Ceramide Regulates Atypical PKCζ/λ-mediated Cell Polarity in Primitive Ectoderm Cells

A NOVEL FUNCTION OF SPHINGOLIPIDS IN MORPHOGENESIS

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In mammals, the primitive ectoderm is an epithelium of polarized cells that differentiates into all embryonic tissues. Our study shows that in primitive ectoderm cells, the sphingolipid ceramide was elevated and co-distributed with the small GTPase Cdc42 and cortical F-actin at the apicolateral cell membrane. Pharmacological or RNA interference-mediated inhibition of ceramide biosynthesis enhanced apoptosis and impaired primitive ectoderm formation in embryoid bodies differentiated from mouse embryonic stem cells. Primitive ectoderm formation was restored by incubation with ceramide or a ceramide analog. Ceramide depletion prevented plasma membrane translocation of PKCζ/λ, its interaction with Cdc42, and phosphorylation of GSK-3β, a substrate of PKCζ/λ. Recombinant PKCζ formed a complex with the polarity protein Par6 and Cdc42 when bound to ceramide containing lipid vesicles. Our data suggest a novel mechanism by which a ceramide-induced, apicolateral polarity complex with PKCζ/λ regulates primitive ectoderm cell polarity and morphogenesis.

Our studies have shown that the membrane sphingolipid ceramide regulates apoptosis during embryonic stem (ES)3 cell differentiation (1–5). This regulation is initiated by direct physical interaction of ceramide with atypical PKCζ or λ (5). Homozygous knock-out of PKCλ in mice results in lethality at embryonic day 9 because of severe defects of post-gastrulation morphology (6). This suggests that atypical PKCs are critical for aspects of early embryonic development, although the degree of functional overlap as well as specific effects and regulation of the two isoforms are not known yet. Our results obtained with ES cells suggest that ceramide-dependent regulation of atypical PKCs contributes to the function of PKCζ/λ during embryonic development.

In the pregastrulation embryo, inner cell mass-derived cells give rise to two adjacent epithelia of polarized cells, the primitive endoderm and the underlying primitive ectoderm. The formation of the primitive ectoderm is followed by the removal of cells that are not in contact with the primitive endoderm derived basal lamina. These cells die by apoptosis giving rise to the proamniotic cavity, a process termed cavitation (7). This developmental process is recapitulated in embryoid bodies (EBs) generated from in vitro differentiating ES cells. ES cell-derived EBs have been used to determine key regulatory factors of primitive germ layer formation and cavitation, and are a bona fide, in vitro model for early embryo morphogenesis (8–10). A key experiment to determine the significance of ceramide for cavitation and primitive ectoderm morphogenesis is depleting EBs of ceramide by disruption of ceramide biosynthesis. Ceramide biosynthesis is initiated and regulated by serine palmitoyltransferase (SPT), an enzyme consisting of the two subunits SPT-1 and SPT-2. Homozygous knock-out of SPT-1 or SPT-2 results in embryonic lethality at a stage of development well before E15 indicating the absolute requirement of ceramide biosynthesis for embryo development (11).

In our study, ceramide biosynthesis was reduced in ES cells and EBs by transfection of SPT-1 and -2-specific RNAi or by incubation with the SPT inhibitor myricin (or ISP-1) (12, 13). To rule out the possibility that the effect of SPT inhibition or depletion was due to the elimination of ceramide biosynthesis precursors rather than ceramide itself, we used a second inhibitor, fumonisin B1 (FB1). FB1 inhibits (dihydro)ceramide synthases, a group of enzymes catalyzing the penultimate step in ceramide biosynthesis. Their inhibition has been linked to neural tube defects in humans and animals caused by the disruption of sphingolipid biosynthesis, indicating the significance of ceramide or its derivatives for developmental processes prior to or during neurulation (14, 15).

Ceramide depletion elevated apoptosis and prevented primitive ectoderm morphogenesis in cultured EBs. Our results suggest that this was because of down-regulation of PKCζ/λ-mediated phosphorylation of glycogen synthase kinase-3β.

1 The abbreviations used are: ES cell, embryonic stem cell; Cer, ceramide; EB, embryoid body; FB1, fumonisin B1; F-actin, filamentous actin; FRET, fluorescence resonance energy transfer; GSK-3β, glycogen synthase kinase-3β; HPTLC, high-performance thin-layer chromatography; HRP, horseradish peroxidase; MACS, magnetic-activated cell sorting; PBS, phosphate-buffered saline; PC, phosphatidylcholine; PI, phosphatidylinositol; PIP2, inositol 1,4,5-trisphosphate.
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(GSK-3β). This study shows, for the first time, that ceramide is asymmetrically distributed to the apicalolateral membrane of primitive ectoderm cells. Further, our results suggest that this distribution initiates the ceramide-induced formation of an apical polarity complex of PKCζ/λ with Cdc42 and, subsequently, inactivation of GSK-3β and formation of cortical filamentous actin (F-actin). Hence, ceramide may act as a pacemaker linking the regulation of apoptosis of inner cell mass-derived cells to primitive ectoderm polarity and morphogenesis.

