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Bafilomycin A_1 Treatment Retards Transferrin Receptor Recycling More than Bulk Membrane Recycling*

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Treatment of Chinese hamster ovary cells with the vacuolar proton pump inhibitor bafilomycin A_1 causes a 2-fold retardation in the rate of recycling of transfected human transferrin receptors back to the cell surface as measured using biochemical assays (Johnson, L. S., Dunn, K. W., Pytowski, B., and McGraw, T. E. (1993) *Mol. Biol. Cell* 4, 1251–1266). We have used quantitative fluorescence microscopy to determine which step(s) in the endocytic recycling pathway are affected. We show that removal of transferrin from sorting endosomes and accumulation in the peri-centriolar endocytic recycling compartment takes place normally in bafilomycin A_1-treated cells. However, the rate constant for exit of transferrin receptors from recycling endosomes (k_e) is reduced from 0.063 min^{-1} in untreated cells to 0.034 min^{-1} in the presence of bafilomycin A_1. This retardation appears to be dependent on the presence of internalization motifs in the cytoplasmic domain since modified receptors lacking these oligopeptide motifs do not show as large a decrease in recycling rate in the presence of bafilomycin A_1. Bulk membrane recycling (measured by efflux of an internalized fluorescent lipid analog, 6-[*N*-[7-nitrobenzo-2-oxa-1,3-diazol-4-yl]-amino]-hexoyl-sphingosylphosphorylcholine) is slowed from an exit rate constant of 0.060 min^{-1} without drug to 0.046 min^{-1} in the presence of bafilomycin A_1. We conclude that bafilomycin A_1 slows bulk membrane flow, but it causes additional inhibition of receptor recycling in a manner that is dependent on a peptide motif on the cytoplasmic domain.

After internalization via clathrin-coated pits, endocytosed material rapidly enters sorting endosomes, organelles that sort recycled molecules such as transferrin (Tf)[1] bound to the transferrin receptor (Tf-R) from lysosomally destined molecules such as low density lipoprotein (1–3). We have provided evidence for a sorting mechanism in which components not carrying special retention or targeting signals are removed from the sorting endosome by a default process based on the geometry of the sorting endosome and an iterative fractionation mechanism (1, 3). Most of the fluid brought into the tubulo-vesicular sorting endosome fills a spherical lumen, whereas most of the surface area is on tubular extensions that are involved in the removal of membrane components from the sorting endosome for export along the recycling pathway (1–3). Tf remains attached to its receptor throughout the recycling pathway, but it is converted to apo-Tf by the low pH of endosomes (4, 5). Occupied Tf-R are transported from sorting endosomes to the endocytic recycling compartment, which in CHO cells is a collection of tubules concentrated near the centriole (6). The Tf-R are returned to the cell surface from the endocytic recycling compartment, and apo-Tf dissociates from the receptor upon return to the surface (5). It has been shown that internalized lipid analogs such as 6-[*N*-[7-nitrobenzo-2-oxa-1,3-diazol-4-yl]-amino]hexoyl-sphingosylphosphorylcholine (C_6-NBD-SM) follow the same endocytic recycling pathway as Tf, indicating that in the absence of specialized signals membrane molecules will recycle efficiently from endosomes to the cell surface (3). Endocytosed fluid and ligands released from their receptors by low endosomal pH are retained in the lumen of the sorting endosome and accumulate. The vesicular portions of the sorting endosomes become the vesicles that fuse with late endosomes (7–9).

Acidification of endosomes by ATP-dependent proton pumps is important for several aspects of endocytic trafficking. Two well established functions of the low pH of endosomes are to dissociate lysosomally destined ligands from membrane-associated receptors and to strip Fe^{3+} from transferrin (4, 5, 10, 11). Two additional roles for endosome acidification have been proposed based on studies of drug-treated cells or mutant cell lines, but these are not well understood at present. Inhibition of endosome acidification has been associated with slowed endocytic recycling and intracellular retention of recycling receptors (12–14). In addition, the delivery of endocytosed molecules to late endosomes or lysosomes is affected by treatments that impair acidification (15, 16). Unfortunately, both of these effects appear to vary among cell lines.

Several studies have shown that weak bases and ionophores

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[1] The abbreviations used are: Tf, transferrin; Tf-R, transferrin receptor; Tx-Tf, Texas Red-labeled transferrin; BSA, bovine serum albumin; C_6-NBD-SM, 6-[*N*-[7-nitrobenzo-2-oxa-1,3-diazol-4-yl]-amino]hexoyl-sphingosylphosphorylcholine; CCD, charge-coupled device; CHO, Chinese hamster ovary; FITC, fluorescein isothiocyanate; F-R-Tf, fluorescein-rhodamine transferrin; NA, numerical aperture; PBS, phosphate-buffered saline.

that collapse transmembrane pH gradients can reduce the fraction of recycling receptors that are expressed on the cell (17–20). However, the degree of inhibition of receptor trafficking varies greatly from study to study. Since weak bases and ionophores have multiple effects, including osmotic effects on organelles, interpretation of these experiments has not been straightforward (21).

In 12-4 cells, a CHO mutant line in the *end*2 complementation group that has partially defective acidification of endosomes, Tf exits the sorting endosome at a normal rate, but it exits the recycling compartment more slowly than in the parental cells (22). However, the rate of bulk membrane recycling, measured by the release of internalized C_6-NBD-SM from the cells, is identical to the rate in the parental cells, suggesting that Tf is retained in the recycling compartment by a specific retention mechanism.

Recently, it was shown that the proton pump inhibitor bafilomycin A_1 raised the pH of sorting and recycling endosomes and slowed recycling of the Tf-R in a Chinese hamster ovary cell line transfected with the human Tf-R (TRVb-1) (14). Internalization of the Tf-R was not affected, but externalization was slowed 2-fold, leading to a net intracellular accumulation of Tf-R.

The effects of bafilomycin A_1 on delivery of molecules to late endosomes and lysosomes have also been investigated (15, 16). In a study of BHK cells it was found that delivery to late endosomes was blocked by treatment with bafilomycin A_1, and in cell homogenates formation of multivesicular body/endosomal carrier vesicles was inhibited by bafilomycin A_1 (15). These multivesicular body/endosomal carrier vesicles are the intermediates between early sorting endosomes and late endosomes (9). In contrast, in HepG2 cells, delivery to late endosomes was slowed somewhat, but delivery to lysosomes was nearly completely blocked (16). Neither the basis for these differences nor the molecular role of endosome acidification in these processes is known. These discrepancies illustrate that even the organelles where bafilomycin A_1 exerts its effects are not well understood.

In this paper, we examine the effects of bafilomycin A_1 on bulk membrane traffic and on normal and mutant Tf-R recycling in wild-type and TRVb-1 cells. Using digital fluorescence microscopy we find that in bafilomycin A_1-treated cells, Tf-R leaves sorting endosomes with normal kinetics, but its exit from the recycling compartment is slowed similar to the observations in 12-4 cells (22). However, we also find that bafilomycin A_1 treatment causes bulk membrane recycling to be significantly slowed. The slowing of membrane recycling accounts for about one-third of the reduction in the rate of Tf-R recycling.

