Direct Binding to Ceramide Activates Protein Kinase Cζ before the Formation of a Pro-apoptotic Complex with PAR-4 in Differentiating Stem Cells* §

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We have reported that ceramide mediates binding of atypical protein kinase C (PKCζ) to its inhibitor protein, PAR-4 (prostate apoptosis response-4), thereby inducing apoptosis in differentiating embryonic stem cells. Using a novel method of lipid vesicle-mediated affinity chromatography, we showed here that endogenous ceramide binds directly to the PKCζ/PAR-4 complex. Ceramide and its analogs activated PKCζ prior to binding to PAR-4, as determined by increased levels of phosphorylated PKCζ and glycogen synthase kinase-3β and emergence of a PAR-4-to-phosphorylated PKCζ fluorescence resonance energy transfer signal that co-localizes with ceramide. Elevated expression and activation of PKCζ increased cell survival, whereas expression of PAR-4 promoted apoptosis. This suggests that PKCζ counteracts apoptosis, unless its ceramide-induced activation is compromised by binding to PAR-4. A luciferase reporter assay showed that ceramide analogs activate nuclear factor (NF)-κB unless PAR-4-dependent inhibition of PKCζ suppresses NF-κB activation. Taken together, our results show that direct physical association with ceramide and PAR-4 regulates the activity of PKCζ. They also indicate that this interaction regulates the activity of glycogen synthase kinase-3β and NF-κB.

In previous studies, we have shown that the simultaneous elevation of the sphingolipid ceramide and the atypical PKCζ inhibitor protein PAR-4 is critical for the induction of apoptosis in differentiating mouse embryonic stem (ES) cells (1–3). We demonstrated for the first time that during cell division, PAR-4 and the neuroprogenitor (NP) marker protein nestin are asymmetrically distributed to the two daughter cells (1). Only the PAR-4(−)/nestin(−) daughter cell survives, whereas its PAR-4(+) /nestin(+) sibling undergoes ceramide-induced apoptosis. In other studies, we have found that PAR-4 sensitizes differentiating mouse as well as human embryoid body-derived cells (EBCs) toward apoptosis induction by ceramide and ceramide analogs (2). We also reported that the novel ceramide analog S18 (N-oleoyl serinol) eliminates residual pluripotent Oct-4(−)/γPAR-4(+) stem cells from EBCs, enriches for NPs, and prevents teratoma formation from stem cell-derived neural transplants in mouse brain (2). It remained to be elucidated, however, which mechanism drives ceramide-induced or ceramide analog-induced apoptosis in the PAR-4(+) cells.

We and others have suggested that ceramide activates atypical PKC but also promotes inhibition of atypical PKC by PAR-4 (1–9). To resolve this apparent paradox, we have analyzed the ceramide-induced or ceramide analog-induced formation of a protein complex between PKCζ and PAR-4 and determined the effect of ceramide analogs on the activation of NF-κB and the degree of apoptosis. Ceramide and ceramide analogs have been shown to enhance phosphorylation of PKCζ in vitro and in vivo, however, without demonstrating direct and specific physical interaction of atypical PKC (4–8). Filter or solid phase binding assays are commonly used to test the affinity of a protein to its lipid ligand. The binding of PKCζ to ceramide, however, has not been reported yet using this or a similar type of binding assay. Most recently, it has been shown that solid phase/overlay binding assays fail to show the specific interaction of mode I of PKCζ with ceramide (10). We developed a novel assay based on mixed ceramide/phospholipid vesicles to determine binding of atypical PKC to ceramide. Our results show that PAR-4 binds to PKCζ that is associated with ceramide and has first been activated due to ceramide-mediated elevation of enzyme phosphorylation. Thus, NF-κB-dependent cell survival is enhanced or suppressed by ceramide, which is dependent on the absence or presence of PAR-4, respectively.

