THE ROYAL SOCIETY

Published online 27 March 2003

Endocytic trafficking of glycosphingolipids in sphingolipid storage diseases

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In this review, recent studies of membrane lipid transport in sphingolipid (SL) storage disease (SLSD) fibroblasts are summarized. Several fluorescent glycosphingolipid (GSL) analogues are internalized from the plasma membrane via caveolae and are subsequently transported to the Golgi complex of normal fibroblasts, while in 10 different SLSD cell types, these lipids accumulate in endosomes and lysosomes. Additional studies have shown that cholesterol homeostasis is perturbed in multiple SLSDs secondary to accumulation of endogenous SLs, and that mis-targeting of the GSLs is regulated by cellular cholesterol. Golgi targeting of GSLs internalized via caveolae is dependent on microtubules and phosphoinositide 3-kinase(s) and is inhibited by expression of dominant-negative rab7 and rab9 constructs. Overexpression of wild-type rab7 or rab9 (but not rab11) in Niemann–Pick C fibroblasts results in correction of lipid trafficking defects, including restoration of Golgi targeting of fluorescent lactosylceramide and endogenous GM1 ganglioside (monitored by the transport of fluorescent cholera toxin), and a dramatic reduction in accumulation of intracellular cholesterol. These results suggest an approach for restoring normal lipid trafficking in this, and perhaps other, SLSD cell types, and may provide a basis for future therapy of these diseases.

Keywords: endocytosis; caveolae; cholesterol; rab proteins; lactosylceramide; BODIPY

1. INTRODUCTION

Fluorescent lipid analogues are useful tools for delineating potential pathways and mechanisms of intracellular transport and sorting of lipids. Several years ago, our laboratory made a surprising observation while studying the endocytosis of BODIPY-labelled lipids in normal HSFs versus SLSD fibroblasts. Namely, we found that an analogue of LacCer is transported from the PM to the Golgi apparatus of HSFs, while in fibroblasts from 10 different SLSDs, the same lipid accumulates in punctate endosomal structures (figure 1; Chen *et al.* 1998, 1999). Lipid accumulation in the endosomal structures of SLSD cells was particularly evident using the BODIPY analogue because this lipid exhibits a spectral shift from green to red wavelengths as its concentration increases in membranes (Pagano *et al.* 1991; Chen *et al.* 1997). Thus, the endosomal structures appeared yellow/orange in colour, indicative of a high concentration of the lipid analogue and its metabolites (Chen *et al.* 1999; Puri *et al.* 1999). Co-localization with fluorescent dextran showed that 30–40% of the vesicles labelled by the LacCer analogue correspond to late endosomes and lysosomes (Chen *et al.* 1999). This accumulation was not the result of a block in hydrolysis of the lipid analogue (except in the case of cells with sap deficiency) since degradation of the lipid was similar to that seen in normal HSFs under the same incubation conditions (Chen *et al.* 1999). However, the extent of LacCer accumulation in different SLSDs varied (figure 2; Puri *et al.* 1999), suggesting that the inherent rates of endosomal transport or intracellular sorting might also vary among the different SLSD cell types. LacCer did not accumulate in endosomal structures of fibroblasts taken from patients with other storage diseases in which lipids do not accumulate, including Hunter disease, Pompe disease, neuronal ceroid lipofuscinosis, Chediak–Higashi syndrome, and mannosidosis, or in two SLSD cell types (Gaucher and Farber; Chen *et al.* 1999; Puri *et al.* 1999). Rather in those cell types, the LacCer was targeted to the Golgi apparatus as seen in normal fibroblasts.

Altered lipid targeting is not restricted to BODIPY-LacCer, but has also been observed using other GSL analogues including BODIPY-labelled globoside and GM1 ganglioside, as well as fluorescent CtxB, which binds to endogenous GM1 ganglioside at the PM (Puri *et al.* 2001; Choudhury *et al.* 2002). This latter result suggests that alterations in lipid trafficking detected with fluorescent GSL analogues also occur for endogenous PM GSLs. To understand this phenomenon better, we have studied the itinerary of GSLs from the PM to intracellular compartments, as described in the following sections.