EXPERIMENTAL PROCEDURES

Materials—Bafilomycin A_1, obtained from Dr. K. Altendorf (University of Osnabrück, Germany), was dissolved in Me_2SO to make a stock solution that was frozen in aliquots. Anti-Tf antibody (B3/25) was obtained from Hybritech Inc. (San Diego, CA). Goat anti-mouse FITC-conjugated antibodies were obtained from Pierce. Human transferrin (Sigma) was iron loaded and purified as described previously (6). Transferrin was conjugated to FITC (FITC-Tf) as described previously (6) and to Texas Red (Tx-Tf) (Molecular Probes Inc., Eugene, OR) according to the manufacturer's instructions. F-R-Tf was made as described previously (23) by reacting iron loaded Tf with succinimidyl esters of rhodamine and fluorescein (8 molecules total of dye per molecule Tf) in a 100 mM sodium borate buffer, pH 9.0, for 1 h, and then removing excess dye by extensive dialysis against phosphate-buffered saline (pH 7.4). C_6-NBD-SM was obtained from Molecular Probes Inc. (Eugene, OR).

Cell Culture and Drug Treatments—TRVb-1 cells, a previously described CHO cell line lacking the endogenous Tf-R but expressing the human Tf-R (24), were grown in bicarbonate-buffered Ham's F-12 (Life Technologies, Inc.) at pH 7.4, supplemented with 5% fetal bovine serum (Life Technologies, Inc.), 100 units/ml penicillin, 100 μg/mg streptomycin, and 200 μg/ml G418 in 100-mm dishes. The 12-4 endosome acidification mutant cell line derived from TRVb-1 (25) was grown similarly. For microscopy experiments, cells were plated in 35-mm coverslip bottom dishes 2 to 3 days prior to experiments and grown to 50% confluence as described previously (26). For biochemical lipid efflux experiments, cells were plated in 60-mm dishes as described previously (3, 22) and grown for 3 days (until 90% confluent). Bafilomycin A_1 (0.5 or 0.25 μM) with a maximum final concentration of 0.1% Me_2SO was used for all drug treatments. Non-drug-treated controls were mock treated with the same concentration of Me_2SO as bafilomycin A_1-treated cells in all experiments. Cells were kept in either HF-12 (Ham's F-12 medium without bicarbonate, but with 20 mM HEPES buffer, pH 7.4) or medium 1 (150 mM NaCl, 20 mM HEPES, pH 7.4, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mg/ml glucose) in experiments carried out outside of a CO_2-buffered incubator.

Fluorescent Lipid Labeling—Labeling with small unilamellar vesicles of the fluorescent sphingomyelin analog C_6-NBD-SM was carried out exactly as described (3, 22). Small unilamellar vesicles were made using C_6-NBD-SM and dioleylphosphatidylcholine (1:1) (3). TRVb-1 cells were labeled with 5 μM C_6-NBD-SM vesicles on ice for 30 min in HF-12 (3). Cells were then washed and warmed to 37 °C for 10 min to allow internalization of fluorescent lipid that had been inserted into the plasma membrane. They were then back-exchanged at 4 °C against several washes of medium 1 containing 5% bovine serum albumin for 1 h. This back-exchange typically removed 98–99% of surface-bound fluorescent lipid (3). Monolayers were then warmed to 37 °C in the presence of medium 1 containing 1% bovine serum albumin to back-exchange effluxed C_6-NBD-SM for varying times as indicated for individual experiments. In biochemical experiments, cell-associated and effluxed lipid was butanol-extracted and quantified by spectrofluorometric analysis exactly as described (3, 22).

Immunofluorescence Staining of Tf-R—TRVb-1 cells on coverslip bottom dishes were fixed with 2% formaldehyde for 30 min and permeabilized with 500 μg/ml saponin in phosphate-buffered saline (PBS), containing 1% BSA and 40 mM methylamine for 20 min. Dishes were then stained with the B3/25 antibody at 4 μg/ml for 1 h and washed in PBS with 1% BSA for 15 min. Cells were stained with a goat anti-mouse polyclonal antibody conjugated to FITC at a 1:100 dilution for 1 h, washed for an additional 15 min, and then placed in Slow-Fade (Molecular Probes, Eugene, OR) and examined by fluorescence microscopy. Under these conditions, the antibody showed a distribution indistinguishable from that of fluorescent Tf at steady state (2.5 h) in the same cells (data not shown).

Quantitative Fluorescence Microscopy—For analysis of approach to steady state of Tf in the recycling compartment, coverslip bottom dishes were pretreated with 0.5 μM bafilomycin A_1 or mock pretreated with 0.1% Me_2SO for 15 min and then labeled with Tx-Tf for varying periods. In each experiment, 3 to 4 dishes were labeled to steady state (2.5 h) with Tx-Tf in the absence of drug. Other dishes were labeled with Tx-Tf for 0–18 min with or without bafilomycin A_1 treatment. Labeling was terminated by acid washing the dishes on ice in mild acid buffer (containing 50 mM sodium citrate, 280 mM sucrose, and 0.01 mM deferoxamine mesylate, pH 4.6) for 2 min followed by a 2-min wash in cold medium 1 on ice (27) and fixation in 2% paraformaldehyde in PBS at room temperature (first 2 min on ice) for 30 min. Dishes were stained with an anti-Tf-R mouse monoclonal antibody (B3/25) and FITC conjugated rabbit anti mouse secondary antibody as described above.

Labeled cells were examined using a 40 ×, NA 1.3 objective on a Leitz Diavert microscope with a cooled charge coupled device (CCD) camera (Photometrics, Inc., Tucson, AZ) using a 660 × 517 pixel array as described previously (3, 22). Images were either viewed and processed on a SPARC/300 workstation (Sun Microsystems, Inc., Mountainview, CA) using software from Inovision Corp. (Durham, NC), or transferred to a micro VAX (DEC, Maynard, MA) and processed on a Gould 8500 image processor (Vicom, Fremont, CA).

Corresponding pairs of Tx-Tf and FITC images (5 to 6 pairs of images per dish, >50 cells) were obtained. The images in each pair were aligned and background corrected using a median filter, and recycling compartments were defined in the FITC (antibody) image using procedures described previously (22). The recycling compartment in the antibody image was used as a mask, so that fluorescence from the antibody image and from the background-corrected Tx-Tf image was quantified from precisely corresponding pixels.

