Chapter 5

Polarized sphingolipid transport from the sub-apical compartment changes during cell polarity development

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SUMMARY

The sub-apical compartment (SAC) constitutes an intrinsic part of the transcytotic pathway and plays an important role in polarized transport of proteins and lipids. In a previous study, we have demonstrated that in polarized hepatoma-derived HepG2 cells, fluorescent (C₆-NBD) analogs of sphingomyelin (SM) and glucosylceramide (GlcCer) are sorted in the SAC (van IJzendoorn, S.C.D. and Hoekstra, D. 1998. *Journal of Cell Biology* **142**, 683-696). Here, we demonstrate that the direction of polarized trafficking of C₆-NBD-SM from the SAC reverses during the development of cell polarity. After plating, HepG2 cells regain their polarized phenotype in a time-dependent manner, reaching half-maximal and maximal polarity after 18 and 72 h in culture, respectively. In 18-h cell cultures, C₆-NBD-SM was efficiently transported from the SAC to apical membrane domain. Intriguingly, this polarized transport of C₆-NBD-SM from the SAC is dramatically altered in cells that had been cultured for 72 h. Thus, in this optimally polarized cell culture, the lipid analogue was efficiently targeted from the SAC to the basolateral domain. C₆-NBD-GlcCer was targeted from the SAC to the apical PM, irrespective of the state of polarity development. Interestingly, transport of SAC-derived C₆-NBD-SM to BC in 18-h cells was abolished by the PKA inhibitor H89, which also inhibited the further polarization of the cells. Neither transport of C₆-NBD-SM from the SAC to the basolateral PM in cells cultured for 72 h, nor apical-directed transport of the GlcCer analogue from the SAC in both 18 or 72 h cultures, was affected by H89. In summary, the present data demonstrate that polarity development is regulated by a transient activation of endogenous PKA. This development includes a transient activation of a specific membrane transport pathway from the SAC to the apical membrane during biogenesis of the apical membrane. This PKA-regulated pathway differs from the apical recycling pathway, which also traverses SAC. After reaching polarity, the direction of the apically activated pathway switches to one in the basolateral direction, without affecting the apical recycling pathway.

INTRODUCTION

Polarized cells have developed distinct plasma membrane (PM) domains, an apical and a basolateral domain. Each PM domain is characterized by a specific protein and lipid composition (Simons and Fuller, 1985; Zegers and Hoekstra, 1998). The establishment and maintenance of such distinct PM domains requires the coordinated vectorial transport (i.e. sorting and targeting), docking, and fusion of selectively targeted vesicles carrying specific cargo molecules to appropriate PM domains. In this way, each membrane domain can be supplied with appropriate proteins and lipids, necessary for the polarized cell to fulfill its specialized tasks at the different extracellular environments. Newly synthesized membrane components can be sorted in the *trans*-Golgi network (TGN) for direct delivery to the correct PM domain (Pelham, 1996). In addition, it is becoming well-recognized (van IJzendoorn and Hoekstra, 1999) that an auxiliary, non-Golgi-related compartment is also engaged in the polarized sorting of proteins (Apodaca et al., 1994; Futter et al., 1998; Zacchi et al., 1998) and, as recently discovered, also of (glyco)sphingolipids (van IJzendoorn and Hoekstra, 1998). This sub-apical compartment, SAC, is located in the hub of intracellular transport routes, and receives and exchanges molecules derived from both the apical and basolateral PM domains (Apodaca et al., 1994; Barosso and Sztul, 1994; Futter et al., 1998; van IJzendoorn and Hoekstra, 1998). Indeed, the SAC appears to be equipped with machineries for protein sorting, such as clathrin/γ-adaptin/AP-1 coat complexes (Futter et al. 1998;
Okamoto et al., 1998) and those involved in (glyco)sphingolipid segregation (van IJzendoorn and Hoekstra, 1998). Hence, in light of continuous transcellular traffic of PM proteins and lipids, this endosomal compartment carries an important part of the sorting burden that secures the specific PM compositions and, thus, cell polarity (reviewed in van IJzendoorn and Hoekstra, 1999).

