrosine phosphorylation is not necessary for the translocation of PKCδ to the Golgi complex, it is possible that PKCδ is tyrosine-phosphorylated after its accumulation in the Golgi complex. To investigate whether PKCδ in the Golgi complex or cytoplasm is tyrosine-phosphorylated after stimulation with C2-ceramide, we visualized the tyrosine phosphorylation in HeLa cells expressing PKCδ-GFP after C2-ceramide treatment for 20 min using anti-phosphotyrosine antibody. Immunoreactivity for phosphotyrosine (pTyr) accumulated in the perinuclear region. However, pTyr was also observed in the cytoplasm did not overlap with the red fluorescence of pTyr. This finding suggests that PKCδ is tyrosine-phosphorylated in the perinuclear region. However, pTyr was also observed in the perinuclear region in ceramide-treated HeLa cells expressing Y311F/Y332F PKCδ-GFP. Because a pTyr immunoreaction was also seen in HeLa cells not expressing Y311F/Y332F PKCδ-GFP (Fig. 6A, arrows), it is possible that the tyrosine phosphorylation of endogenous PKCδ was detected by anti-pTyr antibody. To distinguish between endogenous and exogenous PKCδ, we utilized the siRNA technique, which reduces the expression of human endogenous PKCδ, but does not affect exogenous rat PKCδ (Fig. 6B). In HeLa cells with siRNA from human PKCδ, ceramide-induced accumulation of pTyr in the perinuclear region was detected only in cells expressing wild-type PKCδ-GFP, although pTyr-positive dots on the plasma membrane were seen in all cells. The absence of pTyr immunoreaction in the perinuclear region in Y311F/Y332F PKCδ-GFP expressing cells suggests that PKCδ accumulated in the Golgi complex by ceramide is phosphorylated at Tyr311 and Tyr332.

But, it was still unclear whether the tyrosine phosphorylation of PKCδ occurred before or after translocation to the Golgi complex. It has been reported that H2O2 treatment induces the tyrosine phosphorylation and activation of PKCδ without affecting its localization (35). To determine where PKCδ is phosphorylated, we constructed a PKCδ mutant lacking the C1B domain (ΔC1B), which could be tyrosine-phosphorylated by H2O2 in the cytoplasm, but failed to translocate to the Golgi complex upon ceramide treatment. As shown in Fig. 7A, C2-ceramide treatment did not translocate ΔC1B. H2O2 induced the tyrosine phosphorylation and kinase activation of ΔC1B and wild-type PKCδ to a similar extent; however, ΔC1B was neither tyrosine-phosphorylated nor activated by C2-ceramide treatment (Fig. 7, B and C). These results show that, after ceramide treatment, PKCδ accumulated in the Golgi complex via its C1B domain and then was tyrosine-phosphorylated and activated in the compartment.

Translocation of PKCδ to the Golgi complex occurs upon association of the enzyme with the Golgi complex, but not upon its tight binding to the Golgi membrane (28). The counterbalance of association/dissociation of PKCδ might be regulated by the tyrosine phosphorylation of PKCδ. We examined the effects of the protein-tyrosine phosphatase (PTP) inhibitor vanadate (200 μM) on the activation and accumulation of PKCδ by ceramide. As shown in Fig. 8A, inhibition of PTP increased the intensity of PKCδ in the Golgi complex and decreased that in the cytoplasm. Furthermore, vanadate treatment increased the activity of PKCδ by up to -200% (Fig. 8B).