In the dishes labeled to steady state (*i.e.* 2.5 h labeling), each receptor in the recycling compartment should be occupied with Tx-Tf. Therefore, there should be an equal number of Tx-Tf molecules and Tf-R. If F_{tx} is defined as Tx-Tf fluorescence power and F_a is antibody fluorescence power in recycling compartments, a correction factor (C) that relates the

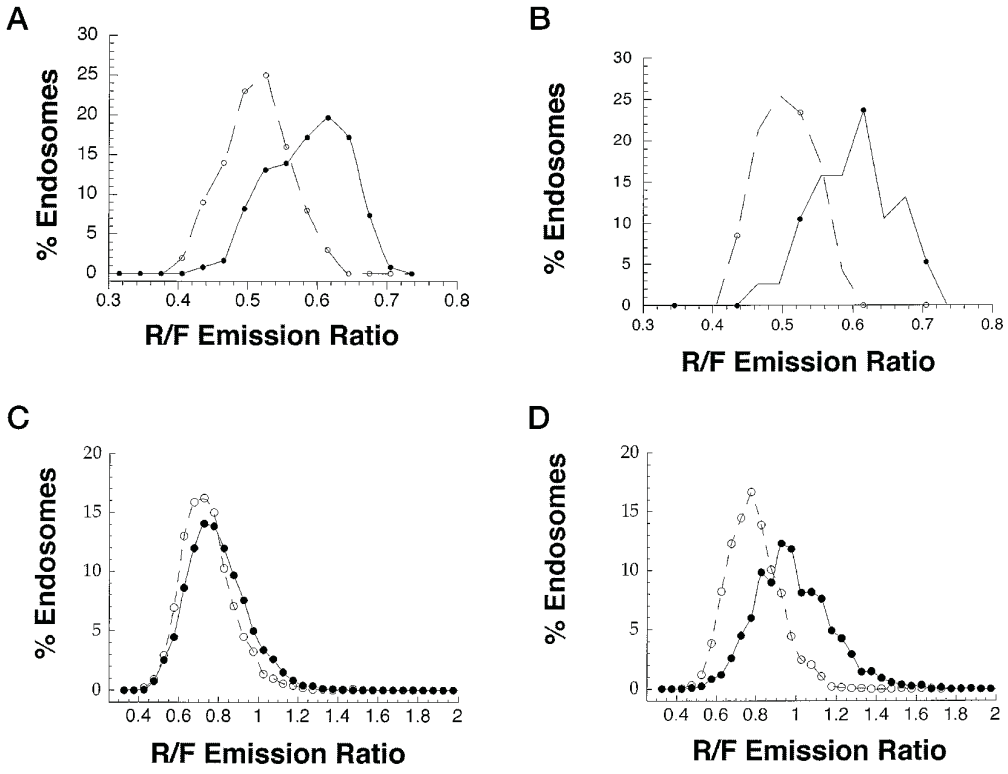

FIG. 1. **The effects of bafilomycin A_1 on pH of sorting and recycling endosomes in TRVb-1 and 12-4 cells.** *A* and *B*, cells were labeled for 10 min with F-R-Tf as described in the text, and images were collected for another 10 min. Histograms show the distribution of rhodamine/fluorescein ratios in R-F-Tf-labeled recycling endosomes in 12-4 (*A*) and TRVb-1 (*B*) cells with and without bafilomycin A_1 treatment. *A*, ●, 12-4 Me_2SO; ○, 12-4 bafilomycin. *B*, ●, TRVb-1, Me_2SO; ○, TRVb-1 bafilomycin. *C* and *D* show the distributions of rhodamine/fluorescein ratios in R-F-Tf-labeled sorting endosomes of 12-4 cells (*C*) and TRVb-1 cells (*D*). *C*, ●, 12-4 Me_2SO; ○, 12-4 bafilomycin. *D*, ●, TRVb-1 Me_2SO; ○, TRVb-1 bafilomycin.

fluorescence from the two labels of Tf-R can be determined from fluorescence measurements at steady state as shown in Equation 1.

$$F_a/F_{tx} = C \qquad \text{(Eq. 1)}$$

This correction factor can then be used in time course experiments to determine the fraction of Tf-R occupied (f_o) and the fraction of unoccupied Tf-R (f_u) by Equation 2 since the antibody will label Tf-R in the recycling compartment at all times.

$$(C \cdot F_{tx})/F_a = f_o \quad \text{and} \quad f_u = 1 - f_o \qquad \text{(Eq. 2)}$$

pH Measurements—The methods for measuring pH of sorting and recycling endosomes were as described in Ref. 22. Briefly, TRVb-1 or 12-4 cells were plated in coverslip bottom dishes 2 days before the experiment and grown until cells were well spread but not yet confluent. Tf-R expression levels were matched by growing 12-4 cells in Ham's F12 medium, which contains iron salts, and TRVb-1 cells in McCoy's 5A medium, which lacks iron salts. Tf-R are up-regulated in TRVb-1 cells grown in McCoy's 5A medium. Dishes were preincubated in medium 1 containing 0.5 μM bafilomycin A_1 for 25 min and then incubated in the same medium with 10 μg/ml Tf labeled with both rhodamine and fluorescein (F-R-Tf). Fluorescence images of sorting endosomes were collected between 5 and 15 min of labeling with a Zeiss 63 × planapochromat NA 1.4 oil immersion objective, and images of recycling compartments were collected between 10 and 20 min of labeling with a Zeiss 40 × plan-Neofluor NA 0.9 water immersion objective. Images were collected with a Zeiss Axiovert microscope using a Bio-Rad MRC-600 confocal attachment, with a 515–545 nm fluorescein and 575 nm rhodamine emission filter sets, and 488 nm excitation. For calibration, cells were fixed and maintained in a range of pH calibration buffers with 10 μM nigericin. The average value of the ratio of rhodamine to fluorescein fluorescence power from endosomes was graphed against pH, and the resulting pH calibration curves were used to assign pH to ratios determined in the experimental dishes.

Image processing was as described previously (22). Sorting endosomes were identified as small punctate structures, and recycling compartments appear as single large structures. Fluorescence from sorting endosomes was quantified after applying a size criterion that eliminated the large recycling compartments. Recycling compartments were identified by visual inspection of processed images. Ratios of rhodamine to fluorescein fluorescence were calculated for individual compartments, and histograms were plotted.

Measurement of Surface Transferrin Receptor—To determine the effect of bafilomycin A_1 on surface expression of receptors, cells grown in 6-well clusters (Becton Dickinson, Lincoln Park, NJ) for 2 days before use were incubated in McCoy's binding buffer (McCoy's 5A medium, without $NaHCO_3$, 100 units/ml penicillin, 100 mg/ml streptomycin, 20 mM HEPES, pH 7.4) for 45 min at 37 °C. The cells were then washed and incubated in 0.25 μM bafilomycin A_1 at 37 °C for the indicated times. The dishes were placed on an ice slurry, washed 3 times with ice-cold medium 1, and incubated with 3 μg/ml ^{125}I-Tf in medium 1 for at least 2 h. The cells were then washed 6 times with ice-cold medium 1 and solubilized. The radioactivity was determined by gamma counting (Wallac Inc., Gaithersburg, MD). One 6-well cluster was preincubated for 45 min in McCoy's binding buffer as described above and then placed on ice with 3 μg/ml ^{125}I-Tf in medium 1 for at least 2 h, and surface Tf binding was determined. The surface Tf binding measured for this plate served as the untreated control value. For each plate the nonspecific Tf binding was determined by including a 200-fold excess of unlabeled Tf in two of the wells during binding on ice. The average surface Tf binding per plate was calculated by computing first an average nonspecific binding from the two competition wells and subtracting this value from the value of each of the 4 experimental wells. Typically, the nonspecific counts were less than 10% of the total. The 4 background-corrected experimental wells were then averaged to give a value for the plate, which could be compared with the similarly determined control value. The data presented are the average values of at least 5 separate experiments ± S.E.

