Ceramide Accumulation Uncovers a Cycling Pathway for the cis-Golgi Network Marker, Infectious Bronchitis Virus M Protein

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Abstract. The M glycoprotein from the avian coronavirus, infectious bronchitis virus (IBV), contains information for localization to the cis-Golgi network in its first transmembrane domain. We hypothesize that localization to the Golgi complex may depend in part on specific interactions between protein transmembrane domains and membrane lipids. Because the site of sphingolipid synthesis overlaps the localization of IBV M, we asked whether perturbation of sphingolipids affected localization of IBV M. Short-term treatment with two inhibitors of sphingolipid synthesis had no effect on localization of IBV M or other Golgi markers. Thus, ongoing synthesis of these lipids was not required for proper localization. Surprisingly, a third inhibitor, d,l-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP), shifted the steady-state distribution of IBV M from the Golgi complex to the ER. This effect was rapid and reversible and was also observed for ERGIC-53 but not for Golgi stack proteins. At the concentration of PDMP used, conversion of ceramide into both glucosylceramide and sphingomyelin was inhibited. Pretreatment with upstream inhibitors partially reversed the effects of PDMP, suggesting that ceramide accumulation mediates the PDMP-induced alterations. Indeed, an increase in cellular ceramide was measured in PDMP-treated cells. We propose that IBV M is at least in part localized by retrieval mechanisms. Further, ceramide accumulation reveals this cycle by upsetting the balance of anterograde and retrograde traffic and/or disrupting retention by altering bilayer dynamics.

The organelles of the classical secretory pathway must maintain their identity despite a large flux of lipids and proteins. Two models have emerged to explain how proteins can be maintained in specific compartments (Machamer, 1993; Nilsson and Warren, 1994). The retention model proposes that proteins are efficiently anchored in the appropriate compartment. The retrieval model proposes that proteins are continually recycled from later compartments. The two models are not mutually exclusive; indeed most proteins within the secretory pathway probably use both mechanisms for localization, albeit to differing extents. An example of this is the localization of the ER resident protein BiP, which contains a KDEL retrieval signal but is only slowly secreted when this signal is removed (Munro and Pelham, 1987). This suggests that other parts of the molecule may contain retention information.

The M glycoprotein from the avian coronavirus, infectious bronchitis virus (IBV), is a model protein for studying localization to the early secretory pathway. Immuno-electron microscopy showed that IBV M expressed in the absence of other IBV proteins was found in the tubulovesicular structures at the entry or cis face of the Golgi stack, as well as the first or second cisterna of the Golgi stack (Machamer et al., 1990; Sodeik et al., 1993). We will refer to this region as the cis-Golgi network (CGN; Mellman and Simons, 1992). Defined in this way, we would consider the CGN to at least partially overlap with the intermediate compartment (IC), defined by such markers as ERGIC-53 or p58 (Schweizer et al., 1988; Lahtinen et al., 1996). The first transmembrane domain of IBV M is sufficient to target chimeric proteins to the CGN (Swift and Machamer, 1991; Machamer et al., 1993). Although the transmembrane domains of other Golgi proteins also contain targeting information, no consensus motif for localization has been identified (for review see Colley, 1997). We are intrigued by the possibility that the targeting of Golgi membrane proteins may in part depend on interactions between their transmembrane domains and specific membrane lipids.

Independent studies demonstrate that the lipid compositions of membranes differ at each stage of the secretory pathway.
Materials and Methods

Materials

FCS was from Atlanta Biologicals (Norcross, GA); fumonisin B1 was from Calbiochem (La Jolla, CA); chromatography plates were from E. Merck (Darmstadt, Germany); endoglycosidase H (endo H) from New England Biolabs (Beverly, MA); N-hexanoylophosphoglycerine (C6Cer), lipid standards, and PDMP were from Matreya (Pleasant Gap, PA); [3H]palmitate (50 Ci/mmol) was from DuPont-NEN (Wilmington, DE); Pro-Mix (1,000 μCi/ml [3H]methionine) was from Amersham Intl. (Arlington Heights, IL); DME, tissue culture-grade trypsin, and penicillin-streptomycin were from Gibco BRL (Gaithersburg, MD); all other reagents were from Sigma Chemical Co. (St. Louis, MO).