EXPERIMENTAL PROCEDURES

Materials—ES-J1 and feeder fibroblasts were purchased from the ES cell core facility (Dr. Ali Eroglu), Medical College of Georgia. Purified bovine brain sphingomyelin was a generous gift from Dr. Somasankar Dasgupta (Medical College of Georgia). Knock-out Dulbecco’s modified Eagle’s medium, knock-out serum replacement, ES qualified fetal bovine serum, N2 supplement, and fibroblast growth factor-2 were from Invitrogen. Dulbecco’s modified Eagle’s medium/P-12 50/50 Mix was obtained from Cellgro (Herndon, VA). Non-enzymatic cell dissociation solution, Hoescht 33258, bovine brain ceramides, protease (P-8340) and phosphatase (P-8726) inhibitor reagents, purified recombinant human PKCζ, and goat anti-rabbit IgG horseradish peroxidase conjugate were obtained from Sigma. N-Acetyl-D-erythro-sphingosine (C2-ceramide) was from Matreya (Pleasant Gap, PA). L-α-Phosphatidylserine (brain and porcine), N-palmitoyl-D-erythro-sphingosine (C16-ceramide), and L-α-phosphatidylcholine (egg and chicken) were purchased from Avanti.
Polar Lipids Inc. (Alabaster, AL). Polyclonal anti-PAR-4 rabbit IgG (catalog number sc-1807), polyclonal anti-SUMO-1 rabbit IgG (catalog number sc-9060), polyclonal anti-PCPKc rabbit IgG (catalog number sc-216), polyclonal anti-PCPKc rabbit IgG (catalog number sc-957), and monoclonal anti-PAR-4 mouse IgG (catalog number sc-1666) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal anti-cleaved caspase 3 rabbit IgG (catalog number 9661S), polyclonal anti-phosphorylated (phospho-Thr38/40) PKCα/β rabbit IgG, and polyclonal anti-phosphorylated (phospho-Ser)** peptide SKG3-3 rabbit IgG were purchased from Cell Signaling (Beverly, MA). Monoclonal anti-ceramide mouse IgM (clone 15B4) was from Alexis (San Diego, CA). Donkey anti-rabbit, anti-rabbit, and anti-goat IgG Cy2, Cy3, and Cy5 conjugates; Cy2-conjugated donkey anti-mouse IgM (Fc-fragment specific); Cy3-conjugated goat anti-mouse IgG (Fcy-fragment specific); goat anti-mouse IgG horseradish peroxidase conjugate; normal rabbit IgGs; and normal donkey serum were purchased from Jackson ImmunoResearch (West Grove, PA). Myristoylated PKCc pseudosubstrate inhibitor peptide, biotinylated PKCc substrate peptide, and NF-κB translocation inhibitor peptide SN50 were from Calbiochem. NBC-6/ceramide/bovine serum albumin conjugate, ER tracker Blue-White DFX, Mitotracker Red CM-H2XPhos, Alexa Fluor 594-conjugated wheat germ agglutinin, Vybrant CM-diI, and Vybrant CFDA-SE were from Molecular Probes (Eugene, OR). Mitotracker Green FM, Mitotracker Deep Red FM, and Annexin V-conjugated magnetic beads was from Miltenyi Biotec (Auburn, CA). Streptavidin-coated membranes (SAM2® Biotin Capture Membrane), pNF-κB-luc reporter plasmid, and the dual luciferase reporter assay were from Promega (Madison, WI). γ**-**P**l**ATP (specific activity, 6000 Ci/mmol) was obtained from ICN Biomedicals (Irvine, CA). The pan-caspase FLICA (fluorochrome inhibitor of caspases) assay kit was from Assay Designs (Ann Arbor, MI). The Lipofectamine 2000 transfection reagent was obtained from Invitrogen. All reagents were of analytical grade or higher.

**Preparation of Lipid Vesicles and PKCc Binding Assays**—Lipid vesicles were obtained from dried mixtures of equimolar amounts of phosphatidylserine (PS) (105 μg) and C16-ceramide (85 μg) following modified procedures for large liposome preparation (10–17). The lipid mixture was dissolved and sonicated to 1:1000 dilution enzyme sites KpnI and XbaI. The PKCc was digested with KpnI and XbaI and cloned into pcDNA3.1 empty vectors as controls. Twenty-four hours after transfection, the cells were harvested by scraping, and the cell lysates were lysed by sonication followed by two-phase/overlay binding assay. The reaction was monitored by scintillation counting. The concentration of 5 or 10 μg/ml, respectively. In case of simultaneous labeling with anti-PAR-4 mouse monoclonal IgG and anti-ceramide mouse monoclonal IgM, we used isotype-specific secondary antibodies (Fc- or chain specific, respectively) for the immunodetection reaction. In control experiments, we ruled out any potential cross-reactivity of the isotype-specific secondary antibody with non-matching primary antibodies (Supplemental Fig. S1). The ceramide specificity of the 15B4 antibody was verified using a pre-adsorption assay with ceramide or sphingomyelin following a protocol as previously reported (18) and shown in Supplemental Fig. S2. Epifluorescence microscopy was accomplished using a Zeiss Axioskop Deconvolution microscope. A Zeiss Axioskop microscope equipped with a Spot digital camera. Confocal fluorescence microscopy and FRET were performed using a Zeiss LSM confocal laser scanning microscope equipped with a two-photon argon laser at 488 nm (Cy2, green fluorescent protein) and 543 nm (Cy3, HiRFP) or 633 nm (Cy5), respectively. Acceptor (Cy5) bleaching was achieved by repetitive scanning at 633 nm during the Cy5 signal was monitored. Data were collected from images at a resolution of 1024 × 1024 pixels, and the fluorescence increase of the Cy3 signal was calculated by densitometry based on pixel-to-pixel counting within selected areas of the images.

