pH-independent retrograde targeting of glycolipids to the Golgi complex

FLORENCIA B. SCHAPIRO,1,2 CLIFFORD LINGWOOD,3,4,5 WENDY FURUYA,1 AND SERGIO GRINSTEIN1,2

Divisions of 1Cell Biology and 2Microbiology, Research Institute, Hospital for Sick Children, and Departments of 2Biochemistry, 3Microbiology, and 4Clinical Biochemistry, University of Toronto, Toronto, Ontario, Canada M5G 1X8

Schapiro, Florencia B., Clifford Lingwood, Wendy Furuya, and Sergio Grinstein. pH-independent retrograde targeting of glycolipids to the Golgi complex. Am. J. Physiol. 274 (Cell Physiol. 43): C319–C332, 1998.—A small fraction of the molecules internalized by endocytosis reaches the Golgi complex through a retrograde pathway that is poorly understood. In the present work, we used bacterial toxins to study the retrograde pathway in vero cells. The recombinant B subunit of verotoxin 1B (VT1B) was labeled with fluorescein to monitor its progress within the cell by confocal microscopy. This toxin, which binds specifically to the glycolipid globotriaosyl ceramide, entered endosomes by both clathrin-dependent and -independent pathways, reaching the Golgi complex. Once internalized, the toxin-receptor complex did not recycle back to the plasma membrane. The kinetics of internalization and the subcellular distribution of VT1B were virtually identical to those of another glycolipid-binding toxin, the B subunit of cholera toxin (CTB). Retrograde transport of VT1B and CTB was unaffected by addition of weak bases in combination with concanamycin, a vacuolar-type ATPase inhibitor. Ratio imaging confirmed that these agents neutralized the luminal pH of the compartments where the toxin was located. Therefore, the retrograde transport of glycolipids differs from that of proteins like furin and TGN38, which require an acidic luminal pH. Additional experiments indicated that the glycolipid receptors of VT1B and CTB are internalized independently and not as part of lipid “rafts” and that internalization is cytoskeletal insensitive. We conclude that glycolipids utilize a unique, pH-independent retrograde pathway to reach compartments of the secretory system and that assembly of F-actin is not required for this process.

Although the recycling and lysosomal delivery of internalized molecules have been extensively investigated, much less is known about the retrograde transport of surface molecules to the secretary pathway. Recent studies have revealed the existence of less prominent routes involved in the delivery of (glyco)proteins and glycolipids from the plasma membrane to the Golgi complex and endoplasmic reticulum (17). The best described of these systems is responsible for the retrieval of proteins such as furin and TGN38 from the plasmalemma to the trans-Golgi network (TGN), where they reside. These proteins are internalized at the plasma membrane via clathrin-coated vesicles and are targeted to the TGN by specific domains of their cytoplasmic tail, which have been defined by mutagenesis studies (13, 14, 36). Like the endocytic pathway, this retrograde route requires luminal acidification for appropriate intracellular traffic. Chapman and Munro (5) showed that delivery of internalized furin and TGN38 to the TGN can be prevented by the weak base chloroquine.

Endogenous retrograde pathways are also utilized by a number of bacterial toxins to reach their target compartment within the cell. Several such toxins share a double-moiety structure: a homopentameric B subunit that binds to cell surface receptors (generally glycolipids) (7) and a toxic A subunit that exerts its activity in the cytosol. After binding to their receptors at the cell surface, these toxins are internalized and retrogradely targeted through the TGN to the Golgi complex and in some cases to the endoplasmic reticulum. The toxic subunit is believed to enter the cytosol by crossing the membrane of these secretory compartments (27). Such is the case for Vibrio cholerae toxin (CT), which specifically binds to the glycolipid monoglycoside 1 (GM1) at the plasma membrane. After reaching the cytosol, the toxic A1 fragment of the A subunit of CT catalyzes ADP ribosylation of the α-subunit of heterotrimeric G proteins, leading to persistent activation of adenylate cyclase (18, 34). Other members of this family are Shiga toxin, the infectious agent in dysentery, which is produced by Shigella dysenteriae (28), and verotoxin (VT). VT, which is synthesized by enterohemorrhagic strains of Escherichia coli, is associated with human vascular diseases such as hemorrhagic colitis and hemolytic uremic syndrome (20). In spite of their different origins, VT and Shiga toxin have a high degree of structural homology and share the same receptor, the glycolipid globotriaosyl ceramide (Gb3). These two toxins also utilize the same intoxication mechanism: after these toxins reach the endoplasm-
mic reticulum, their A subunits presumably translocate to the cytosol where they inactivate the 60S ribosomal subunits by depurination of a specific adenine residue, causing inhibition of protein synthesis (32).

