Glycosphingolipids Internalized via Caveolar-related Endocytosis Rapidly Merge with the Clathrin Pathway in Early Endosomes and Form Microdomains for Recycling*

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We have previously demonstrated that glycosphingolipids are internalized from the plasma membrane of human skin fibroblasts by a clathrin-independent, caveolar-related mechanism and are subsequently transported to the Golgi apparatus by a process that is dependent on microtubules, phosphatidylinositol 3-kinase, Rab7, and Rab9. Here we characterized the early steps of intracellular transport of a fluorescent glycosphingolipid analog, BODIPY-lactosylceramide (LacCer), and compared this to fluorescent transferrin (Tfn), a well established marker for the clathrin pathway. Although these two markers were initially internalized into separate vesicles by distinct mechanisms, they became co-localized in early endosomes within 5 min. These results demonstrate that glycosphingolipid-containing vesicles derived from caveolar-related endocytosis fuse with the classical endosomal system. However, internalization to Tfn, internalization and trafficking of LacCer was independent of Rabβ, a key regulator of transport to early endosomes. By taking advantage of the monomer/excimer properties of the fluorescent lipid analog, we were also able to visualize LacCer segregation into distinct microdomains of high (red emission) and low (green emission) concentrations in the early endosomes of living cells. Interestingly, the high concentration “red” microdomains co-localized with fluorescent Tfn upon exit from early endosomes and passed through Rab11-positive “recycling endosomes” prior to being transported back to the plasma membrane. These results together with our previous studies suggest that glycosphingolipids internalized by caveolar endocytosis are rapidly delivered to early endosomes where they are fractionated into two major pools, one that is transported via late endosomes to the Golgi apparatus and the other that is returned to the plasma membrane via the recycling compartment.

We previously showed that fluorescent GSL1 analogs, lactosylceramide (LacCer) and globoside, are selectively internalized by a dynamin-dependent, clathrin-independent mechanism in human skin fibroblasts (HSFs) and suggested that this mechanism is “caveolar-related” based on multiple criteria (1). A significant portion of the LacCer analog is subsequently transported to the Golgi apparatus in a process that is dependent on microtubules, phosphatidylinositol 3-kinase(s), Rab7, and Rab9 (2). The involvement of Rab7 and Rab9 suggested that vesicles carrying internalized GSLs might join with the well characterized endocytic pathway established for markers internalized though coated pits. However, there is conflicting data on whether there is a “merging” of clathrin-independent and -dependent pathways and, if so, which clathrin-independent mechanisms are involved. For example, cholera toxin bound to GM1 ganglioside at the plasma membrane (PM) and internalized through caveola has recently been reported to be transported by Rab5-independent mechanisms to the Golgi apparatus in vesicles devoid of markers of the clathrin pathway in COS-7 cells (3, 4). However, previous studies (5, 6) have demonstrated that cholera toxin rapidly reaches early endosomes accessible to markers internalized by clathrin endocytosis in multiple cell types. Thus, it is still uncertain whether GSLs internalized from the PM converge with the classical endocytic pathway or are transported to the Golgi apparatus in vesicles, which are distinct from the clathrin pathway. In this study, we examined this possibility in detail using live cell and multi-color imaging and provide evidence that BODIPY-LacCer is rapidly transported to early endosomes where it merges with markers for the clathrin pathway.

A fascinating feature of the early endosome compartment was revealed in a study by Zerial and colleagues (7). They examined the distribution of GFP-tagged Rab4, Rab5, and Rab11 along with fluorescent transferrin (Tfn) in living cells and demonstrated that these Rab proteins are localized in morphologically distinct domains, which can reside on the same endosome. Although it has been speculated that some lipids are also present in discrete membrane microdomains in various endosomal compartments (8–10), there is currently no direct evidence for this possibility. Thus, in this study, we also examined the lateral distribution of the LacCer analog in the early endosome membrane. By taking advantage of the concentration-dependent fluorescence emission characteristics of the GSL analogs, we were able to distinguish discrete, contiguous membrane microdomains that contained high or low concentrations of the lipid analog within individual early endosomes. Interestingly, these microdomains behaved differently with respect to their subsequent intracellular trafficking. Namely, the dominant negative; EEA1, early endosome antigen 1; HHMEM, Hepes-buffered minimum Eagle’s medium; BSA, bovine serum albumin.

