

Polarized Sphingolipid Transport from the Subapical Compartment Changes during Cell Polarity Development

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The subapical compartment (SAC) plays an important role in the polarized transport of proteins and lipids. In hepatoma-derived HepG2 cells, fluorescent analogues of sphingomyelin and glucosylceramide are sorted in the SAC. Here, evidence is provided that shows that polarity development is regulated by a transient activation of endogenous protein kinase A and involves a transient activation of a specific membrane transport pathway, marked by the trafficking of the labeled sphingomyelin, from the SAC to the apical membrane. This protein kinase A–regulated pathway differs from the apical recycling pathway, which also traverses SAC. After reaching optimal 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 subapical 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; Barroso 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 (for review, see van IJzendoorn and Hoekstra, 1999).

It has been proposed that the SAC is not a compartment that is unique to polarized cells. Indeed, the SAC shows remarkable analogy with the pericentriolar recycling compartment in nonpolarized cells (Apodaca *et al.*, 1994; van IJzendoorn and Hoekstra, 1998; Zacchi *et al.*, 1998). For instance, in polarized HepG2 cells, apical PM-derived 6-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-amino]hexanoic acid (C₆-NBD)-labeled sphingolipids and basolaterally derived immunoglobulin A, bound to the polymeric immunoglobulin receptor, 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 nonpolarized cells (Hunziker and Peters, 1998; Zacchi *et al.*, 1998). Also, another epithelium-specific rab protein, rab25, localizes exclusively to the SAC (Casanova *et al.*, 1999). These studies suggest that the SAC is the equivalent of the pericentriolar recycling compartment in nonpolarized cells, but acquires (part of) the functional sorting machinery (e.g., rab17, rab25) when required, i.e., upon de-

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velopment 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 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, which are representative of the apical, bile canalicular PM domain (BC; Chiu *et al.*, 1990; Sormunen *et al.*, 1993; Zaal *et al.*, 1994). 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 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, sphingomyelin (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 (PKA) activation. Because apical membrane recycling via the SAC is unaffected, the data emphasize the importance of the sorting capacity of this compartment in cell polarity development.

MATERIALS AND METHODS

Sphingosylphosphorylcholine, 1- β -glucosylsphingosine, tetramethylrhodamine isothiocyanate (TRITC)-labeled phalloidin and Hoechst 33528 (bisbenzimidazole) were from Sigma Chemical Co. (St. Louis, MO). The monoclonal antibody raised against a BC-specific antigen was bought from Chemicon (Temecula, CA). Albumin (from bovine serum, fraction V) was bought from Fluka Chemie AG (Buchs, Switzerland). C6-NBD was obtained from Molecular Probes (Eugene, OR). DMEM was purchased from Life Technologies (Paisley, Scotland). Fetal calf serum was from BioWhittaker (Verviers, Belgium). Sodiumdithionite was bought from Merck (Darmstadt, Germany). The PKA inhibitor H89 was obtained from Calbiochem-Novabiochem (La Jolla, CA). All other chemicals were of the highest analytical grade.

Cell Culture

HepG2 cells were cultured in DMEM with 4500 mg glucose/liter, supplemented with 10% heat-inactivated (at 56°C) fetal calf serum and antibiotics (penicillin and streptomycin). Media were changed every other day. For experiments, cells were plated onto ethanol-sterilized glass coverslips at low density ($\pm 20\%$ 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; van IJzendoorn and Hoekstra, 1999b). Cells were fixed with -20°C ethanol for 10 s and rehydrated in HBSS. Cells were then incubated with a mixture of TRITC-labeled phalloidin and the nuclear stain Hoechst 33528 at room temperature 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]. Ten 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-labeled phalloidin was used to identify BC.