**Construction of PKCc-EGFP and PAR-4-HiRFP Plasmids**—For the construction of pcDNA3.1-PAR-4, mouse PAR-4 cDNA was amplified using sense primer 5'-attgagctccgactttcctctctc-3' (KpnI site) and antisense primer 5'-caatatgaactgactgactgtcagcaagc-3' (XbaI site) and the PAR-4-HiRFP plasmid as a template (1). The PCR product was digested with KpnI and XbaI and cloned into pcDNA3.1/myc-his (+) B. For the construction of pcDNA3.1-PKCC and PKCc-EGFP, mouse PKCc cDNA was amplified from a PKCc full-length IMAGE clone (ATCC clone 62347) using the sense primer 5'-agattcagcagactgtcagcagcagc-3' (XbaI site) and antisense primer 5'-caatatgaactgactgactgtcagcaagc-3' (XbaI site). The amplification product was ligated into pcDNA3.1/myc-his (+) B vector between the restriction enzyme sites KpnI and XbaI. The PKCc-EGFP vector was obtained by ligation of a HindIII/SacII fragment from pcDNA3.1-PKCC into pEGFP-N1. Transfections were performed using the Lipofectamine 2000 procedure following the manufacturer’s (Invitrogen) protocol. At various time periods of incubation (10, 20, 40, and 60 min), aliquots of 20 μl were taken, and the reaction mixture was centrifuged at 12,000 × g for 20 min at 4 °C. The pellet (large lipid vesicles) was resuspended in 100 μl of buffer A and incubated with 1 nmol of Vybrant CM-dil for 1 h at 37 °C to visualize the vesicle fraction after MACS separation. A detergent-free cell lysate was prepared by sonication of differentiated EBCs or EBCs in 30 μl of hypotonic buffer (10 mM Tris/HCl (pH 7.0) with protease and phosphatase inhibitors) followed by removal of membranous debris by centrifugation. The cleared lysate was added to the lipid vesicle suspension, and the mixture was incubated for 2 h at 4 °C. The reaction mixture was supplemented with 20 μl of Annexin V binding buffer and 50 μl of a solution of 150 mM NaCl, 10 μg/ml bovine serum albumin, and protease and phosphatase inhibitor mixtures at 4 °C. The cell lysate was centrifuged for 15 min at 12,500 × g. For each assay, 100 μl of the supernatant were supplemented with 8 μg of PS pre-dissolved in dimethyl sulfoxide and 40 μl biotinylated PKCc substrate peptide (final concentration) pre-dissolved in de-ionized water. The reaction was started by the addition of 50 μl of non-labeled ATP (final concentration) and 10 μl of [γ-32P]ATP and then incubated at room temperature. At various time periods of incubation (10, 20, 40, and 60 min), aliquots of 20 μl were taken, and the reaction mixture was centrifuged at 12,500 × g. The cell lysate was centrifuged at 4 °C before addition to the medium in a 1:1000 dilution. Controls were obtained with diluted vehicle only. For immunocytochemistry and FRET analysis, cells were fixed with 4% paraformaldehyde/hyde phosphate-buffered saline, followed by permeabilization with 0.2% Triton X-100 for 5 min at room temperature. Nonspecific binding sites were saturated by incubation with 3% ovalbumin and 2% donkey serum in phosphate-buffered saline at 37 °C for 60 min. Cells were stained with primary and fluorescence-labeled secondary antibodies at 37 °C. The presence and quantity of lipid sites were saturated by incubation with 3% ovalbumin and 2% donkey serum in phosphate-buffered saline at 37 °C for 60 min. Cells were stained with primary and fluorescence-labeled secondary antibodies at
ity of proteins to ceramide (10). We attempted to test binding of PKC/ to ceramide by using a lipid vesicle binding assay. The preparation of pure ceramide (C16-ceramide) vesicles, however, failed as verified by microscopic inspection, density gradient centrifugation, and filter exclusion assays (data not shown). Therefore, we developed a novel technique termed lipid vesicle-mediated affinity chromatography (LIMAC) to isolate ceramide-binding proteins. Based on procedures used for phosphatidylserine liposome aggregation assays, we prepared lipid vesicles from a variety of phospholipid and ceramide mixtures (10–17). Lipid vesicles obtained from PS, phosphatidylcholine (PC), and/or ceramide (Cer) were labeled with the fluorescent membrane marker dye Vybrant CM-dil. The labeled vesicles were then subjected to Annexin V MACS to isolate the PS-containing lipid vesicles due to binding of PS to Annexin V-conjugated magnetic beads.

