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Endosome Acidification and Receptor Trafficking: Bafilomycin A_1 Slows Receptor Externalization by a Mechanism Involving the Receptor's Internalization Motif

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To examine the relationship between endosome acidification and receptor trafficking, transferrin receptor trafficking was characterized in Chinese hamster ovary cells in which endosome acidification was blocked by treatment with the specific inhibitor of the vacuolar H^+-ATPase, bafilomycin A_1. Elevating endosome pH slowed the receptor externalization rate to approximately one-half of control but did not affect receptor internalization kinetics. The slowed receptor externalization required the receptor's cytoplasmic domain and was largely eliminated by substitutions replacing either of two aromatic amino acids within the receptor's cytoplasmic YTRF internalization motif. These results confirm, using a specific inhibitor of the vacuolar proton pump, that proper endosome acidification is necessary to maintain rapid recycling of intracellular receptors back to the plasma membrane. Moreover, receptor return to the plasma membrane is slowed in the absence of proper endosome acidification by a signal-dependent mechanism involving the receptor's cytoplasmic tyrosine-containing internalization motif. These results, in conjunction with results from other studies, suggest that the mechanism for clustering receptors in plasma membrane clathrin-coated pits may be an example of a more general mechanism that determines the dynamic distribution of membrane proteins among various compartments with luminal acidification playing a crucial role in this process.

INTRODUCTION

In eukaryotic cells many organelles, including clathrin-coated vesicles, endosomes, multivesicular bodies, lysosomes, the Golgi apparatus, secretory granules, and yeast and plant vacuoles have been shown to be acidified to varying degrees (reviewed by Mellman *et al.*, 1986; Maxfield and Yamashiro, 1991). These organelles, elements of the endocytic and exocytic pathways, contain the vacuolar class of proton-translocating ATPase (reviewed by Forgac, 1989; for recent reviews see Journal of Experimental Biology, 1992, volume 172). The functions served by acidification within each of these organelles have been the subject of considerable study, and several physiological functions have been well established. For example, acidic pH is required for uncoupling of receptor-ligand complexes, removal of iron from transferrin (Tf) and endosome/lysosome degradative processing. In addition, certain toxins and viruses have been shown to make opportunistic use of luminal acidity to gain entry to the cytoplasm. Endosome acidification has been suggested to play a role in the recycling of internalized receptors back to the plasma membrane, but the nature of this role remains unknown (reviewed by Mellman *et al.*, 1986; Forgac, 1989; Ganapathy and Leibach, 1991).

Much of what is known about the functions of intracellular pH has been learned from studies using weak bases or ionophores to dissipate pH gradients (reviewed by Dean *et al.*, 1984; Mellman *et al.*, 1986; Maxfield and Yamashiro, 1991). These reagents have been useful in

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providing an experimental paradigm for studying the functional significance of intravacuolar pH. With respect to the importance of pH in receptor trafficking, studies generally found that treatment of cells with a weak base or ionophore caused, concurrent with endosome alkalinization, a rapid decrease in the number of surface receptors (Tolleshaug and Berg, 1979; Tietze *et al.*, 1980; Basu *et al.*, 1981; Schwartz *et al.*, 1984; Stein *et al.*, 1984; Stoorvogel *et al.*, 1987). Receptor internalization kinetics were found to be unaffected (Tietze *et al.*, 1980; Ciechanover *et al.*, 1983; Harford *et al.*, 1983; Klausner *et al.*, 1983), indicating that the effect of the weak base or ionophore was to disrupt the return of receptors to the cell surface. In the case of the Tf receptor (TR), which is unique in that it normally recycles still carrying its ligand, ^{125}I-Tf efflux was found to be slowed or blocked (Klausner *et al.*, 1983; Stein and Sussman, 1986). Results from studies employing multiple receptor types and multiple cell lines yielded various interpretations as to the possible mechanism underlying the perturbed recycling (Basu *et al.*, 1981; Tietze *et al.*, 1982; Harford *et al.*, 1983; Klausner *et al.*, 1983; Schwartz *et al.*, 1984; Stein and Sussman, 1986). Beyond its requirement for uncoupling of receptors and ligands, no consensus emerged from these studies as to the nature of the role played by endosome acidification in receptor recycling. Interpretation of the results was further complicated by the lack of specificity of weak bases and ionophores, which dissipate pH gradients throughout the cell and cause morphological changes in the vacuolar compartments due to osmotic swelling (e.g., Ohkuma and Poole, 1981; Tartakoff, 1983; Stein *et al.*, 1984). Thus, a specific mechanism underlying the disruption of receptor recycling by these reagents has remained unknown.

