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Polarized Membrane Traffic and Cell Polarity Development Is Dependent on Dihydroceramide Synthase-Regulated Sphinganine Turnover

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Sphingoid bases have been implicated in various cellular processes including cell growth, apoptosis and cell differentiation. Here, we show that the regulated turnover of sphingoid bases is crucial for cell polarity development, i.e., the biogenesis of apical plasma membrane domains, in well-differentiated hepatic cells. Thus, inhibition of dihydroceramide synthase or sphinganine kinase activity with fumonisin B1 or N,N-dimethylphosphoglycine, respectively, dramatically perturbs cell polarity development, which is due to increased levels of sphinganine. Consistently, reduction of free sphinganine levels stimulates cell polarity development. Moreover, dihydroceramide synthase, the predominant enzyme responsible for sphinganine turnover, is a target for cell polarity stimulating cAMP/protein kinase A (PKA) signaling cascades. Indeed, electrospray ionization tandem mass spectrometry analyses revealed a significant reduction in sphinganine levels in cAMP/PKA-stimulated cells. These data suggest that sphinganine turnover is a critical target for and is actively regulated during HepG2 cell polarity development. Previously, we have identified an apical plasma membrane-directed trafficking pathway from the subapical compartment. This transport pathway, which is part of the basolateral-to-apical transcytotic itinerary, plays a crucial role in apical plasma membrane biogenesis. Here, we show that, as a part of the underlying mechanism, the inhibition of dihydroceramide synthase activity and ensuing increased sphinganine levels specifically perturb the activation of this particular pathway in the de novo apical membrane biogenesis.

INTRODUCTION

Epithelial cell polarity depends on the structural asymmetry of apical and basolateral plasma membrane (PM) domains, separated by junctional complexes. The establishment and maintenance of PM domains requires a careful orchestration of extra- and intracellular signals, triggering crucial or- ganellar- and membrane traffic-linked machineries (Veeman et al., 1999; Mostov et al., 2003). Hepatocytes, like all epithelia, display distinct apical, bile canalicular (BC) and basolateral, sinusoidal PM domains. Some of the intracellular sites and molecular components of the mechanisms that contribute to apical BC biogenesis in hepatocytes have been clarified. These include E-cadherin (Terry and Gallin, 1994; Matsui et al., 2002), cAMP/protein kinase (PKA) and PKC activities (Roelofsen et al., 1998; Zegers and Hoekstra, 1998), the cytoskeleton (Mishra et al., 1999), and the sorting of specific sphingolipids and proteins, mediated by the Golgi and endosomal recycling system (Zegers and Hoekstra, 1998; Ait Slimane and Hoekstra, 2002; Maier and Hoekstra, 2003). In particular, the subapical compartment (SAC), the hepatocyte equivalent of the common endosome in other epithelia, plays a central role in the polarized trafficking of proteins (Ihrke et al., 1998; van IJzendoorn et al., 1998; Rahner et al., 2000; Silver et al., 2001; Wüstner et al., 2004), sphingo- lipids (van IJzendoorn and Hoekstra, 1998, 1999b, 2000; Maier and Hoekstra, 2003), and cholesterol (Silver et al., 2001; Wüstner et al., 2001, 2002, 2004).