2. INITIAL INTERNALIZATION OF GSLs IN NORMAL AND SLSD CELLS

To study the initial internalization of PM GSLs, HSFs are incubated with a fluorescent GSL analogue (or with fluorescent CtxB) at low temperature to label the PM, and then briefly warmed (e.g. 5 min) to 37 °C to initiate endocytosis. The samples are then back-exchanged at 10 °C

One contribution of 17 to a Discussion Meeting Issue 'Glycolipids in cell biology and medicine'.

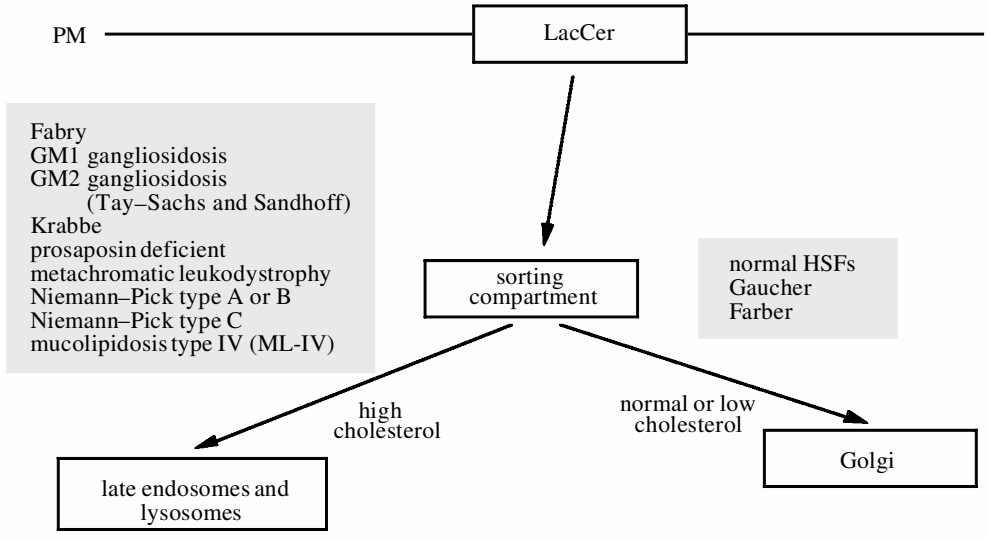

Figure 1. Intracellular targeting of BODIPY-LacCer in normal versus SLSD fibroblasts. Following insertion into the PM, LacCer is internalized by a caveolar mechanism and transported to the Golgi apparatus of normal fibroblasts or to late endosomes and lysosomes in SLSD fibroblasts. Two exceptions to the latter are Gaucher and Farber disease fibroblasts, in which LacCer targeting is indistinguishable from normal cells.

with defatted bovine serum albumin to remove any fluorescent lipid remaining at the PM (Puri *et al.* 2001) (or acid stripped to remove CtxB from the cell surface; Shogomori & Futerman (2001)), and the specimens are viewed under the fluorescence microscope. Internalization can be quantified by image processing or by biochemical analysis of the amount of cell-associated fluorescence.