It has been proposed that the SAC is not a compartment that is unique to polarized cells. Indeed, SAC shows remarkable analogy with the pericentriolar recycling compartment (PCRC) in non-polarized cells (Apodaca et al., 1994; Zacchi et al., 1998; van IJzendoorn and Hoekstra, 1998). For instance, in polarized HepG2 cells, apical PM derived C<sub>6</sub>-NBD-sphingolipids and basolaterally derived IgA, bound to the polymeric immunoglobulin receptor (pIgR), accumulate in the SAC at 18°C, whereas in nonpolarized HepG2 cells, these molecules accumulate in a pericentriolar recycling compartment under otherwise identical conditions (van IJzendoorn and Hoekstra, 1998). In addition, the epithelium-specific small GTPase rab17 localizes to the SAC in polarized cells, where it interferes with polarized sorting, and to the recycling compartment when expressed in non-polarized cells (Zacchi et al., 1998; Hunziker and Peters, 1998). Also another epithelium-specific rab protein, rab25, localizes exclusively to the SAC (Casanova et al., 1998). These studies suggest that the SAC is the equivalent of the PCRC in nonpolarized cells, but acquires (part of) the functional sorting machinery (e.g. rab17, rab25) when required, i.e. upon development of cell polarity. Although the involvement of the SAC in the establishment of cell polarity thus seems evident, it remains yet unclear how, and to what extent, the membrane sorting capacity of the SAC contributes to this process.

In this study, we have investigated the polarized transport of (glyco)sphingolipids from the SAC during HepG2 cell polarity development. HepG2 cells have retained their capability to acquire the polarized phenotype after plating, as evidenced by the formation of microvilli-lined intercellular vacuoles, that are representative of the apical, bile canalicular PM domain (BC, Chiu et al., 1989; Sormunen et al., 1993; Zaal et al., 1993). Polarized HepG2 cells have been proven to be a suitable model for the study of several functional properties of hepatocytes, including metabolism, sorting, polarized transport and secretion (see Zegers and Hoekstra, 1998 and references therein). We have determined the time-dependent advancement of polarity development of HepG2 cells after plating, and present evidence that reveals a concomitant change in the direction of polarized membrane transport from the SAC. Our data demonstrate for the first time that the sorting of a specific sphingolipid, SM, and consequently, its subsequent preferential transport to a specific PM domain depends on the degree of cell polarization. Moreover, this polarity-dependent shift in transport direction appears to be regulated by protein kinase A activation. Since apical membrane recycling via the SAC is unaffected, the data emphasize the importance of the sorting capacity of this compartment in cell polarity development.
RESULTS

Kinetics of HepG2 cell polarity development
In culture, HepG2 cells retain their capability to acquire a polarized phenotype, as indicated by the formation of microvilli-lined intercellular vacuoles (BC) that are reminiscent of the apical, bile canalicular domain (Chiu et al., 1989; Sormunen et al., 1993; Zaal et al., 1993). In order to determine the time-dependent development of HepG2 cell polarity, cells were plated on ethanol-sterilized glass coverslips at low density, and allowed to grow for various time intervals. Cells were then fixed and, as a measure of cell polarity, the ratio [BC/100 cells] was determined as described in EXPERIMENTAL PROCEDURES. As shown in figure 1, the ratio of [BC/100 cells] increased from 2.6 ± 0.3 to 10.9 ± 0.3 in cells cultured for 3 and 18 h, respectively, and reached a maximum of 21.3 ± 0.5 BC/100 cells in cells cultured for 72 h. Since, in general, two cells participate in the formation of one BC, 5%, 20% and 43% of the cells cultured for 3, 18 and 72 hours, respectively, can be considered as being polarized (Fig. 1, right y-axis). After culturing for another 24 h, the ratio [BC/100 cells] decreased again to 15.6 ± 0.6 (Fig. 1). Importantly, very similar results were obtained when BC were identified by indirect immunofluorescent labeling of a BC-specific antigen, using the monoclonal antibody Mab442 (Chemicon, Temecula, CA/USA), or by phase-contrast microscopical analysis. Hence, the data show that after plating, the HepG2 cells regain their polarized phenotype in a time-dependent manner, reaching maximum polarity after 72 h in culture.