EXPERIMENTAL PROCEDURES

Materials

ES-J1 mouse ES cells and feeder fibroblasts were purchased from the ES core facility (Medical College of Georgia, Augusta, GA). ES-RW4 mouse ES cells (ATCC SCRC-1016) were purchased from the American Type Culture Collection (Manassas, VA). The polyclonal mouse anti-ceramide IgM MAS0020 (GlycoTecbiotech, Kukels, Germany) was a generous gift from Dr. Alfred Merrill, Georgia Institute of Technology, Athens, GA. Sphingomyelin was a generous gift from Dr. Somsankar Das-gupta, Medical College of Georgia. Knock-out Dulbecco’s modified Eagle’s medium, knock-out serum replacement, ES-qualified fetal bovine serum, N2 supplement, basic fibroblast growth factor (FGF-2), Alexa Fluor® 546-conjugated goat anti-rabbit IgG and Alexa Fluor® 647-conjugated phalloidin were obtained from Invitrogen. Dulbecco’s modified Eagle’s medium/F-12 50/50 mix was purchased from Cellgro (Herndon, VA). ESGRO from Invitrogen. Dulbecco’s modified Eagle’s medium, knock-out serum replacement, ES-qualified fetal bovine serum, N2 supplement, basic fibroblast growth factor (FGF-2), Alexa Fluor® 546-conjugated goat anti-rabbit IgG and Alexa Fluor® 647-conjugated phalloidin were obtained from Invitrogen. Dulbecco’s modified Eagle’s medium/F-12 50/50 mix was purchased from Cellgro (Herndon, VA). ESGRO leukemia inhibitory factor was from Chemicon International (Temecula, CA). Myriocin (M-1177), Hoechst 33258, protease (P-8340), and phosphatase (P-8726) inhibitor reagents, polyclonal anti-laminin rabbit IgG (L-9393), and goat anti-rabbit IgG horseradish peroxidase conjugate were obtained from Sigma. Polyclonal anti-PKCζ rabbit IgG (sc-216), polyclonal anti-actin goat IgG (sc-1616), monoclonal anti-GSK-3β (sc-7291), and monoclonal anti-Cdc42 mouse IgG (sc-8401) were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-β-catenin rabbit IgG, anti-phosphorylated PKCζ/λ (Thr-410/Thr-403) rabbit IgG, and anti-phosphorylated GSK-3β (Ser-9) rabbit IgG were purchased from Cell Signaling (Beverly, MA). TrueBlot horseradish peroxidase (HRP)-conjugated antibody native IgG was from eBioscience (San Diego, CA). Fumonisins B1 was from Alexis Biochemicals (San Diego, CA). Cy2 conjugated donkey anti-mouse IgG, Cy2-conjugated donkey anti-mouse IgG (γ-chain specific), Cy3-conjugated donkey anti-mouse IgM (μ-chain specific), HRP-conjugated rabbit anti-mouse IgM (μ-chain specific), Cy3 conjugated donkey anti-rabbit IgG, Cy5-conjugated donkey anti-mouse IgG, Cy2-conjugated donkey anti-mouse IgM (μ-chain specific), Cy3-conjugated goat anti-mouse IgG (Fcγ fragment-specific), goat anti-mouse IgG HRP conjugate, and normal goat and donkey serum were purchased from Jackson ImmunoResearch (West Grove, PA). Recombinant GST-Cdc42 was from Cytoskeleton Inc. (Denver, CO). GST-Par6 was custom-made and purchased from Abnova (Chung Li, Taiwan). The immunohistochemical diaminobenzidine staining kit was from Vector Laboratories (Burlingame, CA). Recombinant human PKCζ and indirubin-3’-monoxime was purchased from Calbiochem. The in situ TUNEL fluorescence staining kit was purchased from Oncogene Research Products (San Diego, CA). The annexin V-MACS kit was from Miltenyi (Auburn, CA). High-performance thin-layer chromatography (HPTLC) plates were purchased from Merck (Whitehouse Station, NJ). The novel ceramide analog N-oleoyl serinol (S18) was synthesized in our laboratory as described previously (16). All reagents were of analytical grade or higher.

Methods

ES Cell Differentiation Protocol and Quantification of Proper Cavitation—The in vitro differentiation of mouse ES cells (ES-J1, ES-RW4) followed a previously published protocol (3, 17). Five μM myriocin was added to the EBs 8–10 h after trypsinization and resuspension of feeder-free ES cells and incubated for 4 days. Sixty μM S18 (a novel ceramide analog) was added to the myriocin-incubated EBs for the last 24 h starting at day 3 of the incubation period. For quantitation of primitive ectoderm formation, EBs were observed (blinded test) under the microscope, and those with a well defined layer of primitive ectoderm (as shown in Fig. 1A, 96 h) were scored as positive. In four independent experiments, EBs were counted from eight different fields, and results were expressed as mean ± S.E. Student’s t test was performed on the data; p < 0.05 was considered statistically significant.

Lipid Analysis—Total lipids were extracted from EBs as described previously (3). The organic solvent was dried under a steady stream of nitrogen and the lipid fractions were purified using a silicic acid column following published procedures (18). In brief, the lipid extracts were applied to a silicic acid column (0.4 × 14 cm), and nonbinding lipids were washed out with 15–20 volumes of CHCl3. The ceramide fraction was then eluted with CHCl3:acetone 9:1 (v/v, 15 ml) and dried. The residue was taken up in solvent, and one-half of the solution was subjected to alkaline methanolysis for 30 min at 37 °C. The reaction mixture was neutralized by the addition of 2 N CH3COOH, and the ceramide fraction was recovered by Folch partition. The lower phase was evaporated to dryness and the lipids resolved by HPTLC using running solvents CHCl3:CH3OH:CH3COOH (95:4.5:0.5; v/v) for sphingomyelin and CH3OH:H2O (20:15; v/v) for ceramide and CHCl3:CH3OH:H2O (20:15:2.5; v/v) for sphingomyelin. Individual bands were visualized by staining with 3% cupric acetate in 8% phosphoric acid and comparing them to the migration distance of standard lipids.

