Regulation of primary cilia formation by ceramide[S]

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Abstract The primary cilium is an important sensory organelle, the regulation of which is not fully understood. We found that in polarized Madin-Darby Canine Kidney cells, the sphingolipid ceramide is specifically distributed to a *cis*-Golgi compartment at the base of the primary cilium. This compartment immunostained for the centrosome marker γ-tubulin, the Rho type GTPase cell division cycle 42 (Cdc42), and atypical protein kinase Cζ/λ (aPKC), a kinase activated by ceramide and associated with a polarity protein complex consisting of partitioning defective (Par)6 and Cdc42. Inhibition of ceramide biosynthesis with Fumonisin B1 prevented codistribution of aPKC and Cdc42 in the centrosomal/pericentriolar compartment and severely impaired ciliogenesis. Cilium formation and codistribution of aPKC and Cdc42 were restored by incubation with N-acetyl or N-palmitoyl sphingosine (C2 or C16 ceramide), or the ceramide analog N-oleoyl serinol (S18). Cilium formation was also restored by the glycogen synthase kinase-3β (GSK-3β) inhibitor indirubin-3-monoxime, suggesting that regulation of ciliogenesis depends on the inhibition of GSK-3β by ceramide-activated aPKC. Consistently, inhibition of aPKC with a pseudosubstrate inhibitor prevented restoration of ciliogenesis by C2 ceramide or S18. Our data show for the first time that ceramide is required for primary cilium formation.—Wang, G., K. Krishnamurthy, and E. Bieberich. **Regulation of primary cilia formation by ceramide. *J. Lipid Res.* 2009.** 50: **2103–2110.**

Supplementary key words sphingolipids • cell polarity • primary cilium • Golgi • centrosome

The primary cilium was first described in 1898; however, its critical function for cell polarity and cell signaling was not discovered until recently (1–6). In contrast to motile cilia that usually have an inner structure of 9+2 microtubules, the primary cilium is nonmotile with the two central microtubules missing. Nevertheless, primary cilia are studded with receptors, including those for PDGF, Wnt, and sonic hedgehog cell signaling pathways (7–11). This feature makes the primary cilium one of the most important sensory organelles on the apical surface of epithelial cells, suggesting an essential function as a "cellular antenna" linked to cell polarity. As a consequence, loss or impairment of the primary cilium goes in hand with severe disorders of epithelial cell polarity. Disorders linked to impaired ciliogenesis are known as ciliopathies and encompass a variety of diseases such as polycystic kidney disease, primary ciliary dyskinesia, retinopathies, combined developmental deficiencies (e.g., Joubert and Meckel syndrome), and other neuronal and sensory disorders (3, 5, 6, 11).

Despite the recent progress in determining the structure and protein composition of primary cilia, it is not fully understood how ciliogenesis is regulated. Immunocytochemistry and electron microscopy studies suggest that the primary cilium originates at the centrosome, usually in close vicinity to the Golgi apparatus (12–14). One of the key factors associated with formation of the primary cilium is γ-tubulin, a cytoskeletal protein that is found in the centriole and pericentriolar material (PCM) at the base of the cilium (2, 15–18). Another factor known to regulate centriole morphogenesis and in turn, cilia formation is the *cis* Golgi marker protein GM130 (19). Many other factors, in particular intraflagellar transport proteins are involved in cilia formation and elongation (5). However, it is still unclear what role lipids play in ciliogenesis.

Most recently, we have found that the sphingolipid ceramide is essential for epithelial cell polarity in the primitive ectoderm and the neuroepithelium (20, 21). We have also shown that its function in cell polarity relies on binding and activation of atypical protein kinase Cζ, λ/ι (aPKC), and the subsequent formation of an aPKC-associated polarity protein complex including the Rho type small GTPase cell division cycle 42 (Cdc42) and the polarity

This work was supported in part by National Institutes of Health Grant R01NS046835 and a March of Dimes grant 6-FY08-322. Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the National Institutes of Health.

Manuscript received 3 March 2009 and in revised form 13 April 2009 and in re-revised form 15 April 2009.

Published, JLR Papers in Press, April 16, 2009
DOI 10.1194/jlr.M900097-JLR200

Abbreviations: aPKC, atypical protein kinase C (PKCζ, λ/ι); Cdc42, cell division cycle 42; ER, endoplasmic reticulum; FB1, fumonisin B1; HPTLC, high-performance thin-layer chromatography; GM130, Golgi matrix 130; GSK-3β, glycogen synthase kinase-3β; MDCK, Madin Darby Canine Kidney; Par, partitioning defective; PCM, pericentriolar material; PZI, pseudosubstrate inhibitor of PKCζ (aPKC); S18, N-oleoyl serinol; SPT, serine palmitoyltransferase; WGA, wheat germ agglutinin.

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[S] The online version of this article (available at http://www.jlr.org) contains supplementary data in the form of two figures.

protein partitioning defective (Par)6 (20–22). Therefore, we hypothesized that the ceramide-dependent regulation of the aPKC-associated cell polarity complex is involved in the formation or elongation of the primary cilium. This hypothesis is consistent with reports on the critical function of aPKC, Par6, and Par3 in ciliogenesis (23, 24). Using a pharmacologic inhibitor of ceramide biosynthesis (Fumonisin B1 or FB1) we show here for the first time that ceramide is required for the regulation of ciliogenesis in Madin Darby Canine Kidney (MDCK) cells. We also describe a novel pericentriolar compartment associated with the *cis* Golgi and enriched in ceramide. Because this compartment is at the base of the primary cilium, our results suggest that ceramide in the *cis* Golgi is critical for ciliogenesis via recruitment and activation of aPKC.

