Ceramide Directly Activates Protein Kinase Cζ to Regulate a Stress-activated Protein Kinase Signaling Complex*

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We have previously shown that interleukin 1 (IL-1)-receptor-generated ceramide induces growth arrest in smooth muscle pericytes by activating an upstream kinase in the stress-activated protein kinase (SAPK) cascade. We now report the mechanism by which ceramide activates the SAPK signaling pathway in human embryonic kidney cells (HEK-293). We demonstrate that ceramide activation of protein kinase Cζ (PKCζ) mediates SAPK signal complex formation and subsequent growth suppression. Ceramide directly activates both immunoprecipitated and recombinant human PKCζ in vitro. Additionally, ceramide activates SAPK activity, which is blocked with a dominant-negative mutant of PKCζ. Co-immunoprecipitation studies reveal that ceramide induces the association of SAPK with PKCζ, but not with PKCe. In addition, ceramide treatment induces PKCζ association with phosphorylated SEK and MEKK1, elements of the SAPK signaling complex. The biological role of ceramide to induce cell cycle arrest is mimicked by overexpression of a constitutively active PKCζ. Together, these studies demonstrate that ceramide induces cell cycle arrest by enhancing the ability of PKCζ to form a signaling complex with MEKK1, SEK, and SAPK.

Ceramide, a sphingolipid-derived second messenger molecule, has been implicated in growth inhibition, apoptosis, and cellular differentiation (1, 2). Inflammatory cytokines, including interleukin-1 (IL-1), tumor necrosis factor-α (TNF-α), and interferon-γ activate sphingomyelinases, resulting in increased cellular ceramide concentration (3–5). We have previously demonstrated that the cell-permeable ceramide analogue, C16-ceramide, mimics the effect of IL-1 to inhibit both tyrosine kinase receptor- and G-protein receptor-linked mitogenesis (6, 7). Yet, the mechanisms by which ceramide induce cell cycle arrest currently remain enigmatic.

Protein kinase Cζ (PKCζ) has been identified as a putative ceramide-activated protein kinase involved in growth control (8, 9, 10). Evidence suggests that PKCζ is directly activated by ceramide and not by diacylglycerol (DAG) (8, 9, 11). Several reports also relate ceramide-induced growth arrest to activation of stress-activated protein kinase (SAPK)/c-Jun N-terminal kinase, but the precise mechanism remains to be elucidated (6, 12, 13). Because ceramide has been shown to activate PKCζ and SAPK in the same cell type, it has been inferred, but not proven, that PKCζ is a critical element in ceramide-induced SAPK activation (11). It is likely that the site of action for ceramide is upstream of SAPK, because ceramide does not directly regulate immunoprecipitated SAPK activity in a cell-free system (7). We now demonstrate that ceramide-activated PKCζ recruits upstream components of the SAPK cascade into a signaling complex, resulting in growth arrest.

Activation of the SAPK signaling pathway is characterized by a cascade of protein kinases, which are recruited to the plasma membrane. Specifically, GTP-dependent activation of Rac or Cdc42 leads to recruitment of MEKK1 to the plasma membrane, where it is phosphorylated and activated (14). Activated MEKK1 directly phosphorylates and activates SEK, which in turn directly activates SAPK. Activation of Rac-1 by inflammatory cytokines or ceramides has been postulated to be one mechanism to activate the SAPK cascade (14–16). Because PKCζ does not directly regulate Rac-1 (17), an alternative mechanism for activation of SAPK could be ceramide-induced PKCζ activation of MEKK1 and/or SEK. Thus, it is hypothesized that ceramide regulation of the SAPK pathway also is dependent on direct activation of PKCζ.

In this study, we demonstrate that ceramide directly activates both immunoprecipitated and recombinant human PKCζ. Upon ceramide activation, PKCζ interacts with MEKK1, SEK, and SAPK to inhibit insulin-like growth factor-1 (IGF-1)-induced cell growth. Together, these findings suggest a novel role of ceramide in regulation and assembly of multi-protein signaling complexes.