Synthesis of C6-NBD-labeled Sphingolipids

C6-NBD-glucosylceramide (GlcCer) and C6-NBD-SM 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-labeled Sphingolipids from the SAC

To study the trafficking of lipid analogues from the SAC, SACs were preloaded with lipid analogue as described elsewhere (van IJzendoorn and Hoekstra, 1998, 1999b). In short, cells were labeled with $4\ \mu\text{M}$ of either C6-NBD-SM or -GlcCer at 37°C to allow internalization from the basolateral surface and subsequent transcytosis to the apical BC. Lipid analogue residing at the basolateral domain was then depleted by a back exchange procedure at 4°C (2×30 -min incubation in HBSS + 5% [wt/vol] BSA, 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, 1999b). After washing away the dithionite, transport from the SAC was then examined by incubating the cells in back exchange medium at 37°C . To examine the effect of the PKA inhibitor H89 (*N*-[2-(*p*-bromocinnamylamino)ethyl]-5-iso-quinolinesulfonamide) on sphingolipid trafficking from the SAC, cells were incubated with $10\ \mu\text{M}$ H89 at 4°C for 30 min after the sodiumdithionite incubation, and the compound was kept present during subsequent incubations.

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 -negative under epifluorescence illumination. Note that a BC is categorized as fluorescently labeled, i.e., NBD-positive, when microvilli-like structures characteristic of the BC can be detected, which are seemingly fluorescent in the wake of the fluorescence derived from the lipid analogue present in the apical membrane (van IJzendoorn *et al.*, 1997). Such microvilli-like structures are typically and readily observed upon gradual photobleaching of the BC-associated NBD fluorescence.

Distinct pools of fluorescence are thus discerned at the apical pole of the cells that are present in vesicular structures adjacent to BC, which are defined as SACs (cf. van IJzendoorn and Hoekstra, 1998). Together, the BC and the SAC 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 the BC, the SAC, or both. This also provides a means to describe the movement of the lipid within or out of this region in the cell (van IJzendoorn and Hoekstra, 1999b). Thus, after loading the SAC with lipid analogue and allowing its transport to take place as described above, the direction of movement of the lipid from or within the BCP region is determined after a given time, by establishing the distribution of the NBD-labeled lipid over the various compartments (BC, SAC, or both) that constitute the BCP, relative to the labeling (i.e., primarily SAC), before starting the chase ($t = 0$). For this kind of analysis, at least 50 BCP per coverslip were analyzed. Data are expressed as the means \pm SEM of at least four independent experiments, carried out in duplicate, and Student's *t*-tests were carried out to determine the statistical significance of the data.

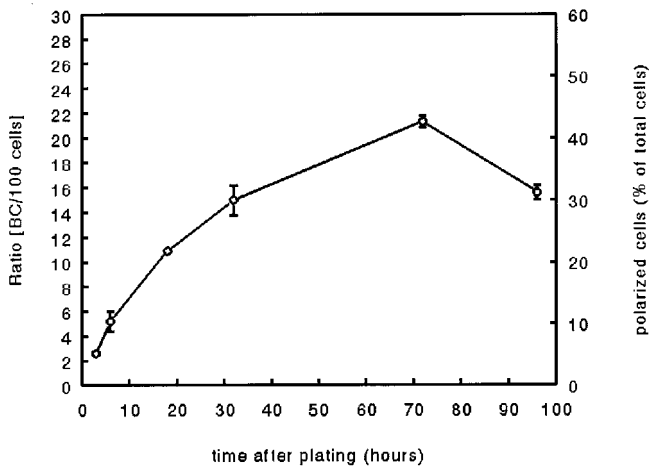


Figure 1. Time-dependent polarity development of HepG2 cell cultures. Cells were plated at low density ($\pm 20\%$ of surface occupied) on ethanol-sterilized glass coverslips and allowed to adhere and grow in normal culture medium. After various time intervals, cells were washed, fixed, and processed for determination of the degree of polarity (see MATERIALS AND METHODS). The left and right y-axes indicate the ratio [BC/100 cells] and the percentage of polarized cells, respectively. Data are presented as means \pm SEM of at least three independent experiments, carried out in duplicate.

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). 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 MATERIALS AND METHODS. 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 h, respectively, can be considered as being polarized (Figure 1, right y-axis). After culturing for another 24 h, the ratio [BC/100 cells] decreased again to 15.6 ± 0.6 (Figure 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, or by phase-contrast microscopic 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, and the SAC is instrumental in governing this preferential distribution (van IJzendoorn *et al.*, 1997; van IJzendoorn and Hoekstra, 1998; 1999b). It was therefore of

interest to examine next whether and how the cells adapted mechanistically to polarity development in terms of this preferred sphingolipid distribution.