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The abbreviations used are: GSL, glycosphingolipid; LacCer, lactosylceramide; HSFs, human skin fibroblasts; PM, plasma membrane; GFP, green fluorescent protein; Tfn, transferrin; WT, wild type; DN, dominant negative; EEA1, early endosome antigen 1; HHMEM, Hepes-buffered minimum Eagle’s medium; BSA, bovine serum albumin.

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enriched domains were subsequently recycled to the PM with Tfn, whereas the remaining lipid was transported further along the endocytic pathway to the Golgi apparatus. These results suggest that GSL microdomains within early endosomes may be an important factor in regulating PM lipid composition by controlling the initial sorting of endocytosed GSLs.

MATERIALS AND METHODS

Cell Culture—Normal HSFs (GM-5659D) were obtained from the Coriell Institute for Medical Research (Camden, NJ) and cultured in Eagle’s MEM with 10% fetal calf serum as described previously (38).

Lipids and Miscellaneous Reagents—Fluorescent Alexa Fluor 594 albumin, EGF, and Tfn were from Molecular Probes (Eugene, OR). BODIPY-LacCer was synthesized as described previously (38). WT and DN Eps15 GFP constructs were from Drs. A. Benmerah and A. Dautry-Varsat (Institut Pasteur, Paris, France) and were used as described previously (1). Early endosome antigen 1 (EEA1) antibody was from Transduction Laboratories (BD Biosciences). Secondary antibodies were from Jackson Laboratories (West Grove, PA). Unless otherwise indicated, all other reagents were from Sigma.

Preparation and Expression of Rab Constructs—DsRed-Rab5a-WT and Rab5a-DN(N133I) constructs were generated by PCR amplification using pcDNA3-Myc-Rab5aWT and pcDNA3-Myc-Rab5aN133I as a template (obtained from Dr. C. Bucci, University of Napoli). BgII and Xhol sites were introduced in the forward and reverse primers, respectively, to allow subcloning of PCR products into the pDsRed2-C1 vector (Clontech, Palo Alto, CA) in-frame with the DsRed moiety. Transfections using FuGENE 6 (Roche Applied Science, Inc., Palo Alto, CA) in-frame with the DsRed moiety. Transfections using FuGENE 6 (Roche Applied Science, Inc., Indianapolis, IN) and Rab GTP overlay assays performed as described previously (2). To characterize the Rab5a constructs, HeLa cells, grown on glass coverslips, were transfected with DsRed-Rab5a-WT or -DN for 48 h. Cells were then incubated for 1 h at 37 °C in serum-free medium containing 10 μg/ml Alexa Fluor 488 Tfn (Molecular Probes), washed, acid-stripped (30 s at 10 °C with HMEM (10 mM Hepes-buffered minimum Eagle’s medium), pH 3.5) to remove surface-bound Tfn, fixed with 4% formaldehyde, and observed by fluorescence microscopy. The DsRed-Rab11-WT construct was previously characterized in our laboratory and used as described previously (2).

Incubation of HSFs with Fluorescent Probes—BODIPY-LacCer was complexed to defatted bovine serum albumin (BSA) as described previously (38). Cells were typically incubated for 30 min at 10 °C with 2.5 μM BODIPY-LacCer/BSA, washed twice with HMEM, and further incubated for various times at 37 °C (see figure legends). Fluorescent LacCer present at the PM was removed by acid stripping as described previously (1), prior to incubation with fluorescent LacCer or Tfn.

For quantitative studies, all photomicrographs in a given experiment were exposed and processed identically for a given fluorophore. For double-labeled experiments, control samples were labeled identically with the individual fluorophores and exposed identically to the dual-labeled samples at each wavelength to verify that there was no apparent image shift using different filter combinations.

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RESULTS

Tfn and LacCer Are Internalized in Separate Vesicles and Then Rapidly Co-localize in Early Endosomes—We used fluorescent Tfn as a marker for the clathrin pathway and compared its initial uptake and transport to that of BODIPY-LacCer, which is internalized by a clathrin-independent, caveolar-related mechanism in HSFs (1). In preliminary experiments to verify that Tfn and LacCer were indeed endocytosed by distinct mechanisms, HSFs were pretreated with nystatin (1) or were transfected with DN Eps15 (11), and internalization of the two markers after 5 min at 37 °C was quantified. Nystatin pretreatment had little effect on Tfn internalization (15 ± 7% inhibition) in contrast to its effect on LacCer uptake (81.0 ± 4.3% inhibition), whereas DN Eps15 blocked Tfn internalization (79.0 ± 2.5% inhibition).
Rab5 on LacCer internalization and transport, we first pre-
shown by several groups to reduce Tfn internalization and
overexpression of DN (N133I or S34N) Rab5 has been
derived endocytic vesicles to form classical sorting endosomes
early endosome marker, EEA1 (Fig. 1
A)

A time course experiment was carried out using Alexa Fluor
fluorescence) and the micrograph at 15 min showing the pres-
and Tfn increased and then decreased. This was apparent from
of Tfn co-localization with endocytosed LacCer and the endosomal marker, EEA1. Samples were labeled as in A and B, and co-localization was
calculated using multiple z-sections of at least 10 cells for each time point in three independent experiments.