Fig. 1A shows that only PS/PC and Cer/PS vesicles were recovered in the elution fraction (E) of the magnetic column, whereas Cer/PC vesicles were found in the flow-through fraction (FT). This result demonstrated that binding of the lipid vesicles relied on the presence of PS and verified the efficacy of the LIMAC procedure to separate Annexin V-binding (PS/PC and Cer/PS) from non-binding (Cer/PC) vesicles. High-performance thin-layer chromatography analysis of the elution fraction showed that the Cer/PS vesicles (Fig. 1B, lane 3) contained equivalent amounts of PS and Cer and verified that the eluted ceramide co-eluted with PS, indicating the integrity of the Cer/PS vesicle preparation. C, lipid vesicles were incubated with human recombinant PKCζ and purified using the LIMAC procedure. The lipid-bound PKCζ was analyzed by SDS-PAGE and immunoblotting using protein from the elution fractions. Lane 1, PS/PC vesicles; lane 2, Cer/PS vesicles; lane 3, Cer/PC vesicles, lane 4, no sample; lane 5, human recombinant PKCζ as positive control; lane 6, Cer/PC vesicles; lane 7, anti-PKCζ antibody incubated with Cer/PS vesicles alone; lane 8, anti-PKCζ antibody incubated with PKCζ prior to the addition of Cer/PS vesicles; lane 9, human recombinant PKCζ as positive control. D, Cer/PS vesicles were labeled with NBD-C6-ceramide, incubated with human recombinant PKCζ, and then immunostained with Alexa 546-linked anti-PKCζ antibody prior to Annexin V-based MACS. NBD-C6-ceramide and PKCζ are co-localized (arrows).
vesicles were composed of the two lipids (Fig. 1B).

To test binding of PKC\(\zeta\) to ceramide, lipid vesicles were incubated with recombinant human PKC\(\zeta\) prior to Annexin V-based MACS. Protein bound to the vesicles in the elution fraction was analyzed by SDS-PAGE and immunoblotting. Fig. 1C shows that PKC\(\zeta\) was only co-purified with Cer/PS vesicles (lanes 2 and 6), but not with PS/PC vesicles (lane 1), indicating that binding of PKC\(\zeta\) was specific for ceramide.

The absence of PKC\(\zeta\) in the elution fraction of PS/PC vesicles suggested that PS by itself did not sustain binding of the lipid vesicles to the enzyme, although PS has been reported to activate PKC\(\zeta\) in vitro (4, 21–23). In contrast to numerous studies showing the specific association of PS with classical PKCa (24–26), binding data for the physical association of PS to PKC\(\zeta\) are not available yet. Absence of binding of PKC\(\zeta\) to PS/PC vesicles may have been due to a low affinity of the enzyme to PS. Alternatively, the combination of lipids in the vesicles may have favored binding of PKC\(\zeta\) to Cer/PS rather than PS/PC vesicles. The binding of PKC\(\zeta\) to Cer/PS vesicles was suppressed by incubation of PKC\(\zeta\) with anti-PKC\(\zeta\) antibody (Fig. 1C, lane 8) prior to the addition of the lipid vesicles. This result indicated that binding of the antibody to PKC\(\zeta\) blocked access of ceramide to its binding site on the enzyme. The antibody itself did not bind to the vesicles as shown by the absence of immunostaining in the elution fraction of Cer/PS vesicles that were incubated with anti-PKC\(\zeta\) IgG alone (Fig. 1C, lane 7). This antibody competition assay clearly demonstrated that PKC\(\zeta\) bound specifically to the Cer/PS vesicles, ruling out nonspecific hydrophobic interaction. To visualize co-localization of ceramide and PKC\(\zeta\), Cer/PS vesicles were labeled with NBD-C6-ceramide prior to the addition of PKC\(\zeta\) and immunofluorescence staining of the vesicle-bound enzyme. Fig. 1D shows that NBD-C6 ceramide was co-localized with Alexa 546-linked anti-PKC\(\zeta\) antibody, indicating that PKC\(\zeta\) specifically bound to ceramide in Cer/PS vesicles (arrows). Our results also suggested that ceramide-containing lipid vesicles could be used to isolate ceramide-binding proteins.

