Transport and sorting of sphingolipids in polarized cells
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Chapter 7

Merging of Biosynthetic and Transcytotic Sphingolipid Transport Pathways in the Sub-Apical Compartments in MDCK Cells

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Abstract

MDCK cells have been extensively exploited to study polarized transport and cell polarity. Biochemical assays have been developed to accurately measure the kinetics of appearance of proteins at the either basolateral or apical PM domain, while fluorescently tagged proteins have proven a valuable tool to map the intracellular transport itinerary and identify the subcellular compartments that are involved in the overall pathway of PM delivery. However, the intracellular transport routes of sphingolipid transport in these cells had thus far been poorly characterized. To address this issue, we have employed fluorescently (C<sub>6</sub>-NBD) labeled sphingolipids to map the compartments that comprise the transcytotic and biosynthetic pathways. The results show that transcytosis of sphingolipids in MDCK cells involves sequential transit through the basolateral early endosome and sub-apical compartments (SAC). Furthermore, evidence is presented that demonstrates for the first time that the transcytotic and biosynthetic pathways are interconnected in the SAC. The SAC has been proposed to contain some well-characterized components that regulate polarity-dependent sorting and targeting of proteins and lipids. Our data show that in the SAC, IgA and sphingolipids are localized to distinct domains or subcompartments. A correlation between the subcompartmentalization of the SAC and its polarized sorting function is discussed.

Introduction

The plasma membrane (PM) of polarized cells is composed of a basolateral and an apical domain, each characterized by a unique protein and lipid composition. In order to generate and maintain such compositions, proteins and lipids are sorted and targeted to the correct PM domains. Apical PM-directed pathways include a direct, post-Golgi biosynthetic pathway for the delivery of newly synthesized apical components, and an indirect pathway involving initial targeting to the basolateral surface. From this membrane domain, the apical components are subsequently internalized and transcytosed to the opposite, apical surface. Different cell types may use specific routes for the apical delivery of newly synthesized molecules. MDCK cells predominantly use the direct route for apically directed transport of newly synthesized proteins and lipids (Mostov and Cardone, 1995). Hepatocytes have been suggested to lack such a direct route for the apical delivery of proteins and predominantly use the indirect pathway instead (Bartels et al., 1987; Schell et al., 1992). However, newly synthesized sphingolipids (Zaal et al., 1994; Zegers and Hoekstra, 1997; Zegers et al., 1998), as well as some proteins (Ali and Evans, 1990), can be transported directly to the apical, bile canalicular PM of cultured HepG2 cells. In addition, sphingolipids are transcytosed in both apical and basolateral direction in these cells (van IJzendoorn et al., 1997). Other cell types such as enterocytic Caco-2 (Hauri and Matter, 1991) and retinal pigment epithelial (Bonilha et al., 1997) cells use both pathways equally for apical targeting. Interestingly, cells can be triggered to employ different pathways (i.e. direct versus indirect) in order to target proteins to the apical surface under certain conditions, e.g. depending on the degree of cell polarity (Zurzolo et al., 1992). Hence, both biosynthetic and transcytotic routes and sorting in these routes appear to be indispensable for the establishment and maintenance of cell polarity.

Although a rough outline of the different pathways to the PM domains is known, information as to the intracellular compartments that are involved in the different transport pathways, especially in the direct route, is still scanty. After endocytosis from the basolateral
surface, most proteins are first confronted with the basolateral early ‘sorting’ endosome (BEE). Here, they are sorted and either (i) tunneled into the late endocytic/lysosomal pathway, (ii) recycled to the original, basolateral PM domain, or (iii) targeted to the sub-apical compartment (SAC). The SAC is an integral part of the transcytotic pathway, and connects the apical and basolateral membrane domains. Moreover, the SAC harbors several mechanisms for the sorting of proteins and sphingolipids, and is believed to orchestrate polarized targeting to the PM during transcellular flow (reviewed in van IJzendoorn and Hoekstra, 1999). Interestingly, also direct Golgi-to-PM trafficking has been reported to involve passage through endosomes (Ali and Evans, 1990; Leitinger et al., 1995; Futter et al., 1995), suggesting that endo-/transcytotic and biosynthetic pathways may be connected. These findings led to suggest that the SAC might also function as a sorting site for the polarized expression of certain receptor proteins in the direct route (Futter et al., 1998). However, the unambiguous involvement of the SAC in the biosynthetic pathway has thus far not been demonstrated.