Targeting of PKCδ Is Indispensable for Apoptotic Effects in HeLa Cells—To explore the functional interaction between apoptosis and PKCδ targeting to the Golgi complex accompanied by its tyrosine phosphorylation-mediated activation, we investigated ceramide-induced apoptotic changes in HeLa cells expressing PKCδ or its mutants. Bisbenzimide staining revealed that HeLa cells overexpressing wild-type PKCδ-GFP or Y311F/Y332F PKCδ-GFP exhibited a robust apoptotic response to C2-ceramide similar to that of untransfected control cells (Fig. 9). However, endogenous PKCδ was tyrosine-phosphorylated in the Golgi complex, as demonstrated in Fig. 6A, and may cause apoptosis that is independent of overexpression of PKCδ-GFP. We examined the effects of rat wild-type or Y311F/Y332F PKCδ on the apoptotic response to C2-ceramide in HeLa cells.
that were treated with siRNA for knockdown of human endogenous PKCδ. The inhibitory effect of siRNA from human PKCδ on ceramide-induced apoptosis was restored by overexpression of rat PKCδ. In contrast, Y311F/Y332F PKCδ-GFP could not restore the apoptotic change induced by ceramide (Fig. 9). These results indicate that the phosphorylation of Tyr311 and Tyr332 in PKCδ is necessary for the apoptotic effect of ceramide. We further studied whether the phosphorylation of Tyr311 and Tyr332 in PKCδ is enough for apoptosis or whether the targeting (translocation and activation) of PKCδ to the Golgi complex is additionally necessary for the apoptotic effect of ceramide. PKCδ-GFP was tyrosine-phosphorylated and activated in the cytoplasm without any translocation upon H$_2$O$_2$ treatment (Fig. 10, A and B). Fig. 10C shows that H$_2$O$_2$-induced tyrosine phosphorylation and activation of PKCδ in the cytoplasm did not induce cell apoptosis.

**DISCUSSION**

Using GFP-tagged PKC subtypes, the dynamic movement of PKC has been visualized in living cells, and the individual function of each PKC subtype in various signaling cascade has been studied (40, 41). Translocation of PKC varies depending on PKC subtypes (41), and extracellular stimuli and PKC translocation to the specific intracellular compartment are necessary for the recognition and phosphorylation of their substrates in the compartment (PKC targeting) (42). As the spatiotemporally different translocation of PKC results in distinct...
cellular responses (35), this strongly suggests that the targeting mechanisms of PKC subtypes determine their individual roles in cell signaling pathways. We have demonstrated that ceramide (generated by transient hydrolysis of sphingomyelin via receptor-mediated stimulation by extracellular ligand) induces translocation of PKCβ from the cytoplasm to the Golgi complex and its activation mediated by tyrosine phosphorylation (28). In this study, we explored the importance of PKCβ and its tyrosine phosphorylation in the induction of apoptosis in HeLa cells upon treatment with ceramide.

Activation of PKCβ via tyrosine phosphorylation has been reported by several investigators. Tyr311, Tyr332, and Tyr312 are candidates for the tyrosine residues phosphorylated by various stimuli (8, 29, 31, 33). Substitution of these tyrosine residues with phenylalanine revealed that two tyrosine residues (Tyr311 and Tyr332) in the hinge region of PKCβ are the tyrosine phosphorylation sites of PKCβ in C2-ceramide-treated HeLa cells, whereas mutation of Tyr187 or Tyr512 did not alter the phosphorylation. It is noteworthy that phosphorylation of both Tyr311 and Tyr332 is necessary for the activation of PKCβ. The present finding is in good agreement with the report by Konishi et al. (34) that phosphorylation of Tyr311 is a critical step in the generation of active PKCβ in response to H2O2 in vitro. However, the relation between the tyrosine phosphorylation of PKCβ and its activation is still controversial, and it is possible that other tyrosine residues are phosphorylated at undetectable levels.

Ceramide has been shown to activate tyrosine kinases, including Src (43) and Lck (44–46), and association of PKCβ with Src family tyrosine kinases has been reported (30, 31, 34, 47–53). The tyrosine phosphorylation of PKCβ by ceramide was inhibited by inhibitors of Src family protein-tyrosine kinases, suggesting that a certain tyrosine kinase in the Src family phosphorylates PKCβ in response to ceramide. Because the existence of Src family kinases in the Golgi complex and the phosphorylation of specific substrates by the kinases have been reported previously (54–56), it is possible that an unidentified tyrosine kinase such as Src phosphorylates PKCβ in the Golgi complex in HeLa cells after ceramide treatment.