MATERIAL AND METHODS

Materials

MDCK II cells were obtained from Dr. Quansheng Du (Medical College of Georgia, Augusta, GA). The anti-GM130 antibody (mouse IgG) was a generous gift from Dr. Richard Cameron (Medical College of Georgia, Augusta, GA). Anti-γ tubulin mouse IgG, anti-acetylated tubulin mouse IgG, and FB1 were purchased from Sigma (St. Louis, MO). Anti-PKCζ (C20) rabbit IgG and anti-Cdc42 mouse IgG was from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Anti-ceramide rabbit IgG was generated in our laboratory as previously described (25). Anti-ceramide mouse IgM (MAS0020) was purchased from Glycobiotech (Kuekels, Germany). Alexa 647-conjugated phalloidin and DMEM were obtained from Invitrogen (Carlsbad, CA). All ceramide species were of the highest purity and purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). N-oleoyl serinol (S18) was synthesized in our laboratory as previously described (26, 27). Myristoylated pseudo substrate inhibitor of PKCζ (PZI) was obtained from Biomol (Plymouth Meeting, PA). High-performance thin-layer chromatography (HPTLC) plates were from Merck (Darmstadt, Germany). 12-well dishes containing 24 mm diameter polyester filters with pores of 0.4 μm diameter were purchased from Corning, Inc. (Corning, NY). All other reagents and solvents were of analytical quality.

Methods

Cultivation of MDCK II cells. MDCK II cells were maintained in DMEM containing 10% fetal calf serum, penicillin, and streptomycin in a 5% CO2, 37°C incubator. Cells were seeded onto 24-mm polyester filters (0.4 μm pore size) at a density of 250,000 cells/filter and cultivated for 72 h to establish apicobasal polarity prior to drug treatment. For further treatment affecting ciliogenesis, cells were incubated for another 72 h in the presence of various drugs (30 μM FB1, 40 μM S18, 10 μM C2 ceramide; 1 μM C16 ceramide, or 50 μM PZI) and then fixed with 4% p-formaldehyde in phosphate-buffered saline (PBS) prior to immunocytochemistry or lipid analysis.

Immunocytochemistry. After fixation with 4% p-formaldehyde/PBS, nonspecific binding sites were blocked with 3% ovalbumin/10% donkey serum/PBS for 1 h at 37°C. Cells were then incubated with primary and secondary antibodies at a concentration of 5 μg/ml or 10 μg/ml in 0.1% ovalbumin as described previously (20–22). Epifluorescence microscopy was performed with an Axiophot microscope (Carl Zeiss MicroImaging, Inc..) equipped with a Spot II CCD camera. Confocal fluorescence microscopy was performed using a Zeiss LSM510 confocal laser scanning microscope equipped with a two-photon argon laser at 488 nm (Cy2), 543 nm (Cy3), or 633 nm (Cy5, Alexa Fluor 647), respectively.

Quantitation of cilia formation and statistics. Cilia length was measured using Zeiss LSM Image Examiner software (Carl Zeiss) modified from (28). Briefly, captured z-stack confocal images (n >50 cilia from at least four separate experiments) were projected at an angle to facilitate visualization and quantitation of the primary cilia. The cilium length was measured by tracing cilia on micrographs using METAMORPH software. Cilia of at least 3 μm in length were counted as elongated cilia. Data were expressed as means ± SD. Statistical significance was determined using Student's *t*-test.

Ceramide analysis. Total lipids were extracted from cells using a mixture of chloroform-methanol (2:1; v/v) as previously described (20, 21, 29, 30). For analysis of subcellular distribution, ceramide fractions of the cytosol and membranes were dissolved in equal volumes of solvent and the lipids were resolved by HPTLC using the running solvent chloroform-methanol-acetic acid (95:4.5:0.5; v/v/v) for the separation of ceramide. Individual bands were visualized by staining with 3% cupric acetate in 8% phosphoric acid and identified by comparing them to the migration