In this study, we have employed fluorescently labeled sphingolipid analogues to monitor trafficking of sphingolipids in both the transcytotic and direct pathways in MDCK cells, and compared the lipid trafficking with that of fluorescently labeled IgA, a well-studied transcytotic marker (Mostov and Cardone, 1995; Apodaca et al., 1994; Barosso and Sztul, 1994). The results show that in MDCK cells, the BEE and the SAC, i.e. the major intracellular compartments, that are well known to comprise the transcytotic pathway for proteins, are also used by transcytosing sphingolipids. In addition, we present evidence that, for the first time, unambiguously demonstrates the involvement of the SAC in the biosynthetic, Golgi-to-PM pathway. These results indicate that the SAC is located in the hub of intracellular sorting pathways and support its proposed function as key sorting center in intracellular traffic in polarized cells. Furthermore, the results suggest a subcompartmental nature of the SAC, which may be instrumental to its function in polarized sorting and targeting.

**Materials and Methods**

**Cell Culture**
MDCK cells that stably express the wild-type rabbit polymeric immunoglobulin (Ig) receptor (Breitfeld et al., 1989) were maintained in MEM (UCSF Cell Culture Facility), supplemented with 10% FBS (Hyclone, Logan/UT) and antibiotics (penicillin and streptomycin) at 37°C in 5% CO2/95% air. For fluorescence microscopy studies, the cells were cultured on 12-mm, 0.4-µm pore size Transwell polycarbonate filters (Corning Costar, Cambridge/MA) so that they reached confluency after 1 day, and were grown for a further 3 days with regular medium changes. Polarity of the cells was verified as described elsewhere (Low et al., 1996). Cells were only used when a confluently grown cell layer was able to withstand the hydrostatic pressure of approximately 1 cm in an overnight ‘leak-test’.

**Synthesis of C6-NBD-Labeled Sphingolipids**
C6-NBD-GlcCer, C6-NBD-SM and C6-NBD-Cer were synthesized from C6-NBD (Molecular Probes, Eugene/OR) and 1-β-glucosylsphingosine, sphingosylphosphorylcholine and D-sphingosine (Sigma Chemical Co., St Louis/MO), respectively, as described elsewhere (Kishimoto., 1975; Babia et al., 1994). The C6-NBD-lipids were stored at -20°C and routinely checked for purity.
Preparation of C₆-NBD-Lipid solutions
C₆-NBD-GlcCer, C₆-NBD-SM or C₆-NBD-Cer was dried under nitrogen, redissolved in absolute ethanol and injected into 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.

Cell Labeling Procedures
C₆-NBD-SM and –GlcCer - Lipid analogue was administered to either the basolateral (1.5 ml) or apical (0.5 ml) side of the cell layer at 4°C for 30 min, in the presence of buffer at the opposite side of the cells. In co-labeling experiments with Texas Red (TxR)-IgA (kindly provided by Dr. Kenneth Dunn, Indiana University Medical Center, Indianapolis/IN), the lipid analogue was inserted into the basolateral PM by transferring the filter support to an ice-cold 100-µl drop of a solution containing both lipid analogue and TxR-IgA (50 µg/ml). 0.5 ml of buffer was added to the upper chamber of the Transwell. Following such an incubation, the filter support was placed back in the 24-wells plate. Non-incorporated lipid analogue was removed by washing the respective side three times with ice-cold buffer. The cells were then incubated at 37°C for 5 min to allow internalization (see Results and Discussion). In some experiments, the remaining pool of lipid analogue residing at the PM was then depleted by a back exchange procedure, which consisted of an incubation of the respective side of the cells with buffer, supplemented with 5% (w/v) BSA (fraction V; Fluka Chemie AG, Bucks/Switzerland), at 4°C for 2 x 30 min. Occasionally, cells were further incubated in the presence of BSA in both apical and basolateral incubation media at 37°C for 25 min.