In polarized HepG2 cells, SM and GlcCer are effectively segregated to the basolateral and apical region of the cells, respectively (van IJzendoorn et al., 1997; van IJzendoorn and Hoekstra, 1998). The SAC is instrumental in governing this preferential distribution. It was therefore of interest to examine next whether and how the cells adopted mechanistically to polarity development in terms of this preferred sphingolipid distribution.

![Graph showing the ratio of BC/100 cells over time after plating](image-url)
Figure 1. Time-dependent polarity development of HepG2 cell cultures. Cells were plated at low density (± 20% of surface occupied) on ethanol-sterilized glass coverslips and allowed to adhere and grow in normal culture medium. After various time intervals, cells were washed, fixed and processed for determination of the degree of polarity (see EXPERIMENTAL PROCEDURES). The left and right y-axis indicate the ratio [BC/100 cells] and the percentage of polarized cells, respectively. Data are presented as mean ± SEM of at least 3 independent experiments, carried out in duplicate.

**Differential targeting of C₆-NBD-SM from the SAC during progression of cell polarity**

The polarized trafficking of lipid analogs from the SAC was investigated in cell cultures that were either sub-optimally (18 h) or optimally polarized, i.e. cultured for 72 h (see Fig. 1). To this end, the basolateral surface of cells was labeled with C₆-NBD-SM at 37°C to allow internalization and transcytotic delivery to the apical, bile canalicular surface (BC, cf. van IJzendoorn and Hoekstra, 1997; Zegers and Hoekstra, 1997). The residual pool of lipid analogue still present at the basolateral membrane domain after this internalization step, was then selectively depleted by a back exchange procedure with BSA at 4°C. Note that in both 18 and 72 h-old cell cultures, 70-80% of the BC remained labeled following the back exchange (not shown, but see below). Hence, since BSA does not have access to the BC membranes, it is concluded that already in 18 h-old cell cultures, a physical separation between the apical and basolateral PM domains was achieved by the presence of tight junctions. Moreover, the ability of BC in both 18 and 72 h-old cell cultures to retain the water-soluble dye rhodamine 123 in their lumen (our unpublished observations) further supports the functional integrity of the BC in 18 h cells. Following the removal of basolateral PM-associated lipid analogue, cells were subsequently incubated in back exchange medium at 18°C for 1 h to chase apical PM derived C₆-NBD-SM into the SAC (van IJzendoorn and Hoekstra, 1998). Finally, NBD-fluorescence associated with the exoplasmic leaflet of BC was abolished using sodiumdithionite at 4°C. At this time, the vast majority of the intracellular lipid analogue is associated with the SAC (Fig. 2 b and c, white bars; cf. van IJzendoorn and Hoekstra, 1998). Transport of C₆-NBD-SM from the SAC was then examined by incubating the cells at 37°C in back exchange medium to prevent re-internalization of lipid arriving at the basolateral membrane. In 72 h-old cell cultures, the percentage of C₆-NBD-SM remaining at the apical pole (BCP, see EXPERIMENTAL PROCEDURES) decreased from ~ 88 to ~ 60 % during a 20 min chase from the SAC, indicating that the lipid analogue had disappeared from the apical region of the cells (Fig. 2 a). Indeed, of the remaining fraction of C₆-NBD-SM in the BCP, the vast majority was found in the SAC alone (Fig. 2 c), consistent with previous observations (see van IJzendoorn and Hoekstra, 1998). In striking contrast, in the 18 h-old cell culture C₆-NBD-SM remained in the bile canalicular pole during the entire chase (Fig. 2 a). Analysis of the distribution of the lipid analogue in the BCP revealed that C₆-NBD-SM labeled BC, SAC or both (Fig. 2 b), indicating that in this case C₆-NBD-SM was redistributed from the SAC to BC, rather than to the basolateral region as observed for the 72 h culture. Hence, the results demonstrate that in cells that are in the process of developing apical PM domains (cf. Fig. 1),
trafficking of C₆-NBD-SM from the SAC is in the *apical* direction, whereas in optimally polarized cell cultures, transport of C₆-NBD-SM from the SAC is in the *basolateral* direction. Apparently, sorting and subsequent polarized targeting of C₆-NBD-SM from the SAC is dictated by the degree of cell polarity development.