The mechanism(s) whereby such bacterial toxins gain access to the Golgi complex and endoplasmic reticulum has not been adequately elucidated. CT has been reported to enter the cells through caveolae (22). In contrast, the process underlying VT internalization is not well defined. Initial studies in Daudi cells showed that monodansyl cadaverine prevents endocytosis of the B subunit of verotoxin 1 (VT1B) (15). This alkyl amine competitively inhibits cytosolic transglutaminase activity, an enzyme involved in the cross-linking of ligand-bound cell surface receptors clustered within clathrin-coated pits. The subsequent steps in the retrograde transport of VT, CT, and Shiga toxin to the Golgi complex have not been explored. In particular, it is unclear whether the toxins utilize the same pathway that mediates retrieval of furin and TGN38 to the Golgi complex.

In the present work, we characterized the internalization and retrograde transport of VT1B and of the B subunit of CT (CTB) in Vero cells. To minimize toxicity, we omitted the A subunits and utilized recombinant B subunits, which dictate the receptor affinity and targeting of the toxins. The results obtained suggest that VT1B can enter the cells by both clathrin-dependent and -independent routes. More importantly, the targeting of VT1B and CTB to the Golgi complex was found to be mediated by a common retrograde pathway, which is distinct from the furin and TGN38 retrieval system.

**MATERIALS AND METHODS**

Materials. Texas red-labeled transferrin and rhodamine-labeled phalloidin were purchased from Molecular Probes (Eugene, OR). Indocarbocyanine (Cy3)-labeled donkey anti-mouse antibodies were from Jackson Immuno Research Laboratories (West Grove, PA). Fluorescein isothiocyanate (FITC)-labeled CTB, filipin, and cytochalasin D were purchased from Sigma Chemical (St. Louis, MO). Concanamycin A was from Kamiya Biochemical (Thousand Oaks, CA), and monodansyl cadaverine was from Fluka Chemie (Buchs, Switzerland). 

Recombinant VT1B was purified by affinity chromatography and labeled with fluorescein by the addition of FITC [1:1 (wt/wt) ratio] in 0.5 M Na2CO3-NaHCO3 (pH 9.5). The mixture was gently rotated for 1–2 h at room temperature, after which free FITC was removed by dialysis. Rhodamine isothiocyanate (RITC)-labeled VT1B was similarly prepared.

Cell culture and incubation with toxins. Vero cells obtained from the American Type Culture Collection (Rockville, MD) were cultured at 37°C in minimal essential medium (MEM; GIBCO, Grand Island, NY) containing 5% heat-inactivated fetal bovine serum (Cansera International, Rexdale, ON, Canada), 0.1% glucose, vitamins, and 1% penicillin-streptomycin (GIBCO) under 5% CO2.

Vero cells, grown to near confluence on 18-mm-diameter glass coverslips, were washed three times in cold Dulbecco's modified buffered saline solution (PBS; Pierce, Rockford, IL) containing 1 mM CaCl2 and 1 mM MgCl2 (pH 7.4). To monitor the distribution of FITC-VT1B or FITC-CTB, the cells were exposed to 10 µg/ml of the appropriate toxin in PBS for 1 h at 4°C to promote binding to the plasmalemmal receptors without endocytosis. After cells were washed twice with PBS, internalization was initiated by incubating the cells at 37°C for the specified periods.

Chinese hamster ovary cells stably transfected with a furin-IgG chimeric construct were kindly provided by Drs. K. Tete and H. P. Moore, Department of Cell Biology, University of California at Berkeley (Berkeley, CA).

Protocols for inhibition of endocytosis. Four different protocols were used to inhibit clathrin-mediated internalization.

1) The first protocol was monodansyl cadaverine treatment: cells were incubated with the appropriate toxin, as described above, in the presence of 500 µM monodansyl cadaverine.

2) The second protocol was K+ depletion: cells were subjected to a hypotonic shock by preincubation in 50% diluted culture medium for 5 min at 37°C. The cells were then washed with PBS and incubated in K+-free medium [140 mM NaCl, 10 mM N-methyl-d-glucamine chloride, 1 mM MgCl2, 1 mM CaCl2, 20 mM N-2-hydroxyethylpiperazine-N'-2-ethansulfonic acid (HEPES), 5.5 mM glucose, and 1% bovine serum albumin (BSA; Boehringer Mannheim), pH 7.4] for 10 min at 37°C. Labeling with the appropriate toxin was performed in the K+-free medium. 3) The third protocol was hypertonic treatment: cells were preincubated in hypertonic medium (in mM: 350 NaCl, 10 KCl, 1 MgCl2, 1 CaCl2, 20 HEPES, and 5.5 glucose and 1% BSA, pH 7.4) for 20 min at 37°C. Labeling with the appropriate toxin was also performed in the hypertonic medium. 4) The fourth protocol was cytoplasmic acidification: cells were preincubated in growth medium supplemented with 5 mM acetic acid (pH 5.0) for 5 min at 37°C. This condition was maintained throughout the labeling of the cells with the appropriate toxin.

