Chapter 4

Polarized Sphingolipid transport from the sub-apical compartment: evidence for distinct sphingolipid domains

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Abstract.

In polarized HepG2 cells, distinct sphingolipids, transported along the reverse transcytotic pathway, are sorted in sub-apical compartments (SAC), and subsequently targeted to either apical or basolateral PM domains (van IJzendoorn, S.C.D. and Hoekstra, D. 1998. *J. Cell Biol.* 142:683-696). In this study, we show that the calmodulin antagonist trifluoperazine (TFP) inhibits apical to basolateral transcytosis of C_6-NBD-sphingomyelin (SM) and - glucosylceramide (GlcCer). TFP does not inhibit apical endocytosis of either lipid analogue or its subsequent delivery to the SAC. Rather, a marked accumulation of C_6-NBD-SM in the SAC was observed. By contrast, apical recycling of C_6-NBD-GlcCer via the SAC remains unaffected. Treatment of the cells with dibutyryl cAMP resulted in a rerouting of C_6-NBD-SM from the SAC to the apical surface and a concomitant hyperpolarization of the cells. In dbcAMP-treated cells, TFP blocked SAC-to-apical transfer of both C_6-NBD-SM and basolaterally internalized pIgR-IgA, but not recycling of the GlcCer analogue via the SAC. These data indicate that dbcAMP-induced SAC to apical transport of SM represents part of the basolateral to apical transcytotic pathway, and trafficking along this pathway appears a prerequisite for stimulated apical PM biogenesis. Interestingly, the discriminating effects of TFP and dbcAMP imply that separate domains of SM and GlcCer exist in the SAC membrane. The fate of these domains, which can exit the SAC by at least three different routes, is regulated by different signals, e.g. those that govern cell polarity development. The consequence of the potential co-existence of such domains within the same compartment in terms of apical targeting, are discussed.

Introduction

Polarized cells have distinct plasma membrane (PM) domains, which are separated by tight junctions. The apical and basolateral domain thus formed, each display a specific composition of proteins and lipids. For the establishment and maintenance of such specific compositions, intracellular sorting machineries are operational that secure the correct targeting and delivery of apical and basolateral proteins and lipids. Following biosynthesis, sorting of proteins and lipids is thought to occur in the trans-Golgi network (TGN), prior to delivery of the molecules to the PM (Traub and Kornfeld, 1997; Matter and Mellman, 1994). In addition, in the presence of continuous transcellular transport, an auxiliary non-Golgi compartment exists that harbors machineries for sorting and subsequent polarized targeting of apical and basolateral proteins and lipids in the endocytic/ transcytotic pathway. Indeed, in the latter pathway sorting of both proteins (Apodaca et al., 1994; Odorizzi et al., 1996; Futter et al., 1998) and (glyco)sphingolipids (van IJzendoorn et al., 1997; van IJzendoorn and Hoekstra, 1998) occurs in a sub-apical endosomal compartment, called SAC (van IJzendoorn and Hoekstra, 1999), which is accessible for molecules derived from either PM domain (Hughson and Hopkins, 1990; Apodaca et al., 1994; Barosso and Sztul, 1994; Knight et al., 1995; Odorizzi et al., 1996; van IJzendoorn and Hoekstra, 1998).

The molecular mechanisms underlying these sorting events are still largely obscure. Yet, instrumental to protein sorting appears to be their clustering, thus giving rise to domains enriched in proteins that are destined for polarized transport. Both coat proteins, such as COP and clathrin (Whitney et al., 1995; Heilker et al., 1996), and adaptins (Pearse and Robinson, 1990) may trigger such a clustering. In fact, clathrin lattices containing adaptins have been identified on the SAC, and may therefore well be implicated in the regulation of polarized trafficking (Futter et al., 1998; Okamoto et al., 1998, 1998b).

Lipids, rather than accompanying proteins in vesicular transport, have been proposed

to play an important role in the sorting of apical resident proteins (Simons and Ikonen, 1997). In particular (glyco)sphingolipids are of interest, because these lipids display a polarized distribution over the basolateral and apical PM domains. Although some important determinants for apical PM directed sphingolipid transport have been identified in polarized cells (reviewed in Zegers and Hoekstra, 1998, Brown and London, 1998), the mechanisms that govern polarized sphingolipid trafficking remain as yet unclear. Importantly, the ability of these lipids to self-associate within a membrane (Schroeder et al., 1994), thus giving rise to sphingolipid-enriched domains and serving in turn as a detergent-insoluble matrix for apical-directed proteins, represents a key issue in the sorting concept (Brown and Rose, 1992; Simons and Ikonen, 1997). In this context it should be noted however that not only the apically enriched glycosphingolipids, such as glucosylceramide (GlcCer) and Forssman antigen (Nichols et al, 1987; van IJzendoorn et al., 1997; van IJzendoorn and Hoekstra, 1998) are highly detergent-insoluble at 4°C, but also sphingomyelin (SM), the ganglioside GM1 and galactosylceramide (GalCer), i.e. basolaterally targeted lipids (van Genderen and van Meer, 1995; van IJzendoorn and Hoekstra, 1998). Evidently, these observations are difficult to reconcile with the notion that sphingolipid domains function exclusively as *apical* sorting platforms.

Recently, we have demonstrated that the SAC, which constitutes an integral part of the transcytotic pathway in polarized cells (van IJzendoorn and Hoekstra, 1999), represents a major intracellular site in polarized sorting of sphingolipids. The compartment harbors sorting devices for the preferential targeting of fluorescently tagged derivatives of SM and GalCer to the basolateral domain, while GlcCer is effectively directed to the apical membrane. Importantly, this sphingolipid trafficking occurs by vesicular means, which implies that the sphingolipids were segregated within the lumenal leaflet of the SAC.

In the present work, we have used the calmodulin antagonist trifluoperazine as a modulator of membrane traffic (Apodaca et al., 1994). The antagonist strongly and selectively interfered with trafficking of SM and GlcCer. We provide unambiguous evidence for the existence of separate sphingolipid domains within the inner leaflet of the SAC in polarized HepG2 cells. Moreover, the trafficking of these domains from the SAC in either apical or basolateral direction can be independently regulated, and depends on cell polarity development.

Materials and Methods

Sphingosylphosphorylcholine, 1-ß-glucosylsphingosine, TRITC-labeled phalloidin, Hoechst 33250 (bisbenzimide), asialofetuin type I, cytochalasin D and geneticin (G-418) were from Sigma Chemical Co., St. Louis, MO/USA. Albumin (from bovine serum, fraction V) was bought from Fluka Chemie AG, Buchs/Switzerland. 6-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]hexanoic acid (C_6-NBD) was obtained from Molecular Probes, Eugene, OR/USA. DMEM was purchased from GIBCO BRL (Life Technologies), Paisley/Scotland. Fetal calf serum (FCS) was bought from BioWhittaker, Verviers/Belgium, and sodiumdithionite ($Na_2S_2O_4$) was from Merck, Darmstadt/Germany. Trifluoperazine dimaleate (TFP) was a product from Calbiochem-Novabiochem Corp., La Jolla, CA/USA. Dibutyryl cyclic AMP (dbcAMP) was obtained from Boehringer Mannheim, Mannheim/Germany. Texas red-labeled IgA was kindly provided by Dr. Kenneth Dunn, Indiana University Medical Center, USA. All other chemicals were of analytical grade.

Cell Culture

HepG2 cells were cultured as described elsewhere (van IJzendoorn and Hoekstra, 1998). For microscopical or biochemical experiments cells were plated onto glass coverslips or in culture dishes (diameter 6 cm), respectively. Cells were used three days after plating. At that time the cells had reached an optimal ratio of polarity versus density, in terms of the number of bile canaliculi (BC), formed between two adjacent cells and representing the apical membrane, versus the number of cells (van IJzendoorn et al., 1997).

Synthesis of C$_6$-NBD-Labeled Sphingolipids

C$_6$-NBD-GlcCer and C$_6$-NBD-SM were synthesized from C$_6$-NBD and 1-ß-glucosylsphingosine and sphingosylphosphorylcholine, respectively, as described elsewhere (Kishimoto., 1975; Babia et al., 1994). The C$_6$-NBD-lipids were stored at -20°C and routinely checked for purity.

Labeling of Cells With C$_6$-NBD-Lipids

Cells were washed three times with a phosphate-buffered saline solution (PBS). C$_6$-NBD-GlcCer or C$_6$-NBD-SM was dried under nitrogen, redissolved in absolute ethanol and injected into Hank's balanced salt solution (HBSS) under vigorous vortexing. The final concentration of ethanol did not exceed 0.5% (v/v). All lipid analogs were administered to the cells at a concentration of 4 μM.

