Brefeldin A and filipin distinguish two globotriaosyl ceramide/verotoxin-1 intracellular trafficking pathways involved in Vero cell cytotoxicity

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In verotoxin 1 (VT1)-sensitive cells, globotriaosyl ceramide (Gb3) bound VT1 is endocytosed and transported retrogradely to the Golgi/endoplasmic reticulum (ER). The importance of the Golgi-dependent retrograde transport of VT1 is now shown to vary as a function of both VT1 exposure time and concentration. Following 3 h exposure to <50 ng/ml VT1, Vero cell cytotoxicity and protein synthesis inhibition is absolutely dependent on intact Golgi structure. However, after 24 h incubation with concentrations of VT1 above 50 ng/ml, a filipin-sensitive (caveolae-dependent) route for cytotoxicity becomes significant. Brefeldin A (BFA), which prevents Golgi-dependent retrograde traffic, protects cells from low VT1 concentrations but not following prolonged toxin exposure at higher VT1 concentrations. Under these conditions, only a combination of BFA and filipin is sufficient to fully protect cells. Intracellular VT1 trafficking monitored using the nontoxic B subunit showed accumulation within BFA-collapsed TGN/endosomes. Considerable VT1 B was retained at the surface of filipin-treated cells, but Golgi targeting was still apparent. Filipin-sensitive VT1 does not colocalize with caveolin 1, a small fraction of endocytosed VT1 is found within caveolin 1–containing vesicles. These studies indicate both a caveolae-dependent and independent pathway for VT1 access to the TGN/Golgi from the cell surface and two noninterconverting pools of membrane Gb3.

Key words: cholesterol/endocytosis/glycolipid enriched microdomains-rafts/Golgi-endoplasmic reticulum/retrograde transport

Introduction

Many toxins enter cells by receptor-mediated endocytosis. Endosomes sort incoming proteins and acid-mediated ligand–receptor dissociation occurs (Schwartz, 1995). However, some protein toxins escape from endosome/lysosomal degradation and enter the biosynthetic/secretory pathway in a retrograde manner (Lord and Roberts, 1998). After receptor binding, toxins enter cells by clathrin-dependent or -independent endocytosis (Montecucco et al., 1994). For most protein receptor–binding ligands, ligand–receptor complexes are sequestered into clathrin-coated pits (Naglich et al., 1992). The receptor cytoplasmic domains recruit adapter proteins, which sort aggregating complexes into the coated pits (Schwartz, 1995). In contrast, glycolipid receptor–binding toxins enter cells via clathrin-independent endocytosis (Montecucco et al., 1994). Ricin, which binds glycoprotein and glycolipid, enters cells by both clathrin-dependent and -independent mechanisms (Sandvig and van Deurs, 1996).

Verotoxins (VTs; also called Shiga toxins) are a family of Escherichia coli AB5 subunit toxins associated with hemolytic uremic syndrome and hemorrhagic colitis (Karmali, 1989). The binding of the B subunit to the cell surface glycolipid, globotriaosyl ceramide (Gb3) (Lingwood, 1993), mediates the entry of the toxin into VT1-sensitive cells, whereas the catalytic subunit A induces cellular toxicity by inhibition of protein synthesis. VT1 remains the only described glycolipid receptor–binding toxin to enters cells by clathrin-dependent receptor-mediated endocytosis (Khine and Lingwood, 1994; Sandvig et al., 1989). However, depending on the cell type, clathrin-mediated internalization may be minor (Nichols et al., 2001).

The cholesterol-binding, caveolin-mediated endocytosis inhibitor filipin also can partially prevent VT1 B subunit internalization (Nichols et al., 2001; Schapiro et al., 1998). Although clathrin-dependent endocytosis can also be sensitive to cholesterol depletion (Subtil et al., 1999), this pathway is not sensitive to filipin/cholesterol binding (Orlandi and Fishman, 1998) or increased cholesterol content (Pagano et al., 2000). Thus the additive protection of filipin and clathrin inhibitors (Schapiro et al., 1998), suggests two distinct mechanisms of VT1 internalization. To intoxicate susceptible cells, it is presumed that the A subunit of the toxin needs to translocate the membrane of intracellular organelles to access ribosomes in the cytoplasm. Some protein toxins, such as diphtheria toxin (DT) and tetanus toxin (TT), dissociate in the acidic endosomal compartment, and catalytic subunits translocate the endosomal membrane mediated by a pH-dependent conformational change in the receptor binding subunit (Montecucco et al., 1994).
Therefore drugs that interfere with the maintenance of endosomal pH protect against such toxins (Umata et al., 1990). In contrast, toxins such as cholera toxin (CT), *E. coli* heat labile toxin (LT), *Pseudomonas aeruginosa* exotoxin A (ETA), Shiga toxin (ST), VT1, and ricin escape endosomal degradation and enter the biosynthetic/secretory pathway in a retrograde manner and presumably translocate across the Golgi or endoplasmic reticulum (ER) membrane (Mallard et al., 1998).