Internalization via caveolae was prevented by treatment with 5 µg/ml filipin immediately before and during incubation with the toxins.

Immunocytochemistry and fluorescence microscopy. Cells were fixed in 4% paraformaldehyde in PBS at room temperature for 30 min, washed with 100 mM glycine, and permeabilized with 0.1% Triton X-100 in PBS. To label the cis-Golgi cisternae, fixed and permeabilized cells were then blocked with 5% normal donkey serum in PBS containing 0.1% BSA and 0.1% Triton X-100 for 20 min at room temperature, washed three times with PBS, and incubated with the monoclonal antibody 10E6 (1:200 dilution in PBS containing 0.1% BSA and 0.1% Triton X-100) for 2 h at room temperature. Samples were subsequently washed three times in PBS containing 0.1% BSA and incubated with Cy3-labeled donkey anti-mouse antibody (1:500 dilution in PBS containing 0.1% BSA and 0.1% Triton X-100) for 1 h at room temperature. After samples were washed three times with PBS-0.1% BSA, samples were treated with Slow Fade (Molecular Probes) and mounted. Control experiments were performed, omitting the primary antibody. F-actin was stained by incubating the fixed and permeabilized cells in the presence of rhodamine-phalloidin (10 U/ml) for 30 min at room temperature.

Analysis of the samples was performed using the ×100 objective of either a Leica TS34D laser confocal microscope or a Leica DMRB fluorescence microscope (Heidelberg, Germany) equipped with a Micromax cooled charge-coupled device (CCD) camera (Princeton Instruments), operated from a Dell computer using Winview software (Princeton Instruments). Digitized images were cropped in Adobe Photoshop and imported to Adobe Illustrator (Adobe Systems, Mountain View, CA) for assembly and labeling.

Quantitative analysis of toxin binding. To analyze the interaction between toxins, cells were incubated with 10
µg/ml of unlabeled VT1B or 10 µg/ml of unlabeled CTB for 1 h at 4°C, washed, and warmed to 37°C for 15 min to allow internalization. The samples were then incubated with either FITC-CTB or FITC-VT1B, respectively, for 1 h at 4°C and washed. After the unbound toxin was washed, the cells were lysed by incubation in 50 mM tris(hydroxymethyl)aminomethane and 1% Nonidet P-40 (pH 8) for 5 min. After a thorough mixing, debris were sedimented in an Eppendorf 5415 microcentrifuge and the fluorescence intensity of the supernatants was quantified in a Perkin Elmer 650-40 fluorescence spectrophotometer. Background fluorescence was determined using nonlabeled cells and subtracted from all other determinations. The results were expressed as the ratio of the fluorescence intensity of toxin-pretreated and control (nonpretreated) cells.

Fluorescence ratio and Nomarski imaging. Simultaneous imaging of fluorescence and of cell morphology was performed using an inverted microscope (Axiovert 135; Zeiss, Oberkochen, Germany) equipped with epifluorescence optics. Excitation at 440 and 490 nm was provided by a xenon arc lamp via a computer-controlled shutter and filter wheel assembly (Sutter Instruments, Novato, CA), whereas continuous 620-nm illumination was achieved by filtering the transmitted incandescent source. The excitation light was attenuated by a neutral density filter and reflected to the cells by a dichroic mirror (510 nm), while the emitted fluorescence (≥510 nm) and the transmitted red light (>620 nm) were separated by an emission dichroic mirror (580 nm). The red light was directed to a video camera, allowing continuous visualization of the cells, while the fluorescent light was directed onto a 542 band-pass 62-nm filter and imaged with a cooled CCD camera (Princeton Instruments). Control of image acquisition was achieved with Metafluor software (Universal Imaging, West Chester, PA), operating on a pentium Dell computer (Dell, Toronto, ON, Canada).

For imaging experiments, the cells were grown on 25-mm diameter glass coverslips (Thomas Scientific, Swedesboro, NJ) that were inserted into a Leiden coverslip dish (Medical Systems, Greenvale, NY), which was in turn placed into a thermostatted perfusion chamber (open perfusion Micro- Incubator, Medical Systems). Regions of interest were selected for measurement by the imaging system. Background fluorescence was subtracted for each wavelength within each experiment. At the end of each experiment, a calibration curve of fluorescence vs. pH was obtained by sequentially perfusing the cells with KCl-rich medium containing (in mM) 125 KCl, 20 NaCl, 10 HEPES, 10 2-(N-morpholino)ethanesulfonic acid, 0.5 CaCl2, and 0.5 MgCl2 in the presence of 5 µM nigericin and buffered to pH values ranging from 5.5 to 7.5. The theoretical basis of this method was described earlier (15). Approximately 3 min were allowed for equilibration at each pH.

All confocal and ratio images are representative of at least three separate experiments.