Bars, 10 μm.

Fig. 1. Endocytosed LacCer is rapidly delivered to early endosomes. A, HSFs were co-incubated with 1.25 μM BODIPY-LacCer (green)
and 5 μg/ml Alexa Fluor 594 Tfn (red) for 1, 5, or 15 min at 37 °C and observed by confocal microscopy. Maximal co-localization (yellow) was seen
at 5 min. In control experiments, no cross-over of LacCer and Tfn fluorescence was detected using these concentrations. B, in a parallel experiment,
cells were incubated with Alexa Fluor 488 Tfn (green) alone for 1, 5, or 15 min at 37 °C and then fixed and immunostained for EEA1 (red). Note
the extensive co-localization (yellow) of Tfn and EEA1 at 5 min. Insets in A and B show an enlargement of the outlined regions. C, quantitation
of Tfn co-localization with endocytosed LacCer and the endosomal marker, EEA1. Samples were labeled as in A and B, and co-localization was
calculated using multiple z-sections of at least 10 cells for each time point in three independent experiments. Bars, 10 μm.

Endocytosed Glycosphingolipids Form Endosomal Microdomains

Caveolar-related Endocytosis of LacCer Also Occurs at
16 °C—When HSFs were incubated with fluorescent Tfn for 1 h
at 16 °C, fixed, and immunolabeled, ~75% of the internalized
Tfn was found in EEA1-positive early endosomes (data not shown), indicating that transport out of this compartment was largely inhibited at this temperature. We also found that BODIPY-LacCer accumulated in early endosomes during a 1-h incubation at 16 °C as shown by its overlap (~50%) (data not shown) with fluorescent Tfn in a double-labeled experiment. This finding suggested that at 16 °C similar to 37 °C, there was a merging of a marker for the clathrin-dependent pathway (Tfn) with LacCer that is internalized by a clathrin-independent
pathway (LacCer) with LacCer that is internalized by a clathrin-independent
mechanism. However, one report (3) suggests that non-
clathrin uptake is inhibited below 20 °C. Thus it is possible
that merging of Tfn and LacCer at 16 °C actually represented internalization of both markers by the clathrin pathway because of “pathway switching” of the LacCer. To test this possi-
bility, HSFs were incubated with fluorescent albumin (a caveo-
Rab5a-independent. HeLa cells (1). LacCer and albumin but not Tfn internalization was significantly inhibited by genistein (Fig. 3A) and nystatin (data not shown). In contrast, Tfn but not LacCer or albumin internalization was blocked by chlorpromazine (Fig. 3A) or in cells expressing DN Eps15 (data not shown). We then examined the co-localization of fluorescent albumin with LacCer versus Tfn after 15 min or 1 h of internalization at 16 °C. At 15 min, albumin co-localized extensively with LacCer but not with Tfn, whereas after 1 h, albumin was also highly co-localized with Tfn (Fig. 3, B and C). These results indicate that LacCer and albumin were internalized by a clathrin-independent caveolar process at 16 °C, apparently identical to that observed at 37 °C, and that the merging of the two pathways also occurred during prolonged incubations at 16 °C.

Live Cell Imaging of Protein Sorting and Lipid Microdomains in Early Endosomes at 16 °C—To further study the endocytic structures formed at 16 °C, HSFs were incubated with Texas Red-labeled EGF and Alexa Fluor 488 Tfn for 1 h at this temperature. Tfn was seen in both spherical and tubular endocytic structures, whereas the EGF was generally seen only in spherical structures (Fig. 4A, bottom panels). When these cells were warmed to 37 °C for 2.5 min, most of the fluorescent Tfn and EGF sorted into separate endosomes (data not shown). When Alexa Fluor 488 EGF (green emission) and Alexa Fluor 594 Tfn (red emission) were used in an identical experiment at 16 °C, a similar result was observed with the exception that the "arms" of the endosomes were predominantly red, whereas the center of the structures was predominantly green (data not shown). The experiments described here demonstrate that subdomains of the sorting endosome could clearly be visualized in HSFs at 16 °C. Such segregation of proteins into distinct endosomal microdomains is in agreement with observations by others at the light and electron microscope levels (7, 16, 17).