**Polarized transport of C₆-NBD-GlcCer from the SAC does not change during progression of cell polarity**

We next investigated the transport of SAC-derived C₆-NBD-GlcCer, which, in fully polarized cells, prefers an apical distribution. The same experimental approach, as described in the previous section, was taken. As shown in figure 3a, C₆-NBD-GlcCer remained associated...
with the BCP during the chase from the SAC in both 18 h- and 72 h-old cell cultures, indicating that this lipid analogue did not leave the apical pole of the cells. The relative distribution of C₆-NBD-GlcCer analogue over the various sites, i.e. the BC, the SAC, or both was indistinguishable (Fig. 3b and c). Hence, it is concluded that in contrast to a polarity-dependent shift in the direction of SM-trafficking, the direction of transport of C₆-NBD-GlcCer, is unaffected by the degree of cell polarity. Presumably, the persistence of an apically-directed flow of GlcCer leaving the SAC in both polarized and polarity-developing cells, reflects an apical recycling pathway (see below; cf. van IJzendoorn and Hoekstra, 1998).

The PKA-Inhibitor H89 inhibits transport of C₆-NBD-SM from the SAC to BC, but not from the SAC to the basolateral PM domain

Previously, we have shown that in optimally polarized HepG2 cells, apical-to-basolateral transcytosis of C₆-NBD-SM is impeded in the presence of dibutyryl cAMP, a cell permeant non-hydrolysable cAMP analogue. Rather, under those conditions, trafficking of C₆-NBD-SM

![Diagram](image-url)
Figure 3. Polarized transport of C₆-NBD-GlcCer is in the apical direction in both 18 h- and 72 h-old cells. The SAC were loaded with C₆-NBD-GlcCer as described in MATERIALS AND METHODS. Transport of the lipid analogue was followed for 20 min in back exchange medium. In a, the percentage of C₆-NBD-GlcCer-labeled BCP was determined in 18 h- (white bars) and 72 h-old (gray bars) cell cultures. In b and c, the distribution of the lipid analogue within the labeled BCP was analyzed. White and gray bars represent the distribution of the lipid prior to and after a 20 min chase from the SAC, respectively.

is redirected to the BC (van IJzendoorn et al., 1997). Moreover, dibutyryl cAMP treatment was also shown to enhance BC-directed sphingolipid transport, in both the direct (biosynthetic) TGN-to-apical route and the basolateral-to-apical transcytotic pathway. Interestingly, a concomitant hyper-polarization of the cells, as evidenced by an increase in the number of BC as well as their circumference, was observed (Zegers and Hoekstra, 1997). The mechanism underlying these events was related to dibutyryl cAMP-induced activation of protein kinase A. These studies thus suggest that apical PM-directed sphingolipid transport and development of polarity of the cells are closely related events in PKA-stimulated HepG2 cells.

Reasoning therefore that the dibutryl cAMP/PKA-mediated hyper-polarization may be of physiological significance in polarity development, we investigated the involvement of endogenous PKA activity in the polarized transport of C₆-NBD-SM from the SAC in sub-optimally and optimally polarized cell cultures. For this, the SAC was first loaded with the SM analogue as described above. Cells were then preincubated with 10 µM of the specific PKA-inhibitor H89 at 4°C for 30 min. Transport of C₆-NBD-SM from the SAC was subsequently determined by incubating the cells at 37°C in back exchange medium, supplemented with H89. In 72 h-old cell cultures, transport of C₆-NBD-SM from the SAC to the basolateral domain was unaffected by H89 (cf. Fig. 2 a, c). Remarkably, H89 strongly inhibited SAC-to-BC transport of C₆-NBD-SM in 18 h-old cell cultures (Fig. 4 b, comp. to Fig. 2 b). Intriguingly, the presence of H89 did not cause a redirection of transport of the SM analogue to the basolateral domain. Rather, the lipid analogue remained associated with the BCP (Fig. 4 a), where it exclusively associated with the SAC (Fig. 4 b). The data thus suggest that during polarity development, as reflected by the 18 h-old cell cultures, the trafficking of C₆-NBD-SM from the SAC to BC is regulated by endogenous PKA activity.