Effect of Bafilomycin A_1 on Surface Tf-R—We assumed that the Tf-R cycle between two pools, a surface and an internal pool, and that exchange between the two pools occurs with simple first order kinetics. Previous studies with CHO cells indicate that internalization and recycling rate measurements are consistent with this assumption within experimental error (1, 3).

Given these assumptions, at steady state surface Tf-R (Tf_s) and internal Tf-R (Tf_i) are related by Equations 3–5.

TABLE I
pH measurements ± bafilomycin A_1

Cell and compartment[a]	Mean pH no drug[b] (ratio ± S.D.)	Mean pH bafilomycin A_1[c] (ratio ± S.D.)
TRVb-1 sorting endosomes	pH 6.1 (0.98 ± 0.19)	pH 6.8 (0.79 ± 0.14)
12–4 sorting endosomes	pH 6.8 (0.79 ± 0.15)	pH 7.1 (0.74 ± 0.13)
TRVb-1 recycling endosomes	pH 6.5 (0.60 ± 0.056)	pH 7.5 (0.50 ± 0.038)
12–4 recycling endosomes	pH 6.6 (0.59 ± 0.0056)	pH 7.5 (0.51 ± 0.045)

[a] From Fig. 1; the ratio is the mean rhodamine:fluorescein emission ratio.
[b] 0.1% Me_2 SO.
[c] 0.5 μM bafilomycin A_1 and 0.1% Me_2SO.

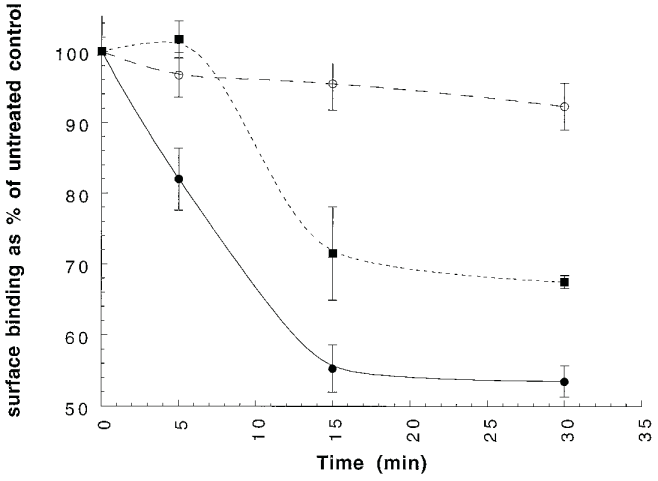

FIG. 2. **Changes in surface expression of Tf-R cells treated with bafilomycin A_1.** TRVb-1 cells containing the human Tf-R or the indicated 12-4 cells were preincubated in McCoy's binding buffer for 45 min at 37 °C. At the end of the preincubation, 0.25 μM bafilomycin A_1 was added for the indicated lengths of time at 37 °C. The cells were then chilled on ice, and surface Tf-R was assayed by binding of ^{125}I-Tf. Values were then compared with control values determined for the Tf-R in untreated 12-4 or TRVb-1 cells at the end of the preincubation. Each point is the average of at least 4 experiments ± S.E. ●, TRVb-1 cell line, Tf-R; ○, TRVb cell line, Δ3–59 Tf-R; ■, 12-4 cell line, Tf-R.

$$k_e\,Tf_i = k_i\,Tf_s \quad \text{(Eq. 3)}$$

$$Tf_s + Tf_i = Tf_{tot} \quad \text{(Eq. 4)}$$

$$Tf_s/Tf_{tot} = k_e/(k_e + k_i) \quad \text{(Eq. 5)}$$

where k_e is the rate constant for externalization, k_i is the rate constant for internalization, Tf_i is the number of internal Tf receptors per cell, Tf_s is the number of surface Tf receptors per cell, and Tf_{tot} is total Tf receptors per cell.

As a rapid assay for the effects of bafilomycin A_1, the surface binding of Tf was measured before and after addition of bafilomycin. It has been shown previously that in TRVb-1 cells the addition of bafilomycin A_1 changes k_e but does not affect k_i (14).

RESULTS

Effects of Bafilomycin A_1 on pH of Recycling Endosomes and Sorting Endosomes in Wild-type and 12-4 Cells—It was shown previously that an *end*2 CHO cell line, 12-4 cells, has alkaline sorting endosomes relative to wild-type cells (0.5 pH units higher than TRVb-1) and more alkaline recycling endosomes (≈0.2–0.4 pH units higher) (22). To compare the effects of bafilomycin A_1 on endosome pH in 12-4 cells and TRVb-1 cells, pH measurements were made (Fig. 1 and Table I). In TRVb-1 cells, both sorting and recycling endosomes were alkalinized by bafilomycin A_1 treatment as described previously (14). Both of these endocytic compartments were also alkalinized by bafilomycin A_1 treatment of 12-4 cells. These results confirm that the mutation in 12-4 cells only partially affects the H^+ATPase-dependent acidification of endosomes.

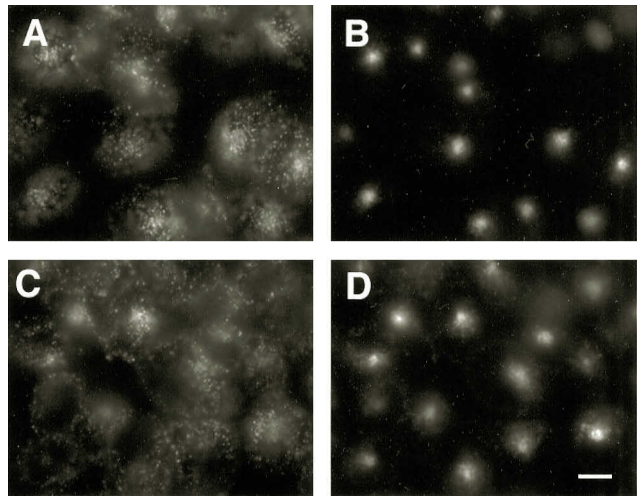

FIG. 3. **Exit of Tf from sorting endosomes of bafilomycin A_1-treated and control cells.** TRVb-1 cells were pretreated for 15 min with HF-12 containing 0.5 μM bafilomycin A_1 and 0.1% Me_2SO or 0.1% Me_2SO only (mock treatment), then labeled for 2 min with Tx-Tf (5 μg/ml), acid-washed as described under "Experimental Procedures," and then chased in HF-12 in the presence of 20 μM deferoxamine for 8 min with FITC-Tf (5 μg/ml) included in the last 2 min of the chase. Cells were fixed and examined using dual labeling Texas Red-fluorescein filter sets described under "Experimental Procedures" and a 63 × NA 1.4 objective. Images were obtained using a cooled CCD as described under "Experimental Procedures." *A* and *B*, mock drug treatment; *C* and *D*, treatment with bafilomycin A_1; *A* and *C* are FITC-Tf; *B* and *D* are Tx-Tf. The large *bright spots* in *B* and *D* are recycling compartments. *Micron bar*, 10 μM.