We tested whether the LIMAC procedure could be used to bind endogenous PKC\(\zeta\) from a detergent-free EBC lysate that was incubated with Cer/PS vesicles. Fig. 2 shows that the Cer/PS vesicles bound to PKC\(\zeta\) (lane 3) and PAR-4 (lane 6).

**PKC\(\zeta\) and PAR-4 protein complexes from stem cells.** EBCs were homogenized in hypotonic buffer, and the detergent-free cell lysate was incubated with Cer/PS vesicles prior to Annexin V-based MACS. SDS-PAGE and immunoblotting were performed with protein recovered in the flow-through (FT) or elution (E) fractions. Lanes 1–3, staining using anti-PKC\(\zeta\) antibody; lanes 4–8, staining using anti-PAR-4 antibody; lanes 7 and 8, eluate from cytosolic protein fraction without (lane 7) or with (lane 8) anti-PKC\(\zeta\) antibody competition prior to the addition of Cer/PS vesicles; lanes 9 and 10, reprobing of the membrane stained for lanes 7 and 8 using anti-SUMO-1 antibody; lanes 11–13, MACS flow-through fraction stained for actin, PKC\(\zeta\), or PKC\(\beta\); lanes 14–16, MACS eluate stained for actin, PKC\(\zeta\), and PKC\(\beta\). T, total protein used for MACS.

**PKC\(\zeta\)** bound to intracellular ceramide is phosphorylated and forms a complex with PAR-4. To determine intracellular binding of PKC\(\zeta\) to endogenous ceramide, we transfected EBCs with vectors encoding PKC\(\zeta\)-EGFP and tested the co-distribution/association with ceramide using a ceramide-specific mouse monoclonal antibody. Although this antibody or a similar antibody has shown a broader specificity toward sphingolipids in solid phase/overlay assays, it was found to react specifically with intracellular ceramide when used with fixed cells (1, 18, 27–34). Our results and those of other groups indicated that solid phase/overlay binding assays are not well suited to test the affinity of proteins to ceramide. The reason for low or absent binding to ceramide may be a different conformation of ceramide incorporated into a lipid membrane as compared with that coated on a solid surface. It is likely that this also applies to ceramide-specific antibodies, which results in a different binding specificity depending on whether ceramide is presented by a lipid membrane or coated on a solid surface. To show ceramide-specific binding, the anti-ceramide antibody was pre-adsorbed to ceramide (18) or sphingomyelin prior to immunocytochemistry. Supplemental Fig. S2. A shows that only pre-adsorption with ceramide (but not sphingomyelin) reduced the antibody-derived fluorescence signal. These results suggested that the anti-ceramide antibody was able to distinguish ceramide from sphingomyelin when used for immunocytochemistry. Our results did not exclude, however, that the antibody may have also bound to dihydroceramide, the precursor of ceramide. Co-staining with markers for the ER and Golgi indicated that the perinuclear region showing elevation of endogenous ceramide was the ER but not the Golgi (Supplemental Fig. S2, B and C). The distribution of the antibody-derived fluorescence signal was consistent with the subcellular site for de novo ceramide biosynthesis (ER), whereas sphingomyelin synthesized in the Golgi was not recognized.

We transfected cells with a vector encoding PKC\(\zeta\)-EGFP to determine the dependence of PKC\(\zeta\) phosphorylation on the expression level of the enzyme and the distribution of ceram-
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Fig. 3. Co-distribution of phosphorylated PKCζ with endogenous ceramide and complex formation of PKCζ and PAR-4 prior to apoptosis. A, EBCs (NP2 stage) were transfected with cDNA encoding PKCζ-EGFP. At 24 h post-transfection, pPKCζ/λ and endogenous ceramide were detected by indirect immunofluorescence microscopy. Note that the intensity of the pPKCζ/λ signal was dependent on the expression level of PKCζ and the concentration and subcellular distribution of ceramide. B, EBCs (NP2 stage) were co-transfected with cDNAs encoding PKCζ-EGFP and PAR-4-HcRFP. At 24 h post-transfection, cells were incubated with 80 μM S18. The fluorescence signals were recorded by time-lapse fluorescence microscopy at 37 °C. Note that PKCζ-EGFP and PAR-HcRFP were co-distributed to a perinuclear region prior to staining of condensed (apoptotic) nuclei with Hoechst dye.