In principle, GSLs at the PM can be internalized by one or more of the mechanisms depicted in figure 3. In addition to the classical clathrin-dependent endocytosis, multiple clathrin-independent mechanisms of uptake have been described. These include internalization through caveolae, phagocytosis, macropinocytosis, pinocytosis and constitutive clathrin-independent endocytosis (Anderson 1998; Nichols & Lippincott-Schwartz 2001; Johannes & Lamaze 2002). While it is relatively easy to determine whether internalization is clathrin dependent or not, the number of distinct clathrin-independent mechanisms that exist and the appropriate molecular criteria for each is not always clear, making it difficult to rigorously identify the specific subtype of clathrin-independent endocytosis that might be operative. For BODIPY-LacCer, internalization is almost completely inhibited in cells expressing DN dynamin-2 but is unaffected by DN Eps15 or by pharmacological inhibitors specific for the clathrin mechanism (Puri *et al.* 2001). These results demonstrate that LacCer internalization occurs by a clathrin-independent process. Additional studies demonstrated that pretreatment of cells with nystatin, filipin or genistein, three agents that disrupt caveolar endocytosis in other cell types (Rothberg *et al.* 1992; Orlandi & Fishman 1998; Aoki *et al.* 1999; Liu & Anderson 1999), severely inhibit LacCer internalization in HSFs (Puri *et al.* 2001). Finally, BODIPY-LacCer and fluorescent CtxB, a marker for caveolae in some cell types (Parton 1994; Orlandi & Fishman 1998), extensively co-localize during their initial internalization from the PM, while fluorescent CtxB, in turn, co-localizes with GFP–caveolin-1 (Puri *et al.* 2001). Based on these results, we suggest that the uptake of the fluorescent LacCer analogue in HSFs occurs by a caveolar-related mechanism.

In preliminary studies, we have extended this work using some of the other markers and inhibitors shown in figure 3, and find that LacCer internalization: (i) was stimulated by expression of caveolin-1; (ii) co-localized extensively with fluorescent albumin, a marker for caveolar internalization (Schubert *et al.* 2001), but not with blue dextran, a fluid phase marker; and (iii) was not altered in cells transfected with DN constructs of RhoA or cdc42 (D. K. Sharma and R. E. Pagano, unpublished observations), two GTPases that inhibit other forms of clathrin-independent endocytosis (Sabharanjak *et al.* 2002; refer to figure 3). These results further strengthen our conclusion that BODIPY-LacCer is initially internalized almost exclusively by caveolae in HSFs. In addition, other GSL analogues (e.g. BODIPY-GM1 ganglioside) and CtxB (a marker for endogenous GM1 ganglioside at the PM) are internalized by the same mechanism as the LacCer analogue in this cell type. Finally, it is important to note that the mechanism of internalization of LacCer is identical in both normal and SLSD fibroblasts (Puri *et al.* 2001).

3. GOLGI TARGETING OF CAVEOLAR-INTERNALIZED GSLs

We further characterized the intracellular transport of LacCer and found that in normal cells, Golgi targeting was dependent on microtubules and phosphoinositide 3-kinase(s), and was inhibited by expression of DN rab7 (mediates early to late endosome, and late endosome to lysosome transport) and rab9 (involved in late endosome to Golgi transport). This inhibition of LacCer targeting to the Golgi in DN rab7- or rab9-expressing cells is not due to the disruption of the Golgi apparatus since staining of this organelle with fluorescent ceramide (Choudhury *et al.* 2002) was normal. Golgi targeting of GSLs is independent of rab11, a rab protein that regulates recycling endosome to PM transport. These results suggest that LacCer passes through a late endosomal compartment en route to the Golgi apparatus.