MATERIALS AND METHODS

Human embryonic kidney 293 (HEK-293) cells were obtained from American Type Culture Collection (Rockland, MD). Anti-PKCα, -PKCδ, -PKCζ, -PCNA, -SAPK, -SEK1/AEK4, and -MEKK1 antibodies were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA). An antibody for the phosphorylated SEK was obtained from New England Biolabs (Cambridge, MA). Purified, recombinant PKCζ was obtained from Panvera Corp. (Madison, WI). Both cell-permeable C16-ceramide and physiological C18:1-ceramide were obtained from Calbiochem (La Jolla, CA) and Avanti Polar Lipids (Alabaster, AL). In addition, the inactive cell-permeable ceramide analogue, dihydro-C16-ceramide (DH), was obtained from Biomol (Plymouth Meeting, PA). Diacylglycerol (1,2-diolein, DAG) was purchased from Serva Research Laboratories (Ontario, Canada). Human IGF-1 and IL-1β recombinant proteins were purchased from Life Technologies, Inc. (Grand Island, NY). The enhanced chemiluminescence (ECL) detection kit was obtained from Amersham Pharmacia Biotech (Arlington Heights, IL).

Human Embryonic Kidney (HEK-293) Cell Culture—HEK-293 cells
are adenovirus-transformed human embryonic kidney cells of tubule epithelial origin. These cells express functional IGF receptors and are an excellent model for growth factor-induced mitogenesis as well as inflammatory cytokine-induced growth arrest (2, 18). Western blot analyses revealed that HEK-293 cells express PKCα, ε, and ζ.

Western blot analysis of the regulatory domain. Transfection efficiency was consistently ~40% as determined by green fluorescence protein co-transfection assay. Cells expressing the wild-type and dominant-negative mutant constructs were subjected to in vitro kinase assays to assess SAPK bioactivity, as described above. In addition, lysates from wild-type or constitutively active PKCζ-expressing cells were subjected to Western blot analysis to assess proliferating cell nuclear antigen (PCNA) expression. To verify that the transfections with constructs for PKCζ did not alter protein levels of other PKC isoforms, Western analyses were performed and PKCε or PKCε expression did not change (data not shown).

**Statistical Analysis**—Independent t tests were used to determine the significant differences between groups. The p value of the individual components was adjusted for multiple comparisons by the Bonferroni method. The data were expressed as mean ± S.E. All non-parametric data were analyzed by the Kruskal-Wallis test. In those experiments where the control optical density values were set to 100%, the S.E. for each of these control values was reported utilizing the non-transformed data.

**RESULTS**

**Ceramide Directly Stimulates PKCζ Bioactivity**—Ceramide has been shown to activate the SAPK cascade. However, the mechanism of activation has not been clearly defined. Because PKCζ has one cysteine-rich domain (CRD), which is suggested to interact with ceramide, but not diacylglycerol (DAG) (21), we hypothesize that ceramide-activated PKCζ regulates the SAPK cascade. Therefore, the ability of ceramide to directly and acutely affect the bioactivity of immunoprecipitated PKCζ from HEK-293 cells was assessed by performing in vitro reconstitution activity assays. The immunocomplexes were treated with physiological ceramide (C18:1-ceramide) and/or physiological DAG (1,2-diolein) for 15 min. Bioactivity was assessed by resolving radiolabeled phosphorylation of Histone IIIS. As shown in Fig. 1, A and B, the bioactivity of immunoprecipitated PKCζ was significantly increased (3-fold) by ceramide but not DAG treatment. When C18:1-ceramide-treated immunocomplexes were challenged with the addition of DAG, the bioactivity of PKCζ was significantly decreased. These results suggest an apparent reciprocal relationship between ceramide and DAG for PKCζ bioactivity.