**H89 does not inhibit SAC-to-BC transport of C₆-NBD-GlcCer**

Evidently, in sub-optimally polarized cell cultures (18 h), both C₆-NBD-SM and -GlcCer are transported from the SAC to BC. Since H89 inhibited SAC-to-BC transport of C₆-NBD-SM in these cells, it was of interest to examine the specificity of this impediment and determine whether this inhibitor also affected SAC-to-BC trafficking of C₆-NBD-GlcCer. Therefore, after loading the SAC with the GlcCer analogue and removal of BC-associated NBD-fluorescence (see above), 18 or 72 h-old cell cultures were preincubated with 10 µM H89 at 4°C for 30 min. Then, cells were incubated in back exchange medium at 37°C in the presence
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Figure 4. SAC-to-BC transport of C₆-NBD-SM from the SAC is inhibited by the protein kinase A inhibitor H89. Cells were cultured for 18 h, after which the SAC were leaded with C₆-NBD-SM. Cells were then incubated with 10 µM H89 or HBSS at 4°C for 30 min and, subsequently, in back exchange medium, with or without (control) H89 at 37°C for 20 min. In a, the percentage of C₆-NBD-SM-labeled BCP in non-treated (white bar) and H89-treated cells (gray bar) is determined. In b, the distribution of BCP-associated C₆-NBD-SM was analyzed in control (white bars) and H89-treated cells (gray bars).

Irrespective of the presence of H89, and in contrast to the observations reported above for C₆-NBD-SM, C₆-NBD-GlcCer was transported from the SAC to BC in both 18 and 72 h-old cells (cf. Fig. 3). The discriminating effect of H89 on SAC-to-BC transport of C₆-NBD-GlcCer on the one hand, and -SM on the other thus suggests that the two lipid analogs travel from the SAC to BC via distinct pathways.

PKA inhibition prevents progression of HepG2 cell polarity

To directly correlate the observed switch of membrane transport, as reflected by C₆-NBD-SM traffic from the SAC in sub-optimally polarized cells (i.e. cells that are in the BC-developing phase), with cell polarity development, we next examined the effect of H89 on polarity development of the cells. Cells were plated and cultured for 18 h. Since at this stage, the cell culture is sub-optimally polarized (see Fig. 1), both progression and loss of cell polarity can be determined. The medium of 18 h-old cell cultures was replaced by medium, supplemented with 10 µM H89, and the cells were cultured for another 18 or 54 h. As shown in figure 5, the presence of H89 in the medium effectively blocked progression of polarity development of the cells, as evidenced by a constant value of approximately 10 in the ratio of BC/100 cells (~20% of the cells are polarized). Note that in control cells (absence of H89), polarity is further increased to a ratio [BC/100 cells] of approximately 21 (~42% of the cells are polarized). Very similar results were obtained when quantitation was carried out by using the BC antigen-specific antibody MAb442 to identify the BC (not shown). Treatment of the 18 h-old cell
cultures with H89 did not cause depolarization of the cells, which would have been reflected by a decrease in the ratio [BC/100 cells]. Taken together, the data strongly suggest that polarized targeting from the SAC, as marked by the trafficking of C₆-NBD-SM, and the acquisition of the polarized phenotype are closely coupled events.