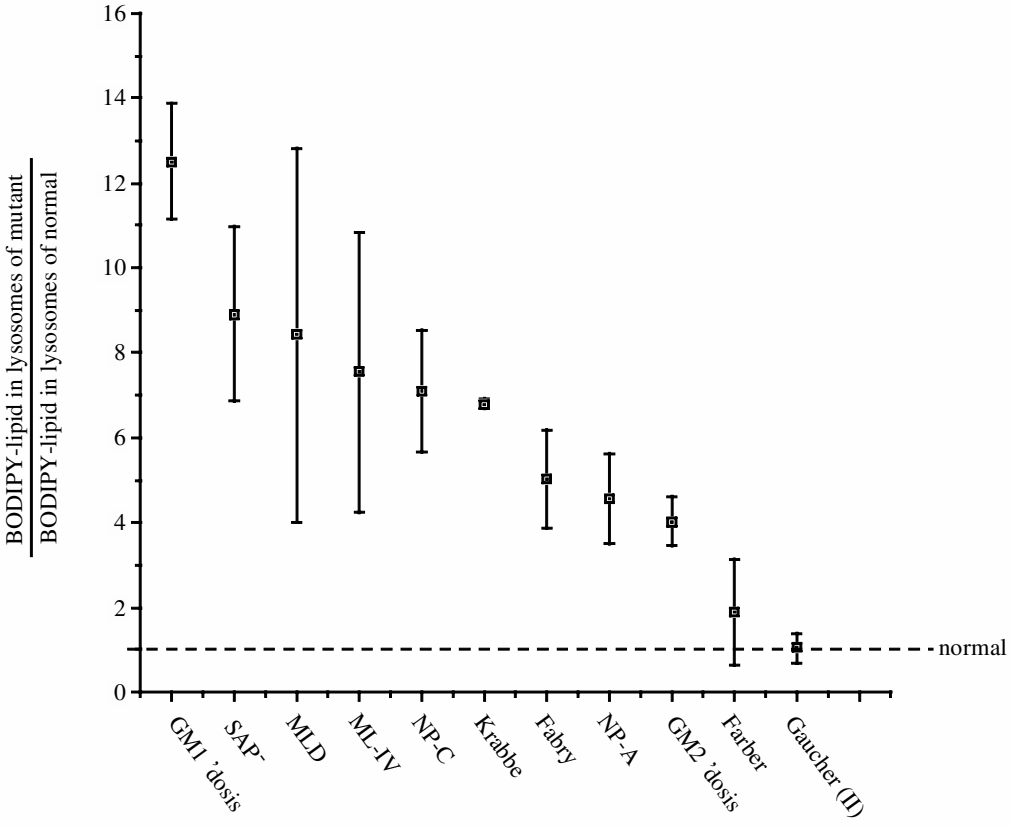

Figure 2. Accumulation of BODIPY-LacCer in endosomes/lysosomes of SLSD cells (Puri *et al.* 1999). Cells were pulse-labelled with BODIPY-LacCer and the amount of fluorescent lipid present in blue-dextran-stained lysosomes, relative to control cells, was quantified by image processing. Values represent the mean and s.d. of measurements of lipid accumulation in several hundred lysosomes in three or more cells for each dataset. Both the Tay–Sachs and Sandhoff forms of GM2 'dosis were used in these experiments. ML-IV, mucolipidosis type IV. (Reprinted, with permission, from *Nature Cell Biology* **1**, 386–388; copyright © 1999, Macmillan Publishers Ltd.)

As noted above, in SLSD cells, Golgi targeting is blocked, leading to LacCer and CtxB accumulation in endosomes. Since each SLSD cell type has a unique biochemical defect with respect to SL degradation (except for mucolipidosis type IV and NP-C cells in which lipids accumulate even though they have no primary deficiency in their lysosomal hydrolases; Bach 2001; Patterson *et al.* 2001), it is not obvious what common feature links the different SLSDs with respect to LacCer targeting. Each SLSD is expected to store various (and different) endogenous SLs. Thus, it seems unlikely that one particular SL would accumulate in all the different SLSDs. However, since SLs interact with cholesterol, another possibility is that the *distribution* and/or *amount* of intracellular cholesterol is altered in SLSD cells, perhaps as a result of such interactions in the late endosomes or lysosomes where the endogenous SLs accumulate. Indeed, there is increased filipin staining (indicative of high levels of intracellular cholesterol) in all of the SLSD cell types that accumulate BODIPY-LacCer, except that filipin staining of GM2 gangliosidosis cells (Tay–Sachs variant) is similar to that of control fibroblasts (Puri *et al.* 1999). To examine the possible effects which cholesterol might have on the intracellular targeting of LacCer, cells were grown under conditions to deplete cellular cholesterol and then pulse-labelled with the fluorescent lipid. Cholesterol depletion was found to dramatically alter the intracellular targeting of the LacCer analogue and CtxB (Puri *et al.* 1999, 2001). In normal fibroblasts, depletion of cellular cholesterol enhanced labelling of the Golgi complex by BODIPY-LacCer, while in the various SLSD cell types, depletion of cellular cholesterol almost completely eliminated the punctate endosomal pattern of fluorescence and enhanced Golgi labelling. Interestingly, incubation of normal HSFs with high amounts of low-density lipoprotein resulted in an elevation of cellular cholesterol, a loss of BODIPY-LacCer Golgi targeting, and a concomitant appearance of punctate endosomal structures similar to those seen in SLSD cells (Puri *et al.* 1999). These experiments suggest that cholesterol plays a major role in modulating the intracellular targeting of the LacCer analogue in normal and SLSD fibroblasts.