To verify the stimulatory actions of physiological C18:1-ceramide on immunoprecipitated PKCζ, we performed direct in vitro kinase activity assay using purified, recombinant PKCζ (Fig. 1C). Consistent with the immunoprecipitated PKCζ kinase assay, physiological ceramide significantly activated recombinant PKCζ activity. Similar to results obtained in the immunoprecipitated PKC kinase assay, DAG treatment had no effect by itself but was able to reduce C18:1-ceramide-induced PKCζ activity. In addition, cell-permeable C6-ceramide mimicked the effect of physiological C18:1-ceramide to stimulate PKCζ activity in the cell-free assays (data not shown). In support of the specificity of the actions of ceramide on PKCζ, the inactive cell-permeable ceramide analogue, dihydro-C6-ceramide, had no effect on PKCζ activity. These studies, utilizing both immunoprecipitated and recombinant human PKCζ protein, confirm the hypothesis that bioactive ceramides directly activate PKCζ activity.

**Ceramide Does Not Change PKCζ Expression**—In addition to directly activating PKCζ, ceramide could also increase PKCζ activity by inducing PKCζ protein expression. We examined the PKCζ protein expression level by performing Western blot analysis using anti-PKCζ antibody. As shown in Fig. 2, when cycling (10% FBS) or non-cycling (serum-deprived, SD) HEK-293 cells were treated with cell-permeable C6-ceramide for
the quantification of the recombinant PKCζ. DAG diolein (no effect on either immunoprecipitated or recombinant PKCζ activity). For both immunoprecipitated and recombinant PKCζ assays, the phosphorylated histone bands were excised and quantified by laser densitometry. For the recombinant protein assays, phosphorylated histone IIIS protein was resolved on a 12% SDS-PAGE gel electrophoresis, visualized by autoradiography, and quantified using enhanced chemiluminescence. No significant changes in PKCζ protein expression were observed among all conditions tested. A, a representative Western blot. B, the quantification of the PKCζ bands (mean ± S.E.; n = 3; p < 0.05, Kruskal-Wallis test). The abbreviations used are: SD, serum deprivation; C6-Cer, C6-ceramide.

24 h, the protein expression level of PKCζ was not altered compared with the control cells without C6-ceramide treatment. These results demonstrate that ceramide regulates HEK-293 cells as a consequence of a direct activation of PKCζ and not up-regulation of PKCζ protein expression.

PKCζ Is a Necessary Component for Ceramide Activation of SAPK Activity—Because we demonstrated that ceramide directly activates PKCζ, we next examined whether ceramideduced SAPK activity is dependent on PKCζ. As shown in Fig. 3, HEK-293 cells overexpressing the WT PKCζ construct resulted in an increase in C6-ceramide-stimulated immunoprecipitated SAPK bioactivity. In contrast, C6-ceramide-induced SAPK activity was blocked with a dominant-negative mutant of PKCζ. These results suggest that PKCζ is a necessary signaling component for modulating ceramide-mediated activation of SAPK bioactivity.

Ceramide Augments PKCζ-SAPK Interaction—To further define the mechanism by which ceramide activates PKCζ leading to SAPK complex formation, we next examined whether PKCζ associates with SAPK. Therefore, to document if ceramide induces a potential interaction between PKCζ and SAPK, we performed co-immunoprecipitation assays. As shown in Fig. 4, HEK-293 cells treated with C6-ceramide specifically increased PKCζ association with SAPK1. Because ceramide induces the translocation of PKCe from the plasma membrane to the cytosol, an event consistent with inactivation (22), we also investigated if ceramide regulates PKCe-SAPK interaction as a negative control. Ceramide did not augment an association between PKCe and SAPK. These results demonstrate that ceramide specifically induces an interaction between PKCζ and SAPK.
IGF-1 induces DAG generation, which we have previously shown to inhibit ceramide-activated PKC\(\zeta\). Thus we investigated whether IGF-1 could diminish ceramide-induced PKC\(\zeta\)SAPK interactions. Our studies document that IGF-1, in contrast to ceramide, does not induce PKC\(\zeta\) association with SAPK. In fact, ceramide-induced association between SAPK and PKC\(\zeta\) was diminished. These data further support the specificity of ceramide-activated PKC\(\zeta\) to form SAPK signaling complexes.