The present results using siRNA and the PKCβ inhibitor show that activation of PKCβ is required for ceramide-induced apoptosis. It has been thought that the apoptotic effect of PKCβ is associated with the cleavage of PKCβ in the hinge region, followed by the production of the constitutively active catalytic domain. Cleavage of the catalytic domain of PKCβ by caspase-3 has been reported in cells treated with DNA-damaging agents (11), Fas ligand (12), and etoposide (8, 13, 14). However, no significant degradation of PKCβ was observed in HeLa cells treated with ceramide by immunoblot analysis (28). In addition, the active form of caspase-3 was undetectable in ceramide-treated cell homogenates, and pretreatment with the caspase-3-specific inhibitor Ac-DMQD-CHO failed to inhibit the ceramide-induced apoptosis in HeLa cells (Fig. 2). These results suggest that the caspase-3 cascade appears not to be involved in this ceramide-induced apoptotic mechanism mediated by PKCβ in HeLa cells, although other caspases such as caspase-9 may play some roles in this pathway. It is noteworthy that the caspase-3 cleavage site in PKCβ is localized between the two tyrosine phosphorylation sites, Tyr311 and Tyr332. It is possible that tyrosine phosphorylation around the caspase-3 cleavage may protect the cleavage, but activates PKCβ without cleavage, although the molecular mechanism of the activation pathway is unknown.

It has been demonstrated that the C1B domain is responsible for the translocation of PKCe to the Golgi complex induced by arachidonic acid and ceramide (57). Deletion of the C1B domain of PKCe abolishes its translocation to the Golgi complex. As PKCe with a C1B domain derived from PKCβ also shows ceramide-induced translocation to the Golgi complex (57), this suggests that PKCβ lacking the C1B domain cannot translocate to the Golgi complex in response to ceramide. ΔC1B is supposed to be activated by phosphorylation of Tyr311 and Tyr332, which are conserved in the deletion mutant. Using the ΔC1B deletion mutant, we have shown that, in addition to PKCβ activation, translocation of PKCβ to the Golgi complex is indispensable for the apoptotic effect of ceramide.

Previous photobleaching studies (28) demonstrated that ceramide modulates the counterbalance of association/dissociation of PKCβ with the Golgi complex. Ceramide translocates PKCβ by increasing its association with the Golgi complex in the
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counterbalance. It is important that inhibition of PTP increased both the activity and accumulation of PKCδ. This finding suggests that PKCδ association with or dissociation from the Golgi complex is regulated by tyrosine phosphorylation. However, as accumulation of PKCδ in the Golgi complex was independent of tyrosine phosphorylation (Fig. 5), this suggests that dissociation of PKCδ from the Golgi complex is regulated by dephosphorylation of PKCδ by PTP. The small increase in PKCδ activity (50% increase) (Fig. 4) after ceramide treatment may be due to the fact that most of the PKCδ in the cytoplasm was inactive and part of the PKC was activated by ceramide because both activation and inactivation of PKCδ occurred only in the Golgi complex.

The species-specific knockdown of PKCδ utilized in this study is a useful technique for analyzing the subtype-specific function of PKC. Overexpression of unphosphorylatable PKCδ failed to show dominant-negative effects on ceramide-induced apoptosis (Fig. 9). This is perhaps due to the fact that a considerable amount of endogenous PKCδ is present in HeLa cells (enough to induce apoptosis). To elucidate the significance of the tyrosine phosphorylation of PKCδ for ceramide-induced apoptosis, it was necessary to reduce the amount of endogenous PKCδ. However, knockdown of human endogenous PKCδ only revealed that endogenous PKCδ is required for the induction of apoptosis. Knockdown of endogenous PKCδ was compensated by rat exogenous PKCδ, which is resistant to siRNA from human PKCδ. This compensation demonstrated that Tyr\(^{311}\) and Tyr\(^{332}\) are tyrosine-phosphorylated in the Golgi complex after ceramide treatment and then induce apoptosis.

In this work, we studied the importance of the PKCδ targeting mechanism (translocation and activation) in the induction of apoptosis in HeLa cells upon treatment with ceramide. We have demonstrated that ceramide induces the translocation of PKCδ to the Golgi complex and its activation via phosphorylation of Tyr\(^{311}\) and Tyr\(^{332}\) by Src-like kinases and that this spatiotemporal regulated activation results in the induction of apoptosis. Activation of PKCδ alone is insufficient for apoptotic events, and the targeting of PKCδ to the Golgi complex is an essential step in ceramide-induced apoptosis. As described previously (28), the activation of receptors such as interferon-γ and tumor necrosis factor-α receptors results in the translocation and activation of PKCδ, suggesting that apoptosis via PKCδ-specific targeting to the Golgi complex occurs under physiological conditions. Although the target molecule of PKCδ in this mechanism is not clear, the results strongly suggest that the targeting mechanism of PKCδ (activation via tyrosine phosphorylation and translocation to the Golgi complex) is indispensable for ceramide-induced apoptosis.