Studies of the intracellular flow of fluorescent lipid analogs indicated the existence of multiple membrane domains in SAC, and the membrane sorting capacity of the SAC was demonstrated to be a target for signals that promote apical-basolateral PM asymmetry, including cAMP/PKA and the interleukin-6 family cytokine oncostatin M (van IJzendoorn et al., 2000; van der Wouden et al., 2002). Thus, during cell polarity development and/or upon stimulation of cAMP/PKA signaling in well-differentiated hepatic HepG2 cells, a specific SAC-to-BC pathway followed by transcytosing IgA and two fluorescent sphingolipid analogs, C6-NBD-sphingomyelin (SM) and -galactosylceramide (GalCer), is activated. Blocking this pathway, e.g., with PKA inhibitors or the microtubule-disrupting agent nocodazole effectively perturbs polarity development. After the establishment of apical-basolateral cell polarity, the trafficking of C6-NBD-SM and -GalCer from SAC switches from an apical to a basolateral destination, whereas the apical recycling of C6-NBD-GlcCer remains unaffected. Fluorescent lipid analogs are thus a useful tool in living cells to monitor intracellular trafficking pathways, and...
alterations in these, that are relevant for cell polarity development. Sphingolipids, together with cholesterol, cluster to form particularly ordered membrane environments or rafts, in which apical proteins and/or signal transduction molecules are coassembled (Simons and Ikonen, 1997; Ait Simane et al., 2003). Therefore, the lateral dynamics and organization of sphingolipids may play a prominent role in the regulation of signaling cascades and cell polarity (Holthuis et al., 2001). Indeed, the segregation of predominately apical PM proteins into sphingolipid-enriched rafts suggests a prominent role for sphingolipids in apical sorting (Simons and Ikonen, 1997). Moreover, the timely regulated activity of enzymes in the sphingolipid biosynthesis pathway is crucial for cellular development, e.g., that of polarized neurons (Futerman, 1998; Schwarz and Futerman, 1998; Ledesma et al., 1999; Boldin and Futerman, 2000). For example, the expression of sphingomyelinase in developing neurons coincides with the segregation of Thy-1 into sphingolipid rafts and its subsequent directed trafficking to the axonal plasma membrane domain (Ledesma et al., 1999).

In addition to achieving specific expression levels of complex (glyco)sphingolipids such as SM, GlcCer, and GalCer, the regulated activity of enzymes that control their biosynthesis also may serve to control the levels and activity of its precursors. Indeed, ceramide (CER) and the sphingoid base precursors, i.e., sphingosine and sphinganine (dihydro-sphingosine), seem highly bioactive compounds that mediate membrane second messenger cascades. For example, the expression of sphingomyelinase in developing neurons coincides with the segregation of Thy-1 into sphingolipid rafts and its subsequent directed trafficking to the axonal plasma membrane domain (Ledesma et al., 1999).

MATERIALS AND METHODS

Hoechst-33258 (bisbenzimide), t-cycloserine, and Fumonisin B1 were obtained from Sigma-Aldrich (St. Louis, MO). β-erythro-Sphinganine, α-threo-sphinganine (safingol), β-erythro-sphinganine-1-phosphate, and N,N-Dimethyl-α-threo-sphingosine were from Avanti Polar Lipids (Alabaster, AL). Dibutyryl cAMP was purchased from Roche Diagnostics (Mannheim, Germany). Albumin (from bovine serum, fraction V) was bought from Fluka (Buchs, Switzerland). D-[6-N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)-amino]hexanoic acid (C6-NBD) and Alexa594-labeled phalloidin were purchased from Molecular Probes (Eugene, OR). Sodium dihydrogenate (Na2S2O4) was from Merck (Darmstadt, Germany). C6-NBD-sphingolipids were synthesized as described previously (Kishimoto, 1975; Babia et al., 2003). For example, the expression of sphingomyelinase in developing neurons coincides with the segregation of Thy-1 into sphingolipid rafts and its subsequent directed trafficking to the axonal plasma membrane domain (Ledesma et al., 1999).

Dihydroceramide (DHC) Synthase Assay

The dihydroceramide synthase synthase assay was performed as described by Merrill and Wang (1992). Cells, control or treated with dibutyryl cAMP (dbcAMP) for 30 min, were rinsed, homogenized in sucrose buffer by sonication, and aliquots of the homogenate were subjected to SDS-PAGE. After separation, the gel was stained with Coomassie Blue R250, destained, and dried. The autoradiogram was scanned, and the band intensity was quantified using a densitometer.