Differential Targeting of C6-NBD-SM from the SAC during Progression of Cell Polarity

The polarized trafficking of lipid analogues from the SAC was investigated in cell cultures that were either suboptimally (18 h) or optimally polarized, i.e., cultured for 72 h (see Figure 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 BC surface (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 after the back exchange (see below). Hence, because 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-old cells. After 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 (Figures 2A and 3B and C, □; 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 reinternalization 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 MATERIALS AND METHODS) decreased from ~88 to ~60% during a 20-min chase from the SAC, reflecting the tendency of the lipid analogue to disappear progressively from the apical region of the cells (Figures 2B and 3A). Indeed, of the remaining fraction of C6-NBD-SM in the BCP, the vast majority was found in the SAC alone (Figure 3C), consistent with previous observations (see van IJzendoorn and Hoekstra, 1998, 1999b). In striking contrast, in the 18-h-old cell culture C6-NBD-SM remained in the bile canalicular pole during the entire chase (Figure 3A). Analysis of the distribution of the lipid analogue in the BCP revealed that C6-NBD-SM labeled BC, SAC, or both (Figures 2C and 3B), indicating that in this case a significant part of 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. Figure 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

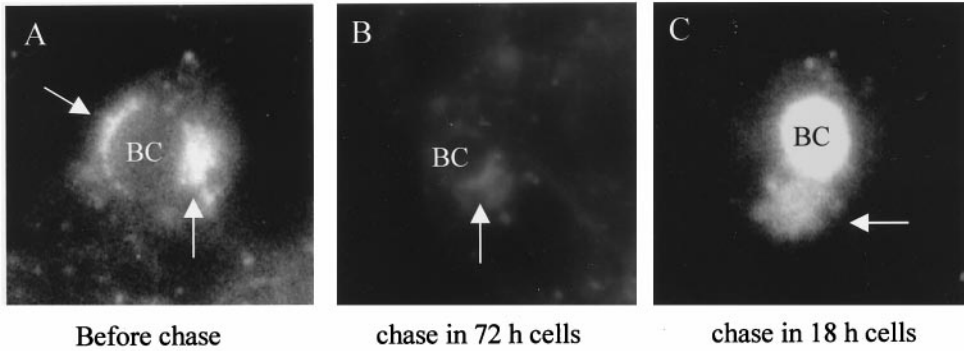


Figure 2. Polarized transport of C6-NBD-SM from the SAC in polarizing cells. The SAC was loaded with C6-NBD-SM as described in MATERIALS AND METHODS. As shown in (A), the lipid analogue predominantly labels the SAC (arrows), whereas the BC is considered “negative,” because the “microvilli criterion” is not met (see METHODS AND MATERIALS). The subcellular distribution of the lipid analogue was then analyzed after a 20-min chase from the SAC in back exchange medium in 72- (B) and

18-h (C) cells. In 72-h cells, the fluorescence intensity strongly diminished after the chase, which is due to extensive basolateral-directed transport and subsequent back exchange to BSA in the medium. Residual fluorescence is primarily observed in the SAC (arrow). In contrast, in 18-h cells the lipid predominantly labels the SAC (arrow) and BC, indicative for SAC-to-BC redistribution of the lipid.