Reduction of Surface Tf-R by Treatment with Bafilomycin A_1—Tf-R recycles between the cell surface and internal endosomal compartments. Consequently, the amount of Tf-R on the cell surface is determined by the rates of internalization and recycling (Equations 3–5 under "Experimental Procedures"), and modification of either rate by bafilomycin A_1 should alter the amount of surface Tf-R in a time-dependent fashion. Surface Tf-R should plateau at a level that reflects the new steady-state rates of recycling and internalization in the presence of bafilomycin A_1. A time course for surface Tf binding following bafilomycin A_1 treatment is presented in Fig. 2. Following bafilomycin A_1 treatment, the amount of surface Tf binding decreases in cells expressing the wild-type Tf-R to a value that is ~54% of the pretreatment value. The new steady-state value is reached within 30 min. In cells expressing the Tf-R with a deletion of 58 of the 61 amino acids of the cytoplasmic domain (Δ3–59), there is only a very small reduction in the surface expression of Tf-R following bafilomycin A_1 treatment (Fig. 2). These results are consistent with previous results showing that bafilomycin A_1 decreases the k_e of Tf-R from TRVb-1 cells without affecting k_i in a manner that depends on the cytoplasmic domain (14).

12-4 cells have a decreased rate of Tf-R recycling that may be related to their defect in endosome acidification. Since bafilomycin A_1 further alkalinizes endosomes in 12-4 cells, we used the surface binding assay to determine whether bafilomycin A_1 can further slow receptor recycling in 12-4 cells. As shown in Fig. 2, surface Tf-R was reduced significantly by bafilomycin A_1 treatment of 12-4 cells. This is consistent with a further reduction in the k_e of the Tf-R, perhaps as a consequence of more complete endosome neutralization induced by bafilomycin A_1 in 12-4 cells (Fig. 1).

Removal of Tf from Sorting Endosomes and Movement to the

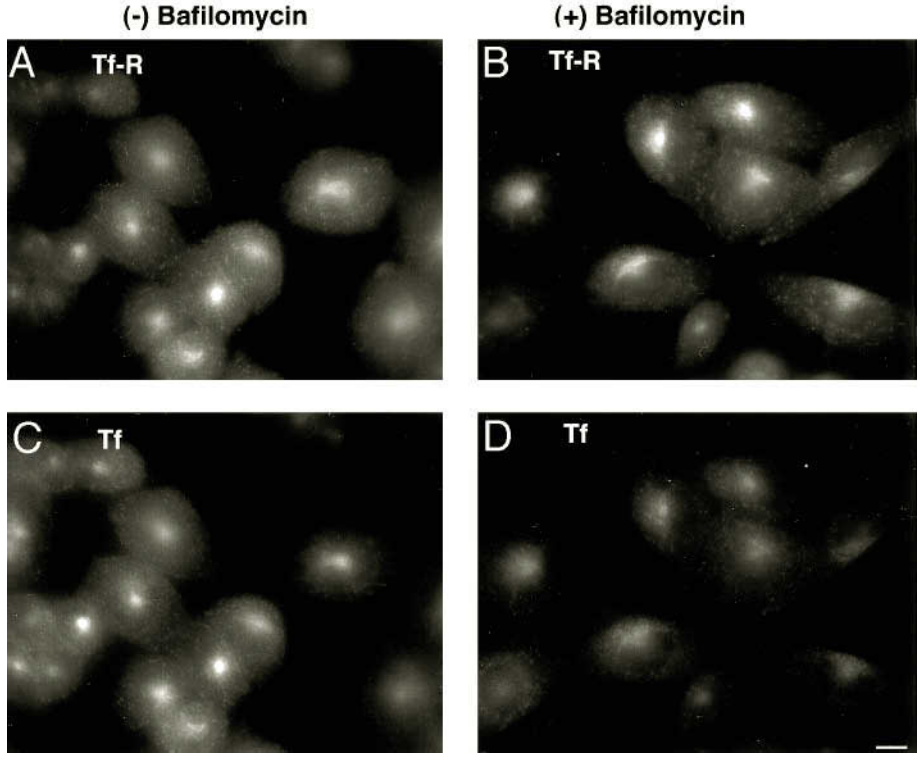

FIG. 4. **Approach to steady state of Tf in bafilomycin A_1-treated and control cells.** TRVb-1 cells in coverslip bottom dishes were pretreated with 0.1% Me_2SO (*A* and *C*) or with bafilomycin A_1 in HF-12 for 15 min (*B* and *D*) and were labeled with Tx-Tf (5 μg/ml) for 12 min in the same medium. Cells were then fixed, permeabilized, and stained with B3/25 and an FITC-labeled goat anti-mouse secondary antibody. Double-labeled images were obtained using a cooled CCD and Texas-Red fluorescein filter set as described under "Experimental Procedures." *A* and *B* show antibody staining for the Tf-R, and *C* and *D* show Tx-Tf. *Micron bar*, = 10 μM.

Recycling Compartment Is Not Significantly Affected in Bafilomycin A_1-treated Cells—While both sorting and recycling endosomes are alkalinized in bafilomycin A_1-treated cells, the point at which trafficking of Tf through the cell is slowed, resulting in net intracellular retention, is unclear. A step-by-step analysis of transit of the Tf-R through the recycling pathway was undertaken in which the sorting of Tf-R from lysosomally directed markers, trafficking of Tf-R to a peri-centriolar recycling compartment, and its exit from the cell were sequentially studied.

Sorting endosomes in TRVb-1 cells (*i.e.* endosomes that contain both lysosomally directed and recycling markers) are observed as punctate structures in the periphery of the cell (1). After short pulses (*e.g.* 2 min), most Tf is colocalized in the sorting endosomes with lysosomally destined markers such as low density lipoprotein (1, 3, 8, 22). Tf and other membrane-associated markers rapidly leave the sorting endosomes and move to the peri-centriolar endocytic recycling compartment with a half-time of ~2 min (3). Thus, if retention in sorting endosomes is contributing in a rate-limiting manner to retention of Tf-R in bafilomycin A_1-treated cells, this retention should be readily detected in a pulse-chase experiment.