To distinguish membrane microdomains enriched in LacCer from other regions of the same membrane containing lower concentrations of the lipid, we utilized the spectral properties of the BODIPY-fluorophore. BODIPY-SL analogs exhibit monomer/excimer fluorescence emitting at green (low mol %) or red (high mol %) wavelengths depending on their concentration in membranes (18). When cells were incubated with 2.5 μM BODIPY-LacCer for 1 h at 16 °C followed by back-exchange with defatted-BSA to remove cell surface fluorescent lipid, many endocytic structures were observed in which both green and red fluorescence were present in the same membrane but appeared to be segregated into discrete regions of the membrane (Fig. 4B), suggestive of microdomain formation. Red/green microdomains were also visible in cells that were incubated briefly (1–5 min) with BODIPY-LacCer at 37 °C; however, because of the rapid and dynamic nature of the transport process at 37 °C, we were unable to identify the compartments in which they were located (data not shown). It is important to note that when lower concentrations of BODIPY-LacCer were used as in Fig. 1 (e.g., 1.25 μM), only green fluorescence was observed, reflecting the concentration-dependent spectral properties of this fluorophore (18, 19). Similar data were also obtained at 16 °C (data not shown).

Tripolar Imaging Reveals That LacCer Lipid Microdomains Travel with Tfn from Early Endosomal Structures to Be Recovered—We next carried out a triple color-imaging experiment to study the distribution of the LacCer microdomains (green and red fluorescence) with respect to internalized Tfn (far-red fluorescence). HSFs were incubated for 1 h at 16 °C with BODIPY-LacCer and Alexa Fluor 647 Tfn. Samples were then washed, back-exchanged, and acid-stripped prior to image acquisition. Images were rendered in pseudocolor using green and red for LacCer fluorescence and blue for Tfn. Fig. 5A shows the distribution of LacCer (green and red) and Tfn (blue) after the 16 °C incubation. Tfn fluorescence overlapped more closely...
with the red emission of LacCer, resulting in a pink overlay of the dual-labeled structures. Upon warming the cells for 5 min at 37 °C (Fig. 5B), (i) the green and red components of LacCer fluorescence were observed to separate and (ii) the red component of LacCer fluorescence co-localized even more extensively with Tfn (in blue). Quantitative analysis of these experiments demonstrated an increase in co-localization between the concentrated red component of LacCer and Tfn upon leaving the early endosome (Fig. 5C).

**LacCer Is Recycled Back to the Plasma Membrane via a Rab11-containing Compartment**—The results in Fig. 5 suggested that upon release of the 16 °C block, the concentrated component of LacCer moved to the recycling endosome and recycled back to the PM along with Tfn. To show the involvement of recycling endosomes, we transfected HSFs with a construct expressing DsRed-Rab11-WT (2) and then incubated these samples with BODIPY-LacCer for 1 h at 16 °C (Fig. 6A). Perinuclear DsRed-Rab11-positive structures were observed in HSFs as reported previously using a GFP-Rab11 construct in HEK 293 cells (20). Samples were then washed and further incubated for various times at 37 °C. There was little co-localization (~10%) between LacCer (green) and Rab11 (red) immediately after the 16 °C incubation; however, this increased to >80% after 5 min and then decreased markedly with longer incubations (e.g., 15 min) at this temperature (Fig. 6A). In separate experiments, we also treated HSFs with AlF4, an agent that results in retention of Tfn in recycling endosomes in Madin-Darby canine kidney cells and Chinese hamster ovary cells (21, 22). HSFs were pulse-labeled with LacCer or Tfn in the presence or absence of AlF4. As shown in Fig. 6B, AlF4 treatment induced pronounced intracellular accumulation of both LacCer and Tfn, and in both cases, some of the internalized fluorescence was concentrated in one region of the cytoplasm reminiscent of recycling endosomes. Together, the results in Fig. 6 suggest that a portion of the LacCer pool moved through the recycling endosomes en route to the PM.