4. MODEL FOR ALTERED SORTING IN SLSD CELLS

The results described above lead us to the working hypothesis that SL accumulation in SLSD cells induces a redistribution of cholesterol which, in turn, leads to an alteration in the intracellular sorting of GSLs endocytosed via caveolae:

accumulation of endogenous lipids →
redistribution of cholesterol →
altered sorting and transport of caveolar-internalized GSLs

Since the strength of SL-cholesterol interactions varies

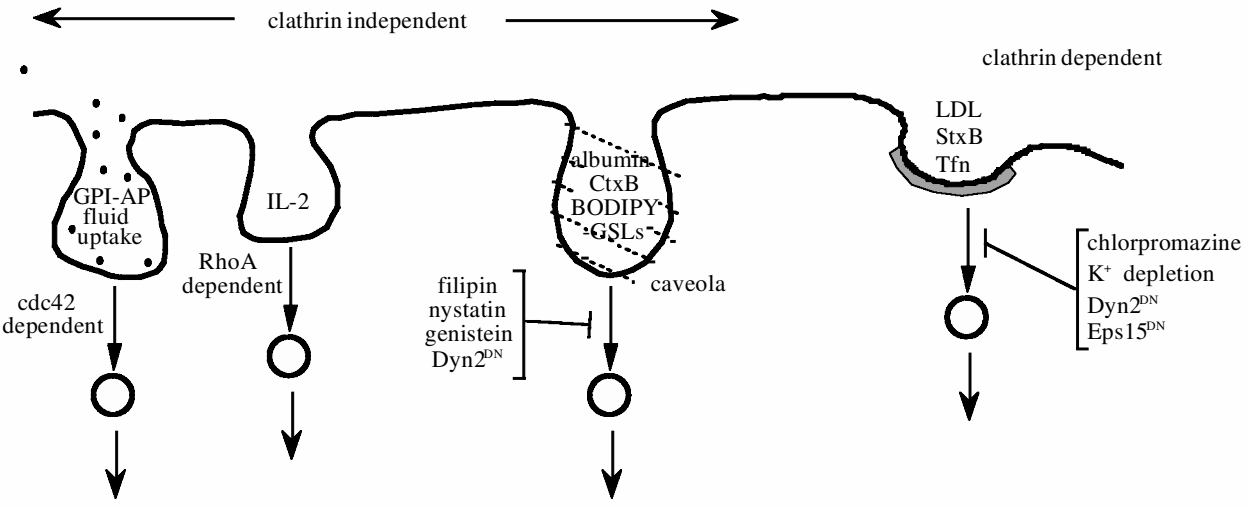

Figure 3. Potential mechanisms of lipid internalization in HSFs. Clathrin-dependent and clathrin-independent mechanisms of internalization are illustrated together with useful markers and pathway-specific inhibitors. Dyn 2DN, DN dynamin 2; GPI-AP, glycosylphosphatidylinositol-anchored proteins; IL-2, interleukin-2; LDL, low-density lipoprotein; StxB, shiga toxin; Tfn, transferrin.