Transport of C$_6$-NBD-Lipids from the SAC

In order to monitor SAC-associated sphingolipid transport, the SAC had to be first preloaded with a lipid analogue. To this end, cells were washed with PBS and incubated with C$_6$-NBD-SM or -GlcCer at 37°C for 30 min to allow internalization of the lipid analogue from the basolateral surface and transcytosis (Zegers et al., 1997; Zegers and Hoekstra, 1998, van IJzendoorn et al., 1997, van IJzendoorn and Hoekstra, 1998). The remaining basolateral pool of lipid analogue was then depleted by a back exchange procedure (5% BSA (w/v) in HBSS, pH 7.4 at 4°C for 2 x 30 min). Then, the lipid was chased from the apical, bile canalicular PM into the SAC by an incubation at 18°C for 60 min in back exchange medium. Finally, NBD-fluorescence remaining at the luminal leaflet of the apical PM was abolished by incubating the cells with 30 mM sodiumdithionite (diluted from a 1 M stock solution in 1 M Tris buffer, pH 10). At this time, the vast majority of the lipid analogue was associated with the SAC (cf. van IJzendoorn and Hoekstra, 1998). In some experiments, cells were subsequently treated with 20 μM trifluoperazine dimaleate (TFP), 100 μM dibutyryl cAMP (dbcAMP) or 10 μg/ml cytochalasin D (cytD) at 4°C for 30 min. Transport of the lipid analogs from the SAC was subsequently monitored by incubation in back exchange medium at 37°C. When required, TFP, dbcAMP and/or cytD were kept present during the transport assay.

In order to quantitate transport of the lipid analogs to and from the apical, bile canalicular (BC) membranes, the percentage of NBD-positive BC membranes 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 classified as 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 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 BC, SAC, or both. This also provides a means to describe the movement of the lipid within or out this region in the cell. At least 50 BCP per coverslip were analyzed. Data are expressed as the mean ± SEM of at least two independent experiments, carried out in duplicate.

Basolateral to Apical Transcytosis of Texas Red-Labeled IgA

HegpG2 cells that stably express the polymeric immunoglobulin receptor (pIgR; van IJzendoorn and Hoekstra, 1998) were washed and asialoglycoprotein receptors were saturated with excess asialofetuin at 37°C for 30 min to prevent uptake of IgA via these receptors (van IJzendoorn and Hoekstra, 1998). Cells were incubated with TxR-IgA (50

μg/ml) at 4°C for 60 min. Cells were then washed with ice-cold HBSS to remove non-bound TxR-IgA and further incubated at 18°C, 37°C or at a combination of both temperatures for various time intervals. In order to investigate the last step of IgA-pIgR transcytosis, i.e. transfer from the SAC to the apical, bile canalicular PM, asialofetuin-pretreated cells were, subsequent to the 4°C binding incubation, incubated with TxR-IgA at 37°C for 15 min. Then, the temperature was lowered to 18°C and cells were incubated for an additional 90 min. In this way, most of the transcytosing TxR-IgA accumulated in the SAC (Fig. 5, Tab. 1; cf. van IJzendoorn and Hoekstra, 1998). In order to examine the effect of TFP on transport of TxR-IgA from SAC to BC, cells were subsequently treated with HBSS, supplemented with 20 μM TFP at 4°C for 30 min and incubated in HBSS with TFP at 37°C.

Microscopical Analysis and Image Processing

Cells were examined microscopically using an *Olympus* Provis AX70 fluorescence microscope. Photomicrographs were taken using Illford HP5-plus films and subsequently scanned and cropped, using imaging software. All images were converted to tagged-information-file format (TIFF) before printing on a *Fujix* P3000 printer.

Results

TFP Inhibits Transport of C_6-NBD-SM from SAC to the Basolateral PM

In order to monitor transport of SM from the SAC, the compartment was loaded with the fluorescent analogue, C_6-NBD-SM, as described in Materials and Methods. The cells were subsequently treated with 20 μM TFP in HBSS at 4°C for 30 min. Note that this treatment did not affect the fluorescence distribution associated with the SAC, when compared to non-treated cells (not shown). Transport of C_6-NBD-SM from the SAC was then activated by an incubation at 37°C in back exchange medium, either in the presence or absence of TFP. In control cells, C_6-NBD-SM rapidly disappeared from the apical, bile canalicular region, defined as BCP (bile canalicular pole, see Materials and Methods), after a 20 min chase (Fig. 1a, insert, dotted line). Significant transfer to the apical, bile canalicular PM (BC) was not observed and the remaining fraction of BCP-associated C_6-NBD-SM was predominantly found in the SAC (Fig. 1a, hatched bars). These results are entirely consistent with our previous observations of the SAC, acting as a traffic center for SM distribution in polarized HepG2 cells (van IJzendoorn and Hoekstra, 1998). By contrast, when the cells had been treated with TFP, transport of C_6-NBD-SM from the apical pole was inhibited. Thus, in the presence of the calmodulin antagonist, the extent of BCP labeling in the cell population remained unaltered (Fig. 1a, insert, dashed line), while the localization of SM was identical to that observed prior to the chase, i.e. the analogue was almost exclusively associated with the SAC. Importantly, note that the TFP-mediated inhibition of basolateral transport neither resulted in a redirection of SM from the SAC to the apical surface (Fig. 1a, cross-hatched bars and Fig. 1e). Hence, the data show that after arrival of apical PM derived C_6-NBD-SM in the SAC, TFP inhibits transport of SM from the SAC to the basolateral area of the cells by preventing its exit from this compartment. Similar results were obtained with other calmodulin antagonists such as W7 and calmodazolium (not shown), suggesting that the observed effect reflected a calmodulin-mediated action rather than a TFP-specific effect.

TFP Does Not Affect Apical Recycling of C$_6$-NBD-GlcCer via SAC

As demonstrated previously (van IJzendoorn and Hoekstra, 1998), the SAC is instrumental in securing the preferential apical distribution of GlcCer in HepG2 cells. To examine the specificity of the TFP block on SM exit from the SAC, we next investigated whether TFP affected transport of C$_6$-NBD-GlcCer from this compartment. To this end, C$_6$-NBD-GlcCer was accumulated in the SAC, similarly as described in the previous section for C$_6$-NBD-SM.
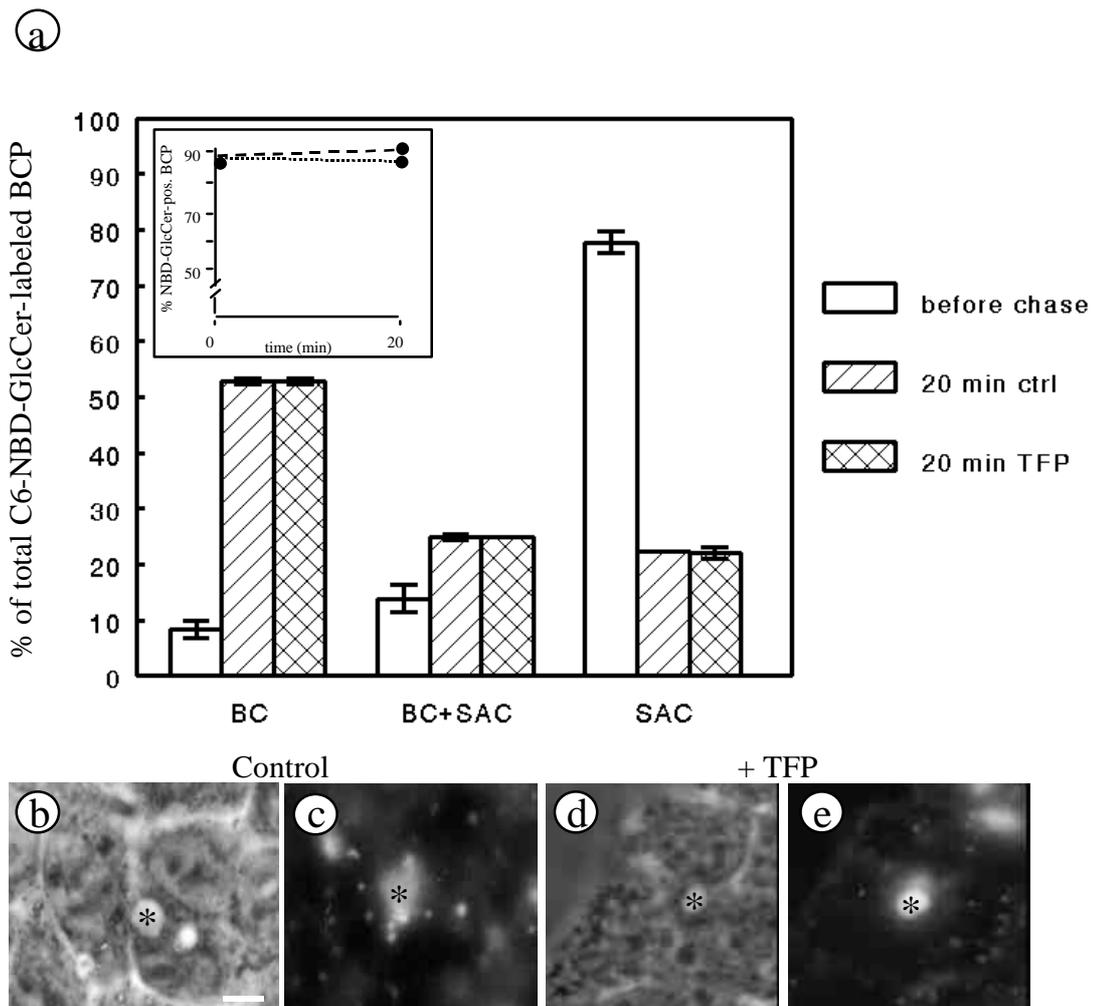