In Vitro Lipid-Protein Polarity Complex—The in vitro reconstitution of a lipid-protein polarity complex was performed following the LIMAC (lipid vesicle-mediated affinity chromatography) procedure as published previously (5). In brief, phosphatidylserine (PS, 420 μg) and C16-ol cereamide (107 μg) were dried from organic solvent and resuspended under sonication in 500 μl of buffer (50 mM Tris/HCl, pH 7.5, 150 mM NaCl). Five μl of 10 mM MnCl2, 1 μl of Vybrant CM-dil, and 500 ng of PKCζ (human, recombinant) was added, and the reaction mixture was incubated under gentle agitation for 60 min at 4 °C. Vybrant CM-dil stained PS/Cer vesicles were recovered by centrifugation at 12,000 × g for 60 min at 4 °C. The pellet (pink) was resuspended in 100 μl of Tris buffer...
supplemented with GTP-γS (100 μM), GDP (1 mM), GST-Par6 (100 ng), or GST-Cdc42 (500 ng) and further incubated for 3 h at 4 °C. Annexin V-buffer (5 μl of a 20× stock solution) and annexin V-conjugated magnetic beads (50 μl) were added, and the reaction mixture was incubated under gentle agitation for another 30 min at 4 °C. Annexin V-MACS was performed following the supplier’s protocol as described previously (2). The elution fraction (1 ml) was supplemented with 10 μg of pure ovalbumin as the precipitation aid. The protein was concentrated by Wessel-Flugge precipitation and analyzed by SDS-PAGE/immunoblotting as described previously (5). The amount of eluted lipid vesicles was quantified by the detection of Vybrant CM-dil (pink) in the organic (chloroform/methanol) phase of the Wessel-Flugge precipitation reaction. The amount of protein was normalized on equal amounts of lipid vesicles.

Immunocyto-/Immunohistochemistry, FRET Analysis, and Quantification of F-actin Formation—EBs were fixed with 4% paraformaldehyde in PBS for 20 min at room temperature. Immunocytochemistry for ceramide was performed with cryosectioned EBs using the MAS0020 anti-ceramide antibody at a dilution of 1:30–1:50. All figures show antibody staining without the use of detergent for permeabilization to avoid loss or artifactual distribution of ceramide. The accuracy of the intracellular antigen distribution was confirmed in a second set of experiments using the same antibody combination after mild permeabilization (0.1–0.2% Triton X-100, 5 min at room temperature) of fixed cells (data not shown). This procedure only slightly reduced the concentration of ceramide (supplemental Fig. S1C). The immunostaining of fixed EBs followed procedures described previously using a blocking solution of 3% ovalbumin, 2% donkey serum in PBS and concentrations of 5 μg/ml primary antibodies or 10 μg/ml secondary antibodies in 0.1% ovalbumin in PBS (5). For immunohistochemistry, incubation with HRP-conjugated anti-IgM, μ-chain-specific secondary antibody was followed by diaminobenzidine staining according to a protocol provided by the supplier (Vector Laboratories). Epifluorescence microscopy was performed with an Axioskop microscope (Carl Zeiss MicroImaging, Inc.) equipped with a Spot II charge-coupled device camera. Confocal fluorescence microscopy and Cy3-to-Cy5 FRET analysis was performed using a Zeiss LSM confocal laser-scanning microscope equipped with a two-photon argon laser at 488 nm (Cy2), 543 nm (Cy3, Alexa Fluor® 546), or 633 nm (Cy5, Alexa Fluor® 647), respectively. Acceptor (Cy5) bleaching was achieved by repetitive scanning at 633 nm until the Cy5 signal was minimized. For quantification of apical F-actin staining, control and myriocin-incubated EBs were fixed and stained with Alexa Fluor® 647-labeled phalloidin. The number of EBs showing apical F-actin staining was counted from micrographs in three independent experiments. More than 50 EBs/group were counted, and the results are expressed as the mean percentage of EBs showing apical F-actin staining ± S.D. Primitive ectoderm formation was quantified in a blinded experiment. Blinding was achieved by coding micrographs and presenting them in random order (controls and treated samples) to three individuals for the analysis of primitive ectoderm morphology and cavitation.

Reverse Transcription and Real-time PCR—Total RNA was prepared from control and myriocin-treated EBs using TRizol® (Life Systems) following the manufacturer’s protocol. First-strand cDNA was synthesized using an Omniscript® RT kit (Qiagen) according to the manufacturer’s protocol. The amount of template from each sample was adjusted until PCR yielded equal intensities of amplification for glyceraldehyde-3-phosphate dehydrogenase. All real-time PCR reactions were performed using iQ SYBR Green Supermix and an iCycler real-time PCR detection system (Bio-Rad). Primers used for real-time PCR were: FGF-5 sense, 5′-gctgcgtacctcaagac-3′; FGF-5 antisense, 5′-ctggaacatgaggtaag-3′. Real-time PCR reactions were normalized to the generation of equal amounts of glyceraldehyde-3-phosphate dehydrogenase amplification product.

Antisense RNAi Transfection of ES Cells and Suppression of SPT-1 and SPT-2 in EBs—ES-J1 cells were cultured without feeder fibroblasts and transected with either Stealth™ RNAi against SPT1 (SPTLC1, MSS218753, RNAi sequence 5′-aaagugcuaagaccucugaga-3′) and SPT2 (SPTLC2, MSS209442, RNAi sequence 5′-aauugacacaccaacgaagc-3′) at a concentration of 100 nm each or Stealth™ RNAi negative control (Invitrogen catalog no. 12935-300, 200 nM) using the Lipofectamine 2000 procedure according to the manufacturer’s instructions (Invitrogen). Stealth™ RNAi was co-transfected with a fluorescein-conjugated double-stranded RNA oligomer (Invitrogen catalog no. 2013, 40 nm) to identify the transfected cells. The efficacy and specificity of the RNAi were confirmed by the transfection of 3T3 fibroblasts, resulting in the reduced expression of SPT1 or SPT2 consistent with the portion of transfected cells (supplemental Fig. S2B). Twelve hours post-transfection, EBs were prepared from the transfected ES cells, and apoptosis and primitive ectoderm morphogenesis were analyzed.