Antibodies were obtained as follows: monoclonal anti-Bip, Stressgen (Victoria, BC, Canada); monoclonal anti-giantin and monoclonal anti-ERGIC-53, Hans-Peter Hauri (Basel, Switzerland); polyclonal anti-giantin and monoclonal anti-vimentin antibodies, Jackson ImmunoResearch (West Grove, PA).

Methods

Cell Culture. Tissue culture cells were grown in DME supplemented with 5% (BHK-21) or 10% (Vero) FCS. Cells were grown at 37°C in an atmosphere of 5% CO2.

Pulse-Chase Labeling. Pulse-chase experiments were performed as previously described (Rosenwald et al., 1992; Weiss et al., 1993). Briefly, cells were plated in 35-mm dishes the night before the experiment to be 90% confluent the next day. VSV (San Juan strain, Indiana serotype) was adsorbed for 30 min in 0.5 ml of serum-free DME. At 4 h after infection, the cells were starved for methionine for 15 min. Cells were then pulsed for 5 min with 50 μCi [35S]Pro-mix and chased for the indicated amount of time in the presence or absence of the indicated drugs. The isopropyl alcohol carrier alone had no effect on the rate of transport of VSV G protein. After the chase, cells were washed once with cold PBS and lysed in detergent. VSV G protein was then immunoprecipitated, treated with endo H as described (Rosenwald et al., 1992), separated by SDS-PAGE, and visualized by fluorography. Endo H-sensitive and -resistant forms of the VSV G protein were quantitated by densitometry.

Indirect Immunofluorescence Microscopy. These experiments were performed as previously described (Swift and Machamer, 1991). Briefly, cells were infected for 30 min with a recombinant vaccinia virus encoding IBV M, and the indicated treatments were begun 4 h after infection. For experiments using exogenous ceramides, the soluble short-chain analogues of ceramide were added to cells as a complex with 0.34 mg/ml defatted BSA as described (Pagano and Martin, 1988). After treatment, cells were fixed, permeabilized, and stained with the appropriate antibody. Images were acquired using a microscope (Axioskop; Zeiss, Inc., Thornwood, NY) equipped with epifluorescence and a CCD camera (Photometrics Sensis, Tucson, AZ) using IP Lab software (Signal Analytics Corp., Vienna, VA). All images shown are the raw data collected at 1×1 binning with a gain of 1.

Lipid Synthesis Assays. Cells were seeded onto 6-cm plastic dishes 2 d before the experiment so that they were 90% confluent the day of the experiment. Cells were incubated in DME supplemented with 50 μg/ml cycloheximide, and half of the dishes were also incubated with 5 mM βCA. 1 h later, fresh medium containing cycloheximide and [3H]palmitate (100 μCi/dish) was added, along with either 1% isopropanol (control and βCA) or 100 μM PDMP and 5 mM βCA if indicated. 1 h later, cells were trypsinized and washed off the plate in 0.8 ml cold PBS. To normalize lipid levels, 50 μl was removed for a protein assay by the method of Bradford (1976). Lipids were then extracted by the method of Bligh and Dyer (1959). Labeled samples were doped with 10 μg cold ceramide to allow for visualization. Samples were run on 10 × 10 cm high performance thin-layer chromatography plates with a mobile phase of chloroform/glacial acetic acid (9:1; Abe et al., 1992). Plates were sprayed with water to visualize the ceramide bands that were scraped and counted after the addition of scintillation fluid. Alternatively, plates were dipped in 10% 4,5-diphenyl-2-oxazol in chloroform for visualization by fluorography (Henderson and Tocher, 1992).