Fig. 4. Perinuclear co-distribution of endogenous ceramide, PKCζ, and PAR-4 in apoptotic cells. Immunocytochemistry was performed on EBCs (NP2 stage) using antibodies against ceramide (Cy2, isotype-specific), PAR-4 (Cy3, isotype-specific), and PKCζ (Cy5, all signals are visualized in different pseudocolors). Apoptotic cells were identified by staining with Alexa 350-conjugated Annexin V. Note that PKCζ and PAR-4 were co-localized in apoptotic cells that also showed a strong signal for ceramide. The perinuclear co-distribution of ceramide, PKCζ, and PAR-4 appears white due to overlay of three pseudocolors.

endogenous ceramide were detected by indirect immunofluorescence microscopy. Note that the intensity of the pPKCζ/λ signal was dependent on the expression level of PKCζ and the concentration and subcellular distribution of ceramide. B, EBCs (NP2 stage) were co-transfected with cDNAs encoding PKCζ-EGFP and PAR-4-HcRFP. At 24 h post-transfection, cells were incubated with 80 μM S18. The fluorescence signals were recorded by time-lapse fluorescence microscopy at 37 °C. Note that PKCζ-EGFP and PAR-HcRFP were co-distributed to a perinuclear region prior to staining of condensed (apoptotic) nuclei with Hoechst dye.

The specificity and authenticity of the FRET signal with PKCζ was also phosphorylated when associated with PAR-4. The two FRET signals co-localized in the perinuclear region and were more intense when cells underwent apoptosis as indicated by the appearance of condensed nuclei. The number of condensed nuclei showing perinuclear staining of a ceramide-pPKCζ-PAR-4 complex was increased by 2-fold when EBCs were incubated with 2 μM C16-ceramide or 80 μM S18. Consistent with our previous observation (Fig. 3A), the co-localization of a strong pPKCζ/λ signal with PKCζ-EGFP suggested that the FRET signal resulted from a complex of ceramide and PAR-4 with pPKCζ. The specificity and authenticity of the FRET
signal were verified by donor de-quenching due to acceptor bleaching of a Cy5-labeled secondary antibody against the anti-pPKC\(\alpha\) antibody (Supplemental Fig. S4). Acceptor bleaching reduced quenching of the Cy3 signals from the secondary antibodies detecting ceramide and PAR-4, indicating that ceramide was associated with a pPKC\(\alpha\) protein complex. In summary, our results suggested that ceramide analogs induced intracellular phosphorylation of PKC\(\zeta\), even at a concentration that resulted in the induction of apoptosis (4). Here, we tested how the degree of PKC\(\zeta\) phosphorylation affected the activity of the enzyme in the presence of PAR-4. Fig. 6B shows that incubation of EBCs with S18 resulted in the elevation of PKC\(\zeta\) phosphorylation, consistent with enhanced phosphorylation of GSK-3\(\beta\), an intracellular substrate of PKC\(\zeta\). In vitro assays of PKC\(\zeta\) activity showed that incubation of EBCs with S18 at a low concentration (40 \(\mu\)M) resulted in a 2-fold increase in the activation of PKC\(\zeta\) (Fig. 6C). At this concentration in the medium, S18 was enriched to \(~70\%\) of the concentration of endogenous ceramide found in EBCs (data not shown). At a higher concentration of S18 in the medium (80 \(\mu\)M), the activation of PKC\(\zeta\) was ablated, concurrent with an increased level of apoptosis (Fig. 6, C and D). Although ectopic expression of PAR-4 by itself was not sufficient to significantly inhibit PKC\(\zeta\), its activity was dramatically reduced when cells were transfected with PAR-4 and at the same time incubated with S18 (Fig. 6C). Induction of apoptosis by incubation of EBCs with a cell-permeable pseudosubstrate inhibitor of PKC\(\zeta\) (PZI) showed that inhibition of PKC\(\zeta\) was sufficient to induce apoptosis (Fig. 6, C and D). PZI has been shown to specifically inhibit PKC\(\zeta\) or PKC\(\alpha\) in various cell systems (35–39). Hence, we cannot exclude that some of the results obtained with PZI may be due to inhibition of PKC\(\alpha\). In summary, our data suggested that binding of ceramide or ceramide analogs induced phosphorylation and activation of atypical PKC. In cells with elevated expression of PAR-4, binding of PAR-4 to activated atypical PKC resulted in the inhibition of the enzyme and the induction of apoptosis.