Brefeldin A (BFA) reversibly disrupts the Golgi by inducing fusion of the cis, medial, and trans cisternae with the ER (Lippincott-Schwartz et al., 1989) and the trans-Golgi network (TGN) with endosomes (Lippincott-Schwartz et al., 1991) and thereby protects cells from retrograde transported toxins (Donta et al., 1993; Yoshida et al., 1991). Although the retrograde transport mechanism of CT, LT, and ETA can be explained by KDEL or KDEL-like ER retention signals, the basis of VT1 retrograde transport (Jackson et al., 1999) is still unknown. Higher VT1 sensitivity can correlate with intracellular retrograde targeting of the toxin±receptor complex to the ER±nuclear membrane as opposed to Golgi (Arab and Lingwood, 1998; Sandvig et al., 1996). This suggests intracellular targeting of the VT1/Gb3 complex can affect VT1 sensitivity.

VT can induce both apoptosis and necrosis (Tetaud et al., 2003; Williams et al., 1999), depending on cell type. Apoptosis often requires protein synthesis and inhibitors of protein synthesis can protect cells from apoptosis (Thakkar and Potten, 1992). VT1 has been shown to increase the half-life of specific mRNA species (Bitzan et al., 1998) and induce the expression of others (Kojima et al., 2000). Thus it is likely that VT1 has mechanisms of action in addition to protein synthesis inhibition.

In this study the relative role of retrograde transport in VT1 cytotoxicity is assessed. Our study indicates an additional non-Golgi-dependent pathway, which increases in significance as a function of VT1 concentration and exposure time.

Results

Effect of BFA on VT1-induced cell death

Because VT1 cytotoxicity assays for cell killing and apoptosis take at least 24 h, the effective dose of BFA in Vero cells for which Golgi disruption was sustained for 24 h was first determined by immunofluorescent staining of Golgi with the trans and medial Golgi marker, Rab6 (White et al., 1999) (data not shown). The effective dose was 0.5–1 μg/ml, which was used for the subsequent experiments. The effect of BFA on VT1-induced cytotoxicity (Figure 1A) and apoptosis (Figure 1B) of vero cells were compared. Cytotoxicity was measured after 3 days, whereas apoptosis was assayed 24 h after VT1 addition. BFA largely protected against cytotoxicity up to 50 ng/ml VT1. Protection was only partial at 100 ng/ml VT1 and higher (Figure 1A). Although effective to prevent apoptosis at lower VT1 concentrations, BFA was largely ineffective to prevent apoptosis at 50 ng/ml VT1 and higher (Figure 1B).

The incomplete protection by BFA at higher dose of VT1 (total cell killing) was distinct from earlier reports that BFA completely protects Vero cells from VT1-induced protein synthesis inhibition after short-term treatment (Donta et al., 1992; Garred et al., 1997; Sandvig et al., 1991). Ten- to 20-fold more BFA was required to protect against VT1 compared with ricin (Garred et al., 1997; Sandvig et al., 1992). BFA protects protein synthesis inhibition induced by 1 μg/ml VT1, but this was not sustained after 6 h toxin treatment (Donta et al., 1992). Therefore the effect of BFA on VT1-induced protein synthesis inhibition after short-term (3 h) and long-term (24 h) treatment with VT1 was compared. Although we found protection was complete for short-term treatment across the entire VT1 dose range, BFA prevention of protein synthesis inhibition was only partial for concentrations of VT1 >10 pg/ml and minimal at >10 ng/ml after long-term treatment (Figure 2). Gb3-negative Vero cells are resistant to higher VT1 dosage (>1 μg/ml) (Pudymaitis et al., 1991). Thus VT1 internalization at both low and high doses is Gb3-dependent.

Effect of BFA on internalization and intracellular targeting of VT1 B in Vero cells

Because VT1 cytotoxicity and inhibition of protein synthesis at higher dosage was maintained despite the BFA-induced
Golgi labeling by FITC-VT1 B was observed (Kim et al., 1996). After 24 h treatment with FITC-VT1 B, and no perinuclear staining was observed (Figure 3A, d). In the presence of BFA, FITC-VT1 B labeling was confined to a juxtanuclear crescent (Figure 3A, a) observed as for FITC-VT1 B (Figure 3A, c). Golgi staining by VT1 was only partially prevented by filipin treatment. Despite this inhibition, only minor protection from VT1-induced cytotoxicity results (Figure 5B). Although the Golgi-dependent retrograde transport is mediated by AP2/clathrin-dependent vesicular transport to early endosomes and then via AP1/clathrin-dependent recycling endosomes to the TGN (Mallard et al., 1998), caveolae can be distributed directly to ER (Abadi and Lingwood, 1998), was used to study internalization. After 3 h incubation with fluorescein isothiocyanate (FITC)–VT1 B at 37°C, internalized VT1 B was found mainly as a juxtanuclear crescent (Figure 3A, a) previously identified as the Golgi (Kim et al., 1996). After 24 h treatment, Golgi labeling was lost and FITC-VT1 B distribution was found to be perinuclear (Figure 3A, c), consistent with further retrograde transport to the ER (Abadi and Lingwood, 1998). In the presence of BFA, loss of distinct Golgi labeling by FITC-VT1 B was observed after short-term incubation. FITC-VT1 B labeling was confined to a single region within the cell (Figure 3A, b) consistent with the collapsed TGN. Such restricted labeling remained after 24 h treatment with FITC-VT1 B, and no perinuclear staining was observed (Figure 3A, d). In the presence of BFA, cell surface labeling was partially retained (Figure 3A, b, d).