VT1B uptake determinations. Cells were incubated with 10 µg/ml VT1B for 1 h at 4°C, followed by a chase at 37°C for the specified periods of time. Samples were fixed as above and then blocked with 5% donkey serum in PBS containing 0.1% BSA for 20 min at room temperature. After cells were washed three times with PBS, cells were incubated with monoclonal anti-VT1B antibody (3) (1:500 dilution in PBS containing 0.1% BSA) for 1 h at room temperature. Samples were subsequently washed three times in PBS containing 0.1% BSA and incubated with 125I-labeled goat anti-mouse antibody (0.1 µCi/ml in PBS with 0.1% BSA) for 1 h at room temperature. After three more washes, the cells were scraped and transferred to counting vials. Radioactivity was quantified using a 1282 Compu-Gamma counter (LKB Wallac, Turku, Finland). Background radioactivity, determined with the omission of the primary antibody, was subtracted from all other determinations.

RESULTS

Time course of VT1B and CTB internalization. The available evidence suggests that VT1B and CTB enter the cells via distinct mechanisms (see introduction). Their rate of internalization and subcellular distribution would therefore be expected to differ. This notion was tested by coincubating Vero cells with the two toxins and monitoring their fate by confocal fluorescence microscopy. The cell surface was labeled with a mixture of RITC-VT1B and FITC-CTB at 4°C (Fig. 1, A–C), the cells were then warmed to 37°C, and samples were taken at increasing times (Fig. 1, D–O). The toxins, which are initially seen lining the surface membrane (Fig. 1, A–C), subsequently assume a punctate distribution and eventually cluster in a tight juxtanuclear complex, likely the Golgi complex. Overlay of the red and green fluorescence (Fig. 1) revealed that the distribution of the two toxins is very similar throughout, suggesting that VT1B and CTB utilize a common pathway.

Several lines of evidence indicate that the morphological redistribution of the toxins is associated with their internalization. First, although VT1B bound to the surface of the cells at 4°C is readily accessible to a specific antibody added externally (Fig. 2A), incubation of the cells for 1 h at 37°C renders the toxin inaccessible to the antibody (Fig. 2B). In fact, the course of disappearance of VT1B from the surface, assessed by binding of anti-VT1B antibody followed by a radiolabeled secondary antibody (Fig. 2D), corresponds well to the redistribution monitored morphologically (Fig. 1). It is noteworthy that, once internalized, VT1B seemingly does not recycle back to the surface membrane. This was concluded from the observation that no labeling was observed even when the anti-VT1B antibody was added to the intact cells for 1 h at 37°C after the toxin was internalized (Fig. 2C). Significant recycling of the toxin to the surface would have resulted in progressive recruitment of extracellular antibody.

The occurrence of internalization is also supported by the observation that, shortly after warming up, both CTB and VT1B colocalize extensively with transferrin receptors, the conventional markers of early and recycling endosomes (Fig. 3). Together, these observations suggest that VT1B and CTB enter the cells by similar pathways and, within the period studied, do not recycle detectably back to the surface membrane.

Clathrin dependence of VT1B internalization. The similarities in the behavior of the two toxins contrast with the notion that they enter via different mechanisms. To better define the pathway utilized by VT, we studied the effects of three protocols routinely used to inhibit clathrin-mediated endocytosis, namely, K+ depletion, hypertonic treatment, and cytoplasmic acidification, on the uptake of FITC-VT1B by Vero cells. Whereas K+ depletion and hypertonic treatment inhibit internal-
ization by dispersing membrane-associated clathrin lattices, cytoplasmic acidification prevents the budding of clathrin-coated vesicles from the plasma membrane (11). To test the efficiency of these treatments in Vero cells, we assessed the uptake of transferrin, which is typically internalized through clathrin-coated pits and vesicles. Cells were preincubated in the presence of Texas red-labeled transferrin for 1 h at 4°C, washed, and incubated at 37°C for 20 min. The distribution of the fluorophore was then determined by confocal microscopy, and representative x vs. z reconstructions are shown in Fig. 4. Whereas transferrin was normally internalized in untreated cells (Fig. 4A), K⁺-depleted cells displayed only labeling on the surface (Fig. 4B). Virtually identical results were obtained in cells treated hypertonically or with acidic solutions (Fig. 4, C and D).

Having confirmed the effectiveness of the treatments used for inhibition of clathrin-mediated endocytosis, we proceeded to determine the role of clathrin in VT1B internalization. As shown above and reported earlier, when allowed to internalize for 1 h, VT1B accumulates in a juxtanuclear location that corresponds to the Golgi complex (Fig. 5A). Figure 5B shows that K⁺ depletion had no noticeable effect on the distribution of VT1B. Similarly, near normal internalization was observed in cells incubated in hypertonic medium (Fig. 5C). These results imply that VT1B can be internalized and reach the Golgi apparatus by clathrin-independent means. In contrast to the effects of K⁺ depletion and hypertonicity, the juxtanuclear accumulation of VT1B was markedly inhibited by cytosolic acidification (Fig. 5D). A substantial portion of the toxin remained on the cell surface, whereas a fraction was detected in small intracellular vesicles. This implies that the harsher acidic treatment is less specific, likely affecting processes other than clathrin-coated vesicle formation.