Subcellular Fractionation, Membrane Translocation, and Co-immunoprecipitation Assay—Cells were rinsed twice in ice-cold PBS, resuspended in HEPES-sucrose buffer containing 250 mM sucrose, 10 mM HEPES buffer (pH 7.4), 2 mM magnesium chloride, 1 mM each of EDTA, EGTA, and phenylmethylsulfonyl fluoride (with protease and phosphatase inhibitors added before use), and homogenized in a Dounce homogenizer. Subcellular fractionation was performed by sequential centrifugation as follows: 1) 100 × g for 5 min to remove unbroken cells (pellet); 2) 900 × g for 10 min to remove nuclei (pellet); 3) 17,000 × g for 15 min to remove mitochondria and lysosomes (pellet); and 4) 110,000 × g for 60 min to obtain the cytosolic fraction (supernatant) and a membranous fraction (pellet) containing fragments of the cell membrane, endoplasmic reticulum, and Golgi. All centrifugation steps were carried out at 4 °C. Co-immunoprecipitation assays were performed as described previously (2).

Flow Cytometry of Apoptotic Cells—For flow cytometry, apoptosis was assayed using Vybrant apoptosis assay kit 2 (Invitrogen) according to the manufacturer’s instructions. Briefly, EBs were trypsinized and passed through a 40-μm mesh screen. Cells were taken up in 100 μl of annexin V
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binding buffer and stained with Alexa Fluor® 488-conjugated annexin V and propidium iodide (PI) for 15 min at room temperature. Stained cells were then analyzed by flow cytometry measuring the fluorescence emission at 530 nm (FL1, annexin V) and >575 nm (FL2, PI).

RESULTS

Ceramide Depletion Disrupts Primitive Ectoderm Morphogenesis—Feeder-free mouse ES cells (ES-J1, ES-RW4) were differentiated in suspension EB culture. Fig. 1A shows that the EBs underwent primitive endoderm formation, cavitation, and primitive ectoderm morphogenesis within 96 h. The effect of ceramide depletion on the composition of sphingolipids and ES cell lineage specification was determined using the SPT inhibitor myriocin (12, 13). Suspension-cultured EBs were incubated with myriocin starting 10 h after reaggregation of tryptin-dissociated ES cells into EBs. Isolation of ceramide by silicic acid gel chromatography followed by HPTLC analysis (Fig. 1B) showed that myriocin incubation reduced ceramide (Cer, lane 3; control in lane 1) and sphingomyelin (SM, lane 9; control in lane 8) by more than 80%. Reduction of ceramide and sphingomyelin was also found when EBs were incubated with the ceramide synthase inhibitor fumonisin B1 (lanes 6 and 10), which confirmed that EBs were depleted of ceramide and sphingomyelin by inhibition of ceramide biosynthesis. After 72 h of cultivation, ceramide-depleted EBs were incubated for 24 h with 60 μM S18, a novel ceramide analog, which was not converted to ceramide or other sphingolipids (2, 5, 16, 19). Novel ceramide analogs have been synthesized in our laboratory and found to mimic many effects of ceramide such as induction of apoptosis in PAR-4-expressing differentiating ES cells, and binding and activation of PKCζ/λ in vitro and in stem cells. Fig. 1B shows that S18 was enriched to a final concentration equivalent to that of endogenous ceramide in control cells (Myr+S18, lane 13; control in lane 11). There was no significant alteration of the level of residual endogenous cera-
mide after S18 treatment, indicating that the novel ceramide analogs did not interfere with ceramide metabolism.

Using real-time reverse transcriptase-PCR we found that the gene expression of the primitive ectoderm marker FGF-5 was not significantly affected by incubation with myriocin (data not shown). This result suggested that the lineage specification of primitive ectoderm cells was not impaired by the inhibition of ceramide biosynthesis. However, 80% of the myriocin-treated EBs did not cavitate and failed to form a distinct layer of columnar primitive ectoderm cells (Fig. 1, C and D). This indicated that ceramide depletion increased the number of EBs with defective primitive ectoderm morphogenesis and cavity formation by 4-fold. After 72 h of cultivation, myriocin-treated EBs were incubated for 24 h with 60 μM S18 or 2 μM C16-ceramide. Replenishment of ceramide or substitution with S18 resulted in partial restoration of the proportion of EBs with well organized primitive ectoderm and defined cavity (Fig. 1, D and E, and supplemental Fig. S1, D and E). These results showed that inhibition of ceramide biosynthesis impaired primitive ectoderm morphogenesis, which was compensated by exogenously added ceramide or a ceramide analog.

Our previous studies provided evidence that ceramide-mediated activation of aPKC promoted aPKC-dependent phosphorylation inactivation of GSK-3β (5). Consistently, formation of the primitive ectoderm layer was partially restored when myriocin-treated cells were incubated with a GSK-3β inhibitor, indirubin-3′-monoxime (Fig. 1, C and D, Myr+Ind). This result was also consistent with the observation that blocking aPKC with a specific inhibitor peptide completely abolished cavitation of EBs (Fig. 1D, PZI). Taken together, these results suggest that ceramide may have regulated primitive ectoderm formation by induction of aPKC-mediated phosphorylation of GSK-3β.

Ceramide Depletion Induces Apoptosis—The pro-amniotic cavity of the pregastrulation embryo and the central cavity of EBs result from apoptosis of cells that do not participate in the formation of the primitive ectoderm layer. Apoptosis during EB formation was quantified by flow cytometry using cells from dissociated EBs that were stained with Alexa Fluor 488-conjugated annexin V and PI and then analyzed by flow cytometry. The control RNAi (scrambled) was detected in cells transfected with feeder-free ES cells or embryos (supplemental Fig. S1, C and D), suggesting that ceramide depletion elevated apoptosis within the EBs concurrent with disrupted primitive ectoderm formation. Myriocin-induced cell death was reduced when ceramide-depleted EBs were incubated with S18, consistent with the restorative effect of S18 on primitive ectoderm morphogenesis in ceramide-depleted EBs (Fig. 1, C and D, and supplemental Fig. S1D).

The morphological appearance of a densely Hoechst-stained cell mass in ceramide-depleted EBs indicated that the apoptotic cells persisted within the EBs (Fig. 2B and supplemental Fig. S1D). Fig. 2B shows that apoptotic cells were localized primarily underneat the emergent primitive ectoderm or within the center of the EBs (arrows pointing at cells with condensed nuclei). These cells also stained for TUNEL and active caspase-3, two assays indicating apoptosis in differentiating stem cells or embryos (supplemental Fig. S1, D and E).