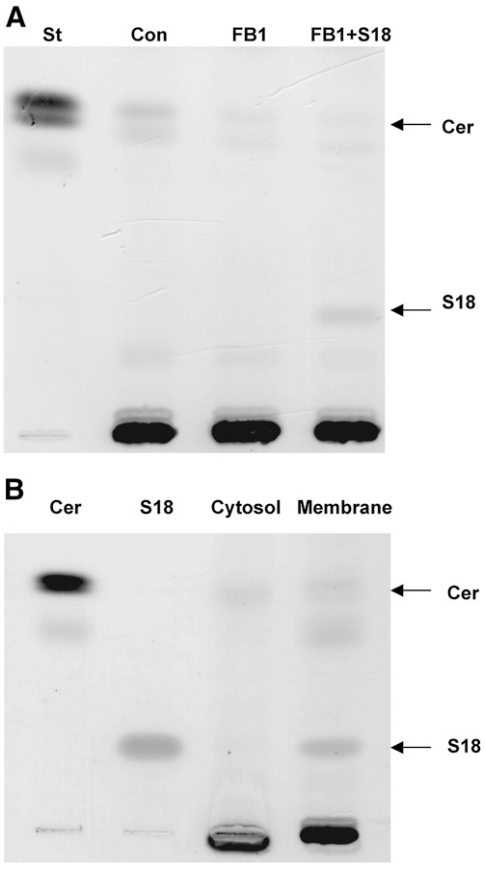

Fig. 1. Ciliogenesis impaired by sphingolipid depletion is rescued by ceramide or S18: lipid analysis. A: HPTLC analysis of ceramide. MDCK cells were incubated with FB1 and/or S18 for 48 h, the lipids extracted, and ceramide and S18 separated by HPTLC. Lane 1, ceramide standard; lane 2, untreated cells; lane 3, FB1-treated cells; lane 4, FB1 and S18-treated cells. B: Subcellular fractionation of MDCK cells to determine the distribution of S18. Cells were treated with S18 and the lipids in the membrane and cytosol analyzed by subcellular fractionation and HPTLC. Note that S18 is exclusively recovered from the membrane fraction. Lane 1, ceramide standard; lane 2, S18 standard; lane 3, cytosolic fraction; lane 4, membrane fraction.

distance of standard ceramide. The identity of the ceramide band was confirmed by alkaline hydrolysis of phospholipids followed by silic acid chromatography (20, 30). The amount of ceramide was quantified by densitometric analysis of the band intensities.

RESULTS

Loss of ceramide prevents ciliogenesis whereas ceramide analogs promote cilia elongation

To determine the role of ceramide for ciliogenesis we used the pharmacologic ceramide synthase inhibitor FB1 to deplete MDCK cells of ceramide, and replenishment with ceramide and ceramide analogs to rescue the phenotype. FB1 was added to the medium 72 h after plating of the cells to deplete the entire cell culture of ceramide (31, 32). Using HPTLC and densitometric analysis of the band intensities, we found that FB1 reduced the ceramide level to less than 50% of untreated controls (**Fig. 1A**, lane 3, lane 2 is control). Cells incubated with FB1 and S18 showed reduction of ceramide levels, but they were enriched with S18 (Fig. 1A, lane 4). Subcellular fractionation of homogenized MDCK cells after incubation with S18 and HPTLC analysis of the individual subcellular fractions showed that S18 was distributed to cellular membranes (Fig. 1B). This result suggested that S18 was able to replace ceramide in the plasma membrane or intracellular membranes of FB1-treated cells.

To confirm that potential effects of ceramide depletion are specific for ciliogenesis we determined the establishment of apicobasal polarity in FB1-treated cells using immunocytochemistry for apical and lateral marker proteins. **Figure 2A** shows that the distribution of F-actin (apical), ZO-1 (apicolateral), and E-cadherin (lateral) of FB1-treated MDCK cells was equivalent to that of control cells, suggesting that the effect of ceramide depletion was specific for ciliogenesis. Primary cilia were visualized using an antibody against acetylated tubulin, a marker for primary cilia (14, 18, 23). Immunocytochemistry for acetylated tubulin showed that FB1 reduced the number and length of primary cilia by 60% or 78%, respectively (Figs. 2B, C, F and **3A**, B). The number and length of cilia was restored by incubation of FB1-treated cells with C2 ceramide, C16 ceramide, or S18 (Figs. 2D–F, 3A, B), indicating that ceramide and not its derivatives were involved in cilia formation. This assumption was also supported by the observation that S18 (without FB1) promoted elongation of primary cilia by 1.5-fold (Fig. 3B). Incubation with sphingosine-1-phosphate had no effect on cilia formation or elongation (not shown). There was no impairment of cilia formation by incubation with sphingosine or dihydrosphingosine, suggesting that FB1 did not prevent cilia formation due to increased levels of these two ceramide precursors. Taken together, this data indicated that ceramide is critical to initiate the formation and promote the elongation of primary cilia in MDCK cells.