Much insight into endosome acidification has also been gained from studies of End1 and End2 mutant cell lines that have been independently isolated by several groups (Robbins *et al.*, 1983; Roff *et al.*, 1986; Colbaugh *et al.*, 1988). These complementation groups of mutant Chinese hamster ovary (CHO) cells, which exhibit pleiotropic defects including impaired endosome acidification, have provided a second valuable experimental paradigm for understanding the functional consequences of endosome acidification. The mutant phenotypes have been extensively characterized (e.g., Klausner *et al.*, 1984; Robbins *et al.*, 1984; Yamashiro and Maxfield, 1987; Schmid *et al.*, 1989; Roff *et al.*, 1990). The results of these studies have been generally in agreement with studies using weak bases and ionophores to such a degree that it has been proposed that the defects in intravesicular acidification explain the phenotypes of these mutant complementation groups (e.g., Robbins, 1988). These mutants were not believed to exhibit altered receptor recycling kinetics (Klausner *et al.*, 1984). That this view was accepted, rather than appearing paradoxical with respect to studies employing weak bases or ionophores, underscores how unclear the role of endosome acidification is in receptor recycling.

We recently reported the isolation of a trafficking defective CHO cell line, 12-4, that was found to belong to the End2 mutant complementation group (Johnson *et al.*, 1993). The 12-4 cell line expresses human TR rather than endogenous hamster TR, which facilitated characterization of TR trafficking kinetics. 12-4 cells internalize receptors from the plasma membrane at 75% of the parental rate and return internalized TR back to the plasma membrane at 50% of the parental rate. We also examined Tf trafficking in another End2 cell line and an End1 cell line, and we determined that slowed internalization and externalization of receptors occur in both of these complementation groups that exhibit defective endosome acidification. These results provided further correlation between endosome pH and receptor trafficking. The most parsimonious interpretation of these studies, when combined with the studies employing weak bases or ionophores, is that the observed defect in receptor recycling is secondary to the defect in endosome acidification. However, because the primary defects in End1 and End2 mutants are unknown, the interpretation of cause and effect from these results is ambiguous. Thus, it is also possible that the trafficking defects in these mutants might cause the acidification defects, perhaps by altering the distribution of the proton pump or a regulator of the proton pump.

To increase our understanding of the relationship between receptor trafficking and vacuolar pH, we have examined receptor trafficking in cells treated with a specific inhibitor of the vacuolar proton pump, bafilomycin A_1. Bafilomycin A_1 is a macrolide antibiotic that has been shown to be a highly potent, specific inhibitor of the vacuolar class of H^+-ATPase in vitro (e.g., Bowman *et al.*, 1988; Hanada *et al.*, 1990). In living cells of several types, bafilomycin A_1 has been shown to inhibit acidification of endosomes, lysosomes, and phagosomes and to inhibit protein degradation (Umata *et al.*, 1990; Lukacs *et al.*, 1991; Yoshimori *et al.*, 1991). In addition, bafilomycin A_1 has been reported not to cause the morphological changes in vacuolar compartments that are characteristic of weak bases and ionophores (Umata *et al.*, 1990; Yoshimori *et al.*, 1991). Thus, bafilomycin A_1 is an excellent pharmacological agent with which to examine the relationship between endosome acidification and receptor trafficking.

In the current study we have found that bafilomycin A_1 alkalinizes endosomes and slows the rate of TR return to the cell surface by twofold. Interestingly, the slowed recycling is dependent upon two amino acids within the receptor's cytoplasmic YTRF internalization motif. Thus, proper endosome acidification is necessary to prevent the receptor's internalization motif from interfering with efficient recycling. The results presented here suggest that intravesicular acidification serves to regulate

cytoplasmic interactions involved in the trafficking of vacuolar membrane proteins.