To determine the effect of the inhibition of ceramide biosynthesis in individual cells, we transfected feeder-free ES cells with RNAi, which suppressed the expression of SPT1 and SPT2 (supplemental Fig. S2B). The cells were co-transfected with a fluorescein-conjugated control oligonucleotide (green) to allow visualization of the transfected cells. Control RNAi-transfected cells are found in the primitive ectoderm layer (arrows, left panel), whereas SPT-RNAi-transfected cells are apoptotic and reside exclusively within the center of the EBs (arrows, right panel). VEn, visceral endoderm; BL, basal lamina; PEC, primitive ectoderm.
EBs but not in the primitive ectoderm layer (arrows in Fig. 2C). This result was consistent with the distribution of cells with condensed nuclei (arrows in Fig. 2B), indicating that elevation of apoptosis and impairment of primitive ectoderm formation was due to the inhibition of ceramide biosynthesis. Flow cytometry showed that the number of fluorescent cells was reduced by 28.3 ± 3.5% in EBs differentiated from RNAi-transfected ES cells as compared with the portion of cells transfected with control RNAi (data not shown). These results suggested that specifically those cells were eliminated in which ceramide biosynthesis was inhibited.

**Ceramide Is Co-distributed with F-actin and β-Catenin at the Apicolateral Membrane of Primitive Ectoderm Cells**—To determine the distribution of ceramide in primitive ectoderm cells, early post-implantation mouse embryos or ES cell-derived EBs were fixed, and immunohistochemistry was performed on cryosections using the mouse polyclonal IgM MAS0020 antibody. This antibody has been shown to be specific for ceramide (20). The specificity of the antibody was confirmed by diminishing the immunofluorescence signal when the antibody was preadsorbed to ceramide (supplemental Fig. S1A). The signal was also reduced when ceramide was depleted by incubation with myriocin but remained unaffected when only glycosphingolipid biosynthesis was inhibited (supplemental Fig. S1B). These results indicated that the MAS0020 antibody is well suited to specifically detect ceramide in cellular membranes.

Confocal laser-scanning immunofluorescence microscopy showed that in the early post-implantation embryo (5.5 days post-coital), ceramide was enriched in the apical membrane of primitive ectoderm cells (Fig. 3A). Cross-sections of the embryo indicated that apical ceramide was, at least in part, co-distributed with F-actin (Fig. 3B).

**Immunohistochemical staining** showed that the strongest signal for ceramide was obtained at the apical membrane of columnar primitive ectoderm cells (Fig. 4A, Ap and PEc). Ceramide elevation was also found in primitive or visceral endoderm cells (a cell layer on top of the basal lamina); however, this elevation was not in a polarized distribution as with primitive ectoderm cells.

This signal pattern was confirmed using confocal laser-scanning immunofluorescence microscopy with whole-mount and cryosectioned EBs (Fig. 4, B and C) and by z-scan (apical to basal) in single primitive ectoderm cells (supplemental Fig. 4D).
Ceramide Associates with PKC\(\zeta/\lambda\) at the Apical Membrane—In previous studies, we and other groups showed that ceramide binds directly to PKC\(\zeta/\lambda\) (2, 5, 19, 21–24). The observation that the distribution of ceramide is polarized suggests that ceramide-mediated apical translocation of PKC\(\zeta/\lambda\) is a regulatory step in primitive ectoderm formation. The importance of PKC\(\zeta/\lambda\) for primitive ectoderm morphogenesis in EBs was indicated by the effect of the pseudosubstrate inhibitor peptide of PKC\(\zeta/\lambda\) preventing proper cavitation of EBs (Fig. 1E). The inhibitor did not affect the viability or proliferation of ES cells cultivated without feeder fibroblasts, which was consistent with the absence of PKC\(\zeta/\lambda\) expression in undifferentiated ES cells (supplemental Fig. S2C). The significance of ceramide for the membrane translocation of PKC\(\zeta/\lambda\) was then determined by immunocytochemistry analyses.

In cryosectioned EBs, total PKC\(\zeta/\lambda\) was detected using an antibody (raised in goat) against the N terminus of the enzyme. A second primary antibody (raised in rabbit) was used to determine phosphorylation of threonine 410/403 (pPKC\(\zeta/\lambda\)), indicating activation of the enzyme. Fig. 5A shows that PKC\(\zeta/\lambda\) was distributed mainly to the membrane of primitive ectoderm and primitive/visceral endoderm cells. There was a moderately higher fluorescence signal for PKC\(\zeta/\lambda\) at the apical membrane of primitive ectoderm cells, whereas primitive/visceral endoderm cells showed a uniform membrane translocation of the enzyme. The antibody against the phosphorylated enzyme, however, detected pPKC\(\zeta/\lambda\) almost exclusively at the apical membrane of primitive ectoderm cells. The fluorescence signal was strictly co-distributed with that of ceramide, suggesting that ceramide was associated with pPKC\(\zeta/\lambda\) (Fig. 5A). This assumption was also supported by the detection of a ceramide-to-pPKC\(\zeta/\lambda\) FRET signal (supplemental Fig. S3A). Taken together, these results suggest that ceramide is associated with PKC\(\zeta/\lambda\) at the apical membrane and that ceramide-bound PKC\(\zeta/\lambda\) is phosphorylated at threonine 403/410.