Ceramide-activated PKC\(\zeta\) Regulates NF-\(\kappa\)B in Dependence on the Level of PAR-4 Expression—It has been suggested that ceramide-mediated complex formation between atypical PKC and PAR-4 reduces the activity of NF-\(\kappa\)B and that this results in the induction of apoptosis (1, 3, 4, 7, 40–45). We have...
previously shown that in differentiating stem cells, the induction of apoptosis by endogenous ceramide and exogenously added ceramide analogs is concentration-dependent and requires the expression of PAR-4 (1, 2). To test the effect of ceramide analogs on the activity of NF-κB, we co-transfected EBCs at different NP stages with a vector encoding the NF-κB 

FIG. 6. Regulation of PKCζ activity and induction of apoptosis by ceramide/S18 and PAR-4. A, EBCs (NP2 stage) were transfected with cDNAs encoding PKCζ-EGFP or PAR-4-HcRFP. Twenty-four hours post-transfection, cells were incubated overnight with 80 μM S18. Cells were trypsinized, labeled with Annexin V-conjugated magnetic beads, and fractionated using MACS. The picture shows immunoblots performed with solubilized protein from the non-apoptotic Annexin V(−) and apoptotic Annexin V(+) cell fractions. Note that endogenous PKCζ was distributed to the non-apoptotic as well as apoptotic cell fraction, whereas endogenous PAR-4 was only found in apoptotic cells, indicating that PKCζ protected against S18-inducible apoptosis unless PAR-4 was also expressed. Caspase 3* denotes activated (cleaved) caspase 3. B, EBCs (NP2 stage) were incubated with or without 50 μM S18 overnight, and PKCζ/λ phosphorylated at Thr410 (pPKCζ/λ) or GSK-3β phosphorylated at Ser9 (pGSK-3β) was immunostained on Western blots obtained with EBC-derived protein. C, protein was solubilized from EBCs and incubated with [γ-32P]ATP and a biotinylated PKCζ substrate peptide (50 μM). The radioactively labeled product peptide was isolated using streptavidin-coated membranes, and the transferred phosphate (dpm/mg cell protein/min) was quantified by scintillation counting. White bars represent means from four independent incubation reactions with S.E. indicated as error bars. D, EBCs were dissociated by trypsinization, and EBs were replated and cultivated for 24 h (NP2 stage) prior to the addition of various apoptosis inducers (40 μM S18, 80 μM S18, 30 μM C2-ceramide, 2 μM C16-ceramide, and 30 μM PZI). Apoptosis was quantified after 15 h using labeling of apoptotic cells with FLICA assays and cell counting. White bars represent the means (percentage of apoptotic cells within the total population of EBCs) from four independent incubation reactions with S.E. indicated as error bars.
enhancer element (κB)-driven luciferase and a vector containing the cDNA of PAR-4 prior to incubation with S18. Fig. 7 shows that the effect of S18 on the NF-κB activity was dependent on the S18 concentration and the differentiation (NP) stage. At the NP2 stage, S18 incubation at low concentration (40 μM) activated NF-κB-driven luciferase expression to a degree that was consistent with that of ceramide-induced activation of PAR-4 (Figs. 6 and 7). At a higher concentration (80 μM) of S18, however, the NF-κB activity was reduced, in particular in cells with elevated expression of PAR-4.

At the NP3 stage, we found NF-κB activation even at a high concentration (120 μM) of S18 (Fig. 7B). This result was consistent with that of our previous study showing that at a later stage of neural differentiation, the degree of S18-inducible apoptosis dropped concomitantly with the reduced level of PAR-4 expression (2). Reduction of the NF-κB activity was only seen at a very high concentration of 250 μM S18 or on co-transfection with PAR-4. Low NF-κB activity concurrent with elevated apoptosis was also observed after incubation of NPs with the PKCζ pseudosubstrate inhibitor (PZI) or a combination of 40 μM S18 and PZI (Fig. 7, A and B). Inhibition of NF-κB with the translocation inhibitor peptide SN50 (50 μM) elevated apoptosis by...
50%, indicating that suppression of NF-κB activation was sufficient to induce apoptosis in NPs. Thus, our results suggested that the effect of the ceramide analog on the activity of NF-κB and the induction of apoptosis was dependent on the expression level of PAR-4. In cells expressing a low level of PAR-4, S18-activated NF-κB, whereas in cells expressing a high level of PAR-4, S18-mediated NF-κB activation was suppressed, and apoptosis was induced.