depending on the particular SL species (Slotte *et al.* 1993; Wang & Silvius 2000), it seems reasonable that redistribution of cholesterol will also vary among the different SLSDs since each one accumulates a different complement of endogenous lipids. This could explain the differences in LacCer accumulation that are observed among the different SLSDs. Weak SL–cholesterol interactions could also be an explanation for our finding that LacCer targeting in Gaucher or Farber cells (accumulate, respectively, endogenous glucosylceramide or ceramide) is indistinguishable from that in normal HSFs. Interestingly, when high levels of glucosylceramide are induced artificially in Gaucher fibroblasts, mis-targeting of LacCer, similar to that seen in other SLSD cell types, is observed (Sillence *et al.* 2002).

Altered sorting and transport of caveolar-internalized GSLs could potentially occur by *physical interactions between SLs and cholesterol.* For example, BODIPY-LacCer could partition into SL/cholesterol-enriched microdomains at the PM or in endosomes, and the presence of high levels of SLs and cholesterol (e.g. in SLSD cells) would cause an increased fraction of SL/cholesterol-enriched microdomain units to be shunted to the degradative pathway. This shunting could occur by exceeding the capacity of the recycling machinery, or by lipid environmental effects on proteins involved in sorting. Another potential mechanism by which altered sorting and transport could occur involves 'molecular trapping' of the LacCer analogue in internal, multilamellar membranes which are present in the endosomes and lysosomes of SLSD cells (Prasad *et al.* 1996). Namely, as the fluorescent SL analogue moves through the endocytic pathway, these lipid stores could become increasingly accessible and represent a 'sink' into which the SL analogue could partition, resulting in 'trapping' of the SL analogue in the internal membranes of the endosomes/lysosomes through interactions with endogenous SLs, cholesterol, and perhaps other stored lipids such as lyso-bis-phosphatidic acid (Kobayashi *et al.* 1999). Another mechanism by which endogenous SL accumulation could cause cholesterol alterations and other alterations in lipid trafficking involves the *possible action of SLs as signalling molecules.* Indeed SLs, either directly through interactions with cell surface proteins or indirectly through the generation of lipid second messengers, have been documented to induce various biological responses (for reviews, see Chatterjee 1998; Kolesnick *et al.* 2000; Shayman 2000; Hannun *et al.* 2001; Hakomori 2002; Spiegel *et al.* 2002). Thus, SLs (or their metabolites) accumulated as a result of defective degradation could initiate a signalling event that results in alterations in the expression or distribution of proteins involved in vesicular transport or cholesterol homeostasis, leading to defects in lipid trafficking.

5. MODULATION OF MEMBRANE TRAFFICKING TO CORRECT THE SLSD PHENOTYPE

The studies cited above demonstrate that high cellular cholesterol can block Golgi targeting of BODIPY-LacCer (and other GSL analogues), while cholesterol depletion of SLSD cells restores Golgi targeting of these lipids. Since DN rab7 or rab9, as well as high levels of intracellular cholesterol, block BODIPY-LacCer targeting to the Golgi apparatus, we speculated that overexpression of WT rab7 or rab9 might overcome the block in Golgi targeting of LacCer seen in SLSD cells.

To test this concept we used NP-C fibroblasts since these cells have high levels of intracellular cholesterol and a severe reduction in vesicular traffic through late endosomes (Neufeld *et al.* 1999; Ko *et al.* 2001; Patterson *et al.* 2001). When NP-C cells were transfected with WT rab7 or rab9 (but not rab11) and then pulse-labelled with fluorescent LacCer, there was pronounced targeting of BODIPY-LacCer to the Golgi apparatus and a reduction in endosomal accumulation of the fluorescent lipid in 60–70% of the transfected cells (Choudhury *et al.* 2002; figure 4*a*). Importantly, similar results to those described above have been obtained using Rh-CtxB, which binds to endogenous GM1 ganglioside at the cell surface and is subsequently targeted to the Golgi complex of normal HSFs or to endosomal structures in SLSD cell types (figure 4*a*). Thus, the restoration of normal lipid traffick-