To quantify recycling of BODIPY-LacCer to the PM, HSFs were incubated with the lipid analog for 1 h at 16 °C (to label early endosomes) and subsequently back-exchanged to remove any fluorescent lipid at the PM. The samples were then incubated for 5–60 min at 37 °C in the absence or presence of defatted-BSA in the medium. Return of LacCer to the PM was readily demonstrated by microscopy of samples warmed ± BSA (Fig. 7A), and recycling could be quantified as described previously (23) by measuring the loss of cell fluorescence when BSA was present in the medium (Fig. 7B). This experiment suggested approximately half of the LacCer analog that was endocytosed at 16 °C was recycled back to the PM in ~20 min. Finally, it should be noted that in addition to recycling, LacCer is also transported to the Golgi apparatus as reported previously (1, 2, 9, 24, 25). Golgi targeting of LacCer can be seen in Fig. 6B following a 60-min chase in the presence of BSA.
In this paper, we show that although Tfn and BODIPY-LacCer are internalized by distinct mechanisms, they rapidly became co-localized in EEA1-positive early endosomes. In addition, we directly visualized LacCer-enriched microdomains in the early endosomes of living cells by taking advantage of the unique spectral properties of the fluorescent lipid, and we showed that the high concentration LacCer microdomains recycled with Tfn, whereas the remaining lipid was transported.
further along the endocytic pathway to the Golgi apparatus. These results suggest that the formation of GSL microdomains within early endosomes may be an important factor in regulating the sorting of lipids for further transport along the endocytic pathway versus recycling to the PM and thus may play an important role in regulating PM lipid composition. In the following sections, we highlight the major findings of this study using the model in Fig. 8 to emphasize some of the main points.

**Initial Internalization of GSL Analogs (Fig. 8, Step I)**—Our previous studies provided evidence that LacCer endocytosis is distinguishable from clathrin-dependent endocytosis by several criteria including cargo, sensitivities to selective inhibitors, dependence on specific proteins (e.g. Eps15), and overlap with specific markers (1). In this study, we first verified that LacCer and Tfn were initially endocytosed into separate vesicle populations by distinct mechanisms using HSFs pretreated with nystatin or expressing DN Eps15. We also showed that LacCer internalization could be further differentiated from clathrin-mediated endocytosis on the basis of its lack of dependence on Rab5a. Namely, we found that although internalization of the clathrin marker Tfn was inhibited by expression of DaRed-Rab5a-DN as would be expected from the literature (14), no effect on LacCer uptake was seen (Fig. 2, C and D). Similarly, the initial step in desensitization of protein kinase C involves its delivery to endosomes via a caveolar-mediated process that is also Rab5a-independent (26). Rab5a is required for homotypic fusion of endocytic vesicles derived from clathrin-mediated endocytosis, and for recruitment of EEA1 to form sorting endosomes (Fig. 8, Step II) (12, 27). Thus, it is curious that endocytic vesicles containing LacCer and derived from a Rab5a-independent process are able to fuse with early endosomes that are presumably Rab5a-positive. One possible explanation for this is that other Rab5 homologs play a role in LacCer transport, leading to fusion with the classical EEA1-positive sorting endosome. Indeed, it has recently been shown that Rab5a and Rab5b have distinct functions in the sorting of endocytic cargo (28). In addition, Rab22a, which has a high degree of sequence homology with Rab5, has recently been shown to interact with EEA1 (29). In the future, we hope to evaluate the potential role of other Rab5 homologs in LacCer internalization and transport to early endosomes.

In this study, we also examined the mechanism of LacCer internalization at low temperature and showed that it was not altered (e.g. to a clathrin-mediated process) by lowering the incubation temperature from 37 to 16 °C (Fig. 3). Nichols et al. (3) have suggested that temperatures below 20 °C inhibit non-clathrin uptake; however, that was not the case in this study using BODIPY-LacCer in HSFs. Thus, the intermixing of the

![Fig. 6. A portion of internalized LacCer moves through recycling endosomes upon release of the 16 °C block. A, HSFs were transfected with a construct expressing DaRed-Rab11-WT (2) and, 12–16 h later, were incubated for 1 h at 16 °C with 0.75 µM BODIPY-LacCer, back-exchanged, and further incubated for 0, 5, or 15 min at 37 °C. Images were acquired at green (LacCer) and red (Rab11-WT) wavelengths and are presented as overlays. Note the increase in co-localization at 5 min. N, nucleus; Bar, 5 µm. B, effect of AlF₄ on LacCer and Tfn internalization. HSFs were separately incubated for 30 min at 10 °C with BODIPY-LacCer (1.25 µM) or Alexa Fluor488 Tfn (25 µg/ml) to label the PM, washed, and further incubated for 30 min at 16 °C. The samples were then chased for 30 min at 37 °C in the presence or absence of AlF₄ (see "Materials and Methods"). Note the accumulation of both LacCer and Tfn in "asterisk"-like structures, presumably corresponding to recycling endosomes, in the presence of AlF₄. Bar, 10 µm.](image)