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 api-

cal distribution. The same experimental approach, as described in the previous section, was taken. As shown in Figure 4A, C6-NBD-GlcCer remained associated with the BCP during the chase from the SAC in both 18- and 72-h-old cell cultures, indicating that this lipid analogue did not leave the apical pole of the cells. The relative distribution of the C6-NBD-GlcCer analogue over the various sites, i.e., the BC, the SAC, or both, was indistinguishable (Figure 4, B and C). Hence, it is concluded that in contrast to a polarity-depen-

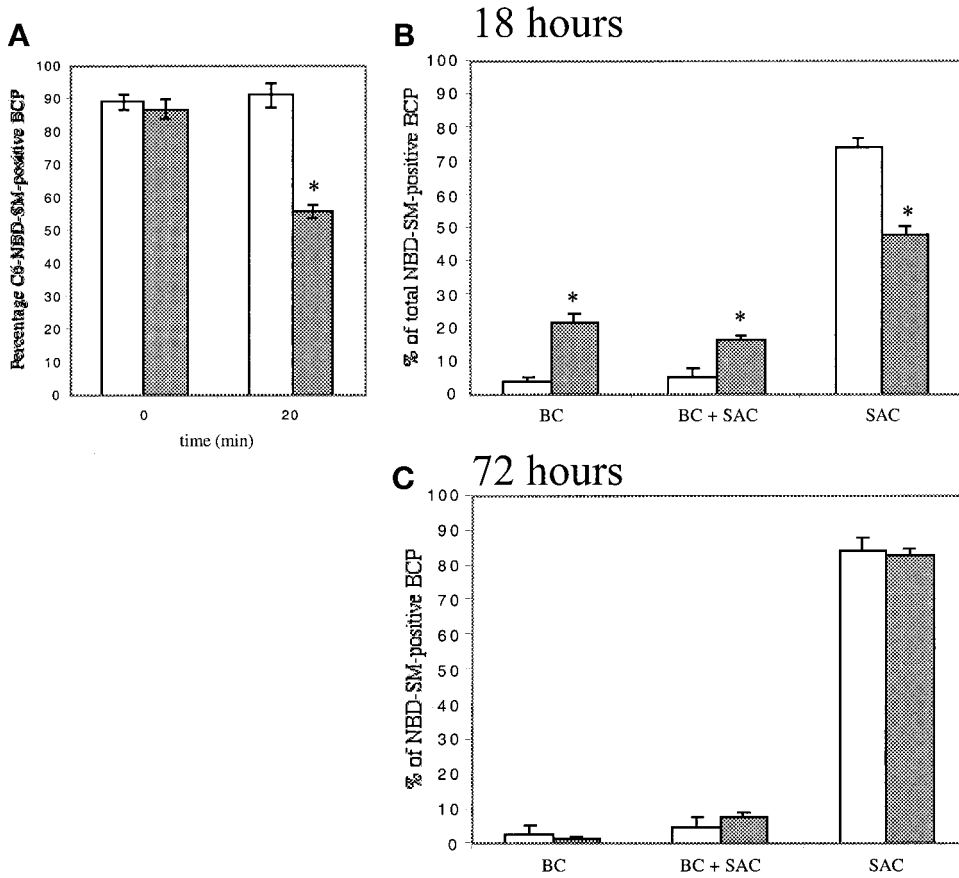


Figure 3. Polarized transport of C6-NBD-SM depends on the degree of cell polarity. The SAC was loaded with C6-NBD-SM as described in MATERIALS AND METHODS. Transport of the lipid analogue from the SAC was then monitored for 20 min in back exchange medium. In (A), the percentage of C6-NBD-SM-labeled BCP was determined in 18- (□) and 72-h-old (■) cell cultures. The asterisk marks the statistically significant (Student’s *t* test, *p* < 0.05) decrease in BCP labeling in 72-h-old cell cultures, when compared with the 18-h-old cell cultures. In (B) and (C), the distribution of the lipid analogue within the labeled BCP of 18- and 72-h-old cell cultures, respectively, was analyzed: the compartmental distribution of the lipid is shown before (□) and after (■) a 20-min chase from the SAC. *Statistically significant differences (Student’s *t* test, *p* < 0.05) between the corresponding BCP compartments (BC, SAC, and BC+SAC) in (B) and (C).