Figure 5. The protein kinase A inhibitor H89 blocks progression of HepG2 cell polarity. Cells were plated and cultured for 18 h. At this time, the cell culture is half-maximally polarized (Fig. 1). Cells were washed with sterile HBSS and cultured in normal culture medium (white bars) or culture medium, supplemented with 10 µM H89 (hatched bars). After a prolonged incubation of 14 and 54 h, cells were fixated and the ration [BC/100 cells] was estimated as described in (EXPERIMENTAL PROCEDURES).

DISCUSSION

Irrespective of the degree of cell polarity, the overall expression of proteins and lipids in differentiating cells is not remarkably different (Bender et al., 1998; Kramer et al., 1997), implying that membrane domain specificity in fully polarized cells is likely governed by specific sorting/targeting and retrieval processes. Indeed, this also holds upon hyperpolarization of HepG2 cells, as induced by exogenous addition of dibutyryl cAMP, which was similarly correlated with a stimulation of apical PM-directed sphingolipid transport (Zegers and Hoekstra, 1997, see below). In the present study, evidence is presented that demonstrates that the degree of cell polarity dictates the polarized targeting of SM to the developing apical membrane. Thus in sub-optimally polarized cells, i.e. cells that had been cultured for 18 h, C₆-NBD-SM was transported from SAC to BC, which was inhibited by H89, whereas in optimally polarized cells (72 h), this lipid was transported from the SAC to the basolateral membrane (Fig. 2). Previously, we observed that inhibition of PKA activation by H89, inhibited apical-directed transport in optimally polarized cells (Zegers and Hoekstra, 1997). In conjunction with these results, the present data indicate that in HepG2 polarity development,
*endogenous* protein kinase A activity is transiently upregulated, which promotes an apical direction of membrane flow. Upon polarity progress in the culture, the activity decreases again (note that 72 h cells are not affected by H89), causing a switch in the flow of SM from an apical to a basolateral direction. Via a mechanism yet to be determined, the intracellular sorting compartment in polarized trafficking, SAC, appears a major target site of endogenous PKA activation. Indeed, as demonstrated previously (Zegers and Hoekstra, 1997), endogenous activation of PKA via dibutyryl cAMP results in neither an enhanced biosynthesis nor an increase in basolateral endocytosis.

Interestingly, the PKA-activated pathway involved in the biogenesis of the apical membrane could be clearly distinguished from the apical recycling route, marked by the flow of GlcCer. The latter is not significantly affected by PKA activation, which is supported by the observation that H89 did not interfere with the recycling pathway. Hence, these observations support the conclusion that the recycling pathway and the pathway involved in the biogenesis of the apical membrane are distinct routes. This notion is further supported by studies on protein trafficking in MDCK cells, which showed that elevated levels of dibutyril cAMP stimulate transcytosing proteins to a much greater extent than that of apical recycling proteins (Hansen and Casanova, 1994). Indeed, more recent evidence (van IJzendoorn and Hoekstra, submitted) demonstrated that the SM-marked pathway between SAC and the apical membrane coincides with that taken by the transcytotic pIgR/IgA marker complex. Since the dbcAMP/PKA activated pathway does not enhance basolateral endocytosis (Zegers and Hoekstra, 1997), this implies that the transcytotic pathway, exiting from SAC, is closely related to the biogenesis of the apical membrane and hence to the development of cell polarity.

An intriguing issue is why inhibition of apical directed trafficking by H89 in 18 h cells does not resemble SM trafficking in maximally polarized cells, which show a basolateral pathway for the lipid. A direct effect of H89 can be excluded, as the inhibitor does not affect the basolateral trafficking in optimally polarized cells. At present we have no clear explanation for this observation, but it is possible that in sub-optimally polarized cells, the proper basolateral sorting machinery has not yet been developed in SAC. The absence of such a pathway from SAC may be reasonable in light of the crucial involvement of the compartment in apical membrane biogenesis during early stages of cell polarity development. In line with this reasoning and given the specific, i.e. polarity-developing conditions, SAC may then favor a retention function, acting as temporal site of storage and providing an immediate supply, when triggered by PKA (re-)activation. Consistent with such an argument would be that transmembrane transporter protein (Katsura et al., 1998), secretory proteins (Ammala et al., 1993) and neurotransmitters (Valtora and Meldolesi, 1994) are also recruited from intracellular vesicular pools to specific PM domains in a cAMP/PKA-regulated manner. Hence, apical targeting of SM from the SAC during cell polarity development, and the process of regulated exocytic transport show some clear similarities. Note that these observations inherently emphasize the significance of (sphingolipid-)sorting capacity in both the SAC and Golgi. Whereas during polarity development SAC appears to play a particular
prominent role in the biogenesis of the apical domain (this study), the obvious (biosynthetic) needs of the basolateral membrane can be met by the Golgi.