Transport of C6-NBD-GalCer from the SAC

To monitor SAC-associated sphingolipid transport, lipid is first accumulated in the SAC, according to a procedure described elsewhere (van IJzendoorn and Hoekstra, 2000). First, cells were washed with PBS and incubated with C6-NBD-GalCer at 37°C for 30 min to allow internalization of the lipid analogue from the basolateral surface. The remaining basolateral pool of lipid analogue was then depleted by a back-exchange procedure [5% (wt/vol) bovine serum albumin in Hanks’ balanced salt solution (HBSS), pH 7.4, at 4°C for 30 min]. Then, the lipid was chased from the apical, bile canalicular PM into the SAC by an incubation at 18°C in back-exchange medium. The chase was done over a 60-min period and at this time, the majority of the lipid analogue was associated with the SAC (cf. van IJzendoorn and Hoekstra, 1998). Any NBD fluorescence still remaining in the apical lumenal leaflet of the 60-min chase was subsequently abolished by incubating the cells with 30 mM Na2S2O4 at 4°C, a condition that prevents intracellular access of the quantum. After 10 min, Na2S2O4 was then removed by extensive washing of the cells with ice-cold HBSS. In some experiments, cells were then treated with 10% heat-inactivated fetal calf serum (56°C, 30 min), and cell polarity development was determined by fluorescence microscopic analysis. The ratio of C6-NBD-GalCer fluorescence in the apical PM to the internalized pool was used to estimate the amount of C6-NBD-GalCer remaining in the SAC.
with dbcAMP and/or sphinganine at 4°C for 30 min. Transport of the lipid analogue from the SAC was subsequently monitored by incubation in back-exchange medium at 37°C. When required, dbcAMP and/or sphinganine were kept present during the transport assay.

To quantitate transport of the lipid analogue to and from the apical, BC membranes, the percentage of NBD-positive BC membranes was determined as described previously (van IJzendoorn et al., 1997; van IJzendoorn and Hoekstra, 1998). Thus, BC were first identified by phase-contrast illumination and then classified as either NBD positive or NBD negative under epifluorescence illumination. Distinct pools of fluorescence were discerned, present in vesicular structures adjacent to BC, which have been defined as SAC (cf. van IJzendoorn and Hoekstra, 1998). Together, BC and SAC thus constitute the bile canalicular, apical pole (BCP) in HepG2 cells. Therefore, within the BCP region the localization of the fluorescent lipid analogues will be defined as being derived from BC, SAC, or both. This also provides a means to describe the movement of the lipid within or out of this region in the cell. Thus, after loading the SAC with lipid analogue and a subsequent incubation as described above, the direction of movement of the lipid analogue from or within the BCP region is inferred from determining the fraction of NBD-labeled BCP (i.e., label in either the BC or SAC or both) at a given time, relative to that labeled when starting the chase (t = 0). At least 50 BCPs per coverslip were analyzed. Data are expressed as the mean ± SEM of at least three independent experiments, carried out in duplicate.

Internalization of Texas Red-labeled IgA
HepG2 cells that stably express plgR were washed with excess asialofetuin at 37°C for 30 min to prevent uptake of IgA via these receptors (cf. van IJzendoorn and Hoekstra, 1998). Cells, treated with sphinganine (2 µM, 37°C for 1 h) were incubated with TxR-IgA (50 µg/ml) at 18°C for 60 min. Cells were then washed to remove nonbound TxR-IgA and further incubated at 37°C for 30 min in the presence or absence of sphinganine.

RESULTS

Sphinganine Accumulation Blocks Polarity Development
To examine the involvement of sphingolipid metabolites in the process of hepatocyte polarity development, we used the mycotoxin fumonisin B1 (FB1), which specifically inhibits DHC synthase activity in the sphingolipid biosynthetic pathway (Figure 1). Quantification of cellular sphingolipids and sphingoid bases was performed by ESI-MS/MS as described in Materials and Methods and in Lieser et al. (2003). As anticipated, treatment of well-differentiated hepatoma HepG2 cells with FB1 (10 µM; 24 h) resulted in decreased levels of ceramides, sphingomyelin, and glucosylceramide (Table 1). Consequently, the level of the sphingoid base sphinganine increased more than fivefold (4.1 ± 1.0–23.0 ± 12.0 pmol/mg cell protein; Table 1; Lieser et al., 2003). To examine potential consequences on HepG2 cell polarity development, i.e., the biogenesis of apical BC, cells were plated on coverslips in the presence of 0–30 µM FB1. Each day, a coverslip was taken and the ratio BC/100 cells was determined as a measure for cell polarity (see Materials and Methods). Control HepG2 cells rapidly acquired polarity, reaching