C₆-NBD-Cer - In order to examine transport of sphingolipids in the biosynthetic pathway, the fluorescent metabolic precursor of SM and GlcCer, ceramide (Cer) was employed. Thus, the basolateral side of the cells was incubated with C₆-NBD-Cer at 4°C for 60 min. As described elsewhere, the Cer analogue accumulates in the Golgi apparatus where it is metabolized to form fluorescent SM and GlcCer (van Meer et al., 1987). Cells were then washed with ice-cold buffer after which both the apical and basolateral sides were subjected to a back exchange procedure and subsequent washing steps. The cells were then incubated in back exchange medium at both sides at 37°C for 30 min to allow for metabolic conversion of the C₆-NBD-Cer to C₆-NBD-SM and C₆-NBD-GlcCer, and subsequent transport of the metabolites. After the final incubation step, both sides of the cells were washed three times and processed for confocal laser scanning fluorescence microscopy.

Confocal Microscopy
The filter was cut from the Transwell and mounted onto a microscope slide immediately before examination. Samples were analyzed using a krypton-argon laser coupled with a BioRad MRC-1000 confocal head attached to an Optiphot II Nikon inverted microscope with a Plan Apo 60X 1.4 NA objective lens. For co-localization studies of NBD- and TxR-fluorophores, samples were sequentially scanned for NBD and TxR emission using appropriate filter blocks. In this way, bleed-through of the NBD fluorescence in the red channel was prevented (see Results and Discussion). Scanning of samples labeled with C₆-NBD-sphingolipids was performed at low scan speed and the collection parameters GAIN and IRIS were set to the point just below from that where noise started to appear. The laser intensity was then reduced to such a level that only a very faint picture could be observed on the screen (typically 0.3% laser intensity), in this way minimizing bleaching of the NBD-fluorophore. Captured images were analyzed using Comos software, converted to information-tagged-file-format (TIFF) and enhanced using NIH Imager software.

Results and Discussion

Sphingolipids and IgA Colocalize in Basolateral Early Endosomes and Sub-Apical Compartments during Basolateral to Apical Transcytosis
To investigate the transport itinerary via which sphingolipids traverse the cells, the basolateral
side of the cells was incubated with C₆-NBD-SM or C₆-NBD-GlcCer at 4°C for 30 min as described in Materials and Methods. Non-incorporated lipid analogue was removed and cells were incubated for 5 min at 37°C to allow internalization. C₆-NBD-SM labeled punctate, vesicular structures primarily located in the basolateral region of the cells (Fig. 1a). In addition, there was significant labeling of the basolateral PM. This fraction of fluorescence could be completely removed when cells were subjected to a back exchange procedure at 4°C, emphasizing the exoplasmic orientation. After this time interval little if any fluorescence was detected in the apical region of the cells (not shown). Given the short time of internalization, the C₆-NBD-lipid-labeled vesicular structures most likely represent basolateral early endosomes (BEE). Indeed, C₆-NBD-SM co-localized with simultaneously endocytosed Texas Red (TxR)-labeled IgA (Fig. 1b) and TxR-labeled transferrin (not shown). Similar results were obtained with C₆-NBD-GlcCer (not shown). Both IgA and transferrin are well known to pass through BEE, where they are sorted from the degradative late endosomal/lysosomal pathway (Apodaca et al., 1994 and references therein). Occasionally, TxR-IgA-labeled punctate structures were observed that did not colocalize with the lipid analogue (Fig. 1b, arrow). These structures may have contained relatively small amounts of lipid analogue that will be rapidly bleached during image collection. On the other hand, we cannot exclude that they may represent distinct TxR-IgA-containing vesicles that lack sphingolipids. Indeed, the relatively more intense labeling of the PM with lipid analogue and the higher frequency of presence of vesicular structures containing TxR-IgA, that were located deeper in the cells, might indicate a difference in transport kinetics between the two probes.