Results

Inhibition of Sphingolipid Synthesis by PDMP but Not βCA or FB1 Causes a Mislocalization of IBV M Protein to the ER

To test whether ongoing sphingolipid synthesis was necessary for the correct localization of the IBV M protein, we used three inhibitors of sphingolipid synthesis: β-choloroalnine (βCA; Medlock and Merrill, 1988), fumonisin B1 (FB1; Wang et al., 1991), and PDMP (Vunnam and Radin, 1980; Inokuchi and Radin, 1987). The biosynthetic pathway for sphingolipids with the sites of inhibition of these drugs is shown in Fig. 1. Both βCA (5 mM) and FB1 (100 μM) inhibited the incorporation of radiolabeled precursors into sphingolipids in BHK-21 cells by >90% (data not shown). Consistent with previous results (Rosenwald et al., 1992), we found that PDMP (100 μM) inhibited GlcCer synthesis by ~40% and SM synthesis by ~50% in BHK-21 cells (data not shown).

To express the IBV M protein, BHK-21 cells were infected with a recombinant vaccinia virus encoding IBV M (Machamer and Rose, 1987). 4 h after infection the cells were treated with cycloheximide for 1 h to chase newly synthesized M protein out of the ER. Sphingolipid synthesis inhibitors were then added and the cells incubated for another 1 h before processing for immunofluorescence (Fig. 2). In control cells, IBV M exhibited a tight, juxtanuclear staining pattern that colocalized with Golgi markers. The Golgi localization pattern of IBV M was unchanged by treatment with either βCA or FB1, suggesting that ongoing sphingolipid synthesis is not required for proper localization of IBV M. In contrast, PDMP-treated cells showed a marked change in the staining pattern of the IBV M pro-
tein after 1 h. The localization of IBV M in the presence of PDMP changed from Golgi to ER based on the absence of strong juxtanuclear staining and the presence of nuclear rim staining and a tubulo-reticular staining pattern that colocalized with ER markers. Treatment of infected cells with lower concentrations of PDMP had no effect on IBV M localization and very little effect on SM synthesis (data not shown). We next tested the kinetics of PDMP-induced mislocalization of IBV M. Cells were infected as before and treated for 1 h with cycloheximide. Then cells were treated with PDMP for varying lengths of time up to 1 h (Fig. 3). Redistribution was first observed at 15 min and became maximal at 60 min. This redistribution was rapidly reversible, such that 30 min after washing out PDMP, IBV M had moved back to the Golgi region.

**PDMP Treatment Does Not Mislocalize Golgi Markers, but Does Mislocalize ERGIC-53**

We next asked if PDMP mislocalized other Golgi markers. Cells were infected and treated as described above and then stained with antibodies to two integral membrane proteins of the Golgi stack, giantin (Linstedt and Hauri, 1993) and mannosidase II (Man II; Moremen and Robbins, 1991; Velasco et al., 1993). Treatment of cells with 100 μM PDMP for 1 h had no effect on the localization of either protein (Fig. 4), suggesting that the morphology of the Golgi was not greatly altered. We also examined the localization of β-COP, a peripheral membrane protein of the stack and CGN involved in vesicular traffic (Oprins et al., 1993). As seen in Fig. 4, the distribution of β-COP appeared unaffected in treated cells.

We then looked at a protein which cycles through the cis-Golgi. As we did not have access to antibodies that crossreact with known markers in BHK-21 cells, we examined ERGIC-53 in Vero cells. IBV M localizes to the CGN in these cells and is also redistributed to the ER by PDMP (data not shown). ERGIC-53 is an IC protein that cycles between the ER, IC, and Golgi stack (Lippincott-Schwartz et al., 1990; Schindler et al., 1993). Vero cells were treated...
Figure 5. PDMP induced the redistribution of the endogenous IC protein, ERGIC-53, to the ER. Vero cells were incubated with cycloheximide for 1 h and then incubated for 1 h in the presence of 1% isopropanol (control) or 100 μM PDMP. Cells were then fixed and prepared for indirect immunofluorescence with antibodies to ERGIC-53. For each experimental group, the Nomarski image is shown on the left and the fluorescence image on the right. Bar, 10 μm.