TRVb-1 cells were treated or mock treated with bafilomycin A_1, pulsed for 2 min with Tx-Tf, acid washed to remove surface-bound Tf, and then chased for 4 or 8 min with FITC-Tf included in the last 2 min of the chase to identify sorting endosomes. In both treated and mock treated cells, Tx-Tf had begun to move to the recycling compartment after 4 min of chase (not shown). After 8 min chase, the initial Tx-Tf pulse was almost entirely redistributed into the peri-centriolar recycling compartment in both treated and mock treated cells (Fig. 3). This indicates that bafilomycin A_1 does not have a large effect on removal of Tf from sorting endosomes and delivery to the recycling compartment.

Tf Receptors Are Retained in the Endocytic Recycling Compartment—Since iron loaded Tf remains tightly bound to its receptor at neutral pH, re-internalization of Tf is a complication of experiments to measure efflux in bafilomycin A_1-treated cells. The rate of approach to steady-state labeling of Tf-R provides a method to measure efflux kinetics that is not affected by futile recycling (14). Here, we adopted this assay to use fluorescence microscopy to measure efflux from the endocytic recycling compartment based on the ratio of Tx-Tf to total Tf-R (detected by antibody labeling). To calibrate the measurements, we measured the ratio at steady-state labeling with a saturating concentration of Tx-Tf. TRVb-1 cells were labeled for 2.5 h with Tx-Tf to ensure full occupancy of all intracellular Tf-R. We stained the same cells for the Tf-R using an antibody against the Tf-R (B3/25) that is not blocked by receptor occupancy and an FITC-labeled secondary antibody. A quantitative fluorescence measurement of both Tx-Tf and the Tf-R was thereby obtained under conditions in which they should be present in all endocytic compartments at the same levels. There was complete colocalization of Tf and Tf-R in these double labeled cells. The ratio of the brightness of the two stains (FITC/Tx) gives a calibration factor (*C*) that when multiplied by the Tx brightness (F_{tx}) in cells pulsed for a short period gives the fraction of occupied Tf-R in the recycling compartment (f_o; see "Experimental Procedures").

Cells were treated or not treated with bafilomycin A_1 and labeled with Tf, followed by immunofluorescence staining of Tf-R. Digital fluorescence images of Tx-Tf and FITC-labeled Tf-R were obtained (Fig. 4). The recycling compartments were identified in the pairs of images using digital image processing (22). Using the calibration factors determined at steady state, the fraction of occupied receptors in the recycling compartment after various labeling periods (0–18 min) was determined. The fraction of unoccupied receptors decayed with time as shown in Fig. 5. This decay could be fit to a single exponential, and rate constants for exit (k_e) were determined. The rate constant for the exit of unoccupied Tf-R from the recycling compartment of mock treated cells was 0.063 ± 0.019 min^{-1}, which agrees well with the rate of 0.057 ± 0.005 min^{-1} obtained using conventional ^{125}I-Tf efflux experiments (28). In bafilomycin A_1-treated cells, the rate of efflux from the endocytic recycling compartment was reduced to 0.034 ± 0.039 min^{-1} (Fig. 5 and Table II).

This reduction in rate is in close agreement with the overall reduction in the rate of efflux from cells treated with bafilomycin A_1 (14) (see Table II). This indicates that the reduced overall rate of exit of Tf-R from the cell is due to slowed exit from the recycling compartment.

Recycling of the Bulk Membrane Marker C_6-NBD-SM Is Slowed in Bafilomycin A_1-treated Cells—In acidification defective 12-4 cells, Tf-R exit from the recycling compartment is slowed, but exit of the bulk membrane marker C_6-NBD-SM is unaffected (22). We wanted to see if the same was true in bafilomycin A_1-treated cells. In drug-treated cells C_6-NBD-SM colocalized with Tf and had a similar distribution to C_6-NBD-SM in non-drug-treated cells (Fig. 6). Using a previously described assay we measured efflux of C_6-NBD-SM from cells (22) with or without bafilomycin A_1 treatment (Fig. 7).

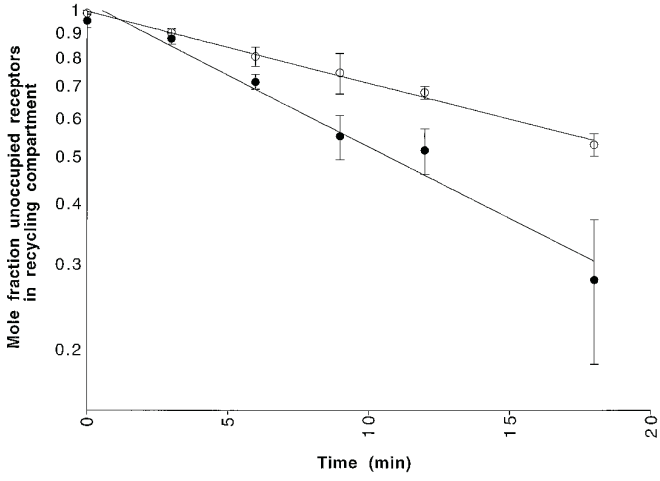

FIG. 5. **Recycling of Tf-R in bafilomycin A_1-treated and control cells.** TRVb-1 cells in coverslip bottom dishes were pretreated with bafilomycin A_1 or mock pretreated with 0.1% Me_2SO for 15 min and then labeled for the indicated lengths of time with Tx-Tf in HF-12. Tf-R in these cells was stained with mouse monoclonal B3/25 and an FITC-conjugated goat anti-mouse secondary antibody, and corresponding Tx-Tf and FITC-Tf-R receptor images were taken of each field. 5–6 fields were taken at random from each dish and were selected using phase contrast. Recycling compartments were isolated in the double-labeled images, and total Tx-Tf and FITC-Tf-R fluorescence in each field was quantified. The ratio of Tx-Tf fluorescence to FITC-Tf-R fluorescence was then normalized as described under "Experimental Procedures" to give mole fraction of occupied Tf-R. The corresponding mole fraction of unoccupied Tf-R was plotted *versus* time and fitted to a single exponential. Thus, this experiment measures the exit of unoccupied receptors. Each curve is an average of 3–4 experiments on different days ± S.D.

As described previously for TRVb-1 and 12-4 (3, 22), efflux kinetics are well fit by a double exponential composed of a major (≈75%) slow component and a minor (≈25%) fast component ($t_{1/2}$ 2–3 min). The nature of the fast component is unknown but may represent direct traffic from sorting endosomes to the plasma membrane. The slow component corresponds to the rate of exit of C_6-NBD-SM from the recycling compartment (3, 22). Double exponential least square fits showed no difference in amplitude or rate of the fast component of lipid exit between bafilomycin A_1-treated and mock treated cells (data not shown). In cells treated with 0.5 μM bafilomycin A_1, the rate of the slow component of lipid exit was 0.042 ± 0.003 min^{-1}. In mock treated cells, the rate of the slow component of lipid exit was 0.059 ± 0.006 min^{-1}, a rate that agrees closely with previously determined values (3, 22). When a lower dose (0.25 μM) of bafilomycin A_1 was used, similar rates were obtained (bafilomycin A_1, 0.060 ± 0.007 min^{-1}; mock, 0.046 ± 0.004 min^{-1}; Table II). Thus, bafilomycin A_1 significantly reduces the recycling of bulk membrane in TRVb-1 cells.