Prima facie, there is an apparent discrepancy between our observations and the results reported in Daudi cells, in which monodansyl cadaverine prevented VT1B internalization. In an attempt to reconcile these results, we tested the effect of this cadaverine derivative in Vero cells. As shown in Fig. 6A, monodansyl cadaverine effectively inhibited transferrin uptake in Vero cells. However, the internalization of VT1B proceeded normally in the presence of the transglutaminase inhibitor (Fig. 6B), confirming internalization by a clathrin-independent route.

As shown in Fig. 7A, CTB also accumulates in the Golgi complex of Vero cells. The internalization of this toxin by A-431 cells is believed to be mediated by caveolae (22). This is likely also to be the case in Vero cells, inasmuch as the uptake and juxtanuclear accumulation of CTB were unaffected by K⁺ depletion, hypertonicity, or monodansyl cadaverine (Fig. 7, B and C, and Fig. 6C), as was the case for VT1B. It is noteworthy that
CTB uptake was markedly inhibited by acid loading. This observation supports the earlier notion that cytosolic acidification exerts multiple effects, inhibiting both clathrin-dependent and -independent processes. Effect of cytochalasin B on VT1B and CTB internalization and retrograde transport. Clathrin-independent internalization of plasmalemmal components can occur by macropinocytosis, which is observed at sites of active
Unlike clathrin-dependent vesicle formation, macropinocytosis requires assembly of filamentous (F) actin and is therefore sensitive to the cytochalasins. To test the possible involvement of macropinosomes in VT1B uptake, Vero cells were pretreated with 5 µM cytochalasin B, which binds to the barbed (plus) end of F-actin, inhibiting its polymerization. The effectiveness of this drug was ascertained by staining F-actin with rhodamine-phalloidin, as illustrated in Figs. 8, A and C, and 9, A and C. Cytochalasin virtually eliminated the stress fibers and decreased the overall content of F-actin, which was largely clustered in irregular aggregates (cf. A and C in Figs. 8 and 9). Figure 8 also shows that VT1B was effectively internalized in cytochalasin-treated cells. It is noteworthy that the morphology of the intracellular compartment where VT1B was accumulated was altered by F-actin disruption. However, this does not reflect a change in the targeting of the toxin but rather an alteration in Golgi morphology. This was determined by comparing the distribution of the toxin with that of a cis-Golgi marker, identified by antibody 10E6 (Fig. 8, E and F). Similar results were obtained using CTB (Fig. 9). These findings indicate that neither macropinocytosis nor other F-actin-dependent processes are important in VT1B internalization. Moreover, they imply that actin polymerization is not required for the retrograde targeting of either VT1B or CTB to the Golgi.

Involvement of caveolae in VT1B internalization. Clathrin-independent internalization can also occur through caveolae, which are 50- to 60-nm plasma membrane invaginations with a characteristic flask shape. These structures are enriched in glycosphingolipids and cholesterol and contain caveolin, a 21-kDa cholesterol-binding protein that cycles between the plasma membrane and the Golgi complex (23). Considering the similar subcellular distribution of VT1B and CTB and their common resistance to antagonists of clathrin-mediated uptake, it appeared likely that VT1B also utilizes caveolae for internalization. This notion was tested with filipin, a cholesterol-binding drug that causes disassembly of caveolae. In cells treated with filipin (5 µg/ml), the internalization of VT1B was...
partially inhibited (Fig. 10A). The toxin displayed a punctate distribution, with moderate perinuclear accumulation.

This finding could reflect incomplete inhibition of caveolae formation or the presence of an alternate internalization pathway. The latter possibility was analyzed by combining the use of filipin with hypertonic treatment, a mild procedure for inhibition of clathrin-mediated endocytosis. As illustrated in Fig. 10B, the inhibition of VT1B uptake was virtually complete under these conditions. This observation implies that VT1B can be endocytosed via clathrin-independent as well as clathrin-independent routes, likely caveolae. The failure to notice significant inhibition when inhibiting only the former suggests that caveolae are the predominant pathway and/or that one route is upregulated when the other is inhibited.