The effect of BFA on internalized unlabeled VT1 B subunit was also studied by indirect immunofluorescence after cell permeabilization (Figure 3B). In control cells, the VT1 B distribution at 3 h is consistent with Golgi restriction in most cells but less, as monitored by this more sensitive method (Figure 3B, a), as compared with direct labeling. Perinuclear ER-like staining was more prevalent (Figure 3B, a) than seen for FITC-VT1 B, which could result from a modification of VT1 B-Gb3 binding by FITC coupling (Chark et al., 2004). At 24 h, however, the intracellular distribution of VT1 B and FITC-VT1 B were indistinguishable. For BFA-treated cells, similar intracellular compartments with restricted VT1 B staining (Figure 3B, d) were observed as for FITC-VT1 B (Figure 3A, d). VT1 B cell surface staining in BFA-treated cells was more apparent by this more sensitive method and accumulated with time (Figure 3B, b, c, d).

Wheat germ agglutinin (WGA) accumulation can be used as a marker of the BFA-fused TGN (Vetterlein et al., 2003). Colocalization of VT1 B and WGA confirmed the BFA-induced accumulation of VT1 in the collapsed TGN (Figure 4). The distinct pattern of TGN/Golgi labeling by Texas red–WGA in control cells (Figure 4A, a) collapsed into an intense fluorescent compartment after BFA treatment (Figure 4A, c) that colocalized with internalized FITC-VT1 B (Figure 4A, b) in double-labeled cells. Labeling of endosomes with FITC-labeled dextran (Figure 4B, a) was not affected by BFA treatment for 3 h (Figure 4B, b), but endosomes (Figure 4B, d) were found to be fused with TGN (Figure 4B, c) after 24 h BFA treatment.

Identification of an alternative route of VT1 internalization

Depending on the cell type, VT1 can enter cells by clathrin-dependent receptor-mediated endocytosis (Khine and Lingwood, 1994; Sandvig et al., 1989) as well as by caveolar-mediated endocytosis (Nichols et al., 2001; Schapiro et al., 1998). The effect of clathrin-dependent or caveolar-dependent endocytosis inhibitors on FITC-VT1 B internalization and VT1-induced cell death was examined. Reducing the internal pH of cells by various protocols prevents coated vesicles pinching off from the plasma membrane (Heuser, 1989). This procedure completely prevents VT1 B internalization (Figure 5A, compare with panel B) and protects Vero cells from VT1-induced cytotoxicity (Figure 5B). Filipin, a sterol-binding drug, inhibits caveolin-mediated endocytosis by disassembling caveolar organization and preventing invagination of caveolae (Rothberg et al., 1992). Treatment of cells with 10 µg/ml filipin significantly (though incompletely) prevents VT1 B internalization (Figure 5A, panel C). Golgi staining by VT1 was only partially prevented by filipin treatment. Despite this inhibition, only minor protection from VT1 cytotoxicity results (Figure 5B). Although the Golgi-dependent retrograde transport is mediated by AP2/clathrin-dependent vesicular transport to early endosomes and then via AP1/clathrin-dependent recycling endosomes to the TGN (Mallard et al., 1998), caveolae can be distributed directly to ER from the cell surface on cholesterol oxidation (Smart et al., 1994) or when Golgi function is compromised (Llorente et al., 2003) and microtubules function as highways for membrane trafficking (Lippincott-Schwartz et al., 1995).

To address the significance of filipin-sensitive endocytosis in VT1 internalization, the VT1 dose-dependent effect of filipin on VT1 cytotoxicity was examined. A synergistic effect of BFA and filipin against VT1 cytotoxicity was observed, especially at higher doses of VT1 when BFA alone cannot protect the cells (Figure 6A), suggesting that VT1 endocytosis is still operating while the Golgi structure is disrupted. Additive protection against VT1 cytotoxicity by filipin and the microtubule-disrupting agent nocodazole was observed (Figure 6B), which was further enhanced in the presence of BFA, indicating the ongoing microtubule-dependent caveolae-mediated VT1 trafficking while Golgi-dependent retrograde transport is suppressed.