From the BEE, IgA is transported to the sub-apical region of the cell where it encounters the sub-apical compartment (Apodaca et al., 1994; Barosso and Sztul, 1994). Also in hepatic cells, IgA passes through the SAC prior to delivery at the apical surface (van IJzendoorn and Hoekstra, 1998; Ihrke et al., 1998). In polarized HepG2 cells, basolateral-to-apical transcytosing IgA co-localizes with C₆-NBD-sphingolipids, internalized from the apical PM domain, in the SAC. Moreover, it was established that in the SAC, sphingolipids are subsequently sorted and targeted by vesicular means to either the basolateral or apical PM domain (van IJzendoorn and Hoekstra, 1998). Indeed, evidence is growing that the SAC functions as a major site in the endocytic routes where the polarized distribution of PM proteins and lipids is orchestrated (reviewed in van IJzendoorn and Hoekstra, 1999). In order to examine whether, in MDCK cells, BEE-derived sphingolipids passed through the SAC en route to the apical PM, similarly as previously demonstrated for IgA (Apodaca et al., 1994; Barosso and Sztul, 1994), cells were co-incubated with C₆-NBD-lipid and TxR-IgA, as described in Materials and Methods. Following the 5-min incubation at 37°C, the cells were cooled to 4°C and lipid analogs residing at the basolateral PM were depleted by back exchange (see Materials and Methods). Subsequently, the cells were warmed to and further incubated at 37°C in HBSS, supplemented with 5% BSA in the apical and basolateral medium, to allow further transport of the BEE-localized TxR-IgA and lipid analogues. After 25 min, TxR-IgA was found almost exclusively associated with the SAC, located subjacent to the apical PM domain (Fig. 1d) in the same confocal plane as the tight junctions (typically 2 microns below the apical PM; not shown), displaying an identical
distribution as previously described (Apodaca et al., 1994). Interestingly, both C₆-NBD-SM (Fig. 1c, arrows) and C₆-NBD-GlcCer (not shown) co-localized with TxB-IgA in the SAC. Given that BSA was present in the apical and basolateral medium during the entire incubation period, thereby prevented any re-internalization of the lipid analogues from either PM domain, SAC-associated C₆-NBD-lipids thus originated from the BEE. Indeed, no C₆-NBD-lipid could be extracted from either PM domain after removal of the incubation media and two subsequent wash steps (not shown). The results suggest that the basolateral-to-apical transport itinerary of C₆-NBD-SM and -GlcCer involves similar subcellular compartments as observed for transcytosing proteins, although individual transport of lipids and IgA between the PM, the BEE and the SAC may slightly differ in kinetics (see above).
Figure 1. Sphingolipids and IgA colocalize in BEE and the SAC. The basolateral side of the cells was incubated with TxR-IgA at 4°C for 30 min as described in Materials and Methods. In some experiments (a,c), C₆-NBD-SM was included during this incubation. In a and b, the subcellular distribution of IgA and C₆-NBD-SM, respectively, following a 5 min internalization step at 37°C, is shown. Occasionally, cells were subsequently subjected to a back exchange procedure to deplete the PM from lipid analogue. Subsequently, the incubation at 37°C was continued in back exchange medium at both sides for another 25 min. In c and d, an image was captured from a confocal plane 2µm below the apical surface of the cells, showing the co-localization of lipid and IgA, respectively. In e and f, co-localization of apical-derived SM and BEE-derived IgA (see text), respectively, is shown at 2µm below the apical surface. Bars 10 µm.