Ceramide Induces PKC\(\zeta\) Association with p-SEK and MEKK1—Our results imply a role for ceramide to modulate PKC\(\zeta\) interactions with upstream elements of the SAPK signaling cascade. Activated SEK is the immediate upstream dual specificity kinase that phosphorylates SAPK on threonine and tyrosine residues. It is possible that bioactive PKC\(\zeta\) may recruit and activate SEK through phosphorylation on Ser-219 and Thr-223. Therefore, we next investigated the ability of ceramide to induce an association between PKC\(\zeta\) and bioactive (phosphorylated)-SEK (p-SEK) by co-immunoprecipitation assays. As shown in Fig. 5, A and B, C\(_6\)-ceramide or IL-1 treatment significantly increased (5-fold) the association of PKC\(\zeta\) with p-SEK in HEK-293 cells. Consistent with the effect of IGF-1 on ceramide-induced PKC\(\zeta\)SAPK interaction, IGF-1 treatment also reduced both C\(_6\)-ceramide- and IL-1-induced association of PKC\(\zeta\) with p-SEK. These results clearly demonstrate that ceramide regulates the activity of PKC\(\zeta\) and its interaction with p-SEK.

To further define the upstream SAPK signaling elements regulated by ceramide-activated PKC\(\zeta\), we also investigated whether ceramide induces an association between PKC\(\zeta\) and MEKK1. As shown in Fig. 5, C and D, we observed a strong association of PKC\(\zeta\) with MEKK1 in response to ceramide or IL-1 treatment. Again, IGF-1 reduced both C\(_6\)-ceramide- and IL-1-induced PKC\(\zeta\)MEKK1 interactions. These results strongly suggest that the stimulatory action of ceramide on SAPK activation are a result of activated PKC\(\zeta\) interacting with MEKK1 as well as SEK.

Ceramide-induced Growth Arrest Is Dependent on PKC\(\zeta\)—The critical role of PKC\(\zeta\) in ceramide- and IL-1-mediated inhibition of cell growth was assessed in HEK-293 cells transiently transfected with either wild-type (WT) or constitutively active PKC\(\zeta\). Cell cycle arrest was evaluated by proliferating cell nuclear antigen (PCNA) expression (Fig. 6). PCNA expression is used as a marker of cells entering the cell cycle at early G1 and S phases. In cells transfected with the WT PKC\(\zeta\)-expressing cells, a similar pattern of PCNA expression was observed in non-transfected or mock transfected cells (data not shown). Together, these re-
Ceramide and PKCζ

FIG. 4. Ceramide enhances PKCζ interaction with SAPK1. Protein interactions between select PKC isoforms and SAPK were assessed by Western analyses utilizing anti-PKCζ or -PKCε antibodies on SAPK1 immunoprecipitates from HEK-293 cells. PKC isoforms, co-immunoprecipitated with anti-SAPK1 antibody, were detected with enhanced chemiluminescence and quantified by laser densitometry. HEK-293 cell monolayers in 12-well plates were made quiescent in Dulbecco’s modified Eagle’s medium supplemented with 0.5% FBS for 24 h. The monolayers were then treated with vehicle control (0.01% MeSO₄), IGF-1 (50 ng/ml), C₆-ceramide (10⁻⁶ M), or IGF plus C₆-ceramide for 5 min. The basal interaction between SAPK/PKCζ (2.19 ± 0.21 arbitrary units) was slightly more than the interaction between SAPK/PKCε (1.45 ± 0.33). C₆-ceramide enhanced interactions of PKCζ, but not PKCε, with the SAPK1 pathway. Equal loading of the samples was assessed by reprobing the blots with anti-SAPK1 antibodies. A. Representative Western blots. B. The quantification of the PKC bands (mean ± S.E.; n = 3). Star, significantly different from vehicle control; p < 0.01, paired t-test. Suc, combination treatment is significantly different from individual treatment alone; p < 0.005, paired t-test.

results suggest that PKCζ is necessary for IL-1 and/or ceramide-induced cell cycle arrest. Moreover, these data imply that PKCζ/SAPK complex formation is required for ceramide-induced growth arrest.