Table 1. Quantification of sphingolipids in control versus FB1-treated cells

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<tr>
<th>Sphingolipid</th>
<th>Control (nmol/mg protein)</th>
<th>FB1 (nmol/mg protein)</th>
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<tr>
<td>SM</td>
<td>21.9 ± 5.4</td>
<td>8.7 ± 0.8</td>
</tr>
<tr>
<td>GlcCer</td>
<td>0.09 ± 0.02</td>
<td>0.03 ± 0.02</td>
</tr>
<tr>
<td>Cer</td>
<td>0.9 ± 0.2</td>
<td>0.2 ± 0.03</td>
</tr>
<tr>
<td>SA</td>
<td>0.0041 ± 0.001</td>
<td>0.023 ± 0.012</td>
</tr>
<tr>
<td>SO</td>
<td>0.042 ± 0.01</td>
<td>0.016 ± 0.004</td>
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Cells were cultured in normal culture medium in the presence of 10 µM FB1 or buffer (control) for 24 h. Cells were then lysed in 0.5% SDS lysis buffer, and lysates were immediately frozen at −20°C. The protein content of each sample was determined, and SM, GlcCer, Cer, sphinganine (SA), and sphingosine (SO) were quantified as described in Materials and Methods. All data are expressed as mean ± SEM nanomoles per milligram of total cell protein of triplicate experiments (n = 3).
a maximum ratio BC/100 cells 2 d after plating (Figure 2A). By contrast, cells grown in the presence of FB1 showed a dramatic inhibition of the formation of BC in a dose-dependent manner, resulting in a reduction of the number of polarized cells by ~75% 2 d after plating (Figure 2, A and B). The inhibitory effect of FB1 on BC formation was reversible. Thus, after 24 h of culturing in the presence of FB1 and subsequent removal of the compound, polarity development was rapidly restored (Figure 2A, dashed line). With the subsequent removal of the compound, polarity development in a dose-dependent manner (Figure 2C), thereby closely mimicking the effect of FB1 (Figure 2A). Addition of 0.5 \( \mu \)M sphinganine for 24 h resulted in an increase in cellular sphinganine levels by >20-fold (from ~4.0–90.0 pmol/mg cell protein; Table 2). Similar to as with FB1, cell polarity was rapidly restored upon removal of the exogenous sphinganine (Figure 2C, dashed line). These data suggest that the accumulation of sphinganine, rather than the inhibition of de novo synthesis of ceramides and/or complex sphingolipids, was responsible for the observed impediment of cell polarity development. Indeed, the inhibitory effect of FB1 on polarity development could not be restored when FB1-treated cells were replenished with 10 \( \mu \)M sphinganine (SA; C), or short-chain ceramides (unpublished data), analogs that were previously shown to restore cell polarity in control or FB1-treated cells (72 h; nuclei in blue and BC in red). Note the absence of BC in FB1-treated cultures. Bars, 5 \( \mu \)m; *p < 0.05 (Student’s t test).

<table>
<thead>
<tr>
<th>Table 2. Quantification of cellular sphinganine levels by ESI-MS/MS</th>
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<tr>
<td>Sphinganine (% of control)</td>
</tr>
<tr>
<td>Control</td>
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<tr>
<td>FB1</td>
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<tr>
<td>Exogenous SA</td>
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<td>LCS</td>
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<td>DbcAMP</td>
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Quantification of sphinganine levels in HepG2 cells. Cells were cultured in normal culture medium in the presence of 10 \( \mu \)M FB1, 0.5 \( \mu \)M exogenous \( \alpha \)-erythro-sphinganine (SA), 250 \( \mu \)M L-LCS, or 100 \( \mu \)M DbcAMP for 24 h. Cells were then lysed in 0.5% SDS lysis buffer, and lysates were immediately frozen at –20 °C. The protein content of each sample was determined and sphinganine levels were quantified by ESI-MS/MS analysis as described in Materials and Methods. Data were calculated as mean ± SEM picomoles per milligram of total cell protein of triplicate experiments (n = 3) and expressed as percentage of control (4.1 pmol/mg protein; set to 100).