Effects of Altering the Internalization Motif of the Tf-R on Its Retention in a Bafilomycin A_1-treated Cell—To explore the role of the cytoplasmic domain of the Tf-R in the bafilomycin A_1-triggered retention, we examined various mutations of the internalization sequence. Since slowed recycling will result in the depletion of surface Tf-R, we examined the effect of bafilomycin A_1 on surface expression of unaltered Tf-R or Tf-R in which the internalization domain on the cytoplasmic tail was altered. Table III shows surface Tf-R on bafilomycin A_1-treated cells as a percentage of the control (mock treated) value after 30 min of drug treatment for various Tf-R.

The surface expression of Δ3–59 Tf-R is only slightly affected by bafilomycin A_1 treatment (Fig. 2 and Table III). The surface expression of a Tf-R containing a mutation in the internalization motif (Y20C Tf-R) is affected to an intermediate extent by bafilomycin A_1 treatment. We examined the effect of bafilomy-

TABLE II
Summary of rates

Assay	Control k_e (min^{-1})	Bafilomycin A_1 k_e (min^{-1})	% of control k_e
^{125}I-Tf approach to steady state[a]	0.069 ± 0.009	0.035 ± 0.006	50.7
Approach to steady state measured in microscope[b]	0.063 ± 0.02	0.034 ± 0.004	54.0
C_6-NBD-SM exit in TRVb-1 cells (0.5 μM bafilomycin A_1)[c]	0.059 ± 0.006	0.042 ± 0.003	71.2
C_6-NBD-SM exit in TRVb-1 cells (0.25 μM bafilomycin A_1)[c]	0.060 ± 0.007	0.046 ± 0.004	76.7

[a] See Ref. 14.
[b] From data shown in Fig. 5.
[c] Methods are described in Fig. 7.

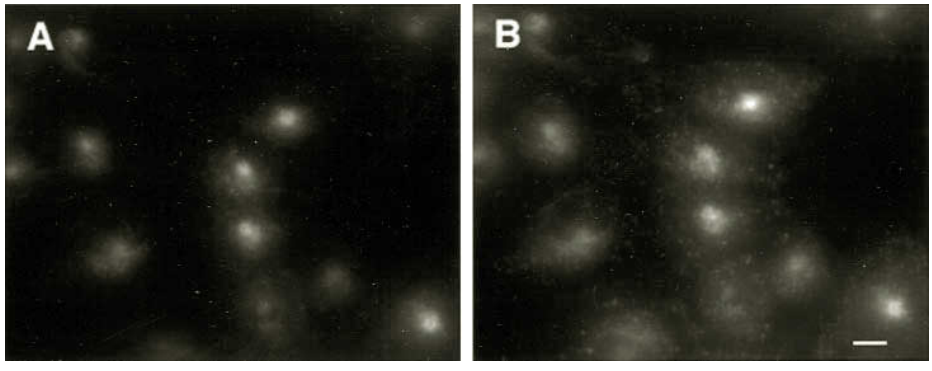

FIG. 6. **Colocalization of C_6-NBD-SM and Tf in bafilomycin A_1-treated cells.** TRVb-1 cells in coverslip bottom dishes were pretreated with bafilomycin A_1 for 15 min, then chilled, and labeled at 4 °C with C_6-NBD-SM vesicles (5 μM total lipid) and 5 μg/ml Tx-Tf, and incubated for 10 min at 37 °C to allow internalization of surface-bound lipid. Cells were then chilled on ice, and remaining surface-bound lipid was removed by back-exchange. Cells were warmed up and chased an additional 6 min to clear sorting endosomes. Cells were visualized using a 40 × objective. *A*, Tx-Tf; *B*, C_6-NBD-SM. *Micron bar*, 10 μM.

cin A_1 on the surface expression of a Tf-R mutant that is more rapidly internalized than the wild-type Tf-R. This mutant Tf-R, G31Y, contains two functional internalization motifs: the native internalization motif, YTRF, at positions 20 to 23, and a second internalization motif at positions 31 to 34 which was created by mutating glycine 31 to a tyrosine (29). The G31Y shows a net loss from the surface similar to that of the wild-type Tf-R which only contains a single internalization motif (29, 30).

The effect of bafilomycin A_1 on the recycling of a Tf-R mutant, Y20C, G31Y Tf-R, containing the artificial G31Y internalization motif but with an alteration in the native motif was also examined. This double mutant is internalized at the rate of the wild-type Tf-R (29). Surface Tf binding in cells expressing the Y20C, G31Y Tf-R is reduced to the same extent as the wild-type Tf-R, demonstrating that a Tf-R containing the G31Y internalization motif is slowly recycled in the presence of bafilomycin A_1 (Table III).

For wild-type Tf-R, the net reduction of surface expression upon addition of bafilomycin A_1 is due to changes in the k_e of the Tf-R with no change in k_i (14). Based on Equation 5 (see "Experimental Procedures"), the percentage reduction in surface Tf-R is dependent on the k_e prior to drug treatment, the new k_e after drug treatment, and k_i. The small redistribution of Δ3–59 from the surface to internal compartments can be accounted for by its slow rate of internalization and small effect of bafilomycin A_1 on k_e for this receptor lacking most of the cytoplasmic domain (14). Interestingly, the Y20C, S34Y Tf-R, and the Y20C, G31Y Tf-R redistribute similarly to the wild-type Tf-R. Since all three receptors have similar internalization rates, these data indicate that they also have similar bafilomycin A_1-induced retention. This indicates that retention is based on cytoplasmic sequences similar to the requirements for rapid internalization.

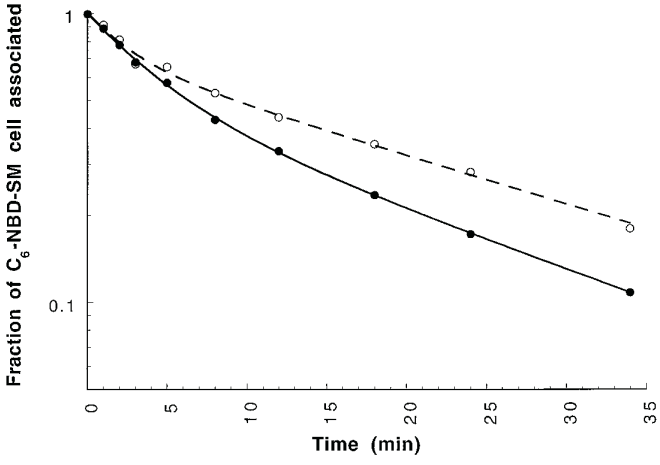

FIG. 7. **Recycling of bulk membrane in bafilomycin A_1-treated and control cells.** TRVb-1 cells were pretreated with 0.1% Me_2SO with or without 5 μM bafilomycin A_1 and were allowed to internalize C_6-NBD-SM for 10 min. Fluorescent lipid was stripped from the cell surface using a BSA back-exchange at 0 °C. Cells were warmed and allowed to efflux lipid into 4 ml of back-exchange medium (medium 1 with 1% BSA) at 37 °C. 400-μl aliquots of medium were removed at intervals with replacement with 400 μl of prewarmed chase medium. After the termination of the experiment cell- and medium-associated lipid was butanol-extracted and spectrofluorometrically quantified as described under "Experimental Procedures." Cells were maintained in drug + Me_2SO or Me_2SO as appropriate throughout the course of the experiment. A representative experiment is shown. For a summary of all experiments see Table II.