MATERIALS AND METHODS

Cells

TRVb-1 is a line of CHO cells that does not express detectable levels of functional hamster TR and has been stably transfected with a cDNA clone encoding the human TR (McGraw *et al.*, 1987). TRVbC20 (McGraw and Maxfield, 1990) and TRVbA23 (McGraw *et al.*, 1991) were derived from the same TR-negative parent as TRVb-1 cells but have instead been stably transfected to express human TR containing single amino acid substitutions in the cytoplasmic domain at positions 20 (Cys for Tyr) and 23 (Ala for Phe), respectively. Cell line TRVbΔ3-59 was derived from the same TR-negative cell line as the others but has instead been stably transfected to express a deletion construct of the human TR missing amino acids 3–59 of the cytoplasmic domain (Pytowski *et al.*, unpublished data).

Media

All cells were carried in Ham's F12 medium supplemented with 2 g/L glucose, 14 mM $NaHCO_3$, 5% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 200 μg/ml G418. The medium 1 used for experimental incubations was Ham's F12 growth medium without serum, supplemented with 2 mg/ml ovalbumin and 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) pH 7.4. Bafilomycin A_1, generously provided by Dr. F.R. Maxfield (Columbia University), was dissolved as a 0.5 mM stock in dimethyl sulfoxide (DMSO) and stored in aliquots at −20°C. During all experiments in which cells were treated with bafilomycin A_1, control cells were treated with the same final concentration of DMSO (0.05–0.1%).

Ligands

Human Tf (Sigma Chemical, St. Louis, MO) was purified further by Sephacryl S-300 gel filtration. Diferric Tf and ^{125}I-Tf (specific activity range 200–400 cpm/ng) were prepared as previously described (McGraw *et al.*, 1987). cy3-Tf was prepared according to the manufacturer's instructions (Biological Detection Systems, Pittsburgh, PA).

Fluorescence Ratio Imaging pH Determinations

For pH measurements Tf was conjugated with both rhodamine and fluorescein by reacting with succinimidyl esters of both carboxytetramethylrhodamine (TMR) and carboxyfluorescein (CF) according to the manufacturer's instructions (Molecular Probes, Eugene, OR) in a ratio of 8 mg Tf to 0.7 mg CF to 0.22 mg TMR. This conjugate yielded a pattern of endosome fluorescence characteristic of Tf in CHO cells. Specificity of F-R-Tf endocytosis was confirmed by inhibition of uptake in the presence of excess unlabeled Tf and loss of fluorescence by labeled cells during a chase period in the presence of 0.5 mg/ml unlabeled Tf and 100 μM desferrioxamine.

The procedure used to measure endosome pH will be described in detail elsewhere (Presley *et al.*, 1993). Briefly, cells were plated 2 d before each experiment onto 35-mm coverslip-bottomed dishes. Experimental dishes were preincubated in medium 1 containing 2 mg/ml of ovalbumin and 10 mM glucose ± 0.25 μM bafilomycin A_1 (control cells were incubated with an equivalent concentration of DMSO) for 30 min then incubated in this same medium containing 20 μg/ml F-R-Tf for 10 min at 37°C on a bench-top warm tray. Fluorescent labeling was continued for approximately another 10 min on the microscope stage of a Zeiss Axiovert microscope (Thornwood, NY) warmed to 37°C, during which time fluorescence images of the cells were collected by means of a Bio-Rad MRC-600 confocal attachment (Richmond, CA). Four hundred eighty-eight nanometers light was used to stimulate both fluorescein and rhodamine fluorescence. Fluorescein emissions were selected using a 515–545-nm bandpass emission filter, and rhodamine emissions were selected using a 600-nm long-pass emission filter (both from Omega Optical, Brattleboro, VT) and collected in the two Bio-Rad imaging detectors simultaneously. Using image processing techniques developed previously (Dunn and Maxfield, 1990), endosome fluorescence was quantified, and the ratio of red to green fluorescence calculated for each individual endosome. For each experiment, calibration curves were constructed by imaging fixed labeled cells that had been equilibrated with a series of pH buffers. These curves demonstrate that the fluorescence emission ratio of the endocytic compartments labeled with F-R-Tf is a sensitive indicator of pH over a range from 5.0 to 7.0.