**DISCUSSION**

We have reported that simultaneous elevation of the sphingolipid ceramide and the atypical PKCζ inhibitor protein PAR-4 induces apoptosis in differentiating ES cells (1–3). Based on this observation, we hypothesized that ceramide may sensitize stem cells toward PAR-4-dependent inhibition of PKCζ. Previous studies have shown that ceramide activates PKCζ in vitro and in vivo, but without providing experimental evidence of sustained binding (4–8). It is well known that activation of enzymes can be due to weak or nonspecific interaction of the effector with the enzyme or even a complex between the substrate and the effector. Solid phase/overlay or filter binding assays, however, failed to demonstrate a specific physical association of ceramide to known ceramide-activated enzymes such as protein phosphatase 2a (10). We show here the specific affinity of PKCζ to ceramide using a novel binding assay based on MACS sorting of lipid vesicles that was developed in our laboratory. The PKCζ-dependent induction of apoptosis was tested by incubation with natural ceramide (C16-ceramide) and the novel ceramide analog S18 that was synthesized in our laboratory and used to determine the significance of ceramide for the induction of apoptosis in stem cells (1–4). We defined the mechanism by which ceramide may induce PAR-4-mediated inhibition of PKCζ and how this inhibition promotes apoptosis. Our results suggest that direct binding of ceramide triggers phosphorylation of PKCζ and its physical association with PAR-4, which results in down-regulation of NF-κB-dependent cell survival and induction of apoptosis.

Fig. 7C depicts a model that incorporates the effect of ceramide on PKCζ and its subsequent effect on binding of PAR-4. It should be noted that this model is likely to apply to other atypical PKC species as well, in particular PKCλ which also has been found to bind to PAR-4 (41, 46, 47). In fact, some of the results obtained in our study using the antibody against pPKCζ/λ or the pseudosubstrate inhibitor of PKCζ (PZI) may involve both atypical PKCs. Cytosolic PKCζ or PKCλ has low activity due to auto-inhibition by binding of the pseudosubstrate domain to the catalytic domain of the enzyme (Fig. 7C, Step 1) (48, 49). It has been suggested that binding of ceramide or structurally related lipids to the C1B domain may trigger a conformational change of the enzyme inducing an activated state (Fig. 7C, Step 2) (50, 51). This activation state is indicated by the ceramide analog-induced phosphorylation of the Thr410 epitope in the activation loop domain of PKCζ (48, 52) and, subsequently, elevated phosphorylation of GSK-3β, an intracellular substrate of PKCζ (53–56). The role of the PKCζ-dependent phosphorylation of GSK-3β for the regulation of stem cell apoptosis remains to be elucidated. The physical association of ceramide with PKCζ is consistent with our observation that phosphorylated PKCζ co-distributes with a ceramide-rich compartment. This model is also supported by the observation that PKCζ binds to ceramide-containing vesicles that show a similar lipid composition as the perinuclear mitochondria-associated membrane subcompartment of the ER (57, 58). At the cytosolic face of this compartment, PAR-4 may bind to ceramide-associated and activated atypical PKC, thereby inhibiting the activity of the enzyme (Fig. 7C, Step 3). A previous model has suggested that ceramide either activates PKCζ or promotes binding to PAR-4 (7). It remained to be elucidated, however, where and how ceramide and PAR-4 interact with PKCζ. Alternatively, ceramide may activate another kinase that phosphorylates PKCζ, or ceramide may first bind to PAR-4, which then inhibits PKCζ. Our model thus extends on the previously suggested mechanism in that ceramide activates PKCζ prior to binding of PAR-4. The ceramide binding assay suggests that a portion of PAR-4 is modified by sumoylation, whose significance for binding of PAR-4 to PKCζ will be investigated in future studies.

Consistent with our model, ceramide or ceramide analogs activate NF-κB-dependent gene expression, unless PAR-4 or another inhibitor of PKCζ (e.g. PKCζ pseudosubstrate) is present. Most inhibitory is the combination of ceramide analogs and PAR-4, which supports our model that pre-activation of PKCζ by ceramide or ceramide analogs sensitizes the enzyme to inhibition by PAR-4. Inhibition of PKCζ is concurrent with enhanced apoptosis, indicating that atypical PKC-dependent NF-κB activation is critical for cell survival. We suggest that ceramide may have beneficial effect as well as detrimental effects, depending on the expression level and distribution of PAR-4. In cells with a low level of PAR-4, direct binding to ceramide activates PKCζ and supports cell survival and/or neural differentiation. In cells with a high level of PAR-4, ceramide induces complex formation of activated PKCζ with PAR-4, which results in the inhibition of the enzyme and down-regulation of NF-κB-dependent cell survival. In future studies, we will determine by which mechanism ceramide association elevates phosphorylation of atypical PKC and how this regulates the activity of its intracellular substrate(s).

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