PDMP but Not βCA or FB1 Slows the Anterograde Traffic of VSV G

The above immunofluorescence results suggested that PDMP might generally alter the distribution of proteins that have a dynamic localization mechanism. Interestingly, it has been shown that PDMP slows the rate of both anterograde vesicular traffic (Rosenwald et al., 1992) and endocytosis (Chen et al., 1995) in CHO cells. We tested whether PDMP also slowed anterograde traffic in BHK-21 cells. We used a plasma membrane protein, the well-characterized G protein from VSV. A pulse–chase experiment was performed in the presence or absence of PDMP, βCA, or FB1. The rate of accumulation of endo H–resistant VSV G was used as an assay for the rate of arrival at the medial-Golgi. Neither βCA nor FB1 had any effect on the localization of ERGIC-53 (data not shown).

The PDMP Effect on Anterograde Traffic Is Ameliorated by Pretreatment with Either βCA or FB1

Fig. 6 shows that neither βCA nor FB1 had any effect on the transit rate of VSV G, suggesting that the decreased rate with PDMP was not due to a block in the ongoing synthesis of sphingolipids. PDMP inhibits the conversion of ceramide into glycosphingolipids and sphingomyelin. One possible explanation for the effects observed with PDMP was an accumulation of the precursor ceramide. βCA and FB1 act upstream of PDMP (Fig. 1), and the intermediates in sphingolipid synthesis between these steps and ceramide production are thought to be short lived (Merrill and Wang, 1986; Medlock and Merril, 1988). To ask if ceramide accumulation might mediate the PDMP-induced slowing of anterograde traffic, cells were treated with βCA or FB1 before PDMP. We performed a pulse–chase labeling experiment with cells pretreated with either βCA or FB1 for 1 h before being chased in the presence or absence of βCA or FB1 and PDMP (Fig. 6). Pretreatment with the earlier inhibitors reduced the PDMP-induced slowing of anterograde traffic by about half, suggesting that accumulation of newly synthesized ceramide at least partially mediates this effect.

Pretreatment with βCA or FB1 Reduces the PDMP-induced Mislocalization of IBV M Protein

To test whether ceramide was likely to mediate the PDMP-induced mislocalization of IBV M, indirect immunofluorescence was performed as above with cells pretreated with either βCA or FB1 for 1 h before the addition of PDMP. To simplify the quantification, the localization of IBV M was classified into one of three staining patterns. Class 1 was the most commonly seen pattern in untreated cells, i.e., tight juxta-nuclear staining that colocalized with Golgi markers. Class 3 was the most commonly seen pattern in PDMP-treated cells, i.e., diffuse staining with prominent nuclear envelope staining that colocalized with ER markers. Class 2 was intermediate between the class 1 and class 3. Whether this pattern represents an overlap of ER and Golgi staining patterns or an increase in IC staining is not clear. For these experiments, coded samples were quantified by counting at least 100 cells for each sample. The experiment was performed five times, with one representative experiment shown (Fig. 7). Interestingly, when cells were quantified in this way, we observed that both βCA and FB1 caused a slight shift from class 1 to class 2. The significance of this observation is not clear. PDMP dramatically shifted the distribution of IBV M from mainly class 1 in the control cells to mainly class 3. Pretreatment with either βCA or FB1 significantly reduced this shift in the staining pattern of IBV M, suggesting that accumula-
tion of newly synthesized ceramide mediates the effects of PDMP on localization of IBV M.