pH dependence of VT1B and CTB retrograde transport. The retrograde transport of furin and TGN38 from the plasma membrane to the Golgi complex requires intraorganellar acidification (see introduction). To determine whether toxins such as VTB utilize a similar pH-dependent pathway, we monitored their distribution under conditions expected to dissipate the electrochemical H⁺ gradient of endomembrane compartments. The luminal pH of endosomes and of components of the Golgi apparatus is maintained as acidic by vacuolar H⁺ pumps, which are selectively inhibited by macrolide antibiotics such as the bafilomycins and concanamycins. Cells were therefore treated with concanamycin A (100 nM) to inhibit active pumping. To ensure elimination of preexisting H⁺ gradients, the cells were additionally exposed to the weak base chloroquine (50 µM). The dissipation of the organelar acidification was initially verified by measuring the pH of endosomes and Golgi complex by imaging the emission of FITC-labeled CTB, a pH-sensitive fluorescent probe. Cells were preincubated with FITC-CTB for 1 h at 4°C, washed, and then incubated for an additional 20 min at 37°C to allow internalization. At this time, the probe is localized largely in endosomes, although some of the juxtanuclear toxin may have entered the TGN (Fig. 11A). Endosomal pH was then monitored by quantifying the fluorescence with excitation at 440 and 490 nm (see MATERIALS AND METHODS). As illustrated in Fig. 11B,
the pH in otherwise untreated cells averaged 6.5 ± 0.03 (mean ± SE of 30 determinations from 3 experiments), in the range reported for early and recycling endosomes (8). The combined treatment with concanamycin and chloroquine elevated pH to 7.1 ± 0.09 (mean ± SE of 30 determinations). Alkalinization was similar in the juxtanuclear and submembranous endosomes.

Having demonstrated the effectiveness of the pH dissipation protocol, we proceeded to test the pH dependence of VT1B retrograde transport. Cells were treated with concanamycin and chloroquine during the last 0.5 h of the preincubation at 4°C with the toxin and throughout the internalization period at 37°C. Figure 12 shows that conditions described above to eliminate intraorganellar pH gradients had little effect on VT1B internalization. The targeting of the toxin to the Golgi complex was confirmed by dual labeling with the marker antibody 10E6 (cf. Fig. 12, A–D). These observations indicate that transport of VT1B from the membrane to the Golgi complex does not require endosomal acidification. Retention of the toxin within the Golgi complex is similarly independent of the activity of the V-ATPase. This was shown in the experiments of Fig. 12, E–F. VT1B was allowed to reach the Golgi under normal conditions, and the pH gradient was then dissipated as above. The distribution of fluorescence was analyzed following an additional 2-h incubation in the presence of concanamycin A and chloroquine. Such a long incubation with the inhibitors resulted in visible swelling of the Golgi, yet the toxin was retained within this compartment. These findings indicate that the toxin does not cycle between the Golgi and other compartments over the time course analyzed or that such recycling occurs in a pH-independent manner. The pH dependence of the retrograde targeting and retention of CTB was analyzed similarly. As summarized in Fig. 13, CTB was directed to the Golgi complex even after dissipation of intraorganellar acidification and was retained therein for at least 2 h in the absence of a pH gradient.

It was important to ascertain that, under the conditions used, targeting of resident proteins to the TGN was impaired, as had been reported (5). To this end, we used cells stably transfected with a chimeric protein consisting of the cytosolic and transmembrane domains of furin, which dictate TGN localization, attached to an extracellular epitope that was readily detectable immunologically (a portion of human IgG). As shown in Fig.
14A, such a chimeric protein concentrates in the juxtanuclear location anticipated for the TGN. More importantly, treatment with the combination of concanamycin (100 nM) and chloroquine (50 µM) resulted in dispersal of furin-IgG in a diffuse vesicular pattern (Fig. 14B), consistent with earlier observations (5).

Under comparable conditions, CTB targeted normally to the Golgi complex, where it retained a compact distribution (cf. Fig. 14, C and D). These observations highlight the differential behavior of the retrograde pathways of proteins and lipids.

Are VT1B and CTB receptors associated? Association among lipids with similar functional and/or structural characteristics in microdomains often called “rafts” has been shown or postulated to exist in different systems. Such lipid-rich microdomains are seemingly involved in sorting and targeting processes (4). Because the receptors for VT1B and CTB (namely, Gb3 and GM1) are both glycolipids, we considered the possibility that they are physically associated in macromolecular complexes, possibly rafts. We reasoned that, if stable association exists, internalization of one of the glycolipids would drive the uptake of the other and vice versa. This was tested experimentally by inducing the uptake

**Fig. 9.** Effect of cytochalasin B on the internalization and targeting of CTB. Vero cells were allowed to internalize FITC-CTB in the absence (A and B) or presence (C–F) of cytochalasin D (5 µM). A and C: actin filaments were stained with rhodamine-phalloidin. B, D, and F: CTB fluorescence. E: indirect immunofluorescence using Ab 10E6, directed to an epitope in the cis-Golgi. Results are representative of 3 similar experiments.