Figure 2. Accumulation of sphinganine inhibits HepG2 cell polarity development. Cells were treated with different concentrations of FB1, FB1 + DHC, or exogenous \( \alpha \)-erythro-sphinganine (SA) in serum-supplemented cell culture medium for 24, 48, or 72 h. At each time point, the cells were fixed and the ratio BC/100 cells was determined as described in Materials and Methods. Effect of 0–30 \( \mu \)M FB1 (A and B), 0.5–2.0 \( \mu \)M sphinganine (SA; C), or 30 \( \mu \)M FB1 + 10 \( \mu \)M DHC (D) on cell polarity development. The dashed lines in A and C (−Rsc) show the course of polarity development after removal of FB1 or exogenous sphinganine, respectively, after 24 h. Data are expressed as mean ± SEM of at least three independent experiments carried out in duplicate. (B) Fluorescence microscopical evaluation of cell polarity in control or FB1-treated cells (72 h; nuclei in blue and BC in red). Note the absence of BC in FB1-treated cultures.

It should be noted that long-term incubation of 3T3 fibroblasts with FB1 was reported to up-regulate the activity of enzymes that produce glucosylceramide and sphingomyelin (Meivar-Levy and Futerman, 1999), both of which have been implicated in differentiation and maturation of specialized plasma membranes in neurons (Futerman et al., 1999; Ledesma et al., 1999). Treatment of HepG2 cells with FB1 under our conditions, however, did not up-regulate glucosylceramide synthase or sphingomyelin synthase (unpublished data). Furthermore, although treatment of cells with FB1 predominantly increases sphinganine levels (Table 1), it has been reported to modestly increase cellular levels of sphingosine (dihydro-sphinganine; Lierse et al., 2003). In HepG2 cells, however, treatment of cells with FB1 (10 \( \mu \)M) resulted in a decrease in sphingosine levels (Table 1). Moreover, the exogenous addition of sphingosine did not alter polarity development of HepG2 cells (Figure 3A), in striking contrast to the exogenous addition of sphinganine (Figure 2C). Importantly, the exogenous addition of equal concentrations of hydrophobic amines NH4Cl and stearylamine did not affect polarity development (Figure 3A), indicating that the exogenously administered sphinganine did not inhibit polarity development via its potential activity as a hydrophobic amine. In addition, the nonnatural stereoisomer of...
sphinganine, L-threo-sphinganine (safingol) did not mimic the effects of FB1 or exogenous (d-erythro)-sphinganine (Figure 3A).

Together, the data indicate that dihydroceramide synthase inhibition and subsequent accumulation of d-erythro-sphinganine inhibits the establishment of HepG2 cell polarity.

**Sphinganine and Not Sphinganine-1-Phosphate Interferes with Polarity Development**

On its accumulation, free sphinganine can be phosphorylated by sphingosine/sphinganine kinase to form sphinganine-1-phosphate. Phosphorylated sphingoid bases are highly bioactive compounds that can elicit various signaling cascades in cells. To determine whether the observed effect of FB1 or exogenous sphinganine on cell polarity development was the result of the phosphorylated product, cells were incubated with FB1 in the presence of N,N-dimethylsphingosine (DMS; 2-5 μM), an inhibitor of sphingosine/sphinganine kinase (Figure 1). As shown in Figure 3B, DMS did not prevent the effects of FB1 on polarity development. Rather, inhibition of sphinganine kinase activity augmented the inhibitory effect of FB1 on polarity development, and treatment of the cells with DMS alone inhibited polarity development to a similar extent as FB1 (Figure 3B). In addition, treatment of the cells with d-erythro-sphinganine-1-phosphate (5 μM) did not mimic the effects of sphinganine (Figure 3B). Together, these data suggest that the accumulation of sphinganine, and not of sphinganine-1-phosphate, perturbs polarity development in HepG2 cells.

**Sphinganine Turnover Promotes Polarity Development**

To obtain further support for a direct correlation between sphinganine level and cell polarization, we next investigated whether decreased levels of this sphingolipid metabolite also affect cell polarity development. A specific inhibitor of serine palmitoyltransferase, 1-cycloserine (LCS), inhibits sphinganine biosynthesis (Figure 1). Indeed, the treatment of HepG2 cells with 250 μM LCS, in contrast to FB1, resulted in a reduction of the cellular sphinganine level with ~27% (Table 2). Interestingly, in striking contrast to the effect of FB1, LCS treatment enhanced cell polarity development, in terms of both kinetics and maximum polarity, as evidenced by the steep increase of the number (Figure 4A), as well as the size (Figure 4B) of the BCs. Similar results were obtained with another specific inhibitor of serine palmitoyltransferase, ISP-1/myriocin (unpublished data; c.f. Figure 4). The opposite effects of LCS and FB1 on polarity development, under the experimental conditions used, point to sphinganine turnover, rather than to decreased levels of (dihydro)ceramide and/or complex sphingolipids as a crucial parameter in polarity development, a decrease promoting and an increase inhibiting polarity development. Indeed, the polarity-stimulating effect of LCS treatment was completely abolished in the presence of increasing concentrations of exogenous sphinganine (Figure 4C), whereas exogenous C6-DHC (Figure 4C) or ceramide (unpublished data) were without effect.