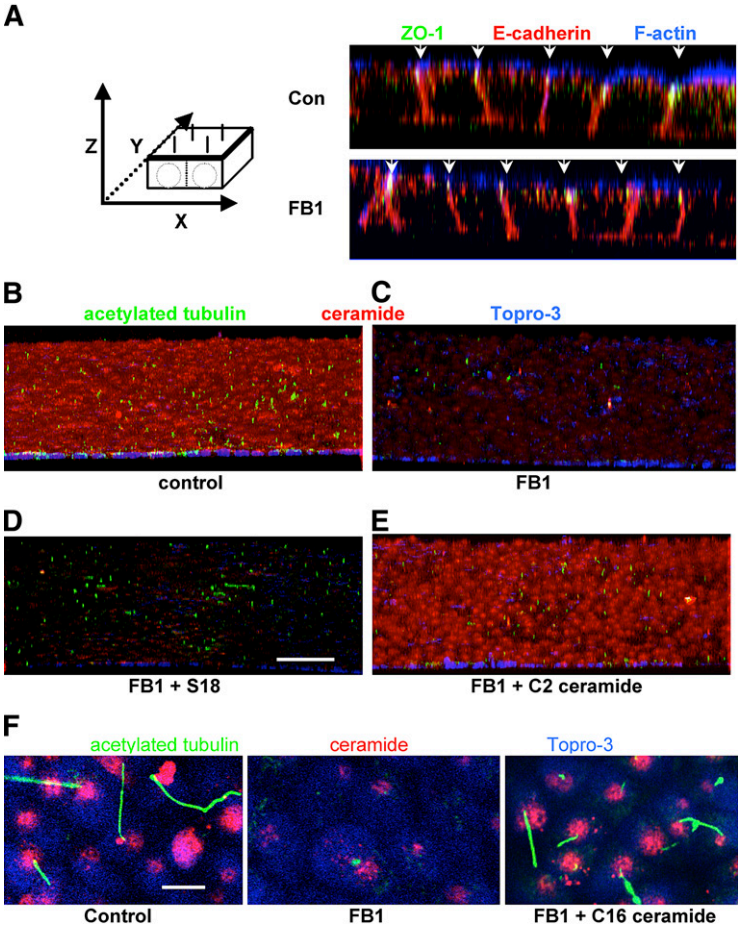

Fig. 2. Ciliogenesis impaired by sphingolipid depletion is rescued by ceramide or S18: immunocytochemistry. A: Confocal immunofluorescence microscopy for ZO-1 (Cy2, green) and E-cadherin (Cy3, red), and staining with Alexa 647-phallodin (Cy5, blue) of control and FB1-treated cells. The figure shows the X/Z plane after z-scan reconstruction. Arrowheads point at the apicolateral distribution of ZO-1 (white in overlay due to partial codistribution with F-actin (apical) and E-cadherin (lateral)), indicating establishment and maintenance of apicobasal polarity in FB1-treated cells. B–E: Projections reconstructed from z-scans as described in C. Secondary antibodies were different from those used in A (acetylated tubulin, Cy2, green; ceramide, Cy3, red). Bar = 50 μm. F: Immunocytochemistry as in B–E, however, top view on apical plane. Ciliogenesis was rescued with C16 ceramide. Bar = 2 μm.

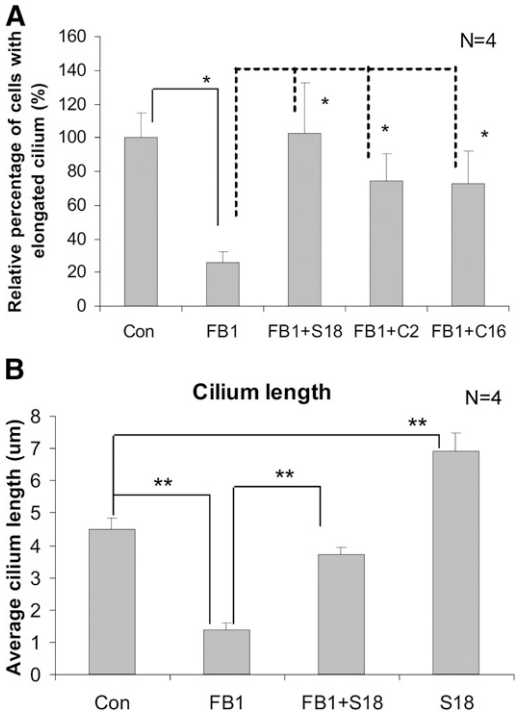

Fig. 3. Ciliogenesis impaired by sphingolipid depletion is rescued by ceramide or S18: quantitative evaluation. A, B: Counting of primary cilia with length >3 μm (A) and determination of the average cilium length (quantitation from N = 4 independent experiments using micrographic images with >800 cells/image). The number of ciliated cells in the control was set to 100% (*, $P < 0.05$; **, $P < 0.01$). Error bars indicate SD.

Golgi-resident ceramide codistributes with γ-tubulin at the base of the primary cilium

The necessity of ceramide for ciliogenesis suggested that it is functionally involved at subcellular sites that participate in cilium formation. To detect ceramide we used two anti-ceramide antibodies (MAS0020 and rabbit IgG) for immunocytochemistry. The cilium base is located in close vicinity to the centrosome and the PCM (15). In these subcellular compartments, γ-tubulin participates in cilium formation by priming the 9+0 microtubule structure, which originates at the basal body (centriole) and protrudes from the apical cell membrane (15–17). **Figure 4A–F** shows that ceramide formed a distinct structure or compartment at the base of the cilium (arrows), which was detected by immunocytochemistry for acetylated tubulin and ceramide. Costaining for F-actin with Alexa 647-phalloidin showed that ceramide was localized at the apical pole of the cell on top of the nucleus (Fig. 4E). At this site, ceramide was codistributed with γ-tubulin in a perinuclear compartment reminiscent of the centrosome and the PCM or the *cis* Golgi ribbon associated with the centrosome (Fig. 4F).

Because the centrosome is localized close to the *cis* Golgi we stained for GM130, a *cis* Golgi matrix protein that regulates centrosome morphology and function (19). Figure 4G shows that ceramide and GM130 were codistributed on top of the nucleus in polarized MDCK cells. Colocalization with GM130 suggested that ceramide was enriched in the *cis* Golgi, one of the first target compartments to which ceramide is transported once its biosynthesis has been accomplished in the endoplasmic reticulum (ER) (33–35). The trans Golgi is another compartment to which ceramide is transported from the ER. However, wheat germ agglutinin (WGA) staining of the trans Golgi did not indicate enrichment of ceramide in this compartment (Fig. 4H). Therefore, our data suggested that *cis* Golgi-derived ceramide is involved in the centriolar or pericentriolar initiation or regulation of primary cilium formation.