DISCUSSION

The concept of signaling complex formation determining the specificity and selectivity of cellular responses is an exciting new area in the field of cellular communication. Recent studies have shown that scaffold/adaptor proteins serve to assemble multiple signaling proteins together in large scale aggregates. The importance of these scaffolding proteins, such as Jun-interacting protein-1, kinase suppressor of Ras, and 14-3-3, in signal transduction pathways is only recently being appreciated (23–26). Adding to this orchestrated regulation, we now elucidate a novel mechanism by which the sphingolipid metabolite, ceramide, can regulate protein-protein interactions between PKCζ and elements of the SAPK cascade, culminating in cell cycle arrest.

We demonstrate that ceramide selectively augments a signal complex formation of PKCζ with MEKK1, SEK, and SAPK. The fact that our co-immunoprecipitation experiments were performed in the presence of a non-ionic detergent (0.2% Nonidet P-40) suggests that direct protein-protein interactions are occurring as a consequence of ceramide activation of PKCζ, and presumably not by ceramide promoting hydrophobic associations of these proteins at the membrane. Based on this evidence, it is suggested that direct binding and activation of PKCζ by ceramide leads to recruitment and activation of upstream elements in the SAPK cascade resulting in cell cycle arrest. Other studies have postulated alternative mechanisms by which ceramide regulates the SAPK cascade. Ceramide has been shown to activate small molecular weight G-proteins that may couple inflammatory cytokine receptors with the SAPK cascade (14, 15). Specifically, ceramide stimulates Rac-1, as well as Vav, a guanine nucleotide exchange factor for Rac (27).

Another SAPK regulatory mechanism may involve ceramide activation of PP1 and PP2A phosphatases (28). Even though overexpression of a dual specificity threonine/tyrosine phosphatase, M3/6, diminishes ceramide-activated SAPK (29), other studies demonstrate that ceramide activates SAPK under conditions where ceramide also activates protein phosphatases (6, 12). The role of ceramide-activated phosphatases to modulate ceramide-dependent PKCζ/SAPK interactions is of potential interest. Thus, ceramide may regulate several mechanisms mediating SAPK-induced cell cycle arrest.

The role of ceramide binding to, and activating, PKCζ is still somewhat controversial. Our studies, using both immunoprecipitated and recombinant human PKCζ, clearly demonstrate that ceramide, but not dihydroceramide, directly induces PKCζ bioactivity. Supporting our findings, ceramide has been shown to bind to PKCζ as determined by kinetic analyses and in vitro phosphorylation studies (9, 30). In contrast, a radiolabeled photoaffinity-labeled ceramide analogue was unable to directly interact with immunoprecipitated PKCζ (31). These apparent contradictions in the literature may be due to structural differences in the ceramide analogues. Alternatively, the ability of ceramide to interact with the cysteine-rich lipid-binding domain (CRD) of immunoprecipitated PKCζ could be altered by co-immunoprecipitating proteins, such as Par-4, that also interact with this domain (21). It has been proposed that the single CRD of PKCζ may interact with ceramide, but not DAG (6, 32). This may be due to the lack of a second CRD, which is observed in conventional and novel PKC classes, or to subtle differences in the loop structure of the PKCζ CRD that respond to the unique polar regions of ceramide (21). Although DAG does not directly activate PKCζ, it is suggested that DAG selectively inhibits PKCζ activity by antagonizing ceramide binding at the CRD. The fatty acyl groups of DAG may block this putative ceramide-binding domain in an analogous fashion to arachidonic acid blocking ceramide binding to PKCζ (9).