**Dihydroceramide Synthase Is a Target for Cell Polarity-Stimulating Signaling Cascades**

Because the data strongly indicate that the establishment of HepG2 cell polarity is critically dependent on the level of sphinganine, we next investigated whether dihydroceramide synthase, the predominant enzyme in sphinganine turnover, functions as a target for cellular signals that contribute to polarity development. For this, we took into account our previous observation that the stable cAMP analog dbcAMP, via activation of PKA, stimulates HepG2 cell polarity development (Zegers and Hoekstra, 1997; van IJzendoorn and Hoekstra, 1999a, 2000). Importantly, quantification of sphinganine from cell extracts by ESI-MS/MS revealed that the cellular sphinganine level was reduced with ~63% in dbcAMP-treated cells, compared with nontreated cells (Table 2), which is in agreement with the observation that reduced sphinganine levels promote polarity development. To measure the dihydroceramide synthase activity, control and dbcAMP-treated HepG2 cells were homogenized, and incubated with [3H]sphinganine and stearyl CoA (at saturating concentrations) for 10 min. During this time interval, the activity of DHC synthase was up-regulated ~1.5-fold in dbcAMP-treated cells, as evidenced by the increased production of radiolabeled (dihydro)ceramides (22.1 ± 2.4–32.6 ± 3.8 pmol/mg protein), whereas treatment with FB1 blocked the activity of the enzyme (Figure 5A). Importantly, whereas dbcAMP typically stimulates polarity development in HepG2 cells (Figure 5B; c.f. van IJzendoorn and Hoekstra, 1999a, 2000), dbcAMP failed to stimulate polarity development in dihydroceramide synthase-inhibited or sphinganine-treated cells (Figure 5B). We conclude that the reduction of cellular free sphinganine, regulated by signal-mediated modulation of acyl CoA-dependent dihydroceramide synthase activity is a crucial parameter in cAMP/PKA-stimulated HepG2 cell polarity development.

**Sphinganine Interferes with Polarized Membrane Traffic from SAC**

Endogenous cAMP/PKA activity is required for HepG2 cell polarity development (van IJzendoorn and Hoekstra, 2000),
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and moreover, it mediates the stimulating effects of interleukin-6 family cytokines on BC biogenesis (van der Wouden et al., 2002). We previously identified a specific BC (apical PM)-directed traffic pathway originating from the SAC in HepG2 cells as a primary target for cAMP/PKA signaling (van IJzendoorn and Hoekstra, 1999; van IJzendoorn et al., 2000). Thus, cAMP/PKA activates a specific SAC-to-BC vesicular traffic route, which is followed by distinct, fluorescently labeled lipids, including sphingomyelin and galactosylceramide (GalCer; Figure 6). Inhibition of this route effectively prohibits polarity development (van IJzendoorn and Hoekstra, 2000). Because the accumulation of sphinganine prevented dbcAMP-stimulated development of HepG2 cell polarity (Figure 2, A and B), we next investigated whether the observed effect was directly correlated with a fracturing of cAMP/PKA signaling to redirect and stimulate SAC-to-BC membrane trafficking.