Figure 1. Effect of TFP on transport of C$_6$-NBD-SM from SAC. C$_6$-NBD-SM was accumulated into SAC as described in Materials and Methods (see also cf. van IJzendoorn and Hoekstra, 1998). After abolishing the remaining BC-associated NBD-fluorescence with sodiumdithionite, cells were treated with HBSS (control) or 20 µM TFP at 4°C for 30 min. Cells were washed and kept in HBSS at 4°C until use (<30 mine; t=o (before chase)) or, alternatively, cells were subsequently warmed to 37°C and further incubated in back exchange medium for 20 min. Cells were then rapidly cooled and kept on ice until use (<30 min). In panel a, a semi-quantitative analysis of transport from or within the BCP is presented. The percentage C$_6$-NBD-SM-labeled BCP (insert; dotted line=control, dashed line=TFP-treated) and the distribution of the BCP-associated C$_6$-NBD-SM (i.e. in BC, in SAC, or in both) was determined as described in Materials and Methods. Data are expressed as mean ± SEM of at least three independent experiments, carried out in duplicate. The photomicrographs presented in b-e illustrate the distribution of the lipid analogue in the BCP after a chase from SAC in non-treated (b, c) and TFP-treated (d, e) cells (b and d: phase contrast to c and e, respectively; asterisk marks the apical, bile canalicular PM). Bar 5 µM.

Subsequently, the cells were treated with 20 µM TFP at 4°C for 30 min and incubated for another 20 min at 37°C in back exchange medium in the presence of the compound. As a control, cells were treated identically but in the absence of TFP. As shown in figure 2a (white bars), prior to the chase, the majority of the lipid analogue was in the SAC. Following the 20 min chase at 37°C, C_6-NBD-GlcCer remained associated with the BCP (Fig. 2a, insert, dotted and dashed lines), irrespective of treatment of the cells with TFP. Interestingly, however, both in the absence and presence of TFP, GlcCer redistributed to the apical surface. Thus, in non-treated cells after the 20-min chase, C_6-NBD-GlcCer was observed in BC alone, BC and SAC, or SAC alone (Fig. 2a, hatched bars, and Fig. 2c), consistent with our previous observation of a preferential apical localization of this analogue (van IJzendoorn and Hoekstra, 1998). Also in TFP treated cells, the lipid analogue remained associated with the BCP (Fig. 2a, insert, dashed line). Remarkably however, in this case, GlcCer was redistributed identically as observed in control cells (Fig. 2a, compare cross-hatched bars vs. hatched bars; Fig. e). Hence, the results demonstrate that apical recycling of C_6-NBD-GlcCer via the SAC, in contrast to basolateral directed trafficking of C_6-NBD-SM via the same compartment, is *not* affected by TFP.

Figure 2. Effect of TFP on transport of C_6-NBD-GlcCer from SAC. C_6-NBD-GlcCer was accumulated into SAC as described in Materials and Methods (see also cf. van IJzendoorn and Hoekstra, 1998). After abolishing the remaining BC-associated NBD-fluorescence with sodiumdithionite, cells were treated with HBSS (control) or 20 μM TFP at 4°C for 30 min. Then, cells were washed and kept in HBSS at 4°C until use (<30 min; t=0 (before chase)) or, alternatively, cells were warmed to 37°C and incubated in back exchange medium for 20 min. Cells were then rapidly cooled and kept on ice until use (<30 min). Panel a shows the semi-quantitative analysis of C_6-NBD-GlcCer transport within the BCP. The percentage C_6-NBD-GlcCer-labeled BCP (insert; dotted line=control, dashed line=TFP-treated) and the distribution of the BCP-associated NBD-GlcCer (i.e. BC, SAC, or both) was determined as described in Materials and Methods. Data are expressed as mean ± SEM of at least three independent experiments, carried out in duplicate. The photomicrographs presented in b-e illustrate the distribution of the lipid analogue in the BCP after a chase from SAC in non-treated (b, c) and TFP-treated (d, e) cells (b and d: phase contrast to c and e, respectively; asterisk marks the apical, bile canalicular PM). Bar 5μM.

TFP Inhibits Apical to Basolateral Transcytosis of C_6-NBD-GlcCer at the Level of SAC

Although the majority of apical PM derived C_6-NBD-GlcCer is efficiently recycled to the apical surface via the SAC, this lipid analogue does have access to the basolateral PM (van IJzendoorn et al., 1997). Presumably, during each round of apical recycling between the apical membrane and the SAC, part of the lipid analogue 'escapes' the recycling route and is transcytosed to the basolateral surface. Indeed, when apical PM associated C_6-NBD-GlcCer is chased from this membrane domain at 37°C for longer time periods in back exchange medium, thus retrieving any basolateral arriving lipid analogue and preventing its re-internalization, nearly the entire pool of the originally apically located lipid analogue can be depleted. Typically, relative to the control (i.e. in the absence of back exchange medium) only 20% of the total BC fraction remains fluorescently labeled over a chase period of 90 min (Fig. 3A). To address the issue of the (lipid-)specificity of the observed TFP effect on basolateral exit, we next examined whether TFP inhibited apical-to-basolateral transport of C_6-NBD-GlcCer, similarly as observed for C_6-NBD-SM. Hence, cells were labeled with C_6-NBD-GlcCer at 37°C for 30 min. In this way, 70-80% of the BC were labeled with the lipid analogue (Fig. 3A, hatched bar). The pool of basolaterally associated lipid analogue was then depleted at 4°C and during the second step in the back exchange procedure (see Materials and Methods), 20 μM TFP was added for preincubation. The BC-labeled cells were then incubated at 37°C in back exchange medium, supplemented with TFP. Control cells were treated identically but in the absence of TFP. In these cells, the percentage of C_6-NBD-GlcCer-labeled BC decreased from about 75% prior to the chase to 20% following a 90 min chase (Fig 3A, upper cross-hatched bar), indicating that the lipid analogue was transported out of the apical area to the basolateral membrane. Indeed, the percentage of C_6-NBD-GlcCer-labeled BCP was reduced from 80% to 35% (Fig. 3A', solid line). Interestingly, in TFP-treated cells, the percentage of C_6-NBD-GlcCer-labeled BC following the 90 min chase was twice as high as the percentage of BC-labeling in non-treated cells (Fig 3A, upper white bar). This would suggest that apical-to-basolateral transport of the lipid analogue was inhibited, while

recycling between the SAC and BC was unaffected (see above). Indeed, in TFP-treated cells, the percentage of C_6-NBD-GlcCer-labeled BCP remained constant, maintaining a level of 80-85 % during the 90-min incubation period (Fig. 3A', solid line). In both control and TFP-treated cells, the remaining fraction of the BCP-associated C_6-NBD-GlcCer was located in BC, SAC, or in both (Fig. 3B), indicating that the C_6-NBD-GlcCer that was remaining at the apical pole, and recycled as usual between the apical PM and the SAC.