Caveolae are characterized by their cytosolic association with one of several caveolins, primarily caveolin 1 (Nabi
and Le, 2003). We therefore compared the surface and intracellular distribution of caveolin1 with that of VT1 during internalization (Figure 7). At 4°C, a general surface staining for VT1 is seen. Cytosolic caveolin 1 is less uniformly distributed (Figure 7a) with no correlation with VT1. On brief incubation at 37°C, VT1 surface staining becomes somewhat more punctuated and caveolin 1 staining more restricted to membrane structures around the

Fig. 3. (top) Effect of BFA on FITC-VT1 B internalization. Vero cells after 3 h (a and b) and 24 h (c and d) after FITC-VT1 treatment in the absence (a and c) or presence (b and d) of 0.5 μg/ml BFA. FITC B is internalized to a juxtanuclear (Golgi) area (a, arrows) after 3 h and undergoes further retrograde transport to ring the nucleus (ER and nuclear membrane) after 24 h. FITC B accumulates in the collapsed TGN (arrows) in BFA-treated cells at both 3 and 24 h. (bottom) Effect of BFA on VT1 B internalization monitored by immunofluorescence. At 3 h (a and b) and 24 h (c and d) in the absence (a and c) or presence (b and d) of 0.5 μg/ml BFA. Juxtanuclear staining (arrows) of Triton permeabilized Vero cells is less prevalent at 3 h than in 3A, but perinuclear ER/nuclear envelope staining (arrows) is increased after 24 h (compare with 3A, c). Punctuated staining of some cells is seen only in the absence of BFA. Condensed staining, consistent with collapsed TGN (arrows), and cell surface staining is seen after BFA treatment at 3 and 24 h. Apparent staining within the nucleus is seen in control but not BFA-treated cells. Bar = 100 μm.
Fig. 4. (top) Effect of BFA on FITC-VT1 B internalization relative to TGN/Golgi. (a) Texas red–conjugated WGA internalized into control Vero cells. Double labeling of FITC-VT1 B (b) and Texas red–labeled WGA (c) of Vero cells in the presence of BFA for 24 h. Twofold magnified insets are shown in (b) and (c). WGA labels the TGN/Golgi (arrows) of untreated cells. After 24 h BFA treatment, WGA labeling collapsed TGN can be seen (c, arrows). Such restricted labeling is colocalized with FITC-VT1 B (compare b, c). (bottom) Effect of BFA on TGN and endosome marker distribution. FITC-dextran internalized into control (a) or 3 h BFA-treated (b) Vero cells. Double labeling of Texas red–labeled WGA (c) and FITC-dextran (d) in the presence of BFA for 24 h. Endosomes were unaffected by 3 h BFA treatment, but after 24 h, the WGA stained collapsed TGN was found to be colocalized with FITC-dextran-stained endosomes. Twofold magnified insets are shown in (c) and (d). Bar = 100 μm.
nucleus (Figure 7b). Again, no correlation of staining is seen. After 1 h at 37°C, VT1 is internalized into structures consistent with Golgi. Caveolin 1 staining is unchanged, but now a few areas of correlation between internalized VT1 and caveolin 1 labeling are seen (Figure 7c, d) in some (but not all) cells consistent with a fraction of VT1 internalization via a caveolin 1–mediated mechanism. By visualizing only the pixels present in both the VT1 and anti-caveolin 1 images a direct assessment of colocalization is possible (Figure 7d). We estimate that <10% VT1 is internalized into caveolin 1–containing vesicles.

Discussion

We have shown that following BFA–induced Golgi collapse, residual cytotoxicity, apoptosis, and long-term VT1 inhibition of protein synthesis were maintained, particularly at higher VT1 concentrations. Apoptosis was maintained at VT1 concentrations at which protein synthesis inhibition was prevented. VT1–induced apoptosis and cytotoxicity are distinct processes (Gordon et al., 2000; Williams et al., 1999), and although the A subunit can be necessary for apoptosis...
VT1 from the cell surface also was compromised. The incomplete protection effect of BFA for VT1 cytotoxicity could be due to a Golgi-independent trafficking of VT1/Gb3. The BFA-resistant VT1 cytotoxicity was largely sensitive to filipin, though filipin alone had little protective effect. Because filipin binds cholesterol and inhibits lipid raft-dependent caveolar-mediated endocytosis, both cholesterol-dependent and -independent pathways are implicated in VT1 Gb3-dependent endocytosis, intracellular trafficking, and cytotoxicity.

Protein toxins usually enter cells after binding to their specific receptors on the cell surface (Montecucco et al., 1994). Depending on the nature of the receptors, the mechanisms of endocytosis vary. Clathrin-dependent endocytosis is usually found to be associated with receptor-mediated endocytosis (Schwartz, 1995). The movement of ligand–receptor complexes into the coated pits is mediated by interactions of adapter molecules in the cytoplasm and cytoplasmic tails of the protein receptors (Schwartz, 1995). Therefore clathrin-dependent receptor-mediated endocytosis becomes the favorable mechanism for protein receptor binding toxins, such as DT and ETA (Montecucco et al., 1994). In contrast, flask-shaped, nonclathrin-coated membrane invaginations, caveolae, are assembled with caveolin protein and lipid microdomains (Parton, 2003). Many surface proteins anchored by glycoinositol phospholipid linkage accumulate in such domains (Anderson, 1993). Consequently, lipid receptors for toxins could also be already preassociated with caveola. Thus caveola-mediated, clathrin-independent endocytosis is an appropriate mechanism for lipid receptor binding toxin internalization. Indeed CT/GM1 ganglioside binding is often used as a marker of this process (Lencer et al., 1999).