To this end, cells were pretreated with FB1 (10 \( \mu M \)), \( \beta \)-erythro-sphinganine (0.5 \( \mu M \)), or nothing for 24 h, and SAC was preloaded with fluorescent GalCer as described in Materials and Methods (c.f. van IJzendoorn and Hoekstra, 1998, 2000). It is important to note that pretreatment of the cells with FB1 or sphinganine did not affect basolateral internalization, apical delivery, or transport of the fluorescent probe from the apical surface to the SAC. After the loading of the SAC with the fluorescent probe, cells were subsequently treated with dbcAMP (100 \( \mu M \)) or buffer at 4°C, warmed to 37°C, and lipid flow from SAC to either apical (BC) or basolateral membrane was followed for 20 min. If appropriate, FB1 or sphinganine were kept present during all incubation steps. In control cells, \( \text{C}_6\text{NBD-GalCer} \) disappeared during this time interval from the BCP, which constitutes SAC (where the lipid is located at \( t = 0 \)) and BC. Indeed, the percentage of BCP that contained \( \text{C}_6\text{NBD-GalCer} \) decreased in time (Figure 6A, 1 and 2). The relative distribution of the lipid analogue in the remaining, faintly labeled \( \text{C}_6\text{NBD-GalCer} \) reveals a corresponding relative distribution of the probe in the BCP (Figure 6B). LCS promoted HepG2 cell polarity development. Cells were plated in the presence of different concentrations of LCS, LCS + DHC, or LCS + sphinganine in serum-supplemented cell culture medium for 24, 48, or 72 h. At each time point, the cells were fixed, and the ratio BC/100 cells was determined as described in Materials and Methods. Effect of 50–250 \( \mu M \) LCS (A and B), 250 \( \mu M \) LCS + 10 \( \mu M \) C6-DHC (C), or 250 \( \mu M \) LCS + 0.5–2.0 \( \mu M \) sphinganine (C) on cell polarity development. Data are expressed as mean ± SEM of at least three independent experiments carried out in duplicate. (B) Fluorescence microscopical evaluation of cell polarity in control or LCS-treated cells (72 h; nuclei in blue and BC in red). Note the increase in BC circumference in LCS-treated cells. Bars, 5 \( \mu m \); *\( p < 0.05 \) (Student’s t test).

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nous sphinganine was added to the cells during the 4°C dbcAMP treatment before the chase (unpublished data). These data strongly suggest that dihydroceramide synthase activity and the subsequent turnover of sphinganine levels is a prerequisite for cAMP/PKA signaling to redirect polarized membrane trafficking from SAC, and in this way, to stimulate polarity development.

**Sphinganine Inhibits Transcytosis of dIgA-pIgR at the SAC**

Many apical PM proteins reach the apical surface via an indirect route that includes initial targeting from the Golgi apparatus to the basolateral surface, followed by transcytosis to the apical domain. The transcytotic pathway involves passage through distinct compartments. Thus, after basolateral endocytosis, proteins are first delivered to basolateral early endosomes. Apical PM proteins are then transported to the SAC, and subsequently, to the apical surface (Rojas and Apodaca, 2002; Tuma and Hubbard, 2003). We have previously shown that the cAMP/PKA-activated SAC-to-apical membrane trafficking pathway also is followed by the transcytosing dIgA-bound polymeric Ig receptor (pIgR; van IJzendoorn and Hoekstra, 1998) and may thus represent the final leg of the transcytotic pathway. To investigate whether accumulation of sphinganine also interfered with dIgA-pIgR exit from the SAC, HepG2 cells that stably express pIgR (van IJzendoorn and Hoekstra, 1998) were treated with exogenous sphinganine (0.5 mM; 30 min or 24 h) instead of FB1, but not in cells treated with t-threo-sphinganine, sphingosine, or sphinganine-1-phosphate (all 0.5–2.0 mM; unpublished data). Asterisks, BC; arrows, SAC. Bottom panels are phase contrast images to top panels. Bars, 5 μm.

**Figure 5.** (A) dbcAMP stimulates FB1-sensitive acyl-CoA–dependent DHC activity. Control and dbcAMP-treated (0–24 h) HepG2 cells were homogenized and incubated with [3H]sphinganine (100 pmol/100 μg of cell protein) and stearyl-CoA (10 nmol/100 μg of cell protein) for 10 or 0 min at 37°C. Lipids were then extracted with CHCl₃:methanol and separated by TLC. Radiolabeled sphinganine and (dihydro)ceramides were scraped from the TLC plates and quantified in a liquid scintillation counter (Veldman et al., 1998). Note that as negative controls, the omission of stearyl (St)-CoA from the reaction or cotreatment of the cells with FB1 prevented dihydroceramide synthase activity. (B) Increased levels of free sphinganine block dbcAMP-stimulated polarity development in HepG2 cells. Cells were cultured in the presence of dbcAMP, dbcAMP + FB1 (10 μM), or dbcAMP + exogenous sphinganine (SA; 0.5 μM) in serum-supplemented culture medium for 24 h. Cells were then fixed and the ratio BC/100 cells was determined as described in Materials and Methods. Note the increase in polarity in dbcAMP-treated cells and the decrease in polarity development in cells cotreated with dbcAMP together with FB1 or exogenous sphinganine, compared with untreated control cells. Data are expressed as mean ± SEM of at least three independent experiments carried out in duplicate.