![Fig. 7. Recycling of endocytosed LacCer to the plasma membrane. HSFs were incubated with 5 µM LacCer for 1 h at 16 °C and then back-exchanged at 10 °C to remove PM fluorescence. Cells were then warmed for 0–60 min at 37 °C in the presence or absence of 5% defatted-BSA to assess the delivery of internalized LacCer back to the PM. A, fluorescence micrographs taken after 0 min (left panel) or 60 min (middle and right panels). Note the presence of PM fluorescence (e.g. at arrow) when cells were warmed in the absence (middle panel) but not the presence (right panel) of BSA. Golgi labeling (G) was also more readily visualized when the 37 °C chase was carried out in the presence of BSA (right panel) to remove LacCer recycled to the PM. N, nucleus; Bar, 10 µm. Graph in B shows quantitative measurements of cell-associated LacCer green fluorescence, which was measured by image analysis. Results are means ± S.D. from three separate experiments and are expressed as the percent of initial total fluorescence.](image)
LacCer analog and Tfn seen during a 16 °C pulse is not the result of clathrin-dependent internalization of the LacCer analog at low temperature.

**Merging of LacCer and Tfn in Early Endosomes**—Another interesting finding pertaining to the distribution of LacCer in the early endosomes concerns its relationship to the distribution of endocytosed Tfn. In dual-labeling experiments, we found that fluorescent Tfn was often present in tubular extensions of the early endosomes, whereas fluorescent EGF was usually localized to the larger spherical regions of this organelle (Fig. 4A), consistent with previous studies of lysosomally targeted ligands (16, 37). We also found that fluorescent albumin, which entered via the caveolar pathway, was translocated to early endosomes at 16 °C where it became highly co-localized with endocytosed Tfn (Fig. 3). However, we were not able to visualize any segregation of albumin into endosomal subdomains (compare Figs. 4A and 3B), suggesting that albumin may be broadly distributed within this compartment. Interestingly, the distribution of Tfn in the early endosomes was similar to that of the high concentration (red) LacCer microdomains in early endosomes. This co-segregation of Tfn and LacCer was further supported by our finding that the high concentration LacCer in early endosomes co-localized with Tfn upon release from the 16 °C block (Fig. 5, B and C), suggesting that the LacCer-enriched microdomains were moving toward the endosomal-recycling compartment with Tfn (Fig. 8, Step V). This was verified by co-localizing LacCer with DsRed-Rab11 (Fig. 6A), a marker for recycling endosomes, and by our finding that AlF4, an agent that results in retention of Tfn in recycling endosomes in Madin-Darby canine kidney cells and Chinese hamster ovary cells (21, 22), caused pronounced accumulation of BODIPY-LacCer and Tfn in punctate endosomal structures in HSFs (Fig. 6B).

Finally, we note that approximately half of the LacCer analog, which was internalized at 16 °C, was subsequently recycled back to the PM in ~20 min (Figs. 6C and 8, Step VI). To our knowledge, this is the first demonstration that a lipid internal-
ized exclusively by the caveolar-related mechanism can function back to the PM. It is important to note that most of the LacCer, which is not recycled to the PM, is subsequently transported to the Golgi apparatus (Fig. 7A) as reported previously (1, 2, 9, 24, 25). This Golgi targeting is independent of Rab5a but is dependent on Rab7 and Rab9 (2). Together with our current results, these data suggest that LacCer traffics through the early and late endosomes en route to the Golgi (Fig. 8, Step VII).

At present, we can only speculate as to why sorting and internalization of PM lipids into distinct endocytic vesicles occurs, only to be followed by intermixing in a common endocytic compartment. One possible explanation is that LacCer non-clathrin endocytosis reflects an important regulatory mechanism to maintain an optimal PM lipid composition for proper cell function. In this model, changes in PM lipids including cholesterol would alter the extent of caveolar-related endocytosis and microdomain formation at the early endosome, thereby modulating the amount of SLs and cholesterol that are recycled back to the PM versus the amount transported further along the endocytic pathway. The latter process could result in transport of lipids and cholesterol to the Golgi apparatus along the endocytic pathway. The latter process could result in transport of lipids and cholesterol to the Golgi apparatus.

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