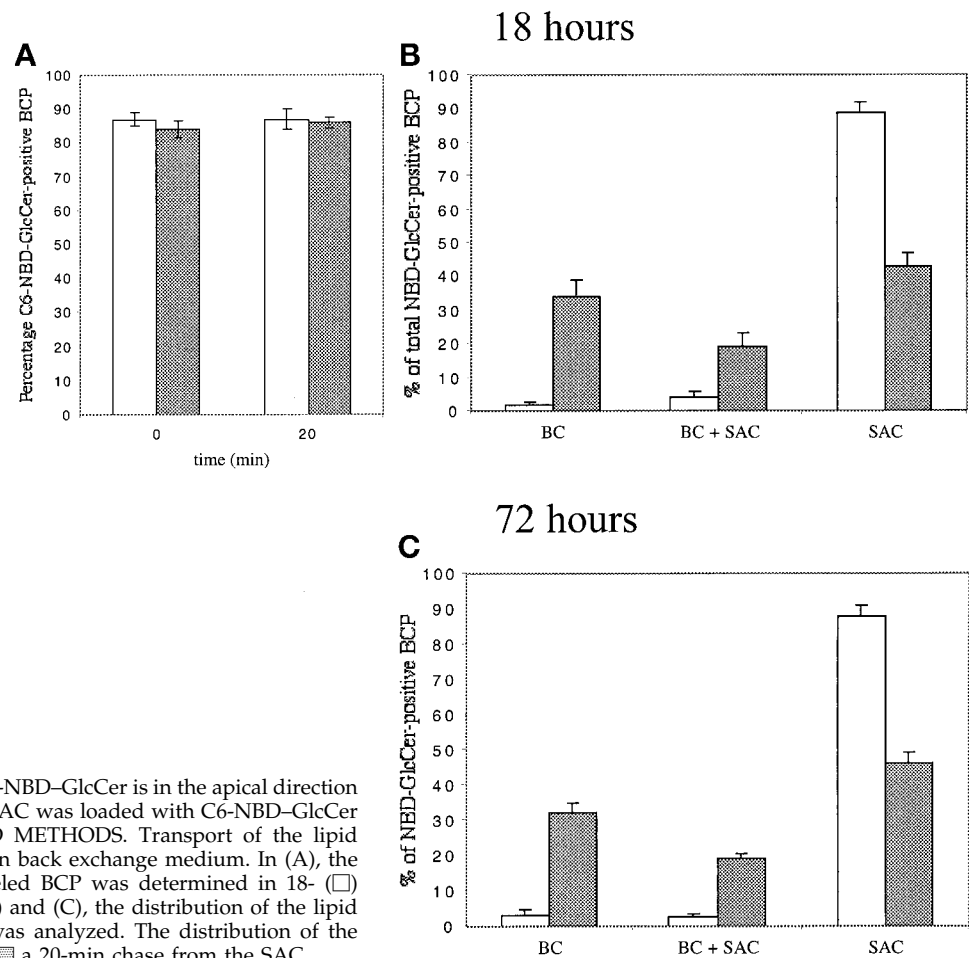


Figure 4. Polarized transport of C6-NBD-GlcCer is in the apical direction in both 18- and 72-h-old cells. The SAC was loaded with C6-NBD-GlcCer as described in MATERIALS AND METHODS. Transport of the lipid analogue was followed for 20 min in back exchange medium. In (A), the percentage of C6-NBD-GlcCer-labeled BCP was determined in 18- (□) and 72-h-old (■) cell cultures. In (B) and (C), the distribution of the lipid analogue within the labeled BCP was analyzed. The distribution of the lipid is shown before (□) and after (■) a 20-min chase from the SAC.

dent shift in the direction of SM trafficking, the direction of transport of C6-NBD-GlcCer is unaffected by the degree of cell polarity. Thus, like in optimally polarized cells (van IJzendoorn and Hoekstra, 1998), also in polarity-developing cells the persistence of an apically directed flow of GlcCer, leaving the SAC, presumably reflects an apical recycling pathway (see below).