**PDMP Increases Cellular Ceramide Levels**

The pretreatment experiments suggested that increased levels of ceramide mediate the PDMP-induced effects on IBV M localization. Ceramide can act as a second messenger (for review see Hannun, 1994) and has been suggested to be the mediator of some of the effects of PDMP in CHO cells (Rosenwald and Pagano, 1993). Furthermore, in several systems, cellular levels of ceramide increased upon treatment with PDMP or one of its more soluble analogues (for example see Rani et al., 1995; Posse de Chaves et al., 1997). We measured the levels of ceramide after various treatments by pulse–labeling with \(^3\)H palmitate. After labeling and treatment, cellular lipids were extracted and run on chromatography plates. The appropriate spots corresponding to ceramide were scraped and counted. Consistent with our hypothesis, Fig. 8 A shows that PDMP treatment increased the levels of ceramide 2.5-fold over control levels. Pretreatment with \(\beta\)CA reduced PDMP-induced ceramide accumulation to roughly half of control levels, but this level was still threefold higher than cells treated with \(\beta\)CA alone. Fig. 8 B is a representative fluorograph showing the ceramide region that was quantified in Fig. 8 A. Interestingly, we noticed the appearance of a lower migrating ceramide band in PDMP-treated cells.

**A Soluble analogue of Ceramide Does Not Affect the Localization of IBV M or ERGIC-53**

The pretreatment experiments as well as lipid analysis implicated ceramide as the mediator of PDMP effects. If this were true, addition of a soluble, short-chain analogue of ceramide might mimic the effects of PDMP. Indeed, one such cell-permeable analogue of ceramide, \(N\)-hexanoylsphingosine (C \(_6\) Cer), reproduces the effects of PDMP on anterograde traffic of VSV G in CHO cells (Rosenwald and Pagano, 1993). We first asked whether C \(_6\) Cer slowed anterograde traffic in BHK-21 cells. A pulse–chase labeling experiment with VSV-infected cells was performed, with cells chased in the absence or presence of 25 \(\mu\)M C \(_6\) Cer. C \(_6\) Cer slowed the rate of anterograde traffic in BHK-21 cells \(\approx 2.5\)-fold (data not shown). We went on to use C \(_6\) Cer in our indirect immunofluorescence assay. As expected, C \(_6\) Cer had no effect on the localization of either Man II or \(\beta\)-COP. However, contrary to our expectation, C \(_6\) Cer did not alter the localization of either IBV M or ERGIC-53. This suggests that exogenously added C \(_6\) Cer and ceramide generated by treatment with PDMP do not have the same effects. Two other soluble analogues of ceramide, C \(_2\) Cer and C \(_8\) Cer, were also tested and found to have no effect on the localization of IBV M (data not shown).

**Discussion**

**Steady-State Localization of IBV M Involves Cycling through the ER**

There are two possible mechanisms to maintain the
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GIC-53. BHK cells (IBV M left and the fluorescence image on the right. Bar, 10 μm. For each experimental series, the Nomarski image is shown on the left and the fluorescence image on the right. Bar, 10 μm.

steady-state localization of proteins to specific sites along the secretory pathway: (a) retention in the particular compartment, and (b) retrieval from other compartments (Machamer, 1993; Nilsson and Warren, 1994). The redistribution of IBV M induced by PDMP was surprising, as we expected that if we disrupted its localization, it would move with “bulk flow” to the plasma membrane. That IBV M was redistributed to the ER suggests that it has specific targeting information for retrieval to the ER. Coupled with earlier work in our laboratory on the role of oligomerization of IBV M chimeras in CGN targeting (Weisz et al., 1993), our findings suggest that IBV M maintains its steady-state distribution by both retention and retrieval (Fig. 10 A). We propose two models to explain the redistribution of IBV M protein induced by PDMP. The simplest model (Fig. 10 B, left arrow) is that the M protein normally cycles between the ER and the Golgi at a significant rate, as has been shown for ERGIC-53 (Lippincott-Schwartz et al., 1990; Schindler et al., 1993). The slowing of anterograde traffic would cause the protein to shift its steady-state distribution to the ER, assuming that retrograde traffic is unaffected or perhaps increased by PDMP. A second possibility is that PDMP disrupts retention of the M protein, which then cycles back to the ER by normal retrieval mechanisms (Fig. 10 B, right arrow). These two models are not mutually exclusive, and both effects of PDMP may be necessary to redistribute IBV M to the extent observed.