Tf Trafficking Assays

In all kinetic experiments a saturating concentration of 3 μg/ml ^{125}I-Tf was used. The kinetic assays were performed at 37°C in 5% CO_2. Washes were done using medium 2 (150 mM NaCl, 20 mM HEPES, 1 mM $CaCl_2$, 5 mM KCl, and 1 mM $MgCl_2$ pH 7.4), and 4°C surface ^{125}I-Tf binding incubations were done in medium 2 containing 2 mg/ml ovalbumin. All kinetic assays were done using cells grown in six-well clusters, in which four experimental measurements were made and corrected for nonspecific binding by subtracting the mean of two other measurements made in the presence of a 200-fold excess of unlabeled Tf.

The Tf efflux assay was performed as previously described (Johnson *et al.*, 1993). Briefly, cells were incubated 2 h at 37°C in medium 1 containing 3 μg/ml ^{125}I-Tf to achieve steady-state TR occupancy with ^{125}I-Tf. For the last 30 min of this labeling period, bafilomycin A_1 (or equivalent DMSO, 0.05%) was added to a final concentration of 0.25 μM. Surface-bound ^{125}I-Tf was removed by incubation for 2 min in a mild acid buffer (0.5 M NaCl, 50 mM 2-(*N*-morpholino)ethanesulfonic acid [MES] pH 5.0) followed by three washes over 1 min in medium 2 containing 3 μg/ml unlabeled Tf and 100 μM desferrioxamine all at 37°C. Cells were then incubated in medium 1 containing 3 μg/ml unlabeled diferric Tf and 100 μM desferrioxamine (to prevent rebinding of released ^{125}I-Tf). After the chase period ^{125}I-Tf was quantified as being in the efflux medium, bound to surface TR or sequestered inside the cell as follows: the plate was placed on ice, the chase media was removed, and the cells were washed once with ice-cold medium 2 that was pooled with the chase media for counting ^{125}I-Tf (efflux). The cells were incubated for 3 min in ice-cold 0.5 M NaCl, 0.5 M acetic acid pH 2.0. Then this acid wash was removed, and the cells were washed with ice-cold medium 2, which was pooled with the acid wash for counting ^{125}I-Tf (surface-bound); finally, the cells were solubilized (1% Triton X-100, 0.1% NaOH) and counted for ^{125}I-Tf (intracellular).

Quantitation of the exocytic rate constant using the ^{125}I-Tf efflux assay is as follows. The change in intracellular Tf with chase time is:

$$dTf_i/dt = -k_e \cdot Tf_i, \quad (1)$$

where Tf_i is the intracellular Tf, and k_e is the exocytic rate constant. This equation assumes that apo-Tf is returned to the plasma membrane where it rapidly dissociates at neutral pH (Dautry-Varsat *et al.*, 1983; Klausner *et al.*, 1983). Solving this equation yields

$$Tf_i(t) = Tf_{i,o} \cdot \exp^{(-ke \cdot t)}, \quad (2)$$

where $Tf_{i,o}$ is internal Tf at time 0 and $Tf_i(t)$ is internal Tf at time t.

In bafilomycin A_1-treated cells the increase in surface-bound ^{125}I-Tf during the chase period (see Figures 6A and 8A) indicates that ^{125}I-Tf externalized to the plasma membrane remains receptor-bound and is, as a result, subject to reinternalization. Therefore, quantitation of k_e in bafilomycin A_1-treated cells requires that $Tf_i(t)$, the Tf remaining internal since time 0, be calculated by correcting the measured internal Tf for the diferric Tf reinternalized from the cell surface during the chase period:

$$Tf_i(t) = Tf'_i(t) - Tf_r(t), \quad (3)$$

where $Tf'_i(t)$ is the experimentally measured internal Tf and $Tf_r(t)$ is

the Tf that has been reinternalized during the chase period. The initial internal Tf and the Tf reinternalized during the chase period can be treated as separate, assuming that the reinternalized Tf does not contribute significantly to the recycling Tf. This assumption is valid over short chase times, because the experimental observation that internalization kinetics remain linear out to 8 min demonstrates that it takes ≥8 min for internalized Tf to be significantly recycled (Figure 8). Thus, treating the reinternalized Tf separately from the internal Tf present at time 0 allows us to combine Equations 2 and 3:

$$Tf_i'(t) - Tf_r(t) = Tf_{i,o} \cdot \exp^{(-ke \cdot t)}. \quad (4)$$

From Equation 4, subtracting the reinternalized Tf from the measured intracellular Tf at each time point allows one to measure k_e. The rate of change in reinternalized Tf over brief periods of time is

$$dTf_r/dt = k_i \cdot Tf_s, \quad (5)$$

where k_i is the internalization rate constant and Tf_s is the surface-bound Tf, which is itself a function of time. Integrating this equation gives the Tf reinternalized to time t:

$$Tf_r(t) = k_i \cdot \int [Tf_s(t)]\, dt. \quad (6)$$

Using equation 6 one can calculate the ^{125}I-Tf reinternalized by bafilomycin A_1-treated cells at early time points using two known values, k_i (Figure 8 and Table 1) and the area beneath the curve representing the surface-bound Tf of bafilomycin A_1-treated cells (Figures 6A and 7A). The area was calculated by fitting the data to a third order polynomial with r^2 typically >0.99. Having measured $Tf_r(t)$ based on Equation 6, one can use this value in Equation 4 and plot the natural logarithm of $[Tf_i'(t) - Tf_r(t)]$ versus time. The slope of this line is $-k_e$ in bafilomycin A_1-treated cells. In the figures presented, the curve representing surface-bound Tf of control cells was subtracted from the corresponding bafilomycin A_1-treated curve, and this difference was used to correct the internal Tf of bafilomycin A_1-treated cells; however, the relative change in k_e induced by bafilomycin A_1 was the same when ke was corrected for both conditions (control and bafilomycin A_1-treated) using their respective curves representing surface-bound Tf. It is unclear why the version of the ^{125}I-Tf efflux assay used in this study tends to yield somewhat curvilinear data; however, the shape of the function tends to be similarly curvilinear for both control and bafilomycin A_1-treated cells and hence should not affect relative values of k_e.

Approach to Steady-State Assay

In this assay cells are incubated in the presence of a saturating concentration of ^{125}I-Tf for varying times until they achieve a maximum, steady-state value of cell-associated label, and the previous values are expressed as a percentage of this steady-state value. The exocytic rate constant, k_e, was quantitated using this assay as follows. Because Tf binding to surface TR is fast (Ciechanover *et al.*, 1983) relative to the time course of the assay and because during the assay cells are kept in a saturating concentration of ^{125}I-Tf, the amount of ^{125}I-Tf bound to surface TR (TR_s^*) can be treated as a constant. The total amount of ^{125}I-Tf bound to TR (TR_t^*) is the sum of this constant and the ^{125}I-Tf bound to TR inside the cell (TR_i^*),

$$TR_t^* = TR_s^* + TR_i^*. \quad (7)$$

The rate of accumulation of ^{125}I-Tf is thus dependent upon the rate at which unoccupied intracellular TR are externalized to the plasma membrane to acquire ^{125}I-Tf, which is described by the equation

$$TR_i^* = TR_{i,ss}^* \cdot [1 - \exp^{(-ke \cdot t)}], \quad (8)$$

where TR_i^* is the ^{125}I-Tf bound to TR inside the cell at time t, $TR_{i,ss}^*$ is the ^{125}I-Tf bound to internal TR at steady state, k_e is the exocytic rate constant, and t is time. Combining Equations 7 and 8,

$$TR_t^* = TR_s^* + TR_{i,ss}^* \cdot [1 - \exp^{(-ke \cdot t)}]. \quad (9)$$

The values of k_e given in the text were obtained from the best fit to this function (Equation 9), where the values of TR_s^*, $TR_{i,ss}^*$, and k_e were the parameters fit by a least squares analysis (Figure 5).