Ceramide activation of aPKC is critical for ciliogenesis

In previous studies, we have shown that ceramide activates aPKC and that this activation is essential for cell polarity in primitive ectoderm cells and neural progenitors (20, 21). Because the aPKC-associated protein complex in cell polarity is similar to that involved in ciliogenesis (23), we hypothesized that ceramide-mediated activation of aPKC will regulate cilia formation. To test this hypothesis, we incubated MDCK cells with myristoylated PZI. PZI is specific for aPKC and has been used to disrupt polarity-related processes in MDCK cells (36). Treatment with PZI by itself reduced the number of primary cilia to 20% of the control value (**Fig. 5**). PZI also reduced the number of cilia restored by C2 ceramide or S18 in ceramide-depleted cells. These results suggested that ceramide-mediated activation of aPKC is essential for ciliogenesis (Fig. 5).

We then tested whether other components of a ceramide/aPKC-associated polarity complex were codistributed with ceramide at the centrosome or the PCM. In our previous studies, we found that ceramide-induced association of aPKC with Par6 and the small GTPase Cdc42 phosphorylates and inactivates glycogen synthase kinase-3β (GSK-3β) and regulates cell polarity in primitive ectoderm cells and neuroprogenitors (20, 21). Likewise, immunocytochemistry showed apical codistribution of ceramide, aPKC, and Cdc42 in polarized MDCK cells (**Fig. 6A,** D, and supplementary Fig. II). This distribution was equivalent to the ceramide-rich Golgi or PCM compartment (Fig. 4). Ceramide depletion with FB1 obliterated the apical codistribution of aPKC and Cdc42 concurrent with the lack of cilia formation (Fig. 6B, D, and supplementary Fig. II). However, the apicolateral distribution of aPKC and Cdc42 was still visible after incubation with FB1, which was consistent with the establishment of apicobasal polarity prior to cilia formation (see also ZO-1 and E-cadherin in Fig. 2A). C2 ceramide and S18 restored the apical codistribution of aPKC and Cdc42 in FB1-treated cells (Fig. 6C, D, and Supplementary Fig. II). S18 also restored posphorylation of GSK-3β, which was almost completely obliterated in FB1-treated cells (**Fig. 7A**). Consistent with a critical function of S18- or ceramide-dependent phosphorylation of GSK-3β in ciliogenesis, the GSK-3β inhibitor indirubin-3′-monoxime restored ciliation of ceramide-depleted MDCK cells (Fig. 7B). Therefore, our results suggest that the centrosomal/pericentriolar ceramide compartment associates with aPKC and Cdc42, which induces phosphorylation of GSK-3β and promotes cilia formation and elongation.