DISCUSSION

Intracellular Routing of the Tf-R on Treatment with Bafilomycin A_1—We find that bafilomycin A_1 does not significantly alter the exit of the Tf-R from early sorting endosomes nor its subsequent accumulation in the peri-centriolar endocytic recycling compartment. Since sorting endosomes are essentially completely neutralized in bafilomycin A_1-treated cells, these data show that proper sorting of recycling membrane components does not require the sorting endosome lumen to be acidic.

The half-time for efflux of internal Tf-R to the cell surface in bafilomycin A_1-treated cells is ~20 min as compared with ~10 min for control cells (14). Exit of Tf-R from recycling compartment was slowed approximately 2-fold relative to nontreated cells. This rate constant (0.034 min^{-1}) was similar to the rate constant for exit of ^{125}I-Tf from drug-treated TRVb-1 cells (0.035 min^{-1}; 14). The rate constant for exit of Tf-R from recycling compartments in non-drug-treated cells was 0.063 min^{-1} (Table II). Thus, exit from the recycling compartment is rate-limiting in bafilomycin A_1-treated cells, and the slowing at this stage of the Tf-R itinerary is responsible for the overall slowdown in Tf-R recycling kinetics.

A notable feature of the endosomal recycling pathway is that the Tf-R normally moves through the entire pathway at the same rate as bulk membrane markers such as C_6-NBD-SM. This indicates that concentrative mechanisms similar to clathrin-coated pits are not required for rapid and efficient recycling of the Tf-R after internalization (3). However, bafilomycin A_1 treatment differentially affects C_6-NBD-SM and Tf-R. Bulk membrane exit rate constants measured with C_6-NBD-SM are reduced to ~75% of non-drug-treated value, and rates for exit of Tf-R are reduced to 50–55% of the control value (Ref. 14 and Table II). Thus, in bafilomycin A_1-treated cells, as in 12-4 cells (22), Tf-R is actively retained relative to bulk membrane.

It was previously reported that Tf-R constructs with a large deletion in the cytoplasmic domain (Δ3–59) or with point mutations in the internalization motif (Y20C or F23A) showed reductions in k_e to 70–90% of control values when treated with 0.25 μM bafilomycin A_1 (14). These values are similar to the reduction in k_e for bulk membrane suggesting that the same or

TABLE III
Reduction of surface Tf-Rs by bafilomycin A_1

MM DQARSAFSNL**F**GGEPLS**Y**TR**F**SLARQVD**G**DN**S**HVEMKLAVDEEENADNNTKANVT KPKR. . .
3 13 2023 31 34 59

Tf-R	k_i (min^{-1})[a]	^{125}I-Tf bound after 30 min in 0.25 μM bafilomycin A_1 as a percentage of control binding
Wild type	0.13	53 ± 2
Δ3–59	0.02	92 ± 3
Y20C	0.03	80 ± 2
Y20C, S34Y	0.13	63 ± 5
Y20C, G31Y	0.13	56 ± 7
G31Y	0.2	60 ± 6

[a] See Ref. 29.

similar sequences may be involved in internalization and in bafilomycin A_1-triggered retention in the endocytic recycling compartment. Consistent with this interpretation mutants Y20C, S34Y and Y20C, G31Y have internalization rate constants that are restored to the wild-type value by creation of a new internalization motif (29), and the constructs are retained in bafilomycin A_1-treated cells almost to the same extent as wild-type receptor (Table III). We cannot rule out that a sequence other than the YTRF could also be involved in retention of the Tf-R in bafilomycin A_1-treated cells.

Mechanism of the Bafilomycin A_1 Effect—Some form of communication between the abnormally neutral lumen of the recycling compartment and the cytoplasmic domain of the receptor tail is required if motifs on this tail are important for slowed recycling of the receptor in bafilomycin A_1-treated cells. One possibility is that alkaline intra-endosomal pH could trigger a change in the distribution or location of Tf-R in recycling compartments, which would allow direct recognition by a cytoplasmic protein of their cytoplasmic tails. This could occur if, for example, receptors are aggregated in regions of an abnormally neutral recycling compartment, allowing adaptins or other proteins to bind the clustered cytoplasmic tails in a nonspecific manner due to the greater avidity of multivalent binding or to other mechanisms leading to active retention. It is also possible that a pH change in the recycling compartment lumen can be transduced to the outside of the recycling compartment through other proteins and trigger an active retention of Tf-R.

We have shown previously that if Tf multimers are made by cross-linking, they are retained in the recycling compartment of TRVb-1 cells expressing either the wild-type Tf-R or Δ3–59, indicating that retention of multimers involves either slow diffusion of the aggregate or retention of aggregates of Tf-R via their lumenal domains (31). The recycling compartments of TRVb-1 cells containing multimeric Tf are functionally normal, since a pulse of monomeric Tf can still enter and leave the recycling compartment at the normal bulk rate (31). Thus, there are at least two mechanisms by which Tf-R can be retained in the recycling compartment of TRVb-1 cells, one requiring the cytoplasmic domain and one independent of the cytoplasmic domain.

The effect of bafilomycin A_1 on bulk membrane trafficking is less than on recycling of the Tf-R. This reduction in bulk flow (to ~75% of pretreatment k_e values) could result from slowed fusing of vesicles with the recycling compartment, slowed formation of exit vesicles, or formation of smaller exit vesicles. Currently, we have no evidence to distinguish these possibilities.

Most previous studies have used weak bases or ionophores to collapse intracellular pH gradients. These agents generally cause vacuolization of endosomal compartments and have other nonspecific effects. In our assays, the weak base primaquine causes a much larger net internalization of surface Tf-R than bafilomycin A_1, and this large net internalization was not dependent on the cytoplasmic tail domain (data not shown). The difference between primaquine and bafilomycin A_1 is probably due directly to the additional changes (*e.g.* organelle swelling) that treatment with weak bases causes in endosomal compartments.

Conclusions—Bafilomycin A_1 reduces the externalization of Tf-R from wild-type TRVb-1 cells by slowing the exit of Tf-R from the recycling compartment in a rate-limiting manner. This is partly affected by a slowdown of the rate of bulk flow from the recycling compartment and partly by active retention of the Tf-R in the recycling compartment by a mechanism dependent on the YTRF internalization motif on the cytoplasmic tail.

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