An interesting lipid-species-dependent distinction became apparent when examining similarly the effect of TFP on apical to basolateral transcytosis (originating from BC) of C_6-NBD-SM. In control cells, the percentage of C_6-NBD-SM-labeled BC decreased from about 80% prior to the chase to ± 20% (Fig. 3A, lower cross-hatched bar) following a 90 min incubation in back exchange medium. In TFP-treated cells, a very similar percentage of approximately 20% of fluorescently labeled BC was observed (Fig. 3A, lower white bar). However, in this case the same low percentage of labeled BC did not reflect transport of C_6-NBD-SM out of the apical pole. Thus, in TFP-treated cells, the percentage of C_6-NBD-SM-labeled BCP was still approximately 80%, whereas in control cells this percentage was about 30 % (Fig. 3A', dotted lines). Analysis of the distribution of the remaining fraction of BCP-associated C_6-NBD-SM revealed that the majority was located in the SAC alone (Fig. 3C). Evidently, and in accordance with the data on transport of the SM analogue from the SAC (Fig. 1a, cross-hatched bars), SM became trapped, and apparently did not enter the apical recycling pathway instead. It is finally important to note that no metabolism of the lipid analogs applied occurred during the time span of the experiments (not shown).
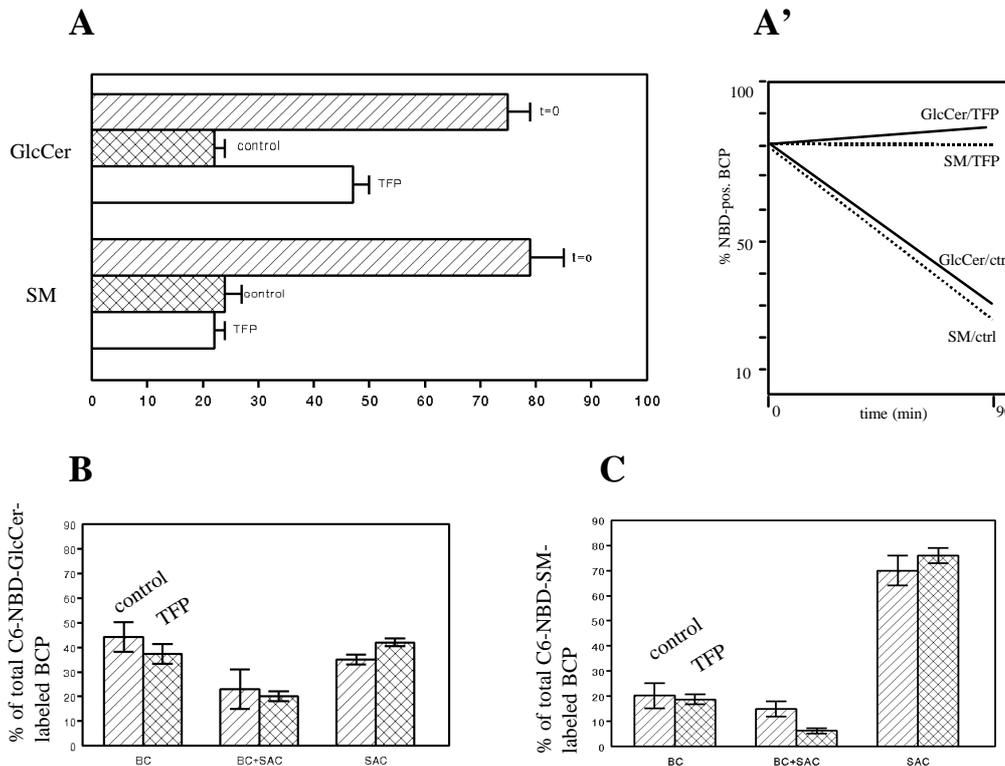
Figure 3. Effect of TFP on apical to basolateral transcytosis of fluorescently tagged GlcCer and SM. Cells were labeled with 4 µM C_6-NBD-GlcCer or -SM at 37°C for 30 min. Cells were then incubated in HBSS, supplemented with BSA, at 4°C for 2 x 30 min to deplete the basolateral pool of lipid analogue (back exchange). During the last 30 min of the back exchange procedure 20 µM TFP was added to the cells. As a control, cells were treated identically but in the absence of TFP. Cells were then warmed to 37°C and incubated in back exchange medium, with or without TFP, for 90 min. In A, the percentage NBD-SM- or -GlcCer-labeled BC is determined after the 90 min chase. In A', the percentage NBD-lipid-labeled BCP is assessed. Thus, a BCP is NBD-positive when either labeling of the BC, SAC, or both BC and SAC is observed (see Materials and Methods, cf. van IJzendoorn and Hoekstra, 1998). In B and C, the distribution of the (remaining) fraction of BCP-associated C_6-NBD-GlcCer and -SM, respectively, is indicated.

Taken together, the data clearly show that TFP inhibits apical-to-basolateral transcytosis of both C_6-NBD-GlcCer and C_6-NBD-SM at the level of the SAC. As a result, basolateral PM-directed transport of both lipid analogs is blocked, whereas apically targeted transport of C_6-NBD-GlcCer was unaffected. Interestingly, TFP does not discriminate between C_6-NBD-SM and -GlcCer during the transport of these analogs from BC to the SAC, nor is sphingolipid transport as such affected. However, when present in the SAC, C_6-NBD-GlcCer and C_6-NBD-SM pools are distinctly recognized by the membrane traffic-modulating compound TFP. Hence, the data strongly suggest that within the SAC membranes C_6-NBD-GlcCer and C_6-NBD-SM are segregated into distinct pools or domains.

If such pools exist, an intriguing possibility would be that lipids, to be retrieved from these pools, might be distinctly regulated. Such a distinct regulation could be reflected by a difference in the traffic pathway by which a particular lipid would reach the same target membrane. To examine this possibility we next studied the effect of dibutyryl cAMP (dbcAMP) and TFP on the trafficking of SM.

DbcAMP Reroutes C_6-NBD-SM from SAC to the Apical PM domain

Previously, we have shown that treatment of the cells with the stable cAMP analogue, dibutyryl cAMP (dbcAMP) abolishes apical-to-basolateral transcytosis of C_6-NBD-SM (van IJzendoorn et al., 1997). Instead, analogous to the fate of GlcCer, C_6-NBD-SM remained associated with the apical pole of the cell. A subsequent study suggested that dbcAMP stimulated apical recycling of C_6-NBD-SM via the SAC, but the site of action was not determined in detail (van IJzendoorn and Hoekstra, 1998). To further examine this issue, the SAC was loaded with apical PM derived C_6-NBD-SM as described (see Materials and Methods), followed by addition of 100 µM dbcAMP. Transport of SAC-associated C_6-NBD-SM was then activated by shifting the temperature to 37°C and incubating the cells in back exchange medium, supplemented with dbcAMP. Whereas in non-treated control cells the percentage of C_6-NBD-SM-labeled BCP decreased from >80% to ~ 60% after a 20 min incubation (cf. Fig 1a, insert, dotted line), in dbcAMP-treated cells the percentage of C_6-NBD-SM-labeled BCP remained constant (Fig. 4a, insert, dotted line), suggesting that elevated levels of cAMP prevented transport of the lipid analogue out of the apical pole. Moreover, the BCP-associated C_6-NBD-SM did not exclusively label the SAC as observed in non-treated
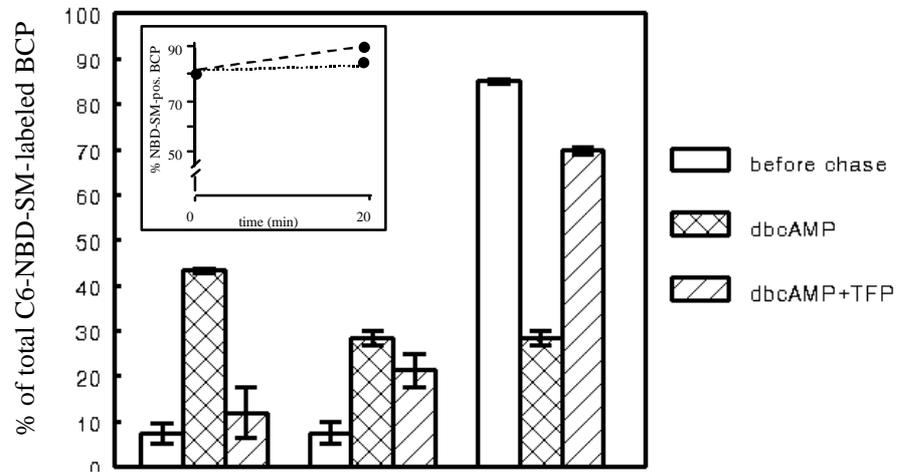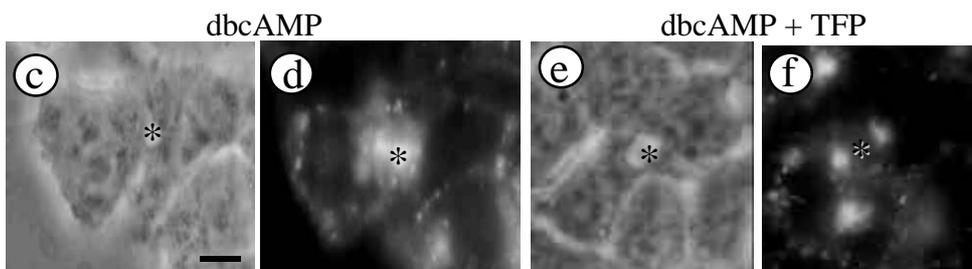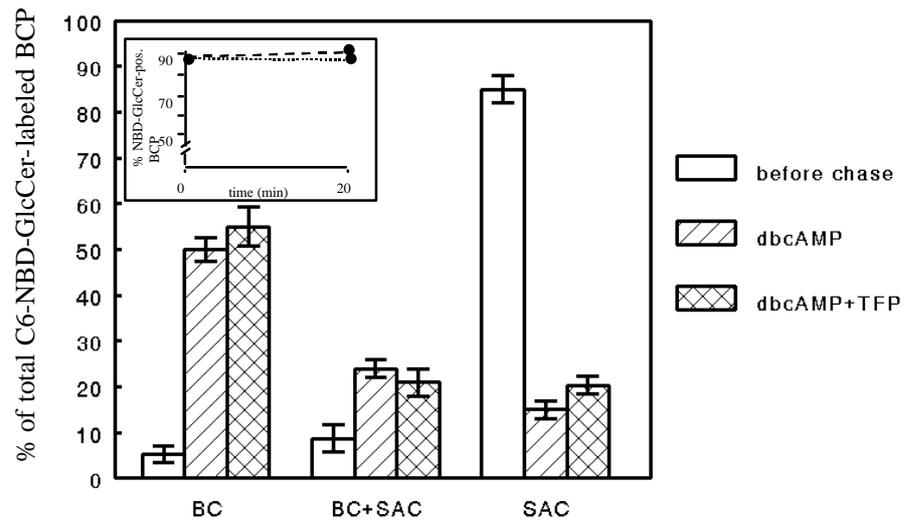
Figure 4. Effect of dbcAMP and a combination of dbcAMP and TFP on transport of C_6-NBD-SM (a) or C_6-NBD-GlcCer (b) from the SAC. C_6-NBD-SM was accumulated into SAC as described in Materials and Methods (see also cf. van IJzendoorn and Hoekstra, 1998). After abolishing the remaining BC-associated NBD-fluorescence with sodiumdithionite, cells were treated with HBSS (control), 100 μM dbcAMP or 100 μM dbcAMP + 20 μM TFP at 4°C for 30 min. Then, cells were washed and kept in HBSS at 4°C until use (<30 min; t=0 (before chase)) or, alternatively, warmed to 37°C and incubated in back exchange medium, with either only dbcAMP or both compounds, for 20 min. Cells were then rapidly cooled and kept on ice until use (<30 min). The percentage C_6-NBD-SM-labeled BCP (insert; dotted line=dbcAMP-treated, dashed line=dbcAMP+TFP-treated) and the distribution of the BCP-associated NBD-SM (i.e. BC, SAC, or both) was determined as described in Materials and Methods. Data are expressed as mean ± SEM of at least two independent experiments, carried out in duplicate. The photomicrographs presented in c-f are illustrating the distribution of the lipid analogue in the BCP after a chase from SAC in dbcAMP-treated (c, d) and dbcAMP+TFP-treated (e, f) cells (c and e: phase contrast to d and f, respectively; asterisk marks the apical, bile canalicular PM). Bar 5μM.