Ceramide-associated PKC\(\zeta/\lambda\) Binds to Cdc42—It has been shown that PKC\(\zeta/\lambda\) interacts with Cdc42 and that this interaction regulates epithelial cell polarity (25–27). Immunocytochemistry was performed to determine the subcellular localization of Cdc42, pPKC\(\zeta/\lambda\), and ceramide. Fig. 5, B–D, shows that these molecules were co-distributed at the apical membrane of primitive ectoderm cells. Ceramide, pPKC\(\zeta/\lambda\), and Cdc2 were also found to be co-distributed at the plasma membrane of primitive/visceral endoderm cells but not in a polarized fashion. The apical co-distribution of these three molecules was confirmed using confocal z-stack scanning with whole-mount EBs (Fig. 5C) and FRET analyses (supplemental Fig. S3B). In particular, the detection of ceramide-to-pPKC\(\zeta/\lambda\) and Cdc42-to-pPKC\(\zeta/\lambda\) FRET signals suggested that ceramide, pPKC\(\zeta/\lambda\), and Cdc42 formed a lipid-protein polarity complex at the apical membrane of primitive ectoderm cells.

The significance of ceramide for the membrane translocation and association of pPKC\(\zeta/\lambda\) with Cdc42 was tested by depleting EBs of ceramide. Fig. 6A shows that myricoin treatment did not inhibit the phosphorylation of PKC\(\zeta/\lambda\) at threonine 410/403, but it completely abolished the co-distribution of pPKC\(\zeta/\lambda\) and Cdc42. After ceramide depletion, pPKC\(\zeta/\lambda\) and Cdc42 were not even detectable in the same cells. This result was confirmed by co-immunoprecipitation studies showing that myricoin treatment reduced the amount of Cdc42 that could be co-immunoprecipitated with PKC\(\zeta/\lambda\) (Fig. 6B). The signal for immunoprecipitated PKC\(\zeta/\lambda\) was not significantly altered, indicating that ceramide depletion did not interfere with PKC\(\zeta/\lambda\) expression but with the formation of a complex containing PKC\(\zeta/\lambda\) and Cdc42. Loss of this complex may then have resulted in the distribution of PKC\(\zeta/\lambda\) and Cdc42 to dif-
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Ceramide affects the binding of PKCζ/λ and Cdc42 to the membrane, which regulates cell polarity.

**FIGURE 6.** Ceramide induces a polarity complex of pPKCζ/λ with Cdc42, stabilizing apical F-actin. A, confocal laser-scanning microscopy with myriocin-treated, whole-mount EBs using antibodies against ceramide (Cy2, green), Cdc42 (Cy3, red), and pPKCζ/λ (Cy5, blue). In ceramide-depleted EBs, pPKCζ/λ and Cdc42 are segregated into different cells. B, EBs were homogenized in detergent-supplemented buffer, and the immunoprecipitation reaction was performed using an antibody against PKCζ/λ. Co-immunoprecipitated Cdc42 was detected by SDS-PAGE and immunoblotting of the immunoprecipitated protein. Con, control; Myr, myriocin. C, EBs were mechanically disrupted in detergent-free buffer, and the membrane and cytosolic fraction were separated by differential centrifugation at 100,000 × g. The distribution of PKCζ/λ was determined by SDS-PAGE and immunoblotting of protein from the two fractions. D and E, annexin V-MACS was performed with in vitro reconstituted polarity complexes using PS-containing lipid vesicles. Lane 1, recombinant human PKCζ (standard protein, 2 ng) detected with anti-PKCζ/λ; lane 2, molecular weight markers; lane 3, PS/Cer vesicles; lane 4, PC/Cer vesicles; lane 5, PS/PC vesicles; lane 6, GST-Par6 (2 ng) and GST-Cdc42 (2 ng) standard protein detected with HRP-conjugated anti-GST; lane 7, molecular weight markers; lane 8, PS/Cer vesicles; lane 9, PC/Cer vesicles; lane 10, PS/PC vesicles; lane 11, molecular weight markers; lane 12, PS/Cer vesicles, only GTPγS and GST-Cdc42; lane 13, PS/Cer vesicles, GTPγS, GST-Cdc42; and PKCζ; lane 14, GDP, GST-Par6, GST- Cdc42, and PKCζ; F, immunoblotting performed with EB-derived protein as indicated in the figure. The blotting membranes were first stained for the phosphorylated proteins and then reprobed with antibodies recognizing both the phosphorylated and the nonphosphorylated form of the proteins. Finally, the membranes were reprobed for actin. G, F-actin formation quantified from the number of EBs with cortical distribution of Alexa Fluor 647 phalloidin (left panel) shows control, arrows point at F-actin ring. Results show means ± S.D. from at least three independent experiments quantifying the F-actin staining from at least 50 EBs in each experiment.
retention of these vesicles in the MACS column. None of the proteins was co-eluted with PS/PC vesicles (Fig. 6, D and E, lanes 5 and 10), clearly showing that they did not bind to PS or PC but specifically to ceramide. Cdc42 by itself was not eluted with PS/Cer vesicles (Fig. 6E, lane 12), indicating that binding of Cdc42 required association of PKCζ with ceramide. Cdc42 was co-eluted with PKCζ only in trace amounts when Par6 or GTPγS was omitted (Fig. 6E, lanes 13 and 14), indicating that the ceramide-associated in vitro polarity complex required the presence of Par6 and activation of Cdc42. In summary, these results suggested that the asymmetric distribution of ceramide in primitive ectoderm cells mediates association of PKCζ/λ with the apicolateral membrane and, subsequently, co-distribution and association of ceramide-bound PKCζ/λ with Cdc42.