**Fig. 10.** Role of caveolae in VT1B internalization. A: Vero cells were preincubated with FITC-VT1B for 1 h at 4°C in the presence of 5 µg/ml of filipin and then were incubated for another hour at 37°C in filipin-containing solution. B: cells were preincubated with hypertonic medium as in Fig. 1C and then labeled at 4°C with FITC-VT1B in the same medium with added filipin. These conditions were maintained throughout the internalization chase (1 h at 37°C). Representative x vs. y scans and x vs. z reconstructions are shown for each condition in top and bottom, respectively. Results are representative of 3 similar experiments.
of one of the lipid receptors by addition of its unlabeled cognate toxin and measuring the degree of surface exposure of the other with the respective tagged toxin. To confirm that a sizable fraction of the first lipid was internalized, we initially performed experiments measuring the effect of each toxin on the ability of a second pulse of the same toxin to bind to the surface. Pretreatment with unlabeled CTB was followed by incubation

Fig. 11. Effect of concanamycin and chloroquine on the pH of endomembrane compartments. A: Vero cells were preincubated with FITC-CTB for 1 h at 4°C, washed, and then incubated for 20 more min at 37°C to allow internalization of the toxin. A representative fluorescence micrograph, obtained using the imaging setup described in MATERIALS AND METHODS, is illustrated. B: pH of the endomembrane compartments labeled with FITC-CTB as in A was measured by fluorescence ratio imaging in otherwise untreated cells (control) and in cells incubated with 100 nM concanamycin (CCM) and 50 µM chloroquine (CLQ) during the last 30 min of the incubation at 4°C and throughout the incubation at 37°C. Data are means ± SE of 30 determinations.

Fig. 12. pH dependence of VT1B internalization. Vero cells were labeled with FITC-VT1B in the cold and then incubated in the absence (A and B) or presence (C–F) of concanamycin (100 nM) and chloroquine (50 µM) as indicated. At the end of the experiment, cells were fixed, permeabilized, and labeled by indirect immunofluorescence using Ab 10E6. Representative x vs. y confocal scans are illustrated. Results are representative of 3 similar experiments.
for 15 min at 37°C, to allow internalization, and then by exposure to FITC-CTB, which was finally quantified fluorometrically (see MATERIALS AND METHODS). As shown in Table 1, upwards of 60% of the GM₁ became inaccessible after incubation with 10 µg/ml CTB. Similarly, nearly 55% of the Gb₂ was inaccessible after treatment with 10 µg/ml of VT₁B. In contrast, pretreatment with VT₁B had no detectable effect on the amount of GM₁ available at the plasma membrane and CTB only modestly decreased the surface-exposed Gb₂, as measured by VT₁B binding. These observations indicate that GM₁ and Gb₂ are internalized independently and not as part of tightly coupled macromolecular complexes.

DISCUSSION

Endogenous retrograde pathways directing molecules from the plasma membrane to compartments of the secretory pathway can be subverted by bacterial toxins, which travel through endosomes to the Golgi complex and sometimes as far back as the endoplasmic reticulum and nuclear envelope. Several lines of evidence indicate that such retrograde translocation is essential for the toxins to exert their biological effects. In particular, it has been shown that cells are protected against the toxic effects of these bacterial polypeptides by brefeldin A (30) and by overexpression of inactive mutants of Rab₁, Sar₁, and Arf₁ (33), which preclude retrograde transport between the Golgi and the endoplasmic reticulum.

Although different aspects of the uptake of toxins have been investigated (29, 31), the nature of the retrograde route mediating their transport from the plasma membrane has not been elucidated. In this report, we compared the internalization and targeting of VT₁B and CTB and showed that they share steps of a common retrograde pathway that differs, at least in part, from the one employed by furin and TGN38.

VT₁B internalization. VT₁B appears to be internalized in Vero cells by two distinct pathways: one that is clathrin dependent and one (or more) clathrin-independent route(s). Endocytosis was partially inhibited by filipin, suggesting the involvement of caveolae, whereas the combination of this drug with hypertonicity resulted in complete inhibition, implying a contribution from clathrin-coated vesicles. We were unable to determine whether both routes are constitutively active or whether the inhibition of one of them caused upregulation of the other. In addition, the specificity of the pharmacological agents used may not be absolute, so that alternative pathways cannot be ruled out. It is clear, however, that macropinocytosis and other F-actin-
dependent processes do not contribute importantly to toxin uptake in Vero cells.

The involvement of cavedae in VT1B uptake is consistent with their reported role in endocytosis of CT, tetanus, and heat-labile toxin (22, 24, 34). Importantly, a dual-internalization mechanism like the one reported here was also observed for ricin, a toxin that binds terminal galactose residues of glycolipids and glycoproteins (32, 33). In contrast, endocytosis of Shiga toxin, which is almost identical to VT1B, was believed to occur primarily via clathrin-coated pits (26, 28). The observed differences may be due to subtle structural differences between these toxins or may reflect differences in the cell types or experimental protocols used.