Implicit in both models is the idea that IBV M contains retrieval information. PDMP-induced ER redistribution was also observed for ERGIC-53, a dilesine-containing IC marker known to cycle through the ER (Lippincott-Schwartz et al., 1990; Schindler et al., 1993). Double-label immunofluorescence in HeLa cells showed that the distribution of ERGIC-53 and IBV M overlapped significantly, though not completely (Sodeik et al., 1993). This suggests that IBV M may in part use the same, as yet unknown, cellular machinery used by IC proteins to maintain its steady-state localization. Using deletion mutants and chimeric molecules, dissection of IBV M retention and retrieval information should be possible using PDMP as a tool. The pathway followed by IBV M during retrieval will also be important to decipher. Experiments in nontreated cells suggest that the protein may move through later Golgi compartments even though its steady-state distribution is the CGN, because its oligosaccharides are slowly processed (Machamer et al., 1990).

Interestingly, we did not observe redistribution of two Golgi stack markers (Man II and giantin) to the ER with PDMP treatment. Golgi membrane proteins were recently suggested to be highly mobile (Cole et al., 1996b) and to cycle through the ER (Cole et al., 1996a). Our results with PDMP suggest that cycling of these proteins does not occur during the time scale of our experiments, and/or that its predominant effect on IBV M is a loss of retention.

Figure 10. Current models for how IBV M maintains its steady-state distribution and how PDMP might alter this distribution. (A) Evidence presented here shows that IBV M can be induced to redistribute to the ER, indicating that IBV M has information necessary for traffic to the ER. We propose that IBV M normally maintains its steady-state distribution by cycling through the ER at some basal rate. (B) If IBV M is cycling through the ER normally, then its localization would depend on the balance of anterograde and retrograde traffic rates. As PDMP slows anterograde traffic, IBV M could accumulate in the ER when the balance of membrane traffic is disrupted (left arrow). Alternatively, PDMP may act by disrupting the retention of IBV M, causing it to move out of the Golgi (right arrow). It is also possible that redistribution of IBV M requires both effects of PDMP.

Ceramide Mediates the Effects of PDMP

Interestingly, it has been shown that myriocin, an inhibitor of sphingolipid synthesis thought to inhibit the same step as βCA, reduces the rate of transport of a glycosylphosphatidylinositol-linked protein but not other proteins out of the ER in yeast (Horvath et al., 1994). It would be interesting to test whether inhibition of ceramide synthesis in mammalian cells also slows the rate of transport of glycosylphosphatidylinositol-linked proteins in mammalian cells. However, neither βCA nor FB1 had an effect on the rate of anterograde traffic of VSV G or the localization of IC, CGN, or Golgi stack proteins. When we quantified the effects of these two inhibitors on the immunofluorescence
staining pattern of IBV M, we did detect a slight shift toward the intermediate staining pattern (Fig. 7). Whether this represents an altered distribution pattern or subtle effects on Golgi structure caused by these inhibitors is unclear. From these results we conclude that ongoing sphingolipid synthesis is not required for either normal rates of anterograde traffic or proper localization of Golgi proteins. However, the glucosylceramide analogue PDMP, at a concentration that inhibits both GlcCer and SM synthases, causes a redistribution of IBV M to the ER and a slowing of anterograde traffic in BHK-21 cells. Why does PDMP have these effects while BCA and FB1 do not? It is likely that PDMP exerts its effects by causing the accumulation of ceramide. Other groups have found that the various effects of PDMP and its active analogues are concomitant with (Uemura et al., 1990; Shayman et al.; 1991; Rani et al., 1995) or actually caused by increases in ceramide concentrations (Abe et al., 1996; Posse de Chaves et al., 1997). In BHK-21 cells treated with 100 μM PDMP for 1 h we measured an increase in newly synthesized ceramide (Fig. 8 A), showing at least a correlation between levels of newly synthesized ceramide and the effects of PDMP on protein traffic and localization. The fact that pretreatment with either BCA or FB1, both of which block the synthesis of ceramide, can ameliorate the effects of PDMP demonstrates that ceramide is at least in part the mediator of these effects. However, the effects of PDMP are not completely abolished by pretreatment, even though BCA pretreatment reduced the level of newly synthesized ceramide by 50% compared to control. One explanation is that ceramide accumulation is only one of the ways that PDMP exerts its effects. Another possibility is that ceramide is being generated by the degradation of preexisting sphingolipids, e.g., at the cell surface or in lysosomes. This ceramide cannot be made back into sphingolipids because the enzymes SM and GlcCer synthases are inhibited in the presence of PDMP. Interestingly, the fluorograph of the ceramide bands generated after the various treatments (Fig. 8 B) shows different relative amounts of ceramide species. We suspect that these ceramides differ in acyl chain lengths (Abe et al., 1992) and that this may be an indication of different sources of ceramide, i.e., de novo synthesis or sphingolipid degradation. We are currently investigating this possibility.