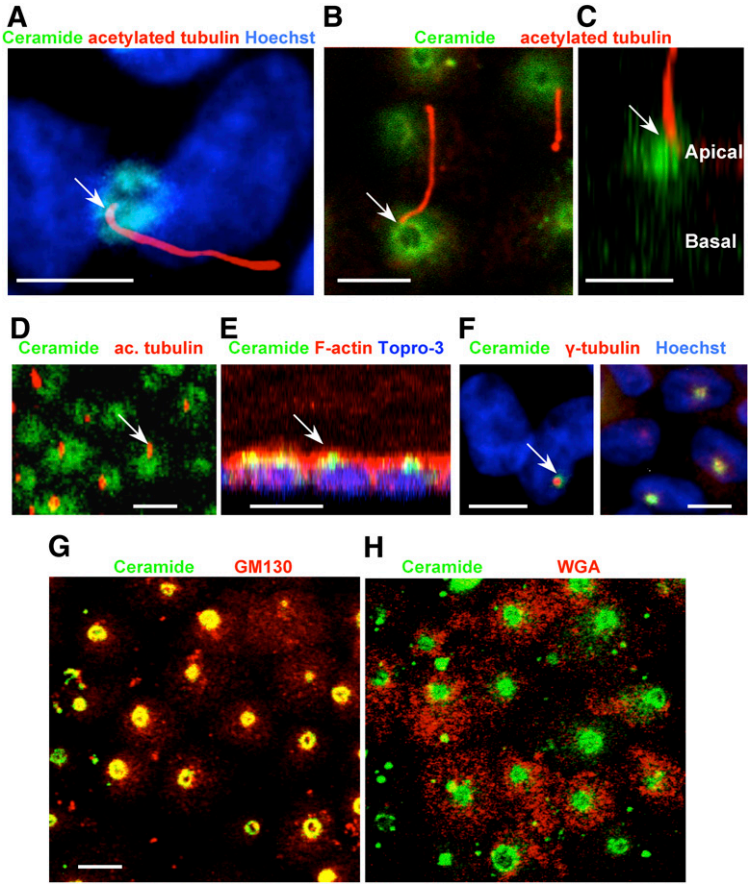

Fig. 4. Ceramide is enriched in a cis Golgi compartment associated with the centrosome at the base of the primary cilium. A–D: Epifluorescence (A) and confocal (B–E) immunofluorescence microscopy using antibodies against acetylated tubulin (Cy3, red) and ceramide (Cy2, green). A, B: X/Y plane; C: X/Z-plane; D: 3D projection, view from top front. Note that the cilium originates on top of the ceramide-rich compartment (arrows). E: X/Z-plane reconstruction using confocal immunofluorescence microscopy for ceramide (Cy2, gren), F-actin (Alexa 488-phalloidin, red), and Topro 3 (blue). F: Epifluorescence microscopy after immunocytochemistry for ceramide (Cy2, green) and γ-tubulin (Cy3, red). G, H: Confocal immunofluorescence microscopy for ceramide (Cy2, ceramide) and the *cis* Golgi marker GM130 (Cy3, red in left panel) or the trans Golgi marker WGA (Cy3, red in right panel). Note that only the distribution of GM130 overlapped with that of ceramide (yellow). Images show view from top onto apical (X/Y) confocal plane. Bars = 2 μm.

DISCUSSION

The sphingolipid ceramide has long been implicated in the induction of apoptosis. Most recently, our group has shown that ceramide has additional, nonapoptotic functions (20, 21). In particular, the regulation of cell polarity in primitive ectoderm and neural progenitor cells critically relies on ceramide (20, 21). We have found that this regulation is determined by the interaction of ceramide with aPKC and its associated polarity complex(es) (22, 26). Because aPKC is also involved in the regulation of ciliogenesis (23), we tested the effect of ceramide on this process. We show here, for the first time, that ceramide is a key regulatory factor for the formation and elongation of primary cilia.

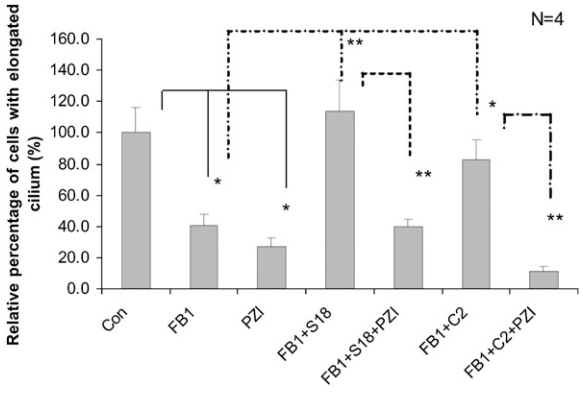

Fig. 5. Ciliogenesis requires ceramide-dependent activation of aPKC. MDCK cells were cultivated in the presence of the reagents as indicated in the figure and the number of primary cilia counted as described in the legend for Fig. 3A. The number of ciliated cells in control was set to 100% (FB1 (30 μM), aPKC inhibitor PZI (50 μM), S18 (40 μM), C2 (C2 ceramide, 10 μM). Note that S18 and C2 ceramide restored cilium formation, whereas PZI blocked ceramide or S18-mediated cilium restoration (*, $P < 0.05$; **, $P < 0.01$). Error bars indicate SD.

To define a specific role of ceramide for ciliogenesis it is indispensable to confirm that apicobasal polarity was not affected during cilia formation in FB1-treated cells. This was achieved by showing the apical, apicolateral, or lateral distribution of F-actin, ZO-1, and E-cadherin, respectively. ZO-1 is a tight junction protein that is regulated by aPKC independent of ciliogenesis (37–39). Hence, the apicolateral distribution of ZO-1 indicates that apicobasal polarity was established before FB1 treatment and then maintained in ceramide-depleted cells.

Several lines of evidence support the hypothesis that ceramide is essential for ciliogenesis: *1*) FB1, an inhibitor of ceramide biosynthesis, dramatically reduces the number of ciliated cells and the length of primary cilia; and *2*) C2 ceramide, C16 ceramide, and the novel ceramide analog S18 restore cilia formation in FB1-treated cells. In particular, restoration of primary cilia by S18 is important as this ceramide analog is not converted to any metabolic derivative of ceramide (20, 21). The rescue effect of S18 may be due to enrichment of this ceramide analog in the membranous ceramide compartment and activation of aPKC.