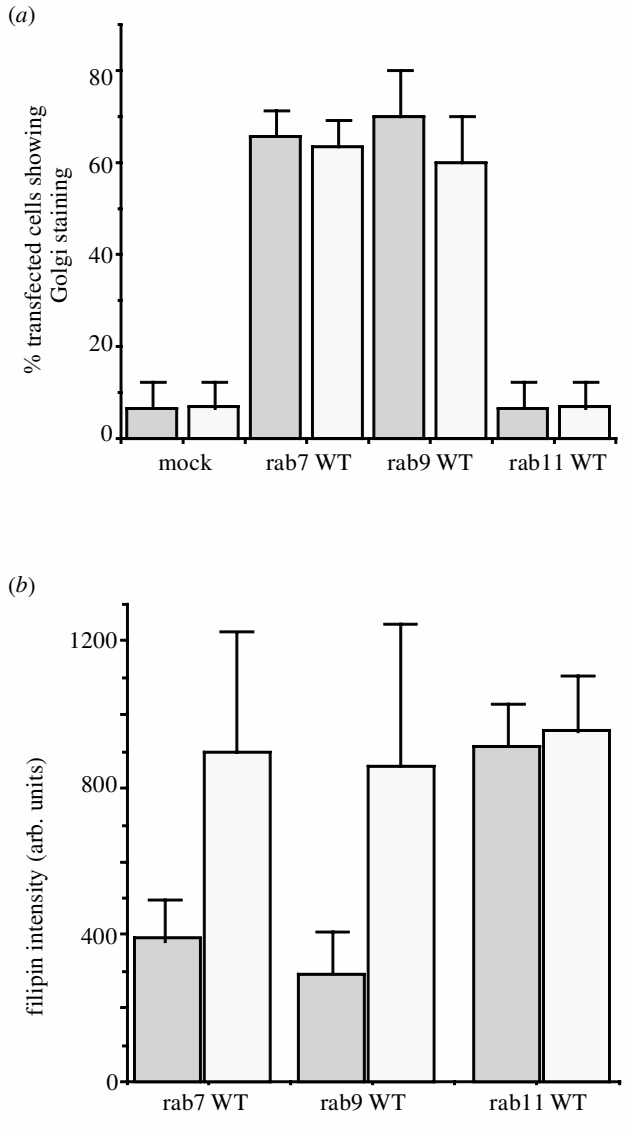

Figure 4. Overexpression of WT rab7 or rab9 (but not rab11) restores normal lipid trafficking and reduces cholesterol accumulation in NP-C fibroblasts. NP-C cells were transfected with plasmids encoding red or green fluorescent-fusion proteins of WT rab7, 9, or 11. Forty-eight hours later the samples were (*a*) pulse-labelled with BODIPY-LacCer (dark grey) or Rh-CtxB (light grey), or (*b*) stained with filipin to examine the distribution of free cholesterol. In (*a*), transfected cells were identified by red fluorescence and then LacCer fluorescence (observed at green wavelengths) was scored as Golgi-positive or -negative. Values are means of three independent experiments ($n = 10$) for each condition. In (*b*), transfected cells (dark grey) were identified by green fluorescence and total filipin fluorescence in individual transfected, or adjacent non-transfected (light grey), cells was measured by image analysis. Values are mean ± s.d. from a typical experiment ($n = 50$). Similar results were obtained in each of three independent experiments. (Reprinted, with permission, from *Journal of Clinical Investigation* **109**, 1541–1550; copyright 2002.)

ing in SLSD cells by rab overexpression also occurs for endogenous SLs at the PM.