Accumulation of Endogenous Ceramide by PDMP Treatment and Exogenous C6Cer Do Not Have the Same Effects

Based on the pretreatment results, we expected that soluble analogues of ceramide would mimic the effects of PDMP. Rosenwald and Pagano (1993) showed that like 100 μM PDMP, 25 μM C6Cer decreased the rate of transit of an itinerant protein to the medial-Golgi in CHO cells. Consistent with these results, 25 μM C6Cer also slowed anterograde traffic in BHK-21 cells. However, 25 μM C6Cer had no effect on the localization of either the M protein or ERGIC-53. Other short-chain ceramide analogues (C2- and C3-ceramide) also had no effect on IBV M localization. How does this fit with our model that ceramide mediates PDMP effects? One explanation is that the effect on protein localization is independent of the effect on anterograde traffic rates. In this case, it may be that C6Cer and endogenous ceramide have similar ability to bind and affect proteins that regulate anterograde traffic, but C6Cer is unable to bind or affect proteins that regulate protein localization. It has been shown that some short chain analogues of ceramide do not have the same effects as endogenous ceramide (e.g., Wolff et al., 1994). A second possibility is that effects of ceramide are limited to the bilayers in which its concentration increases, and the difference seen between endogenous ceramide and exogenous C6Cer is due to the different intracellular distribution of these molecules. C6Cer would be unlikely to accumulate in compartments where C6Cer accumulates, possibly the trans-Golgi and TGN (Pagano et al., 1989). Consistent with this idea, the rate of movement of VSV G through the late Golgi (measured by sialylation) was slowed to a much greater extent by C6Cer than by PDMP (Rosenwald and Pagano, 1993).

It is not yet clear how ceramide induces the changes in anterograde traffic and protein localization. Recent studies have shown that ceramide (Hannun, 1994) and its metabolites (Hakomori, 1990) play a major role as second messengers. Preliminary experiments with both the ceramidase inhibitor N-oleoylthanolamine and exogenously added sphingosine suggest that the ceramide breakdown product sphingosine does not play a role in the localization of IBV M (Maceyka, M., and C. Machamer, unpublished observations). Ceramide activates a cytosolic protein phosphatase (Dobrowsky and Hannun, 1992; Wolff et al., 1994) that can be inhibited by okadaic acid. Preliminary experiments suggest that okadaic acid does not block the PDMP-induced changes (Maceyka, M., and C. Machamer, unpublished observations). Ceramide could be activating a protein kinase (Mathias et al., 1991), but this kinase has not been fully characterized. Another possibility is that increased ceramide within the lipid bilayer directly affects the localization of IBV M. Earlier work from our laboratory showed that the first transmembrane domain of IBV M can target chimeras to the CGN (Swift and Machamer, 1991; Machamer et al., 1993). It is possible that CGN bilayers have distinct lipid domains, and that these domains contain different sets of proteins, analogous to glycosphingolipid rafts. Segregation of membrane proteins involved in vesicular traffic, such as SNAREs, would lead to mobile and immobile domains. We hypothesize that under normal conditions, IBV M would be targeted to an immobile domain and escaped molecules would be cycled back through the ER. Elevated levels of ceramide could disrupt these immobile lipid domains, inducing IBV M to cycle. Alternatively, perhaps ceramide binds to IBV M transmembrane domains, preventing an interaction with immobile lipid domains.

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