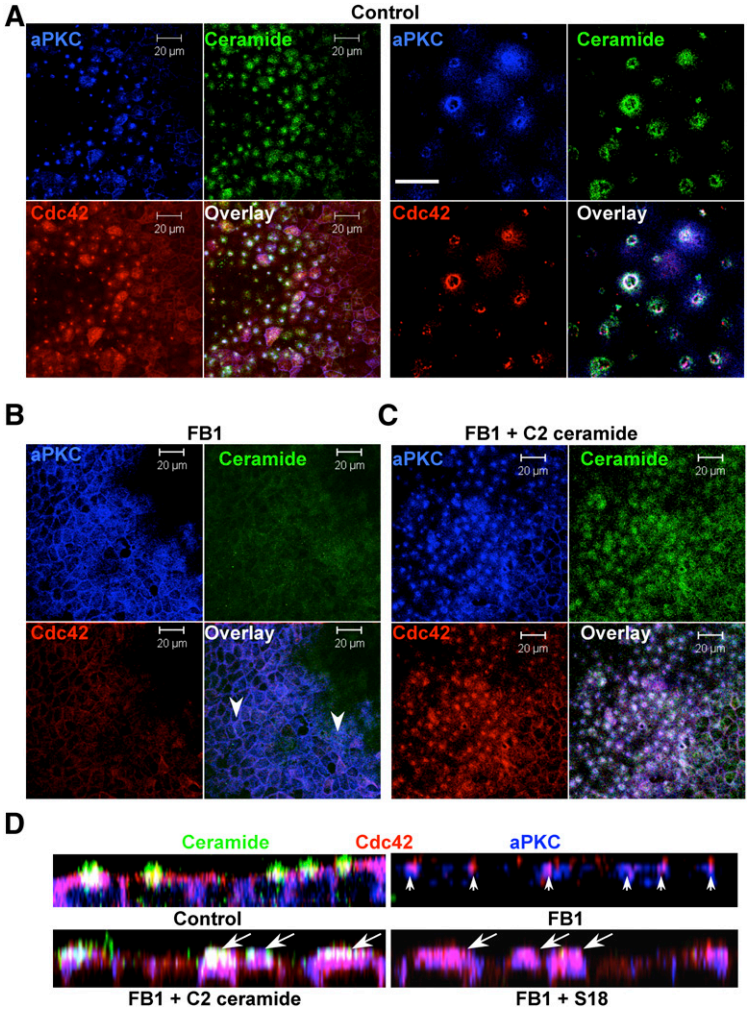

Fig. 6. Ceramide codistributes with aPKC and Cdc42. A–D: Confocal immunofluorescence microscopy for ceramide (Cy2, green), Cdc42 (Cy3, red), and aPKC (Cy5, blue). Bar (right panel) = 5 μm. B, C: Immunocytochemistry as in A, however, after incubation with FB1 or FB1+C2 ceramide. D: X/Z plain reconstruction from z-scan. Note that the apical codistribution of aPKC and Cdc42 (white in overlay due to codistribution with ceramide) was obliterated by incubation with FB1, and restored by C2 ceramide and S18. Also note that FB1 did not prevent apicolateral codistribution of aPKC and Cdc42, indicating that apicobasal polarity was established and sustained throughout the incubation reaction. Bar = 10 μm. See Supplementary Fig. II for top view on apical and apicolateral plane.

Taken together, impairment of cilia formation by ceramide depletion and restoration of primary cilia by C2 ceramide, C16 ceramide, and S18 strongly suggests an essential function of ceramide in ciliogenesis.

Previous studies from our and other laboratories have shown that ceramide and its analogs associate with and activate aPKC (22, 26, 40–44). This association takes place in the cell membrane, a perinuclear ER and/or mitochondria-associated compartment, and the Golgi apparatus (21, 22). Our previous studies are consistent with the observation that S18 distributes to the membrane fraction after subcellular fractionation. We have also shown that S18 activates aPKC in in vitro assays and in living cells (26). Thus, it is likely that ceramide or S18-induced activation of aPKC at a membranous compartment is critical for the function of ceramide in ciliogenesis. This hypothesis is supported by two observations: *1*) inhibition of aPKC prevents restoration of primary cilia by S18, C2, or C16 ceramide in ceramide-depleted cells; and *2*) immunocytochemistry shows codistribution of aPKC and aPKC-associated proteins with ceramide in a membranous compartment at the base of the cilium.

The codistribution of ceramide with GM130 suggests that this membranous compartment is derived from the *cis* Golgi. Ceramide enrichment in this compartment is consistent with the de novo sphingolipid biosynthesis pathway, which involves ceramide transport from the ER to the *cis* Golgi (34, 45). Another transport pathway using the ceramide transport protein CERT mediates traffic of ceramide from the ER to the trans Golgi compartment (46). As the ceramide signal is not codistributed with the trans Golgi marker WGA, it is rather unlikely that ceramide in the trans Golgi is involved in ciliogenesis.

GM130 is not only a *cis* Golgi marker but has also been found to actively regulate the biogenesis and morphology of the centrosome (19). For a long time, it has been known that there is a close vicinity and interaction between the centrosome and the Golgi in polarized cells (12). In epithelial cells with apicobasal polarity, the centrosome and PCM are located on top of the nucleus and linked to an apical *cis* Golgi ribbon. GM130 and γ-tubulin are involved in generating a microtubule-associated network that physically links the centrosome to this apical *cis* Golgi ribbon (12–14, 19). γ-tubulin is also essential for nucleating the primary cilium at its basal body within the region of the centrosome and PCM. Therefore, it appears reasonable to speculate that ceramide in the *cis* Golgi may regulate ciliogenesis via the interactive network between the centrosome and the Golgi ribbon. At present, we cannot exclude that other organelles, such as pericentrosomal