For cell lines that express internalization-defective TR, the approach to steady-state TR occupancy is insensitive for measuring the externalization rate constant, k_e. This is because most of the total cycling TR are on the surface of internalization-defective cells, and therefore the cells attain most of their maximum steady-state ^{125}I-Tf binding by the earliest time point in the assay.

AP-2 Immunofluorescence

For fluorescent Tf uptake experiments, cells were incubated with 20 μg cy3-Tf per ml medium 1 for 1.5 h at 37°C. Either 0.25 μM bafilomycin A_1 or equivalent DMSO (0.1%) was included for the final 30 min of the incubation. Cells were fixed in 3.7% formaldehyde and prepared for indirect immunofluorescence. Cells were incubated in medium 2 containing 100 μg/ml saponin, 2 mg/ml ovalbumin, and a monoclonal antibody (mAb) against α-adaptin (AP-6, kindly provided by Dr. Francis Brodsky, University of California San Francisco). The primary antibody was visualized by incubation with fluorescein-labeled rabbit anti-mouse antibody (Cappel, Malvern, PA). Cells were examined using a Leitz fluorescence microscope equipped with a 63× objective (Leitz, Wetzlar, Germany), and images were collected using a charged coupled device camera (Photometrics, Tucson, AZ).

RESULTS

Bafilomycin A_1 Raises the pH of Endosomes

In CHO cells Tf encounters a number of acidic compartments during a round of internalization and recycling (e.g., Figure 1A) (Maxfield and Yamashiro, 1991). Tf is initially internalized into sorting endosomes in which luminal pH reaches a value of ~6.0. From this compartment ligands destined for lysosomal delivery are sorted from those that are recycled (e.g., Dunn *et al.*, 1989; Dunn and Maxfield, 1992). These sorting endosomes are distributed throughout the cell in a punctate pattern. Tf and other recycled molecules move from the sorting endosome to a compartment concentrated near the centrioles. This pericentriolar recycling compartment is comprised of small diameter tubules and vesicles and has a pH of ~6.4 (Yamashiro *et al.*, 1984). From this compartment recycling molecules are delivered back to the plasma membrane. The rate limiting step in recycling in CHO cells is exit from this compartment (e.g., Mayor *et al.*, 1993).

The effect of bafilomycin A_1 on the pH of Tf-containing endosomal compartments was determined by means of confocal emission ratio imaging of cells labeled with Tf conjugated to both fluorescein and rhodamine (F-R-Tf). The use of image analysis to measure endosome pH in intact cells provides the required spatial resolution to determine the pH of both the sorting endosomes and the pericentriolar recycling compartment. Confocal microscopy was used, because its narrow focal sectioning permits collection of fluorescence images of living cells in the presence of extracellular fluorescent ligand that, in wide-field microscopy, completely obscures cellular fluorescence. Because Tf is rapidly removed from sorting endosomes (with a half-time of 2–