The PKA Inhibitor H89 Inhibits Transport of C6-NBD-SM from the SAC to the 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 nonhydrolyzable cAMP analogue. Rather, under those conditions, trafficking of C6-NBD-SM is redirected to the BC (van IJzendoorn *et al.*, 1997; van IJzendoorn and Hoekstra, 1999b). Moreover, dibutyryl cAMP treatment also was 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 hyperpolarization of the cells, as evidenced by an increase in the number of BC as well as their circumfer-

ence, was observed (Zegers and Hoekstra, 1997). The mechanism underlying these events was related to dibutyryl cAMP-induced activation of PKA. 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 dibutyryl cAMP/PKA-mediated hyperpolarization 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 suboptimally 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 μ M of the specific PKA inhibitor H89 at 4°C for 30 min. Note that we have previously shown that H89 effectively inhibits the increment of PKA activity, induced upon addition of dibutyryl cAMP (Zegers and Hoekstra, 1997). 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, and the transport data were essentially the same as those presented

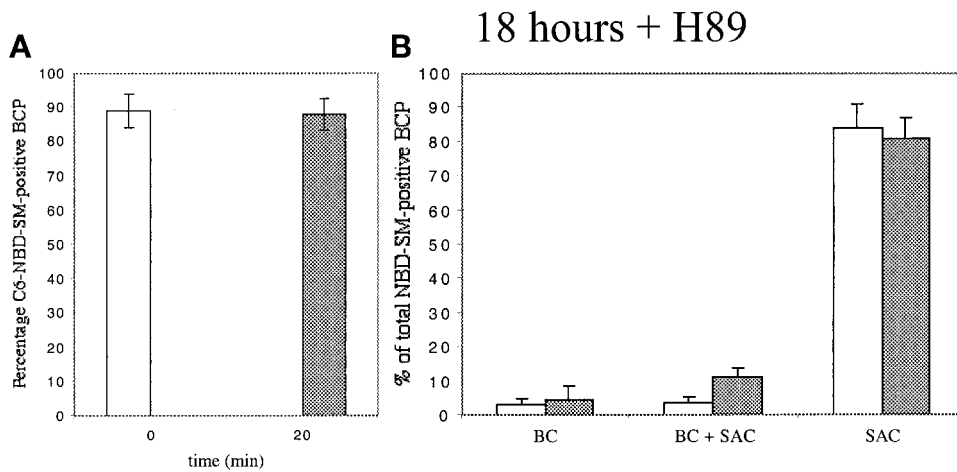


Figure 5. SAC-to-BC transport of C6-NBD-SM from the SAC is inhibited by the PKA inhibitor H89. Cells were cultured for 18 h, after which the SAC was loaded with C6-NBD-SM. Cells were then incubated with 10 μ M H89 or HBSS at 4°C for 30 min and, subsequently, in back exchange medium, with H89 at 37°C for 20 min. In (A), the percentage of C6-NBD-SM-labeled BCP before (\square) and after (\blacksquare) the chase in H89-treated cells was determined. In (B), the distribution of BCP-associated C6-NBD-SM was analyzed before (\square) and after (\blacksquare) the chase in H89-treated cells.

in Figure 3, A and C, obtained in the absence of the inhibitor. Remarkably, H89 inhibited SAC-to-BC transport of C6-NBD-SM in 18-h-old cell cultures (Figure 5B; cf. Figure 3B). 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 (Figure 5A), where it exclusively associated with the SAC (Figure 5B). 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 suboptimally polarized cell cultures (18 h), both C6-NBD-SM and -GlcCer are transported from the SAC to BC. Because 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 μ M H89 at 4°C for 30 min. Then, cells were incubated in back exchange medium at 37°C in the presence of H89. 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. Thus, the lipid distribution patterns in the BCP area, both in a quantitative sense and with respect to the distribution over SAC and BC, were indistinguishable from those obtained in the absence of H89 (cf. Figure 4). 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 analogues 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 suboptimally 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. Because at this stage, the cell culture is suboptimally polarized (see Figure 1), both progression and loss of cell polarity can be determined. The media of 18-h-old cell cultures were replaced by media, supplemented with 10 μ M H89, and the cells were cultured for another 18 or 54 h. As shown in Figure 6, the presence of H89 in the medium effectively blocked progression of polarity development of the cells, as evidenced by a constant value of \sim 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