VT1 is the only lipid receptor binding toxin that can enter cells by clathrin-dependent receptor-mediated endocytosis (Khine and Lingwood, 1994; Sandvig et al., 1989). Gb3 analogs in which the fatty acid is substituted by a fluorophore are internalized in skin fibroblasts by a clathrin-independent, caveolae-dependent mechanism (Puri et al., 2001). Endocytosis of endogenous Gb3 in the same cells, via ligation by VT1 B subunit was, in contrast, clathrin-dependent (Puri et al., 2001). Thus changes in the lipid moiety of Gb3 may determine whether internalization is clathrin- or caveolae-dependent. This is consistent with our suggestion that the fatty acid chain length can affect Gb3 intracellular trafficking (Arab and Lingwood, 1998).

Gb3 might be recruited into coated pits via association with transmembrane cell surface proteins. Two transmembrane proteins may associate with Gb3 on the surface of the cell—the B lymphocyte cell differentiation antigen, CD19 (Maloney and Lingwood, 1994) and IFNAR1 chain of the α2-interferon receptor (Lingwood and Yiu, 1992), the extracellular domains of which have amino acid sequence similarity to VT1 B subunit (Lingwood, 1996). Recently, a related similarity has been found in MHC class II antigens (George et al., 2001). Gb3 can be in detergent-resistant membrane domains in untreated cells (Mori et al., 2000). Despite the fact that Gb3 is only in the outer membrane leaflet, VT1 binding to such domains can signal across the membrane to activate cytosolic kinase activity (Katagiri

Fig. 7. Double labeling of caveolin 1 and VT1. Cy3-VT1 confocal staining (right) at 4°C (a) of the Vero cell surface is compared to cytosolic caveolin 1 (left) immunodetected after permeabilization. Staining after 15 min (b) or 1 h (c) at 37°C is compared. Panel d is a combination of the images in c in which only coincident pixels are shown. Caveolin 1 labeling does not correspond to VT1 cell surface staining, but a small fraction of VT1 is internalized at 37°C into caveolin 1–containing intracellular structures (c, d, arrows) in some cells.
Such raft-dependent signaling could initiate clathrin-mediated internalization. Gb3-mediated VT1 intracellular routing may play a pivotal role in VT1 susceptibility. Monocytes, macrophages, and dendritic cells express Gb3, but are not susceptible to VT1 cytotoxicity (Falguieres et al., 2001; Ramegowda and Tesh, 1996; Tesh et al., 1994; van Setten et al., 1996). Internalization of VT1 into these cells, as compared with highly VT1-susceptible HeLa cells, did not result in retrograde transport to the Golgi/ER, but rather transport to late endosomes/lysosomes. In HeLa cells but not macrophage/dendritic cells VT1 B was associated with detergent insoluble membrane domains (Falguieres et al., 2001), suggesting that Gb3 membrane organization may predominate intracellular routing and hence toxin susceptibility (Hoey et al., 2003).

Our studies indicate VT1 internalization in Vero cells is partly clathrin-mediated and partly due to filipin-sensitive, likely caveolae-mediated endocytosis (Schapiro et al., 1998). Unlike in Daudi cells (Khine and Lingwood, 1994; Fra et al., 1994) (lymphocytic cells, lacking caveolae), monodansyl cadaverine was unable to inhibit clathrin-mediated Vero cell VT1 endocytosis (data not shown). Cytosolic acidification prevented VT1 B internalization, consistent with clathrin dependancy (Hansen et al., 1993; Heuser, 1989) and protected cells from VT1 cytotoxicity. This suggests heterogeneity in Gb3 clathrin internalization among cell lines. Such differences may relate to the organization of plasma membrane Gb3.

CT is endocytosed separately from VT1 in Vero cells (Schapiro et al., 1998), despite the finding of both GM1 and Gb3 in the detergent resistant membrane (DRM)/raft fraction (Falguieres et al., 2001), indicating the heterogeneity of such detergent-resistant microdomains (Iwabuchi et al., 1998). In contrast to VT1, CT is internalized independent of clathrin (Puri et al., 2001), primarily by a caveolar mechanism (Le and Nabi, 2003). Fluorescence Resonance Energy Transfer (FRET) studies have shown energy transfer between cell surface glycolipid-bound CT and VT1 (Kovbasnjuk et al., 2001), indicating either the very close apposition of VT1 and CT containing rafts or that ligand binding can initiate raft subdomains. Both VT1 (Khine and Lingwood, 1994) and CT (Kellie et al., 1983) have been shown to undergo a process of patching/capping prior to endocytosis, implying extensive lateral reorganization. The relative GSL content of rafts may also vary between cell types. In this regard, it is of interest to note that CT endocytosis was originally reported as clathrin-dependent in lymphocytes (Revesz and Greaves, 1975).