Apically-Derived C₆-NBD-Sphingolipids Colocalize with TxR-IgA in the Sub-Apical Compartments

In order to investigate whether also sphingolipids that were internalized from the apical surface were delivered to the SAC, as observed previously in HepG2 cells (van IJzendoorn and Hoekstra, 1998), the following experiments were performed. The basolateral PM of the cells was incubated with TxR-IgA as described in Materials and Methods. After removal of non-receptor bound ligand by washing three times with ice-cold buffer, cells were incubated at 37°C for 30 min to allow transport of the TxR-IgA to the SAC (cf. Fig. 1d). During the last 5 min of this incubation, the buffer in the upper chamber of the Transwell was replaced by one containing C₆-NBD-SM or –GlcCer. As shown in figure 1e and f (arrows), basolateral PM-derived TxR-IgA and apical PM-derived C₆-NBD-sphingolipids co-localized in the SAC. Co-localization in the SAC was also observed when both lipid analogue and TxR-IgA were simultaneously internalized from the apical PM for 5 min (not shown). Interestingly, in the latter experiment, there was also a significant divergence in the localization of the lipid analogue and TxR-IgA, even when the incubation was reduced to 2-3 min, a time-span in which the pool of recycling probes can be considered insignificant (not shown). Hence, in contrast to differences in kinetics, as noted above for internalization at the basolateral membrane, such a kinetic distinction is less likely for the differences still seen upon apical internalization of SM and pIgR/IgA within such a small time frame. This would imply that different transport vesicles might be involved. Indeed, evidence indicates (Barosso and Sztul, 1994; van IJzendoorn, S.C.D. and Hoekstra, D., submitted) that the trafficking between the SAC and the apical membrane consists of at least two pathways, i.e. an apical recycling pathway and a transport pathway that is specifically involved in the biogenesis of the apical membrane, representing the final leg in the overall transcytotic pathway. The present data suggests that reversal of this latter pathway, followed by SM and leading to its deposition at the basolateral membrane (van IJzendoorn and Hoekstra, 1998) is also taking place, which appears separated from the apical membrane-to-SAC recycling pathways, taken by apically delivered pIgR/IgA. In fact, this notion is very much appreciated by the observation that overexpression of ADP-ribosylating factor (ARF)-6 mutants causes a 6-fold increase in apical IgA endocytosis (Y. Altschuler and K. Mostov, personal communication), but has no significant effect on apical sphingolipid endocytosis (S. van IJzendoorn, Y. Altschuler, K. Mostov and D. Hoekstra, unpublished observations).
Taken together, the data emphasize that the SAC is accessible for both basolaterally and apically internalized proteins and sphingolipids, and constitutes an integral part of both the basolateral and apical directed transcytotic pathway of sphingolipids in MDCK cells. Combined with the fact that C₆-NBD-SM and –GlcCer display a preferential basolateral and apical PM distribution, respectively (van IJzendoorn et al., 1997) and are subject to extensive transcellular flow, a function of the SAC in maintaining lipid polarity in these cells is readily anticipated, as has been demonstrated for proteins (Apodaca et al., 1994; Odorizzi et al. 1996; Futter et al., 1998; Gibson et al., 1998). Indeed, that transport of the different molecules to and from the SAC is likely to occur via different carrier populations and/or routes emphasizes the central position of the SAC in intracellular traffic. However, whereas SAC’s capacity to sort sphingolipids has recently been demonstrated in HepG2 cells (van IJzendoorn et al., 1997; van IJzendoorn and Hoekstra, 1998), sorting of C₆-NBD-SM and –GlcCer was not observed to occur during transcytosis in MDCK cells (van Genderen and van Meer, 1996). In contrast to MDCK cells, hepatic cells have been proposed to rely solely on transcytosis for the delivery of apical resident proteins (Schell et al., 1992), and therefore might be highly dependent on compartments that orchestrate polarized targeting in this route. Interestingly, direct apical delivery of newly synthesized sphingolipids in hepatic HepG2 cells can occur, independently of transcytosis (Zaal et al., 1994; Zegers and Hoekstra, 1997; Zegers et al., 1998). Moreover, evidence is emerging that also proteins can reach the apical surface of hepatocytes in a transcytosis-independent manner (Ali and Evans, 1990). Hence, the discrepancy in transcytotic sphingolipid sorting between the two cell types cannot simply be attributed to a cell type-dependent predominant use of different apically directed pathways. A more likely explanation may stem from the fact that different experimental approaches were used in both studies. Further experiments will have to reveal the sphingolipid sorting capacity of the SAC during transcytosis in MDCK cells.