cells (Fig. 1a, hatched bars), but was redistributed within the BCP, and also labeled BC (Fig. 4a, cross-hatched bars). The distribution of C_6-NBD-SM in the BCP is depicted in figure 4d. Hence, these data indicate that in the presence of dbcAMP C_6-NBD-SM recycled between the SAC and the apical PM domain, similarly as observed for C_6-NBD-GlcCer in non-treated cells (Fig. 2a, hatched bars). Taken together, the data show that dbcAMP reroutes SAC-associated C_6-NBD-SM from the basolateral to the apical PM. Hence, the question can now be raised as to whether SM participates in the same recycling pathway as that observed for GlcCer. We therefore examined the effect of TFP on the apically directed flow of SM and GlcCer, exiting from the SAC.

TFP Inhibits SAC-to-Apical Transport of C_6-NBD-SM, But Not of -GlcCer, in DbcAMP-Treated Cells.

In order to examine the effect of TFP on dbcAMP-induced transport of the C_6-NBD-lipids from the SAC to the apical PM, 20 μM TFP was included during those incubation steps where dbcAMP was present (see Materials and Methods). As shown in figure 4a (insert, dashed line), the presence of TFP did not affect the pool of C_6-NBD-SM that remained associated with the apical pole (BCP) of the cell in the presence of dbcAMP alone (Fig. 4a, insert, dotted line). However, in contrast to the cells that had been treated with dbcAMP alone, which show a distribution of the SM analogue over both BC and the SAC, TFP/dbcAMP-treated cells display a distribution in which the vast majority of C_6-NBD-SM in the BCP remained in the SAC alone. Only a relative small fraction of the lipid analogue had been transported from SAC to BC (Fig. 4a, hatched bars). The BCP distribution of C_6-NBD-SM in TFP-treated cells, as revealed by fluorescence microscopy, is shown in figure 4f. Interestingly, apical recycling of C_6-NBD-GlcCer via the SAC was unaffected by TFP (Fig. 4b) in both control and dbcAMP-treated cells. These data strongly suggest, therefore, that in dbcAMP-treated cells C_6-NBD-GlcCer and -SM remained in distinct pools or subdomains. Apparently, dbcAMP

does not interfere with the segregation of the lipid analogs in the SAC. Furthermore, the discriminating effect of TFP on SAC-to-BC transport of C_6-NBD-SM and -GlcCer implies that at least two independent pathways from the SAC to BC exist. By monitoring the trafficking of the polymeric Ig receptor/IgA complex, a well-established transcytotic marker, the nature of these different pathways was further investigated.

TFP Inhibits the Final Step of Basolateral to Apical Transcytosis of TxR-IgA, Its Delivery from SAC to the Apical Surface.

In a previous study, we have shown that basolaterally endocytosed receptor-bound IgA (pIgR-IgA) passes through the SAC prior to delivery at the apical surface of HepG2 cells, while the complex accumulates in the SAC when the temperature is lowered to 18°C (van IJzendoorn and Hoekstra, 1998). In order to investigate whether TFP affected transport of pIgR-IgA from SAC to the apical, bile canalicular PM domain, HepG2 cells that stably express the pIgR were washed, pretreated with excess asialofetuin to saturate asialoglycoprotein receptors, and incubated with 50 μg/ml Texas-red-labeled IgA (TxR-IgA) at 4°C for 60 min. Then, non-bound TxR-IgA was removed by rinsing the cells with ice-cold HBSS and the cells were incubated in HBSS at 37°C to allow internalization. After 15 min, the cells were cooled to 18°C and incubated for another 90 min. This incubation procedure caused the vast majority of the transcytosing TxR-IgA to accumulate in the SAC, while the amount reaching the apical PM domain was essentially negligible (Fig. 5a and Tab. 1). Cells were then treated with 20 μM TFP or HBSS at 4°C for 30 min and subsequently incubated in HBSS with or without TFP at 37°C for 30 min. Whereas in non-treated cells, TxR-IgA was readily transported from the SAC to BC (Fig. 5b), apical delivery of TxR-IgA from the SAC was inhibited by TFP treatment (Fig. 5c). Indeed, the percentage of TxR-IgA-positive BC in TFP-treated cells was significantly decreased when compared to HBSS-treated cells (Tab. 1). Hence, the data show that TFP inhibits trafficking of TxR-IgA from the SAC to BC, suggesting that pIgR-IgA and C_6-NBD-SM are transported along the same pathway from the SAC to BC in dbcAMP-treated cells. Apparently, this pathway differs from that followed by the GlcCer analogue, traveling between the SAC and BC, as its trafficking is unaffected by TFP.