The role of PKA activation in the biogenesis of cell polarity in HepG2 cells appears to be primarily restricted to biogenesis itself, rather than to maintaining polarity. This is suggested by the dramatic apical-to-basolateral shift in SM transport, once the cells have reached optimal polarity. Moreover, as noted above the artificial reactivation as triggered by adding dibutyryl cAMP reverses this pathway once more, culminating in hyperpolarization. In this respect, it is interesting to note that in fully polarized MDCK cells, H89 abolished cAMP/PKA-stimulated, but not basal levels of SAC-to-apical transport (Hansen and Casanova, 1994). Consistently, in 72 h HepG2 cells, H89 abolished hyperpolarization but did not affect the polarity of non-stimulated cells (Zegers and Hoekstra, 1997). Similarly, H89 effectively impeded cell polarity development in 18 h cells, but did not cause a depolarization.

An issue that remains unresolved is what causes the PKA-mediated switch in polarized targeting from the SAC in cells that are actively engaged in polarity development. In intercalated epithelial cells, the polarized PM distribution of band 3 was shown to be switched from apical to basolateral. This feature was dependent of cell density and correlated with the secretion of specific extracellular matrix (ECM) proteins (van Adelsberg et al., 1994). The activity of these proteins has been related to activation of PKA (Lochter and Schachner, 1997; Fushimi et al., 1997; Katsura et al., 1997). Interestingly, a correlation between epithelial polarity development and the employment of different targeting pathways has also been demonstrated in Fisher rat thyroid (FRT) cells. Thus, in 1 day-old polarized FRT cell monolayers, targeting of apical proteins was accomplished by use of an indirect pathway (i.e. involving transcytosis), whereas in 7 days old monolayers apical delivery was via the direct TGN-to-apical route (Zurzolo et al., 1992). Importantly, additional to a possible role of developmental stage-regulated secretion of signaling molecules, this strongly emphasizes the importance of the transcytotic pathway, most likely involving the SAC, in the process of apical PM biogenesis. The classic upstream effector in the cAMP/PKA signaling cascade includes heterotrimeric G protein alpha subunits, proposed to be involved in maintenance and biogenesis of epithelial cell tight junctions (Saha et al., 1998) and apical PM directed transport (Bomsel and Mostov, 1993; Pimplikar and Simons, 1994; Barosso and Sztul, 1994). Possibly, HepG2 cells secrete ECM molecules following their plating which, upon cell-cell contact (note that secreted ECM associate with the cell’s exterior rather than that they travel relative long distances as do secreted hormones), interact with cell surface receptors. Interestingly, it was recently demonstrated that ligand-receptor binding at the surface of MDCK cells initiated intracellular signaling that resulted in a stimulated SAC-to-apical transport (Luton et al., 1998). Since the apical targeting of the SM analogue is evidently not maintained, additional mechanisms are probably operational that cause a down-regulation or desensitization of the signaling cascade. Such mechanisms are most likely located upstream of cAMP, since treatment of 72 h-old HepG2 cells with dibutyryl cAMP induces a similar rerouting of SAC-associated C6-NBD-SM and hyper-polarization (S. van IJzendoorn and D. Hoekstra, manuscript submitted), as proposed to occur during natural development of cell polarity (this
Although the involvement and nature of possible extracellular signals, as well as target molecules of cAMP/PKA, remain as yet elusive, this study provides the first evidence that the polarized targeting of specific molecules from a single organelle, the SAC, changes during the development of cell polarity and that endogenous cAMP/PKA-mediated signaling plays a central role in this process.