De Novo-Synthesized Golgi-Derived Sphingolipids and Transcytosing IgA Meet in the Sub-Apical Compartments En Route to the Plasma Membrane

In comparison to PM derived pathways, little is known about compartments that function as intermediates in the biosynthetic PM-directed pathway in living cells, which is mainly due to the difficulty of visualization of proteins traveling along this route. In this study, we have employed the C₆-NBD-labeled Cer, which is readily transferred to the Golgi apparatus at 4°C by monomeric flow (Lipsky and Pagano, 1985) and metabolically converted to C₆-NBD-SM and –GlcCer upon warming the cells to 37°C (Zaal et al., 1994; Putz and Schwarzmann, 1995). Hence, by following the transport of the newly synthesized sphingolipid analogs, intermediate compartments in Golgi-to-PM trafficking can be mapped and characterized. To this end, the cells were incubated with C₆-NBD-Cer and TxR-IgA, included in the basolateral medium, at 4°C for 30 min. Both sides of the cells were then washed and subjected to a back exchange procedure to remove all PM-associated C₆-NBD-Cer. The cells were subsequently warmed to 37°C, and incubated for 30 min in the presence of BSA in both apical and basolateral media. Simultaneously, TxR-IgA is endocytosed and transported to the SAC en route to the apical PM (Fig. 2a; cf. Fig. 1b). Interestingly, the newly synthesized sphingolipids
co-localized with the transcytosing IgA in the SAC, while no co-localization was observed in
the Golgi (not shown), the latter being consistent with other studies (Futter et al., 1998). It is
important to emphasize that, given the presence of BSA in the media that extracted any lipid
analogue arriving at the surface, the SAC-associated lipid analogue originated from the Golgi
(cf. Zaal et al., 1994). In summary, the data thus suggest that the SAC connects the
transcytotic and biosynthetic pathway in living cells, and further support the hypothesis that
the SAC is a central site located in the hub of polarized traffic routes (van IJzendoorn and
Hoekstra, 1999).

It has been proposed that the polarized distribution of newly synthesized proteins that
are sorted by clathrin-mediated mechanism might originate from endosomes (Futter et al.,
1998). Also glycosylphosphatidylinositol (GPI)-anchored proteins were reported to pass
through endosomal compartments en route to the apical, bile canalicular surface of
hepatocytes (Ali and Evans, 1990), suggesting that PM-directed pathways that involve
passage through endosomes might also be used by proteins that are sorted by other
mechanisms. In this respect it is interesting to note that sorting mechanisms including clathrin
coated lattices (Futter et al., 1998; Okamoto et al., 1998) and sphingolipid segregation (van
IJzendoorn and Hoekstra, 1998) have both been demonstrated to be harbored in the SAC.
Although the endosomal compartments through which these proteins traveled en route to the
PM have so far not been identified as SAC, our observation that the SAC constitutes part of
the biosynthetic route suggests that this may indeed be the case. The intriguing question then
arises whether, and if so how and why, two distinct sorting organelles, the Golgi and the SAC,
operate in series in the process of polarized targeting.