DbcAMP Causes Hyperpolarization of HepG2 Cells Which is Abolished by TFP

While analyzing sphingolipid trafficking in dbcAMP-treated cells, it was readily observed that the number of apical PM domains, BC, as well as their size was increased. To define the parameters that affected the degree of cell polarity and to appreciate the relevance of distinct pathways involved, the following experimental conditions were examined. First, the cells were treated with 100 μM dbcAMP at 4°C for 30 min and subsequently at 37°C for 30 min. Alternatively, *i)* cells were treated with 20 μM TFP, *ii)* cells were simultaneously treated with TFP and dbcAMP, *iii)* cells were first incubated with dbcAMP for 30 min at 4 and 37°C and subsequently incubated with TFP at 37°C for 30 min, *iv)* cells were first incubated with TFP for 30 min at 4 and 37°C and subsequently incubated with dbcAMP at 37°C for 30 min or finally, *v)* the cells treated with buffer. After fixation and permeabilization in -20°C ethanol

for 10 s and rehydration in HBSS, the cells were subsequently double-stained with Hoechst and TRITC-labeled phalloidin and the ratio [BC/100 cells] was determined as described in Materials and Methods. The results are presented in table 2. DbcAMP treatment caused an

t=0   t=30   t=30 + TFP
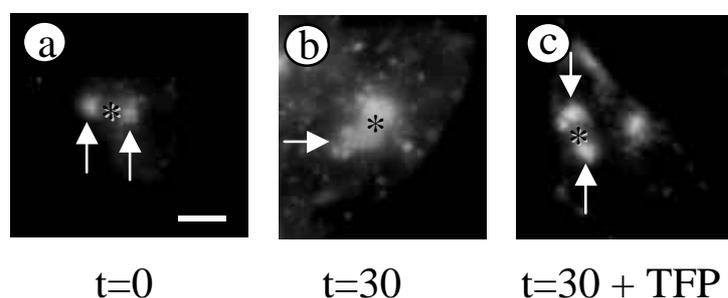
increase of the ratio [BC/100 cells] and, thus, enhanced cell polarity by approximately 200%

Figure 5. Effect of TFP on SAC to BC transport of TxR-labeled IgA. Cells that stably express the polymeric immunoglobulin receptor (van IJzendoorn and Hoekstra, 1998) were washed and incubated with excess asialofetuin to saturate asialoglycoprotein receptors. Subsequently, cells were incubated with 50 μg/ml TxR-IgA at 4°C for 1 h. After rinsing the cells to remove non-bound ligand, cells were warmed to 37°C and incubated for 15 min to allow internalization and partial transcytosis. Then, cells were rapidly cooled to 18°C and further incubated for 90 min. In a, basolateral PM derived TxR-IgA has accumulated in SAC (arrows). Following a subsequent incubation at 37°C TxR-labeled both SAC and BC, indicative of transport to BC (b). However, in the presence of TFP, SAC to BC transport of TxR-IgA was severely inhibited. Most of the fluorescently labeled IgA was still in SAC alone (arrows) with little if any TxR-IgA transported to BC. A semi-quantitative analysis is presented in table 1. Asterisks mark the apical, bile canalicular PM. Bar 10μM.

Table 1. Accumulation of transcytosing TxR-labeled IgA in SAC and subsequent delivery to BC; effect of TFP.

	BC	BC + SAC	SAC
15' 37°C ⟶ 90' 18°C	0 ± 0	4 ± 2	96 ± 2
15' 37°C ⟶ 90' 18°C ⟶ buffer ⟶ 30' 37°C	19.1 ± 1.7	62.4 ± 7.4	18.6 ± 5.6
15' 37°C ⟶ 90' 18°C ⟶ TFP ⟶ 30' 37°C (+TFP)	2.5 ± 1.5	22.5 ± 2.5	75 ± 5

Cells were washed and incubated with excess asialofetuin to saturate asialoglycoprotein receptors (see Materials and Methods). Subsequently, the cells were incubated with 50 μg/ml TxR-IgA at 4°C for 1 h. After rinsing the cells to remove non-bound ligand, cells were warmed to 37°C and incubated for 15 min to allow internalization and partial transcytosis. Then, the cells were rapidly cooled to 18°C and further incubated for 90 min. As shown

in the first row, after this incubation scheme about 96% of the label located in the BCP was due to the exclusive labeling of SAC. Subsequently, the cells were incubated in HBSS (control) or HBSS, supplemented with 20 μM TFP, at 4°C for 30 min and for an additional 30 min at 37°C in the presence or absence of TFP. In buffer-treated cells, a clear shift of TxR-IgA-labeling from SAC to BC was seen to have occurred, leaving only 18% of the ligand in SAC alone (second row). In TFP-treated cells, this shift was effectively inhibited, as judged by the presence of the vast majority of BCP-associated label in SAC. Only a relatively minor fraction of the TxR-IgA was transported from SAC to BC (third row). Data are expressed as mean ± SEM of at least 4 independent experiments.

Table 2. Effect of dbcAMP and/or TFP or a sequential incubation of a combination of both on polarity of the cells.

	Ratio [BC/100 cells]
Control	10.2 ± 1.5
DbcAMP	21.3 ± 0.5
TFP	9.5 ± 1.0
DbcAMP + TFP	10.8 ± 0.5
DbcAMP $\longrightarrow$ TFP	19.2 ± 1.0
TFP $\longrightarrow$ dbcAMP	9.7 ± 1.5

For incubation schemes see text. After the incubations, the cells were fixated and permeabilized with -20°C ethanol for 10 s, rehydrated in HBSS and incubated with TRITC-labeled phalloidin and Hoechst to determine the ratio [BC/100 cells] (cf. Zegers et al., 1997). Data are presented as mean ± SEM of 2-3 independent experiments.

when compared to buffer-treated cells. However, when the cells had simultaneously been treated with TFP, this increase in polarity was abolished. Importantly, TFP alone did not affect the polarity of the cells. Since the calmodulin antagonist inhibited apical directed transport from the SAC in dbcAMP-treated cells (see Fig. 5), and did not cause depolarization as such, the results thus suggest that dbcAMP-induced hyperpolarization is due to a stimulation of apical directed transport via the SAC. Indeed, when cells were treated with TFP, prior to dbcAMP, no change in the ratio [BC/100 cells] was observed, suggesting that the dbcAMP-induced hyperpolarization is closely correlated to an enhanced membrane flow, typified by the pathway followed by C_6-NBD-SM and pIgR-IgA from the SAC. Totally in

agreement with this, TFP was unable to counteract the hyperpolarized state of the cells when administered after dbcAMP.

Transport of C$_6$-NBD-Lipids from SAC Does not Depend on An Intact Actin Filament Network.

The exiting of C$_6$-NBD-SM and -GlcCer from the SAC, either in basolateral or apical direction in dbcAMP-treated cells, is vesicle-mediated (van IJzendoorn and Hoekstra, 1998). It is possible therefore that the calmodulin antagonist inhibits the formation of C$_6$-NBD-lipid-containing vesicles (see Discussion). Calmodulin functions as a light chain for myosin (Geli et al., 1998), which is an actin/ATP-dependent molecular motor. Both myosin (Müsch et al., 1997) and actin (Gottlieb et al., 1993) have been suggested to be important factors in vesicle formation. Hence, calmodulin might play a role in the regulation of cytoskeleton rearrangements and, thus, vesicle formation. Interestingly, myosin II has been suggested to interfere with the transport of secretory vesicles across the cortical actin meshwork at a late, postmicrotubular stage (Ayscough et al., 1997). Moreover, recycling of PM components from the perinuclear recycling endosome, the proposed SAC equivalent in non-polarized cells (Zacchi et al., 1998; van IJzendoorn and Hoekstra, 1999), is regulated by ADP ribosylating factor (ARF)-6 GTPase (D'Souza-Schorey et al., 1998) which, in turn, is also implicated in modeling the cortical actin cytoskeleton (Song et al., 1998). To test whether transport of the lipid analogs from the SAC required intact actin filaments, the SAC was loaded with either lipid as described in Materials and Methods. Subsequently, cells were incubated with the actin filament-disrupting drug cytochalasin D (cytD) at 4°C for 30 min. Then, the cells were rewarmed to 37°C and incubated for an additional 20 min in the presence of cytD. Although cytD effectively disrupted actin filaments as evidenced by staining with fluorescently labeled phalloidin (cf. Zegers et al., 1998, Fig. 1D), no effect on SAC-to-basolateral nor SAC-to-apical transport of C$_6$-NBD-SM and -GlcCer, respectively, was observed (Fig. 6a and b, compare with Fig. 1a and 2a, respectively). Hence, it is unlikely that TFP inhibited C$_6$-NBD-SM-containing vesicle formation from SAC by perturbing actin filament organization.

Discussion

C$_6$-NBD-SM and -GlcCer are in distinct domains in SAC in polarized HepG2 cells.