NP-C cells transfected with WT rab7 or rab9 (but not rab11) constructs show dramatically reduced filipin staining compared with non-transfected cells, while DN constructs of rab7 or rab9 have little or no effect on filipin staining in NP-C cells (Choudhury *et al.* 2002). Quantitative analysis showed that filipin fluorescence was consistently reduced more than 50% in cells transfected with the WT rab7 or rab9 constructs compared to adjacent non-transfected cells in the same field (figure 4*b*). By contrast, overexpression of EGFP–rab11WT fusion protein showed almost no difference in filipin staining compared with non-transfected cells. Consistent with the reduction in intracellular free cholesterol illustrated by lower filipin staining, cells transfected with WT rab7 or rab9 (but not WT rab11) also showed an almost twofold increase in Nile Red staining of cholesterol esters (and other neutral lipids) (Choudhury *et al.* 2002). These results suggest that these rab proteins might accelerate transport of cholesterol to the endoplasmic reticulum for subsequent esterification.

Thus, overexpression of WT rab7 or rab9 corrects defective lipid trafficking and abrogates cholesterol storage in NP-C cells. Similar results have been obtained using GM1 gangliosidosis cells; however, we do not yet know if WT rab7 or rab9 overexpression will correct defective LacCer targeting and lipid accumulation in other SLSD cell types.

The mechanism(s) by which rab overexpression corrects the SLSD phenotype is unknown. One potential mechanism may be through *modulation of endosomal lipid composition*. As noted above, high concentrations of cholesterol and SLs that accumulate in the endosomes of NP-C cells could perturb endosomal sorting and reduce the amount of SL that can be transported to the Golgi. Overexpression of WT rab7 or rab9 may increase the transport of SLs and/or cholesterol from the compartment where aberrant sorting occurs, thereby changing its composition. This change would be sufficient to restore normal sorting and trafficking. Another potential mechanism by which overexpression of WT rab proteins may correct lipid trafficking defects involves *cholesterol interactions with rab-GTPases or rab-associated proteins*. In this scheme, high levels of cellular cholesterol might alter the distribution of rab proteins, affect a critical post-translational modification (e.g. prenylation) of the rab proteins required for optimal activity, or affect the state of activation (e.g. GDP- versus GTP-bound) of the rab protein (Cavalli *et al.* 2001). Thus, the GSL targeting defect reviewed here may be caused by a deficit of properly targeted or GTP-charged rabs, and overexpression of WT rab7 or rab9 may provide sufficient levels of active rabs to overcome this defect.

6. SUMMARY AND FUTURE DIRECTIONS

Most strategies for treating SLSDs deal either with enhancing degradation of the stored lipids (e.g. by gene therapy, enzyme replacement therapy, or by 'substrate deprivation'; Abe *et al.* (2000), Butters *et al.* (2000), Cabrera-Salazar *et al.* (2002), Schiffmann & Brady (2002), Brady (2003), Gieselmann *et al.* (2003), Butters *et al.* (2003)). In the latter, GSL synthesis is reduced by inhibition of glucosylceramide synthase, a key enzyme in the synthesis of higher-order GSLs. Although the mechanism by which rab overexpression abrogates the SLSD phenotype requires further study, our findings suggest a possible alternative for therapy in which vesicle trafficking and sorting along the endocytic pathway are modulated to pro-

mote the clearance of stored lipids. We are currently exploring various methods for optimizing the efficiency of correction *in vitro*, including protein transduction (Schwarze *et al.* 1999) and viral transformation, and hope to be able to extend these approaches to animal models of SLSDs in the near future.

This work was supported by USPHS grants GM-22942 and GM-60934 and a grant from the Ara Parseghian Medical Research Foundation.

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GLOSSARY

BODIPY: 4,4-difluoro-4-bora-3a,4a-diaza-*s*-indacene
CtxB: cholera toxin B subunit
DN: dominant negative
GFP: green fluorescent protein
GSL: glycosphingolipid
HSF: human skin fibroblast
LacCer: lactosylceramide
NP-C: Niemann–Pick C
PM: plasma membrane
Rh: rhodamine
SL: sphingolipid
SLSD: sphingolipid storage disease
WT: wild-type