**Ceramide Depletion Prevents Phosphorylation of GSK-3β and F-actin Formation**—It has been reported that the atypical PKC-Par6-Cdc42 polarity complex phosphorylates GSK-3β, a protein kinase in which inactivation by phosphorylation at serine 9 is critical for the establishment of cell polarity (25–27). Immunoblot analysis was performed to determine the time-dependent phosphorylation of PKCζ/λ and GSK-3β in control and myriocin-incubated EBs. Fig. 6F shows that the level of pPKCζ/λ was highest at 24 h after initiation of EB formation (lane 2). Reprobing of the membrane for total PKCζ/λ indicated that its expression level was highest at 24 and 48 h. The ratio of pPKCζ/λ to total PKCζ/λ, however, showed that that elevation of pPKCζ/λ was not entirely due to increased levels of protein but to activation of a mechanism that elevates its degree of phosphorylation. We then determined the phosphorylation of GSK-3β at serine 9 (phospho- or pGSK-3β). The level of pGSK-3β was highest at 48 h after initiation of EB formation (Fig. 6F, lane 3), suggesting that its phosphorylation followed activation of PKCζ/λ. There was no alteration in the protein level of GSK-3β, indicating that its phosphorylation was due to prior activation of PKCζ/λ. At this time, the primitive endoderm and basal lamina were established, and cavitation and primitive ectoderm formation were initiated (Fig. 1A). Myriocin treatment reduced the phosphorylation of PKCζ/λ by 50% as determined from the ratio of pPKCζ/λ to total PKCζ/λ (Fig. 6F, lanes 6 and 7). Consistently, phosphorylation of GSK-3β (Fig. 6F, lane 7) was also reduced. These results suggest that ceramide induced PKCζ/λ-mediated phosphorylation and inactivation of GSK-3β.

Formation of F-actin at the apicolateral membrane is known to be essential for the polarization of epithelial cells (25). To determine ceramide-dependent F-actin formation, immunocytochemistry was performed in the presence and absence of myriocin. F-actin was visualized and quantified by staining with fluorescence (Alexa 647)-labeled phalloidin. The formation of cortical F-actin was prevented or significantly reduced when EBs were depleted of ceramide (Fig. 6G). When EBs were incubated with S18, phalloidin binding due to F-actin formation was restored. In summary, these results suggest that ceramide-mediated formation of a polarity complex is critical for the formation of F-actin at the apicolateral membrane and, subsequently, for the morphogenesis of the primitive ectoderm epithelium.

**DISCUSSION**

Results from our previous studies show that the elevation of ceramide induces apoptosis in a portion of EB-derived cells (Oct-4(+)/PAR-4(+)) that are at the point of differentiating into neural progenitor cells (2, 3). In the present study, evidence suggests that at earlier differentiation stages, ceramide prevents apoptosis and promotes primitive ectoderm morphogenesis. Our studies have shown that ceramide binds to atypical PKCζ/λ in EB-derived cells (5). The ceramide-PKCζ/λ complex may then associate with PAR-4, which results in the inhibition of PKCζ/λ and the induction of apoptosis. PAR-4, however, is not expressed in the early stages of ES differentiation, ruling out the possibility that binding of ceramide will result in the PAR-4-mediated inhibition of PKCζ/λ (3, 4). On the contrary, results presented here suggest that in the absence of PAR-4, ceramide mediates association of PKCζ/λ with Cdc42, a small GTPase that has been shown to activate PKCζ/λ in a polarity complex with Par6 (25). Ceramide may thus have detrimental or beneficial effects for cell survival and differentiation, depending on whether it promotes complex formation of PKCζ/λ with inhibitor or activator proteins, respectively.

The model shown in Fig. 7A outlines a molecular mechanism by which ceramide may prevent apoptosis and, at the same time, promote morphogenesis of primitive ectoderm cells in cavitating EBs. In step 1 (Fig. 7A), up-regulation of SPT results in the elevation of ceramide by de novo biosynthesis. Ceramide binds to PKCζ/λ at the cell membrane. This association with ceramide is consistent with recent results from our laboratory showing the association of PKCζ/λ with ceramide-containing lipid vesicles (5). It is also shown by the effect of myriocin or myriocin plus S18 on depleting or replenishing the portion of PKCζ/λ in the membrane fraction. Membrane-bound PKCζ/λ is phosphorylated at threonine 410/403, an observation that is supported by the results from the immunoblot and ceramide-to-pPKCζ/λ FRET analysis. It is consistent with our previous studies showing that PKCζ/λ is phosphorylated and activated when associated with ceramide or novel ceramide analogs (5, 19). The significance of binding to ceramide for the sustained phosphorylation of PKCζ/λ is indicated by the lower phosphorylation level in the presence of myriocin. From our results, however, we cannot exclude the possibility that at least a portion of the membrane-resident ceramide was derived from sphingomyelin. Sphingomyelin may have first been synthesized from ceramide and then hydrolyzed to ceramide by sphingomyelinase at the apicolateral cell membrane.

In step 2 of this model (Fig. 7A), ceramide-bound PKCζ/λ forms a complex with Cdc42, a small GTPase known to activate the kinase via binding to PKCζ/λ-associated Par6 (26, 28). Consistent with our model, ceramide depletion reduces the formation of this protein complex, as shown by co-immunoprecipitation assays and immunocytochemistry/FRET analysis. Interestingly, ceramide depletion even results in phosphorylated PKCζ/λ and Cdc42 ending up in different cells within the growing EB. The ceramide-mediated association appears to ensure the distribution of the two proteins...
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A model for ceramide-induced cell polarity and primitive ectoderm morphogenesis in EBs. A, ceramide in which biosynthesis is initiated and regulated by SPT binds to atypical PKCζ/λ at the cell membrane (step 1) and may induce complex formation with polarity proteins such as Par6 and Cdc42 (step 2). Activation of PKCζ/λ results in phosphorylation/inactivation of GSK-3β (step 3). Inactivation of GSK-3β results in the formation of F-actin, possibly interacting with β-catenin via actin-associated proteins (X). Ceramide depletion due to inhibition of SPT (myriocin) or ceramide synthase (FB1) induces apoptosis and disrupts primitive ectoderm morphogenesis. Morphogenesis and cavitation is restored by incubation of ceramide-depleted EBs with endogenous ceramides (e.g. 16:0-ceramide), novel ceramide analogs (e.g. S18), or inhibition of GSK-3β. B, cells within the EB up-regulate and then polarize the distribution of ceramide, Cdc42 and PKCζ/λ, and F-actin toward the apicolateral membrane (ap). Cells that fail to up-regulate ceramide undergo apoptosis and form a cavity underneath the newly formed primitive ectoderm layer (PEC). VEn, visceral endoderm. C, expansion of the primitive ectoderm (red arrows) due to cell division increases the surface of the cavitating EB (green arrows). Removal of dead cells within the EB gives rise to the cavity.