IgA and Sphingolipids are Present in Distinct Domains or Subcompartments of the SAC
In a previous study, it was shown that the sub-apical compartment where molecules
accumulate at low temperature are clustered around the microtubule-organizing center, while
their spatial organization was found to depend on intact microtubules (Apodaca et al., 1994).
In order to examine whether this was also the case for the SAC where IgA co-localized with
the sphingolipids, the following experiment was performed. First, C_6-NBD-sphingolipid and
TxR-IgA were chased into the SAC, as described in figure 1. The cells were then incubated
with nocodazole at 4°C for 60 min to disrupt the microtubule network. As shown in figure 3,
such treatment caused the SAC to disperse within the same confocal plane. Interestingly, the
SAC dispersed into compartments that showed only partial co-localization. Indeed, clear
structures labeled with either sphingolipid (green) or IgA (red) are observed, and thus
suggests that both probes were (at least in part) present in distinct subcompartments.

Subcompartmentalization could well be instrumental to the function of the SAC in the
polarized sorting and targeting of proteins and lipids (see van IJzendoorn and Hoekstra,
1999). For instance, clathrin-coated lattices on the SAC have been implicated in both
basolateral (Odorizzi et al., 1996; Futter et al, 1998) and apical (Okamoto et al., 1998)
transport. In addition, of the distinct sphingolipid domains that exist in the SAC, those
enriched in SM can be subject to either apical or basolateral targeting (van IJzendoorn and
Hoekstra, submitted). Based on the available data, we propose that the SAC represents a heterogeneous collection of subcompartments, similarly as reported for the recycling endosomes (Teter et al., 1998), the proposed SAC equivalent in non-polarized cells (van IJzendoorn and Hoekstra, 1999). Such subcompartments may have specialized functions with respect to the processing of molecules destined for a specific PM domain. This is supported by the following observations. In MDCK cells, it was demonstrated that from a sub-apical compartment that is accessible to both IgA and the transferrin receptor, the apically targeted IgA enters a separate sub-apical compartment from which the basolateral recycling transferrin receptor is excluded (Gibson et al., 1998; Brown et al., unpublished observations). In addition, in HepG2 cells, SM can be rerouted from the SAC to the apical surface via distinct rab11-positive sub-apical compartments in a signal-dependent manner (S.C.D. van IJzendoorn, M. Jonker and D. Hoekstra, submitted). At present, we have no information about the nature of the distinct IgA- and lipid-enriched sub-apical structures that are observed after the nocodazole-induced dispersion of the SAC (Fig. 3). Future experiments that include more markers for specific transport routes and/or SAC subcompartments (e.g. transferrin receptor, rab proteins) are imperative to unravel the morphological and functional subcompartamentalization of the SAC. Finally, it will be a challenge to elucidate the relation

Figure 2. IgA and newly synthesized sphingolipids meet in the SAC. Cells were incubated with C6-NBD-Cer and TxR-IgA at 4°C for 30 min, washed and subjected to a back exchange procedure. The cells were then incubated at 37°C in back exchange medium for 30 min. In a and b, colocalization (arrows) of IgA and sphingolipid in the SAC, respectively, is demonstrated. In c, an x-z section clearly shows that transcytosing IgA (red) and newly synthesized sphingolipid (green) merge in the SAC (yellow). Bar 10 µm.
between the sorting capacities of the Golgi on the one hand, and that of the multicompartamental SAC on the other, in the complex process of cell polarity biogenesis.

Figure 3. Transcytosing IgA and SM partly locate to distinct sub-compartments of the SAC. The basolateral side of the cells was incubated with TxR-IgA and C₆-NBD-SM at 4°C for 30 min as described in Materials and Methods. The cells were subsequently subjected to a back exchange procedure to deplete the PM from lipid analogue. Then, cells were incubated at 37°C in back exchange medium at both sides for an additional 25 min to allow transport from the BEE to the SAC (see text and Fig. 1). The cells were subsequently treated with nocodazole at 4°C and 18°C for 60 and 30 min, respectively. An image was then captured from a confocal plane 2 µm below the apical surface of the cells, showing IgA- (red) and lipid- (green) labeled structures. The co-localization of both markers is reflected by the yellow-colored structures.

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