Our study indicates that clathrin-dependent endocytosis and retrograde Golgi transport is the major internalization route at low VT1 concentrations. Other studies have shown VT1/Gb3 internalization via clathrin-coated pits in Vero and HeLa cells (Sandvig et al., 1989; Sandvig and van Deurs, 2002). In HeLa cells, the subsequent retrograde transport of VT1 B is a function of cholesterol-dependent Gb3 raft organization, as monitored by methyl β cyclodextrin (Falguieres et al., 2001). Though methyl β cyclodextrin depletion of cholesterol can affect clathrin-mediated internalization (Subtil et al., 1999), recent studies indicate lipid rafts, as monitored by CT/GM1 binding, are excluded from coated pits (Nichols, 2003). This may reflect a fundamental difference between Gb3- and GM1-containing rafts.

In Vero cells, we show cholesterol dependent endocytosis, as monitored by filipin sensitivity, is an alternative pathway, more apparent at higher VT1 concentrations and/or when Golgi retrograde transport is inhibited. The retention of cell surface VT1 B after filipin treatment indicates that these pathways are not in any equilibrium in the plasma membrane. The concentration-dependent role these pathways play in VT1 cytotoxicity necessitates care in the interpretation of trafficking studies with the higher levels of VT1 B subunit required for subcellular detection.

Unlike methyl β cyclodextrin-cholesterol depletion, filipin has had no reported effect on clathrin-mediated endocytosis (Smart and Anderson, 2002). VT1 B trafficking to the Golgi in the presence of filipin, though reduced, is still clearly evident, consistent with separate clathrin and caveolae-mediated retrograde transport routes to this organelle. However, despite the protective effect of filipin, we found no correlation between VT1 surface binding and caveolin 1. Although caveolin 1 may be involved in stabilization of membrane caveolae to inhibit internalization (Nabi and Le, 2003), the lack of correlation at 4°C we observed suggests that this cholesterol-dependent fraction of VT1 internalization might not be caveolae-mediated. In contrast, CT surface binding and caveolin 1 are colocalized (Puri et al., 2001), and this is retained after internalization (Le and Nabi, 2003), quite distinct from our VT1 B results. Nevertheless, lack of caveolin 1–VT1 B colocalization does not necessarily exclude caveolae (Nabi and Le, 2003).

It is clear that the relationships between GSL/cholesterol-enriched rafts and coated pits and rafts and caveolae/caveolin 1 are complex and not yet generally understood. Even the CT raft marker can be internalized by clathrin-dependent as well as independent mechanisms (Shogomori and Futerman, 2001; Torgersen et al., 2001). Thus, although our studies identify two separate VT1/Gb3 internalization mechanisms, one BFA-sensitive intracellularly and one filipin-sensitive at the cell surface, consistent with clathrin and caveolae dependency, respectively, other interpretations remain. Our studies imply two noninterconverted pools of Gb3 within the plasma membrane with distinct fates for bound ligand.

In the presence of BFA, only VT1 B internalization to the collapsed TGN is seen. The mechanism of the BFA-resistant, filipin-sensitive VT1 cytotoxicity remains unclear. Because BFA prevents Golgi/ER and nuclear VT1 targeting (Facchini and Lingwood, 2001), these pathways are not involved. A remaining possibility is that filipin-sensitive VT1 cytotoxicity results from direct transmembrane, likely raft-mediated, activation of cytosolic signal transduction cascades (Katagiri et al., 1999). Such signaling could occur at the cell surface, but because filipin does not prevent cell surface VT1 binding and inhibition of VT1 internalization protects against cytotoxicity, it is more likely to occur across intracellular membranes, in which Gb3 is still found in detergent-resistant raft structures (Falguieres et al., 2001). Indeed, filipin has been shown to prevent VT1/Gb3 raft binding-mediated transmembrane cytosolic kinase activation (Katagiri et al., 1999). Gb3 raft-associated protein signaling may be important (Katagiri et al., 2002).
Two routes for VT1/Gb3 internalization