It is not clear how glycolipids localize to coated pits. It is conceivable that they are specifically associated with transmembrane proteins that bear endocytic targeting sequences that bind to adaptor complex 2. It is easier to rationalize the internalization of toxin receptors via cavedae, where the glycolipids are originally abundant. It remains to be determined if the glycolipids cycle constitutively from the plasma membrane to the secretory pathway or if internalization is triggered by binding to the toxins. The latter is pentavalent, raising the possibility that cross-linking of multiple glycolipids and their associated proteins and lipids induces active internalization.

Characterization of VT1B and CTB targeting to the Golgi complex. Glycosphingolipids have been shown to form microdomains at the plasma membrane and TGN. In polarized cells, TGN microdomains are involved in sorting and specific targeting of secreted proteins through the formation of rafts (4). However, the involvement of such rafts in retrograde transport from the plasma membrane has not been determined. Indeed, comparatively little is known about the lipid retrograde transport pathways. Martin and Pagano (21) showed that, in fibroblasts, glucosylceramide fluorescent analogs are internalized from the plasma membrane by an ATP-independent and temperature-insensitive saturable mechanism to the Golgi complex, where they can be targeted to the Golgi complex. In the case of Gb3 and GM1, uptake is clearly temperature sensitive and mediated by endosomes.

Table 1. Interaction between VT1B and CTB during internalization

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Toxin binding, %control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unlabeled CTB → FITC-CTB</td>
<td>37 ± 7.5</td>
</tr>
<tr>
<td>Unlabeled VT1B → FITC-VT1B</td>
<td>47 ± 3.6</td>
</tr>
<tr>
<td>Unlabeled CTB → FITC-VT1B</td>
<td>82.8 ± 5.8</td>
</tr>
<tr>
<td>Unlabeled VT1B → FITC-CTB</td>
<td>103.2 ± 10</td>
</tr>
</tbody>
</table>

Values are means ± SE of 3 separate experiments. Results are expressed as percent of the fluorescence intensity of a parallel sample treated with the fluorescein isothiocyanate (FITC)-labeled toxin only. Vero cells were incubated with 10 µg/ml of unlabeled B subunit of verotoxin 1 (VT1B) or 10 µg/ml of unlabeled cholera toxin B subunit (CTB) for 1 h at 4°C, washed, and warmed to 37°C for 15 min to allow internalization. Samples were then incubated with either FITC-CTB or FITC-VT1B, as indicated, for 1 h at 4°C and washed. After unbound toxin was washed, cells were lysed and the fluorescence intensity of the supernatants was quantified fluorometrically (see MATERIALS AND METHODS for details).
Ligands internalized through coated and uncoated pits have been found within the same endosomal compartments, suggesting that these structures are a meeting point for molecules entering by different routes. The ligands and their receptors are then sorted and directed to their final destinations. Delivery of early endosomal proteins to late endosomes and lysosomes involves a step that is dependent on the maintenance of an acidic luminal pH. The precise nature of the pH-sensitive step is ill-defined, but assembly of coat proteins before budding of the endosomes has been proposed as the critical acidification-dependent event (1). Importantly, retrieval of proteins like furin and TGN38 from the membrane to the TGN is comparably pH sensitive. It is therefore remarkable that retrograde targeting of neither VT1B nor CTB was affected by a combination of chloroquine and concanamycin. Parallel experiments confirmed that this combination of agents neutralized the pH of the compartments involved in the translocation of the toxins to the TGN (Fig. 11) and of the TGN itself (data not shown) and altered the targeting of TGN-resident proteins (Fig. 14). This result is consistent with observations made earlier with other toxins. Treatment with either NH₄Cl, nigericin, or monensin was unable to prevent the intoxication of Vero cells with Shiga toxin, a process that requires retrograde transport (26). Similarly, in T84 cells, dissipation of the pH gradient with chloroquine, nigericin, or concanamycin did not alter internalization of CT or the subsequent signal transduction events (19). We therefore conclude that at least two different retrograde pathways exist for delivery of plasmalemmal macromolecules to the TGN: one that requires luminal acidification, which is used by molecules such as furin and TGN38, and another one that is pH independent and mediates retrograde transfer of glycolipids and their ligands.

Finally, data were presented that suggested that internalization of GB₁ and GM₁ occurs independently, arguing against mediation by stable glycolipid rafts. It is possible that the glycolipids exist within the membrane as monomeric entities or in association with specific proteins. These may cycle constitutively between the Golgi and the plasma membrane. However, no significant forward transport was detected for hours after the lipid-toxin complexes reached the Golgi region (e.g., see Figs. 2C, 12, E and F, and 13, E and F), arguing against constitutive cycling. Alternatively, it is conceivable that addition of the pentavalent toxins may induce cross-linking of the lipids and their associated proteins, thereby signaling internalization. The detailed mode of internalization remains to be defined.

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