To the same progeny cells. It has been suggested that Par6 inhibits PKCζ/λ unless the two proteins form a complex with Cdc42 (25, 26). Thus, it is likely that ceramide-bound PKCζ/λ is associated with Par6, which binds to Cdc42. This assumption is consistent with the results of the lipid vesicle binding assays showing that in the absence of Par6, only trace amounts of Cdc42 bind to PKCζ-associated PS/Cer vesicles. In living cells, Cdc42 may sustain activation of PKCζ/λ, probably by enhancing its phosphorylation by PDK1 (3-phosphoinositol-dependent kinase-1) or by auto-phosphorylation. PDK1 is recruited to the membrane and activated by PIP3 (29). Consistent with this model, genetic ablation of Cdc42 results in similar effects on primitive ectoderm formation as observed with ceramide depletion in our study (30). These results suggest that the sphingolipid/ceramide and phosphoinositol/PIP3 pathways may act synergistically in a lipid-protein complex consisting of ceramide-bound PKCζ/λ-Par6-Cdc42 and PIP3-bound PDK1, which phosphorylates and further activates PKCζ/λ.

In step 3 (Fig. 7A), the ceramide-associated polarity complex promotes phosphorylation and, therefore, inactivation of GSK-3β. The dependence of GSK-3β phosphorylation on the intracellular ceramide concentration/distribution is shown by the reduction of its phosphorylation level in ceramide-depleted EBs. It is also consistent with our most recent study showing that incubation of EB-derived cells with S18 results in the increased phosphorylation of GSK-3β (5). Phosphorylation at serine 9 has been shown to inactivate GSK-3β and to dissociate the enzyme from PKCζ/λ (25). The inactivation of GSK-3β may involve association with ceramide-bound PKCζ/λ at the membrane, as shown in Fig. 7A, but is also likely to involve steps independent of this association. This assumption is supported by our results showing that inactivation of GSK-3β with a pharmacological inhibitor can restore primitive ectoderm formation in ceramide-depleted EBs. Therefore, the model in Fig. 7A illustrates only one possible mechanism by which ceramide may regulate primitive ectoderm formation via GSK-3β. Ceramide may very well initiate activation of PKCζ/λ at the apical membrane, whereas downstream effects on GSK-3β are independent of the association with ceramide. At this point, the potential interaction of ceramide-associated PKCζ/λ with Cdc42 or GSK-3β and its function for primitive ectoderm morphogenesis needs to be further established, which will be part of our future research. Among candidate proteins regulating F-actin formation, many downstream targets of GSK-3β may be affected, including adenomatous polyposis coli (APC), β-catenin, and various actin or microtubule-associated proteins (25, 31, 32). Most recently, we have suggested that sphingolipids induce the formation of protein complexes termed SLIPS (sphingolipid-induced protein scaffolds) (1). Our results indicate that the ceramide-induced formation of apicolateral polarity complexes regulating actin polymeriza-
tion may be an example of the function of polarized SLIPS during embryo morphogenesis.

Our data suggest that ceramide links primitive ectoderm morphogenesis to the regulation of apoptosis in cavitating EBs. Fig. 7, B and C, shows that this regulation may determine the epithelization of primitive ectoderm cells on top of the emergent cavity. Primitive ectoderm morphogenesis is linked in an orderly manner to apoptosis, perhaps as the result of asymmetrical cell division and/or shared cell signaling pathways. Previous studies have suggested that pro-survival or pro-apoptotic signals derived from primitive or visceral endoderm cells determine the fate of the presumptive primitive ectoderm (33, 34). Our results are compatible with the previous model in that these extrinsic signals may regulate the generation of ceramide or its distribution in cells within the EB. However, our results also suggest that primitive ectoderm morphogenesis and apoptosis is regulated by intrinsic signals such as elevation and asymmetrical (apicomedial) distribution of ceramide. This hypothesis is supported by the observation that ceramide depletion prevents cell polarity and induces apoptosis specifically in cells that are transfected with the SPT-RNAi but not in adjacent cells or in cells transfected with the control RNAi.

The critical role of sphingolipids for morphogenesis has been suggested by previous studies showing that incubation of embryos with FB1 results in deficient neural tube closure (14, 29). The embryonic lethality of knock-out mice for sphingosine kinases or glycosyltransferases clearly shows that the expression of particular ceramide derivatives is vital for embryo development (35, 36). The phenotypes of the recovered embryos, however, rather suggest post-gastrulation or neurulation defects that occur later than cavitation or primitive ectoderm formation. In the SPT knock-out mouse, embryos die well before embryonic day 15; early embryos have not been recovered yet, indicating that ceramide itself is critical for early embryo development (11). This is consistent with our observation that ceramide is highly enriched in the apical membrane of primitive ectoderm cells in the early post-implantation embryo. It is also supported by the observation that the novel ceramide analog S18, which is not converted to ceramide derivatives, can partially rescue cavitation and primitive ectoderm morphogenesis in ES-cell derived EBs. However, because our interpretation relies on the use of this ceramide analog it remains to be investigated whether other structurally related compounds or ceramide derivatives may also critically partake in morphogenesis.

Our data show, for the first time, that ceramide is apically distributed in primitive ectoderm cells and that this distribution may be critical for primitive ectoderm morphogenesis. Cells that fail to achieve this polarization will undergo apoptosis. Cells that show apical ceramide distribution may form a PKCζ/α-dependent polarity complex that establishes cell polarity in the newly formed primitive ectoderm layer. These results thus provide evidence for a possible link between ceramide biosynthesis and actin polymerization and the exciting possibility that ceramide is a “morphogenetic lipid,” the polarized distribution of which may regulate the polarity of embryonic epithelia.

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