In the present work, we have obtained novel insight into the molecular sorting of distinct sphingolipids in the reverse transcytotic pathway in polarized HepG2 cells. In particular, the direct involvement and the significance of the previously identified sub-apical compartment, SAC, as a major sorting compartment in generating cell polarity, was further highlighted. Thus, evidence was obtained which reveals that at least three different membrane flow pathways originate from SAC, leading to either basolateral or apical membrane, while each route is likely controlled by a different traffic regulating machinery. In part, this regulation may involve the molecular organization of relevant compounds into separate (membrane)domains, as illustrated in the present study, in which we demonstrate the
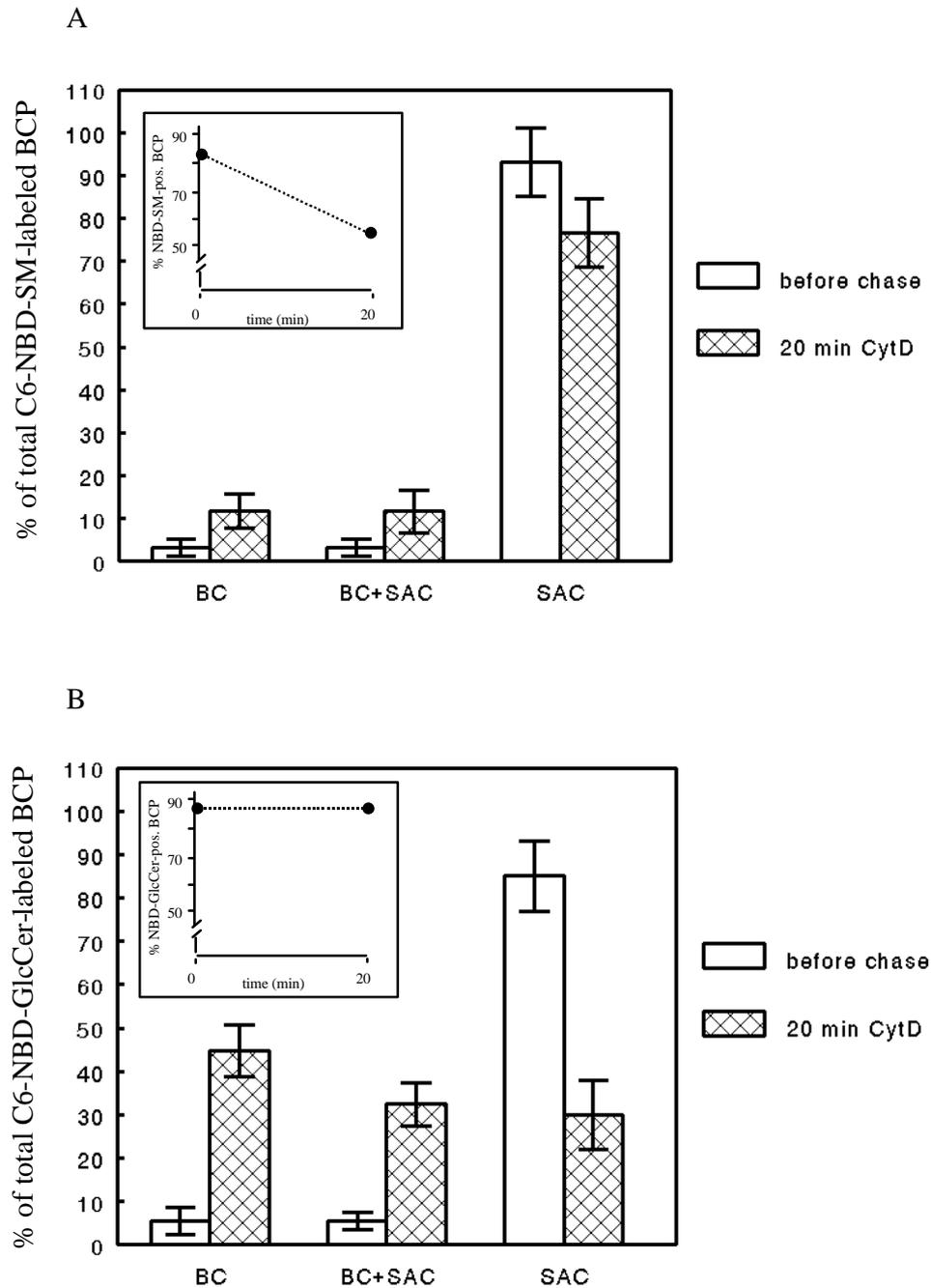
A

B

Figure 6. Effect of the actin filament disrupting agent cytD on transport of C_6-NBD-SM (A) and -GlcCer (B) from SAC. C_6-NBD-lipid was accumulated into SAC as described in Materials and Methods. After abolishing the remaining BC-associated NBD-fluorescence with sodiumdithionite, cells were treated with 10 μg/ml cytD at 4°C for 30 min. Then, they were washed and kept in HBSS at 4°C until use (<30 min; t=0 (before chase)) or, alternatively, warmed to 37°C and incubated in back exchange medium, in the presence of cytD for 20 min. Cells were then rapidly cooled and kept on ice until use (<30 min). The percentage of NBD-lipid-labeled BCP (insert) and the distribution of the BCP-associated NBD-lipid (i.e. BC, SAC, or both) before (white bars) and after the chase from SAC (cross-hatched bars) was determined as described in Materials and Methods. Data are expressed as mean ± SEM of at least two independent experiments carried out in duplicate.

existence of separate domains of C_6-NBD-SM and -GlcCer in SAC membranes. Evidently, the fate of such a domain can, in addition, be subject to control. As mirrored by the flow of fluorescent SM, this element of control may involve a shift in trafficking direction, as induced by an elevation of the level of cAMP, when departing from the SAC. In general, the need for such a regulation is likely related to specific biological demands, in this particular case a change of cell polarity.

Our data demonstrate that the calmodulin antagonist TFP neither affected apical endocytosis of either SM or GlcCer, or their transport to the SAC (Fig.3). Rather, *after* having reached this compartment, the antagonist strongly interfered with the subsequent fate of the sphingolipids. As shown previously (van IJzendoorn and Hoekstra, 1998), sorting occurs in this compartment, SM being directed to the basolateral membrane, whereas GlcCer returns from the SAC to the apical plasma membrane. In the presence of TFP, the latter pathway still occurred (Fig. 2). However, the antagonist selectively and strongly inhibited the exit of C_6 -NBD-SM from the SAC (Fig. 1). Interestingly, being barred from exiting in a basolateral-

directed pathway, participation, as an alternative, in the apical pathway marked by GlcCer flow, neither occurred. Such an apical flow of C_6-NBD-SM could be activated, however, upon treatment of the cells with dbcAMP. Remarkably, in contrast to the recycling pathway of the GlcCer analogue, the dbcAMP-controlled apical pathway of SM could be completely blocked, when TFP was included in the medium. In this context it should be noted however, that a minor effect on GlcCer recycling cannot entirely be excluded, since in the presence of cAMP a small increment in the lipid's trafficking to the apical membrane has been noted (van IJzendoorn et al., 1997). However, demonstration of such an effect on top of the regular level of apical trafficking, is beyond the limit of detection (by fluorescence microscopy or sodiumdithionite quenching; see Methods and Materials). It implies that the cAMP-sensitive pathway contributes only to a very minor extent to the 'normal' recycling pathway of GlcCer. It is evident therefore, that the discriminatory effect of TFP would suggest that the apical membrane directed pathways of both lipids, exiting from SAC, are different. Specifically, since TFP also inhibited transport of basolaterally endocytosed IgA/pIgR from the SAC to BC, the evidence supports the view that in dbcAMP-activated cells, SM is transported to the apical membrane via the same TFP-sensitive basolateral-to-apical transcytotic pathway.

Whereas SM is apparently effectively excluded from the GlcCer recycling pathway, minor fractions of the latter lipid can 'escape' from the apical directed route from the SAC, and instead, recycle via the basolateral membrane (van IJzendoorn et al., 1997). In the presence of TFP, this phenomenon is also completely inhibited, without any apparent effect on the recycling of the lipid between the SAC and BC (Fig. 3). Interestingly, in polarized MDCK cells, calmodulin antagonists have similarly been shown to inhibit the basolateral recycling of transferrin (Apodaca et al., 1994), thus impeding trafficking between basolateral membrane and sub-apical compartments that are closely related to or inherently part of SAC (Odorizzi et al., 1996; Futter et al., 1998; van IJzendoorn and Hoekstra, 1999). Taken together, the data indicate that overall basolateral membrane directed transport is effectively inhibited by TFP,

whereas apically directed trafficking from SAC is selectively inhibited. Most importantly, the distinct response of the SM and GlcCer analogs to TFP strongly suggests that C_6-NBD-SM and -GlcCer were located in different pools or domains in the SAC membranes. Moreover, since transport of both analogs from the SAC is vesicle-mediated (van IJzendoorn and Hoekstra, 1998), topological requirements dictate that these distinct sphingolipid domains must be located in the inner leaflets of the compartment.

Distinct sphingolipid domains: implications for polarized transport.