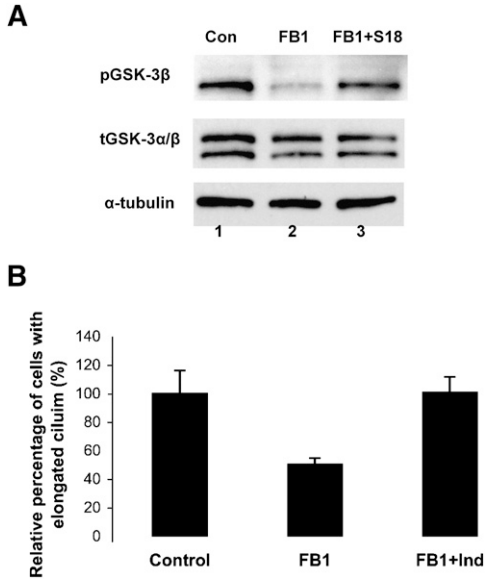

Fig. 7. Ceramide biosynthesis inhibition reduces GSK-3β phosphorylation, which is critical for ciliogenesis. A: MDCK cells were cultivated for 3 days on filter membranes and then for another 2 days incubated with FB1. The immunoblot shows that the phosphorylation of serine 9 in GSK-3β was dramatically reduced. Incubation with S18 restored phosphorylation and inactivation of GSK-3β. B: Treatment with FB1 reduced primary cilium formation, which was restored by incubation with the GSK-3β inhibitor indirubin-3′-monoxime. Error bars indicate SD.

recycling endosomes, participate in the ceramide-rich compartment. Further characterization of this compartment will be part of our future studies.

Figure 8 depicts the structural arrangement by which the regulation of ciliogenesis may be mediated after recruitment and activation of aPKC to the ceramide compartment. Ceramide depletion by treatment with FB1 obliterates codistribution of aPKC with Cdc42, which is restored by S18. This result clearly indicates that ceramide in the *cis* Golgi (ribbon) associates with aPKC and Cdc42, potentially leading to a vectorial activation of cilia formation or elongation. It remains to be elucidated how ceramide regulates aPKC at the Golgi and how this regulation promotes ciliogenesis. It has been suggested that aPKC phosphorylated, microtubule-associated proteins or aPKC-associated polarity proteins such as Par3 or Par6 are involved in cilia formation/elongation (23). Our group has shown that in primitive ectoderm and neural progenitor cells, ceramide is essential for the formation of an aPKC-Par6-Cdc42 complex, which could sustain ciliogenesis in a similar fashion as it regulates cell polarity (20–22). Another regulatory factor involved in ciliogenesis is GSK-3β, a protein kinase phosphorylated by aPKC. Inactivation of GSK-3β by phosphorylation of serine 9 has been shown to be critical for cilium formation sustained by van Hippel-Lindau protein (47). Consistent with this role of GSK-3β inactivation, we have found that ceramide depletion reduces and S18 treatment restores GSK-3β phosphorylation, concurrent with loss and restoration of primary cilia, respectively. Therefore, we assume that a ceramide-activated complex of aPKC with Par6 and Cdc42 phosphorylates GSK-3β, which induces or sustains primary cilia formation or maintenance (Fig. 8).

This is the first report showing that ceramide is critical for the regulation of primary cilia formation and elongation. We will now investigate how ceramide promotes ciliogenesis via aPKC and whether lack or excess of ceramide may disturb this process in ciliopathies. In the case of a lack of ceramide, analogs such as S18 may pave the way to treat ciliopathies. To further define the role of ceramide in ciliogenesis is, thus, a novel path to understanding the regulation of ciliogenesis and treating disorders related to it.

The authors are grateful to Drs. Richard Cameron and Somsankar Dasgupta, Medical College of Georgia, Augusta, GA, for their assistance with cell culture and ceramide analysis. We thank the imaging core facility under the supervision of Drs. Paul McNeil and Katsuya Miyake, Medical College of Georgia, Augusta, GA, for assistance. The authors also thank Dr. Robert K. Yu for institutional support.

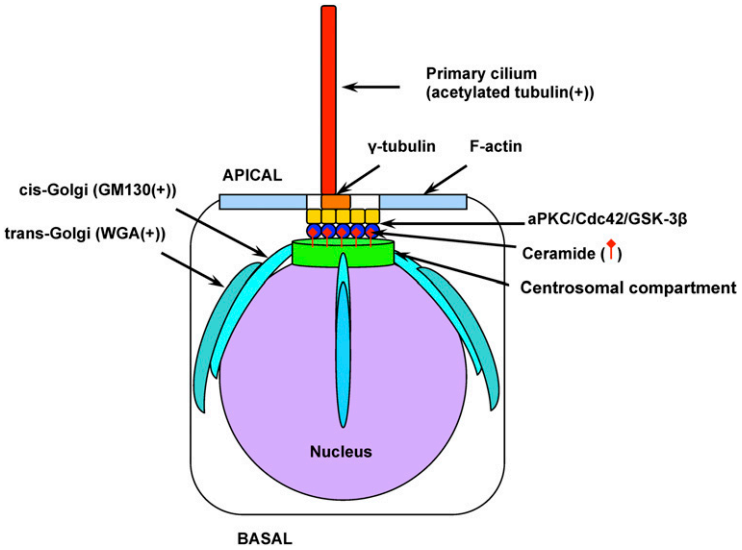

Fig. 8. Model for ceramide-dependent regulation of ciliogenesis. Ceramide is enriched in a centrosomal/pericentriolar compartment derived from the *cis* Golgi underneath the apical membrane and on top of the nucleus of polarized epithelial cells. The compartment recruits and/or activates aPKC and Cdc42, which promotes elongation of the primary cilium, most likely mediated by a polarity complex acting on microtubule or γ-tubulin-associated proteins. Ceramide depletion by incubation with FB1 prevents apical codistribution of aPKC and Cdc42, which impairs ciliogenesis.

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