**EXPERIMENTAL PROCEDURES**

**Cell culture**

HepG2 cells were cultured in Dulbecco’s modified essential medium (DMEM) with 4500 mg of glucose per liter, supplemented with 10% heat-inactivated (at 56°C) fetal calf serum (FCS) and antibiotics (penicillin and streptomycin). Media were changed every other day. For experiments, cells were plated onto ethanol-sterilized glass coverslips at low density (± 20% of surface occupied). The cells were used for experiments after various time intervals after plating.

**Determination of HepG2 cell polarity**

Accurate estimation of the degree of HepG2 polarity was performed as described elsewhere (Zegers and Hoekstra, 1997). Cells were fixed with -20°C ethanol for 10 s and rehydrated in HBSS. Cells were then incubated with a mixture of tetramethylrhodamine isothiocyanate (TRITC)-labeled phalloidin and the nuclear stain Hoechst-33528 at RT for 20 min. The cells were then washed and the number of BC (identified by the presence of dense F-actin staining around BC) per 100 cells (identified by fluorescently labeled nuclei) was determined and expressed as the ratio [BC/100 cells]. 10 fields (each containing >50 cells) per coverslip (at least 2 coverslips per condition were studied) were analyzed. Identical results were obtained when a monoclonal antibody raised against a BC-specific antigen (MAb442) in stead of TRITC-phalloidin was used to identify BC.

**Synthesis of C6-NBD-labeled sphingolipids**

C₆-NBD-SM and C₆-NBD-GlcCer were synthesized from C₆-NBD and 1-β-D-glucosylsphingosine and sphingosylphosphorylcholine, respectively, as described elsewhere (Kishimoto, 1975; Babia et al., 1994). The lipids were stored at -20°C and routinely checked for purity.

**Analysis of transport of C₆-NBD-Sphingolipids from the SAC**

In order to study the trafficking of lipid analogs from the SAC, SAC were preloaded with lipid analogue as described elsewhere (van IJzendoorn and Hoekstra, 1998). In short, cells were labeled with 4 µM of either C₆-NBD-SM or -GlcCer at 37°C to allow internalization from the basolateral surface and subsequent transcytosis to the apical, bile canaliclar PM domain (BC). Lipid analogue residing at the basolateral domain was then depleted by a back exchange procedure at 4°C (2 x 30 min incubation in HBSS + 5% (w/v) BSA, cf. van IJzendoorn et al., 1997), and BC-associated lipid analogue was chased into the SAC at 18°C for 1 h in back exchange medium. Then, the NBD-fluorescence at the exoplasmic BC leaflet was abolished using sodiumdithionite at 4°C, leaving the vast majority of the intracellular lipid analogue in the SAC (van IJzendoorn and Hoekstra, 1998). Transport from the SAC was then examined by incubating in back exchange medium at 37°C. To examine the effect of the protein kinase A inhibitor H89 (N-[2-(p-bromocinnamylamino)ethyl]-5-iso-quinolinesulfonamine) on sphingolipid trafficking from the SAC, cells were incubated with 10 µM H89 at 4°C for 30 min following the sodiumdithionite incubation and the compound was kept present during subsequent incubations.

In order to quantitate transport of the lipid analogues to and from the BC, the percentage of NBD-positive BC was determined as described elsewhere (van IJzendoorn et al., 1997; van IJzendoorn and Hoekstra, 1998). Briefly, BC were first identified by phase contrast illumination, and then categorized as NBD-positive or NBD-
negative under epifluorescence illumination. Distinct pools of fluorescence were discerned at the apical pole of the cells, present in vesicular structures adjacent to BC, which are defined as sub-apical compartments (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 analogs will be defined as being derived from the BC, the SAC, or both. At least 50 BCP per coverslip were analyzed. Data are expressed as the mean ± SEM of at least four independent experiments, carried out in duplicate.

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