Sphingolipid domains, referred to as rafts, have been proposed to play a pivotal role in the transport of apical proteins (Brown and Rose 1992; Simons and Ikonen, 1997). Typically, these domains are identified by their isolation as Triton X-100-insoluble fractions (at 4°C), enriched in distinct proteins, cholesterol and sphingolipids such as GlcCer and SM (Brown and Rose 1992). The present observations raise some intriguing questions concerning the composition and specificity of raft-mediated trafficking. First of all, although acyl chain saturation has been mechanistically related to raft formation (Ahmed et al., 1997; Simons and Ikonen, 1997; Brown and London, 1998; Zegers and Hoekstra, 1998), the hydrophobic parts of the fluorescent analogs are remarkably similar, implying that their sorting into distinct domains must be largely driven by head group specificity. The intriguing question arises to what extent these lipids, being located in separate domains, each can participate in the formation of such rafts. Could they possibly participate in the formation of *distinct* rafts, each directed to a different, i.e. apical or basolateral target membrane? In this context, it is not unlikely that the distribution of rafts (and their functioning) may be more ubiquitous than revealed thus far (this study; Brown and London, 1998; Mayor et al., 1998). Evidently, further work is obviously required, but these issues may have important consequences with respect to the exclusiveness of raft-specific (i.e. apical) targeting, and claims concerning their composition when isolated by detergent extraction. In this respect it is important to emphasize that both C_6-NBD-SM and C_6-NBD-GalCer are targeted from the SAC to the basolateral membrane (van IJzendoorn and Hoekstra, 1998), although we have not yet determined whether these analogs are randomized within the same domain. It is apparent though, that the SM and GlcCer domains do maintain their specific identity when the membrane flow pathway of SM is reversed from basolateral to an apical direction upon treatment of the cells with dbcAMP.

It is furthermore of particular interest to note that in the present cell system, evidence has been provided that several apical GPI-linked proteins reach this membrane in an indirect pathway that leads via the basolateral membrane (Ihrke et al., 1998; Schell et al., 1992). Since newly synthesized SM and GlcCer also appear to travel through SAC prior to reaching their final destination (S. van IJzendoorn, K. Mostov and D. Hoekstra, unpublished observations), the present data might therefore suggest that different raft-like domains with different destinations are in fact available for trafficking. It could also explain why several non-apical proteins are found in the detergent insoluble fraction (Weimbs et al., 1997). Hence, the present observations indicate the potential existence of multiple sphingolipid-enriched

domains in the same membrane fraction, which cannot be distinguished as separate entities in detergent extracts. It is apparent, that a detailed analysis of these domains, closely related to sorting and targeting governed by as yet unknown mechanisms, paves the way for identifying novel concepts in membrane cell biology in general, and polarized trafficking in particular.

TFP inhibits the formation of C_6-NBD-SM-enriched vesicles from SAC

Apart from the intriguing consequences of TFP's action on SAC-originating lipid trafficking and membrane flow, another question concerns the underlying mechanism of its action. Our data (Fig.1) suggest that the mechanism of TFP in inhibiting sphingolipid flow relates to an interference with an early step in the transport pathway from SAC, rather than to an interference with sorting or polarized targeting, such as docking/fusion of transport vesicles. Indeed, in the presence of TFP, no randomization of the distinct sphingolipid pools in SAC is observed. Furthermore, in MDCK cells, TFP has been shown to inhibit basolateral to apical transcytosis of IgA/pIgR, resulting in an intracellular accumulation of the complex in large early endosomal compartments filled with internal membranes (Apodaca et al., 1994). From these compartments, no enhanced basolateral recycling was observed. Recently, Enrich et al. (1996) identified pIgR as the major calmodulin-binding protein in a rat liver endosomal fraction enriched in recycling receptors, while Chapin et al (1996) showed that although calmodulin binds to the autonomous basolateral targeting signal of pIgR, it is neither necessary nor interferes with basolateral targeting of pIgR. Taken together, these observations would be consistent with an effect of calmodulin antagonists on membrane vesiculation, rather than on sorting. In fact, calmodulin has been implicated in several processes that might be required in vesicle biogenesis, including actin-myosin interaction, actin-PM association, and coat protein recruitment. Disruption of actin filaments with cytD has been reported to (partly) inhibit endocytic pathways (Gottlieb et al., 1993; Jackman et al., 1994; Lamaze et al., 1997), including basolateral endocytosis of C_6-NBD-SM and -GlcCer in HepG2 cells (Zegers et al., 1998). However, cytD did not inhibit exiting of these sphingolipid analogs from SAC (Fig. 6). In addition, significant changes in actin filament organization were not apparent after TFP treatment (van IJzendoorn en Hoekstra, unpublished observations), implying that the mechanism of TFP action on vesiculation is not mediated via perturbation of actin filaments.

Calmodulin binds to clathrin heavy and light chains (Merisko et al., 1988), and has been implicated to interfere with the recruitment of clathrin to membranes (Salisbury et al., 1980). Moreover, clathrin-coated lattices, involved in vesicle formation and vesicle transport (Kirchhausen et al., 1997; Futter et al., 1998; Okamoto et al., 1998), have recently been demonstrated on SAC tubules in polarized epithelial cells (Futter et al., 1998; Okamoto et al., 1998, 1998b). Also in HepG2 cells, clathrin partly localizes to the SAC (our unpublished observations), while calmodulin has been shown to tightly bind to a rat liver endosomal fraction highly enriched in SAC membranes and transcytotic vesicles (Enrich et al., 1988, 1996). Although calmodulin antagonists might interfere with other proteins required for vesicle formation (Wang et al., 1993), it will be of interest to examine a possible involvement of clathrin in SAC-derived vesicular trafficking.

Distinctly regulated sphingolipid trafficking pathways exit from SAC: a correlation with apical PM biogenesis.

Activation of different steps in the adenylate cyclase-cAMP-protein kinase A signal transduction cascade stimulates transport to the apical PM domain in polarized cells (Mostov and Cardone, 1995; Hansen and Casanova, 1994; Zegers and Hoekstra, 1998). In agreement with this notion, treatment of HepG2 cells with dbcAMP enhances apical PM directed transport of C_6-NBD-SM and –GlcCer involving a protein kinase A-mediated mechanism, and causes a concomitant hyperpolarization of the cells (Zegers and Hoekstra, 1997). Here, we demonstrate that the traffic machinery in the SAC acts as a target site for the stable cAMP analogue. Thus in contrast to a basolateral direction in control cells, an apical pathway is taken by C_6-NBD-SM, after initial accumulation in the SAC and when, subsequently, lipid trafficking is resumed in the presence of dbcAMP. In this case, the lipid remains in the apical pole of the cells, its direction of trafficking being apical rather than basolateral (Fig. 4). Concomitantly, hyperpolarization is taking place, as reflected by the increase in the number (Tab. 2) and size (not shown) of the BC. As indicated above, the sensitivity of this pathway towards TFP explicitly discriminates this route, which is also taken for apical delivery of pIgR/IgA, from the apical GlcCer recycling pathway.

From these observations several important conclusions can be drawn. First, the data indicate that the lateral segregation of the SM and GlcCer is maintained upon dbcAMP treatment of the cells. Second, since hyperpolarization was completely abolished upon simultaneous treatment of the cells with dbcAMP and TFP, a close correlation between the transport pathway from the SAC to BC, as marked by the trafficking of C_6-NBD-SM in cAMP-treated cells, and the biogenesis of apical PM is suggested. Furthermore, as dbcAMP was unable to cause hyperpolarization in TFP-pretreated cells, while TFP did not reverse dbcAMP-induced hyperpolarization (Tab. 2), it is apparent that transport along this pathway from the SAC to BC precedes apical PM biogenesis. Indeed, elsewhere we have demonstrated that dbcAMP treatment mobilized sphingolipid trafficking from an intracellular pool, rather than stimulating endocytosis at the basolateral membrane or the biosynthesis of sphingolipids (Zegers and Hoekstra, 1998). The data presented here point to the SAC as a likely candidate for dbcAMP-induced, i.e. signal-mediated stimulation of apical transcytosis, and the importance of this pathway, as marked by the TFP-sensitive SM/IgA-pIgR route, in polarity development.

Concluding remarks.

This study has demonstrated that separate sphingolipid domains exist in SAC, which pathways of trafficking can be distinctly regulated. Thus dbcAMP can reroute SM from a basolateral to an apical membrane destination, without perturbing its lateral segregation. A differential regulation is also observed for TFP, an apparent modulator of membrane trafficking, presumably at the level of vesiculation. This interference is observed at the level of basolateral membrane directed trafficking and transcytotic transport of SM and IgA/pIgR from SAC to BC. Intriguingly, no effect is seen in case of recycling of GlcCer between SAC

and BC. This emphasizes the specificity of the process, presumably reflecting an interference with *domain-specific* signals, as part of a trafficking machinery. This level of control could possibly be related to the involvement of specific coat-complexes, giving rise to formation of vesicles that participate in specific trafficking pathways, mediating different subcellular destinations. The goal of current work in our laboratory is to exploit this experimental model further in an effort to obtain molecular insight into these events.