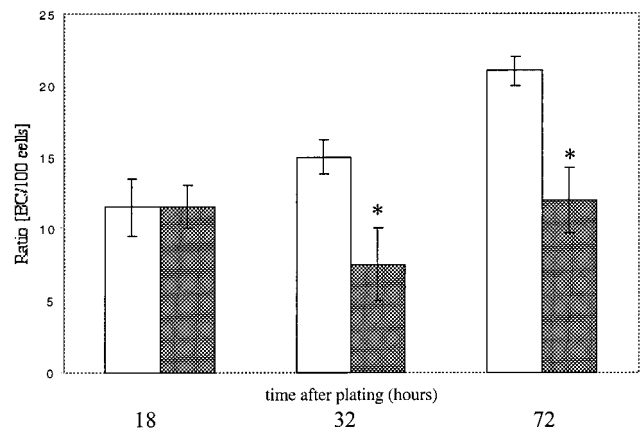


Figure 6. The PKA inhibitor H89 blocks progression of HepG2 cell polarity. Cells were plated and cultured for 18 h. At this time, the cell culture is half-maximally polarized (Figure 1). Cells were washed with sterile HBSS and cultured in normal culture medium (\square) or culture medium supplemented with 10 μ M H89 (\blacksquare). After a prolonged incubation of 14 and 54 h, cells were fixed and the ratio [BC/100 cells] was estimated as described in MATERIALS AND METHODS. *Statistically significant (Student's *t* test, $p < 0.05$) inhibition of cell polarity progression in H89-treated cell cultures at each indicated time point, when compared with nontreated cells.

[BC/100 cells] of ~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 (our unpublished results). 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.

DISCUSSION

Irrespective of the degree of cell polarity, the overall expression of proteins and lipids in differentiating cells is not remarkably different (Krämer *et al.*, 1997; Bender *et al.*, 1998), 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 suboptimally 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 (Figures 2 and 3). Previously, we observed that apical-directed transport in optimally polarized cells is regulated by PKA activity. Thus, exogenous addition of various PKA-specific modulators, which either inhibit or stimulate the kinase's activity, similarly inhibited or stimulated, respectively, membrane transport to the apical membrane, thereby affecting the state of polarity (Zegers and Hoekstra, 1997; van IJzendoorn and Hoekstra, 1999b). In conjunction with those results, the present data are entirely consistent with the notion that in HepG2 polarity development, endogenous PKA 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, the SAC, appears to be 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 Madin-Darby

canine kidney (MDCK) cells, which showed that elevated levels of dibutyryl cAMP stimulate the flow of 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, 1999b) demonstrated that the SM-marked pathway between SAC and the apical membrane coincides with that taken by the transcytotic polymeric immunoglobulin receptor-immunoglobulin A marker complex. Because the dibutyryl cAMP/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, because 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 suboptimally 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 (Valtorta and Meldolesi, 1994) also are 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 nonstimulated 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 (Fushimi *et al.*, 1997; Katsura *et al.*, 1997; Lochter and Schachner, 1997). Interestingly, a correlation between epithelial polarity development and the employment of different targeting pathways also has been demonstrated in Fisher rat thyroid cells. Thus, in 1-d-old polarized Fisher rat thyroid cell monolayers, targeting of apical proteins was accomplished by use of an indirect pathway (i.e., involving transcytosis), whereas in 7-d-old monolayers apical delivery was via the direct TGN-to-apical route (Zurzolo *et al.*, 1992). Importantly, in addition 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 α -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; Barroso and Sztul, 1994; Pimplikar and Simons, 1994). Possibly, HepG2 cells secrete ECM molecules after their plating, which upon cell-cell contact (note that secreted ECM molecules 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). Because the apical targeting of the SM analogue is evidently not maintained, additional mechanisms are probably operational, which cause a downregulation or desensitization of the signaling cascade. Such mechanisms are most likely located upstream of cAMP, because treatment of 72-h-old HepG2 cells with dibutyryl cAMP induces a similar rerouting of SAC-associated C6-NBD-SM and hyperpolarization (van IJzendoorn and Hoekstra, 1999b), as is proposed to occur during natural development of cell polarity (this study). Although the involvement and nature of possible extracellular signals, as well as target molecules of cAMP and 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.

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