During the course of retrograde transport through the Golgi and ER, the VT1 A subunit has to translocate the membrane to reach the ribosomes in the cytoplasm or cytoplasmic face of the Golgi/ER to directly inhibit protein synthesis. However, cytosolic VT1 translocation has yet to be reported, either from Golgi or ER. The protection against VT1 inhibition of protein synthesis by BFA indirectly suggests that Golgi or ER could be a site of VT1 membrane translocation (Donta et al., 1993; Garred et al., 1997; Yoshida et al., 1991). In the presence of an intact Golgi complex, VT1 B was mainly found in Golgi at the 3-h time point and progressed to a perinuclear ER-like structure and nucleus on longer incubation. Although the VT1 B subunit is a convenient acytotoxic model (Arab and Lingwood, 1998) of holotoxin internalization, and VT1A subunit lacks known trafficking motifs (Jackson et al., 1999), the A subunit might modify holotoxin transport via unknown motifs. Nevertheless, initial internalization will be A subunit-independent and the two pathways identified applicable to holotoxin endocytosis. The VT1 B trafficking pathways identified in the present study are likely based for the most part on the intracellular movement of the VT1 receptor, Gb3, and could involve the differential organization and/or lipid heterogeneity of this molecule (Arab and Lingwood, 1998; Falguieres et al., 2001). The longer chain fatty acid homologs of Gb3 were found to preferentially mediate cell surface α2-interferon antisignal transmission (Khine and Lingwood, 2000), suggesting such isoforms might play a greater role in the filipin-sensitive VT1 pathway.

In conclusion, because early VT1 1 inhibition of protein synthesis is prevented by BFA, protein synthesis may be initially inhibited via a Golgi-dependent, A subunit cytosolic translocation pathway. In contrast, filipin-sensitive inhibition of protein synthesis in the longer term and at higher VT1 concentrations may involve direct transmembrane signaling via Gb3 ligation. These studies imply two separate membrane Gb3 pools.

Materials and methods

Cell culture

Vero cells from American Type Cell Culture (Rockville, MD) were grown in α-minimum essential medium with 5% fetal calf serum and 40 μg/ml gentamycin at 37°C in the presence of 5% CO2.

VT1 cytotoxicity assay

Cells grown to confluency were trypsinized, and 100 μl 1.5 × 10^7/ml cell suspension was seeded in 96-well cell culture plates for 24 h at 37°C prior to the experiment. Then serial dilutions of VT1 were added, and cell culture plates were further incubated at 37°C for another 48 h, when cell survival was assessed. In some experiments, cells were pretreated with the indicated concentration of BFA (Sigma, St. Louis MO), filipin (Sigma), nocardazole (Sigma), or acetic acid containing medium for 30 min at 37°C before addition of toxin and maintained throughout. At the end of the incubation period, the plates were fixed with 2% formaldehyde in phosphate buffered saline (PBS),

relative importance of this pathway may vary between cell types, because other studies have shown that filipin can completely inhibit VT1 B Golgi labeling (Nichols et al., 2001).

Following clathrin-dependent endocytosis, VT1 escapes degradation in the lysosomal system and directly enters the TGN from early endosomes via clathrin-coated vesicles (Mallard et al., 1998). From the TGN, the toxin traverses the biosynthetic pathway—the Golgi complex and ER in a retrograde manner (Mallard et al., 1998). Because drugs such as BFA (Garred et al., 1997; Sandvig et al., 1991), nocardazole (Johannes et al., 1997), and ilmaquinone (Nambiar and Wu, 1995), which disrupt the Golgi structure, protect cells from VT1-induced protein synthesis inhibition, the Golgi and/or ER are believed to be the site of cytosolic translocation. In the present study, although the disruption of Golgi structure was sustained throughout the 24-h period of the VT1 cytotoxicity assay, BFA did not completely protect the cells from VT1-induced cytotoxicity or protein synthesis inhibition at higher doses of VT1.

Vesicular-mediated intracellular transport requires microtubule-guided traffic (Lippincott-Schwartz et al., 1995). Partial protection of VT1 cytotoxicity by filipin and nocardazole, and the additional protection these offer in the presence of BFA-induced Golgi disruption, indicates (provided the selectivity of these drugs is maintained in combination) the existence and/or up-regulation of caveola-mediated, microtubule-dependent targeting for VT1-induced cytotoxicity, particularly when Golgi-dependent retrograde transport was disturbed or down-regulated. Our finding that BFA effectively prevented Golgi/ER VT1 B trafficking without complete protection from cytotoxicity while the full protection afforded by BFA + filipin was correlated with increased VT1 B remaining at the cell surface could be explained if this second trafficking pathway does not require cytosolic toxin access for cytotoxicity. A filipin-sensitive transmembrane signal across the plasma membrane, endosome or TGN (+collaps) may occur, primarily for apoptosis induction. This would be consistent with the findings that VT1-induced apoptosis does not correlate with Golgi/ER retrograde transport of the toxin (Tetaud et al., 2003).

In earlier work we have shown that increased sensitivity to VT1 is associated with retrograde transport to the ER, nuclear envelope/nucleus, as opposed to the Golgi (Arab and Lingwood, 1998; Lala et al., 2000; Lingwood et al., 1998). These studies monitored retrograde transport within 1 h after VT1 addition. Under these conditions, internalized FITC-VT1 B is restricted to the Golgi of Vero cells (Kim et al., 1996; Schapiro et al., 1998) and this is now shown to be maintained at least until 3 h. Our present results, however, show that on prolonged incubation (24 h) retrograde transport continues, such that the ER/nucleus is stained even in Vero cells. Similar results were seen for ECV304 cells (Heath-Engel and Lingwood, 2003). Thus the rate at which retrograde transport to the ER/nucleus occurs, rather than the subcellular end target, is likely the correlate with VT1 sensitivity. In highly VT1-sensitive cells, rapid ER/nuclear targeting is seen, and we have proposed that this plays a role, as yet unknown, in VT1 cytotoxicity (Arab and Lingwood, 1998) which might relate to the efficacy of A subunit translocation.
and residual substrate attached cells stained with crystal violet as described (Petric et al., 1987). The percentage of live cells was calculated from absorbance of destained cells read at 570 nm using a microtiter plate reader (Dynatech Laboratories, Chantilly, VA).