**Figure 6.** Accumulation of sphinganine interferes with polarized membrane trafficking from SAC. SAC of control (A–C, 1–3) or FB1-treated (24 h; A–C, 4) HepG2 cells were preloaded with C₆NBD-GalCer, as described in detail previously (van IJzendoorn and Hoekstra, 1998, 2000). After a subsequent chase, the percentage of C₆NBD-GalCer–positive BCP, comprising BC and/or SAC, was determined (A), as well as the corresponding relative distribution of the fluorescent probe in the BCP, i.e., in BC, SAC, or both (BC + SAC) (B). 1. Before the chase (t = 0); 2. 20-min chase in HBSS (control cells); 3. 20-min chase in HBSS + 1 mM dbcAMP (control cells); 4. 20-min chase in HBSS + 1 mM dbcAMP (FB1-treated cells). Data are expressed as mean ± SEM of at least three independent experiments carried out in duplicate. (C) Representative images showing the distribution of the fluorescent probe in the BCP area marked by the dashed rectangle (i.e., BC alone, BC + SAC, or SAC alone). Similar results were obtained when cells were treated with exogenous sphinganine (0.5 μM; 30 min or 24 h) instead of FB1, but not in cells treated with t-threo-sphinganine, sphingosine, or sphinganine-1-phosphate (all 0.5–2.0 μM; unpublished data). Asterisks, BC; arrows, SAC. Bottom panels are phase contrast images to top panels. Bars, 5 μm.
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**Figure 7.** Sphinganine inhibits transcytosis of dIgA-pIgR. HepG2 cells that stably express pIgR were treated with 2.0 μM sphinganine (C) or buffer (A and B) for 30 min at 37°C and incubated with Texas Red-labeled dIgA at 18°C for 60 min to accumulate the ligand in SAC (A). Cells were subsequently shifted to 37°C and incubated for another 30 min in buffer in the absence (B) or presence (C) of 0.5 μM sphinganine. Note that most of the dIgA-pIgR associated with SAC (A, arrows) is readily transported to the BC (B, asterisk), whereas in cells treated with exogenous sphinganine, dIgA-pIgR is not transported to the BC and is retained in SAC (C, arrows; see text). Treatment of the cells with sphingosine, l-threo-sphinganine, or sphinganine-1-phosphate for 30 min at 37°C gave similar results as observed in control cells (unpublished data; c.f. B). Bars, 5 μm.

Discussion

In this study, we implicate regulated dihydroceramide synthase activity as a prominent parameter in cell polarity development. The profound effects that an altered level of sphinganine has on cellular levels of sphinganine, rather than the reduced levels of higher sphingolipids, have recently emerged as important, but poorly understood modulators of membrane trafficking (Friant et al., 2000; Zanolari et al., 2000; de Hart et al., 2002). Here, we show that elevated levels of sphinganine dramatically perturb polarized membrane traffic from the SAC (Figures 6 and 7), a sub-

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Figure 8. Working model that describes the relationship between sphinganine metabolism, polarized membrane traffic from SAC, and apical PM biogenesis in response to cAMP/PKA signaling in HepG2 cells. First, cAMP/PKA signaling reorganizes polarized membrane traffic from the SAC, which includes the rerouting of a fluorescent lipid probe from a basolateral direction into the transcytotic pathway, leading to the apical surface. Activation of this pathway is crucial for cAMP/PKA-stimulated polarity development. cAMP/PKA signaling also increases the activity of dihydroceramide synthase, thereby stimulating the turnover of sphinganine. The inability to turn over sphinganine and the consequent accumulation of free sphinganine inhibits cAMP/PKA-induced activation of the apical PM directed traffic route from SAC and, hence, polarity development.

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