In our studies, we found that the actions of ceramide on PKCζ/SAPK interactions were inhibited by IGF-1 treatment. This somewhat surprising finding has several possible explanations. IGF-1-generated DAG may compete with ceramide at the putative ceramide binding site on PKCζ (21) or activate other PKC isotypes linked to mitogenesis. In addition, IGF-1 has also been shown to stimulate PKCζ through a phosphatidylinositol 3-kinase-dependent mechanism (33). This alternate mechanism to stimulate PKCζ may couple PKCζ to other mitogen-activated protein kinases (MAPK), such as extracellular signal-regulated kinases (ERK), which are more closely linked to mitogenesis. The role of sphingolipid- and polyphosphoinositol-derived second messengers to differentially regulate PKCζ/MAPK signaling complexes is an attractive theory that is beyond the focus of the current studies. Alternatively, ceramide may become phosphorylated by IGF-1 treatment, generating
ceramide-1-phosphate, a pro-mitogenic lipid (34). Another possibility suggests that IGF-1 treatment induces the activation of ceramidase, an enzyme that catalyzes the deacylation of ceramide to form the pro-mitogenic lipids, sphingosine or sphingosine-1-phosphate (35). Regardless of mechanism, IGF-1 co-treatment reduces the actions of inflammatory cytokines or ceramides to activate SAPK activity. However, this compensatory mechanism does not supersede the ability of ceramides to induce cell cycle arrest.

Our finding that ceramide-induced cell growth inhibition is a consequence of activated PKCζ coupling to elements of the SAPK cascade clearly suggests the critical role that specific PKCζ MAPK signaling complexes play in cell cycle arrest. This is particularly intriguing, because PKCζ can be activated by both mitogenic and anti-mitogenic stimuli. Thus, the ability of activated PKCζ to interact with distinct MAPK signaling elements could explain the contradictory actions of PKCζ as a regulator of cell growth. For example, it was initially documented that PKCζ is required for maturation of Xenopus oocytes and for DNA synthesis in fibroblasts (36). Interactions between ceramide and PKCζ protein levels in the complex, respectively (mean ± S.E.; n = 3). Star, significantly different from vehicle control; p < 0.05, paired t-test. Sun, significantly different from IGF-stimulated condition; p < 0.05, paired t-test.

FIG. 5. Ceramide induces PKCζ association with p-SEK and MEKK1. HEK-293 cell monolayers in 6-well plates were used to assess PKCζ interaction with bioactive phospho-SEK (p-SEK) (A and B) as well as MEKK1 (C and D). Cells were treated with C6-ceramide (C6-Cer, 1 μM), IL-1 (20 ng/ml), and/or IGF-1 (50 ng/ml) for 5–10 min. To assess PKCζ/p-SEK interactions, the lysed cells were immunoprecipitated with the anti-PKCζ antibody and immunoblotted with an antibody that detects phosphorylated SEK. To assess PKCζ-MEKK1 interactions, the lysed cells were immunoprecipitated with an anti-MEKK1 antibody and immunoblotted with the anti-PKCζ antibody. Re-probing the blots with the appropriate antibody (anti-PKCζ for A and anti-MEKK1 for C) assessed equal loading. Positive and negative controls included whole cell lysates and cell-free samples, respectively (data not shown). Both C6-ceramide and IL-1 treatment led to a significant interaction between PKCζ and p-SEK as well as MEKK1, which was reduced by co-treatment with IGF-1. A and C, representative Western blots of three such experiments each. B and D, the quantification of p-SEK and PKCζ protein levels in the complex, respectively (mean ± S.E.; n = 3). Star, significantly different from vehicle control; p < 0.05, paired t-test. Sun, significantly different from IGF-stimulated condition; p < 0.05, paired t-test.
Ceramide and PKCζ

between PKCζ and the pro-mitogenic ERK cascades have been suggested, as a dominant-negative mutant of PKCζ suppressed activation of MEK and ERK by TNFα (37). However, recent studies suggest a growth inhibitory role for PKCζ. NIH-3T3 fibroblasts transfected with wild-type PKCζ are not tumorigenic (38). In fact, PKCζ has been reported to actually suppress the neoplastic transformation of fibroblasts mediated by the v-raf oncogene (39) and does not activate Raf-1 activity, an upstream kinase in the ERK cascade in vitro (40, 41). These observations offer an explanation for inhibition of ERK bioactivity by ceramide (6, 7, 12, 42). Thus, the ability of ceramide to selectively induce PKCζ and regulate MEKK1-SEK1-SAPK interactions is a novel hypothesis by which inflammatory cytokine receptor-induced ceramide formation may limit cellular proliferation in models of non-proliferative immunological renal diseases.

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