**Cytochemical staining of apoptotic cells**

To examine the morphological changes in the nuclear chromatin, cells undergoing apoptosis were identified by acridine orange/ethidium bromide staining of nuclei (Vasconcelos and Lam, 1994). After incubation of Vero cells with VT1 in the presence or absence of BFA for 24 h at 37°C, cells in the incubation medium (only seen for toxin-treated cultures) and trypsinized attached cells were pelleted. After washing with PBS, cells were resuspended in the nuclear stain (100 mg/ml acridine orange and 100 mg/ml ethidium bromide) and examined under UV illumination. Cells with condensed chromatin and apoptotic bodies were scored as apoptotic cells. A minimum of 200 cells were counted for each experiment.

**Protein synthesis inhibition assay**

Vero cells were prepared in 96-well plates for the experiment. At the end of indicated experiment, medium was removed and cells were incubated in 200 μl of the same medium containing 1 μCi/ml 3H-leucine (Amersham, Little Chalfont, U.K.) for 10 min at 37°C. After washing, cells remained in the plate were washed twice with 100 μl 5% trichloroacetic acid, and precipitated protein was solubilized with 100 μl 0.1 M KOH. Acid precipitable radioactivity was measured by using a Beckmann LC 3800 C counter.

**Internalization of fluorescent VT1/VT1 B**

VT1 B subunit was labeled with FITC (Molecular Probes, Eugene, OR). FITC (1:1 [w/w] to VT1 B) was added directly to the B subunit in 0.5 M carbonate/bicarbonate buffer, pH 9.5, and the mixture was gently rotated for 2 h at room temperature (Johnson and Holborow, 1986; Khine and Lingwood, 1994). Free FITC was removed by dialysis against 10 mM PBS, pH 7.4. Cy3-labeled VT-1 was prepared using Cy3 nonreactive dye according to manufacturer's instructions (Amersham Pharmacia Biotech). Vero cells grown on coverslips were incubated with medium containing 5 μg/ml FITC-VT1 B at 37°C for 3 h or 24 h and fixed at room temperature with 4% paraformaldehyde in PBS for 15 min. In some experiments, cells were pretreated with the indicated concentration of BFA (Sigma), or acetic acid containing medium for 30 min at 37°C before addition of toxin B subunit. Images were recorded using a Polyvar fluorescence microscope.

For caveolin1/VT1 double labeling, cells were treated with 1 μg/ml Cy3-VT1 for the times indicated. After fixation as described, cells were permeabilized with 0.1% saponin solution followed by incubation with anti-caveolin-1 antibody (Santa Cruz Biotechnologies, Santa Cruz, CA), followed after washing with FITC-goat anti-rabbit secondary antibody (Sigma). The coverslips were mounted on the microscope slide, using fluorescent mounting medium (Dako, Copenhagen) and examined using a Zeiss LSM510 confocal microscope. Zeiss Image Examiner was used to visualize colocalized pixels.

**Indirect immunofluorescence labeling**

Cells were incubated with VT1 B (5 μg/ml) rather than FITC-VT1 B. At the end of indicated experiments, cells were fixed and permeabilized using 0.1% Triton X-100 in PBS at room temperature for 10 min. After blocking of nonspecific protein binding by using 1% bovine serum albumin and 0.1% Triton X-100 in PBS, the cells were immunostained using polyclonal primary antibody to Rab6 (SantaCruz Biotechnology) or PH1 monoclonal antibody to VT1 B subunit (Boulanger et al., 1990) followed by FITC-conjugated secondary antibodies (Sigma). As control, rabbit preimmune serum or mouse IgG1 (Sigma) was used in place of primary antibody incubation. For organelle staining, cells were incubated with 5 μg/ml Texas red–conjugated WGA, to label TGN, or 50 μg/ml FITC-conjugated dextran, to label endosomes, at 37°C for 45 min. The mounted coverslips were examined under a Polyvar fluorescence microscope.

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**Abbreviations**

BFA, brefeldin A; CT, cholera toxin; DT, diphtheria toxin; ER, endoplasmic reticulum; ETA, Pseudomonas aeruginosa exotoxin A; FITC, fluorescein isothiocyanate; LT, Escherichia coli heat labile toxin; PBS, phosphate buffered saline; TGN, trans-Golgi network; TT, tetanus toxin; VT, veroxin; WGA, wheat germ agglutinin.

**References**