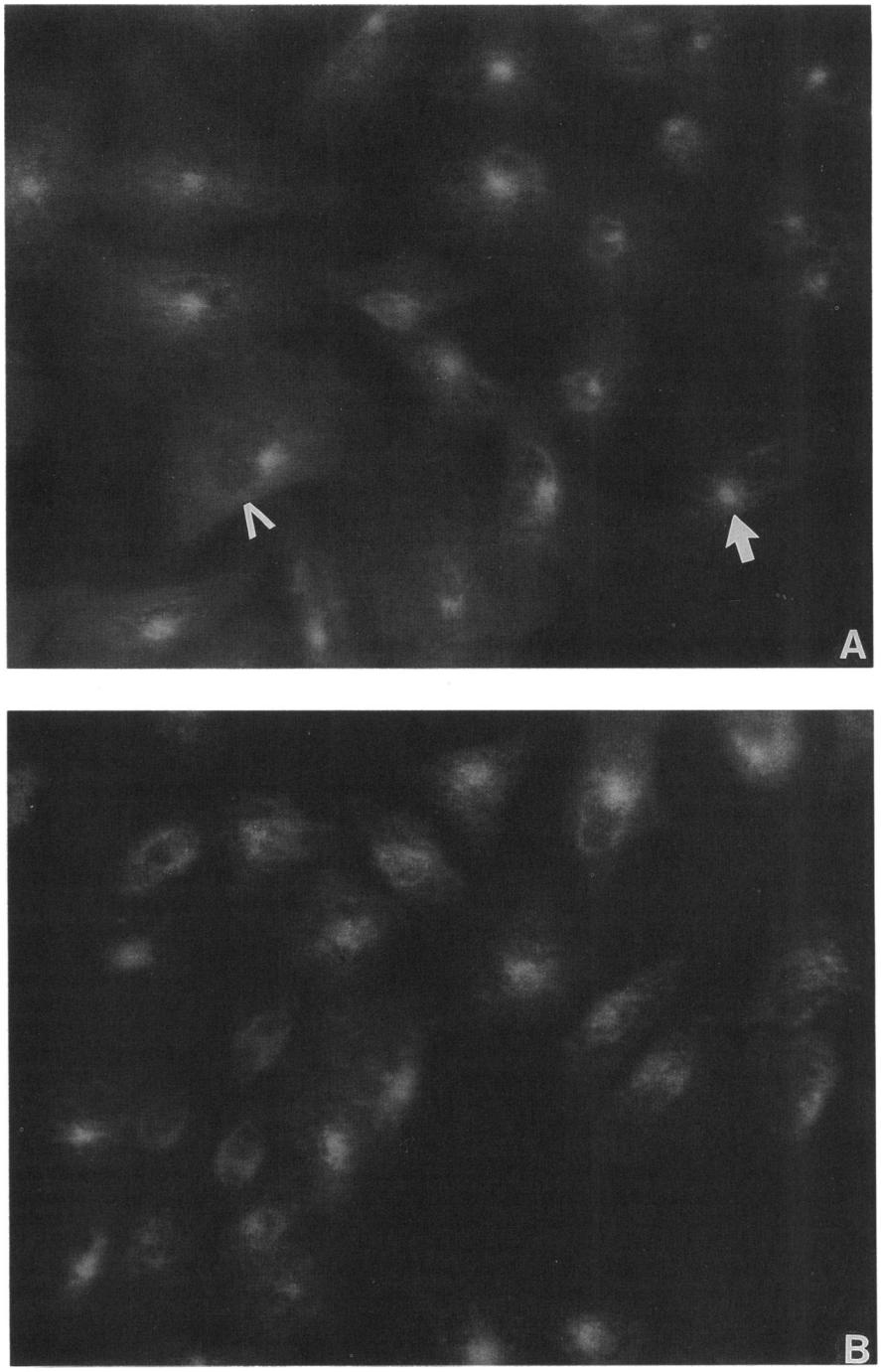

Figure 1. Effect of bafilomycin A_1 on steady-state transferrin labeling of CHO cells. TRVb-1 cells were labeled by incubation in the presence of cy3-Tf for 1.5 h with either 0.1% DMSO (A) or 0.25 μM bafilomycin A_1 (B) included during the final 30 min of incubation. In both cases the majority of the internalized Tf is concentrated in the pericentriolar recycling compartment (arrow). Additionally, the pattern of the more peripheral, smaller, punctate structures labeled with Tf is also unaffected by bafilomycin A_1 treatment (arrowhead). These results suggest that bafilomycin A_1 treatment does not alter the trafficking pathway of internalized Tf. These images were collected using standard wide-field fluorescence microscopy.

3 min.) (e.g., Dunn *et al.*, 1989), imaging of sorting endosomes requires the continuous presence of extracellular ligand. Bafilomycin A_1 was used at a concentration of 0.25 μM, which has been found to block endosome acidification in living cells of several types (Umata *et al.*, 1990; Yoshida *et al.*, 1990; Yoshimori *et al.*, 1991; and see below). This treatment with bafilomycin A_1 did not alter the morphology of Tf-labeled endosomal compartments, suggesting that Tf traffics through the same compartments in control and bafilomycin A_1-treated cells (Figure 1). Bafilomycin A_1 treatment significantly shifted the pH of both F-R-Tf–labeled punctate endosomes (sorting endosomes) and the pericentriolar recycling compartment toward neutral (Figure 2). The median pHs of the sorting endosomes in control versus bafilomycin A_1-treated cells were 6.0 and 6.6, respec-

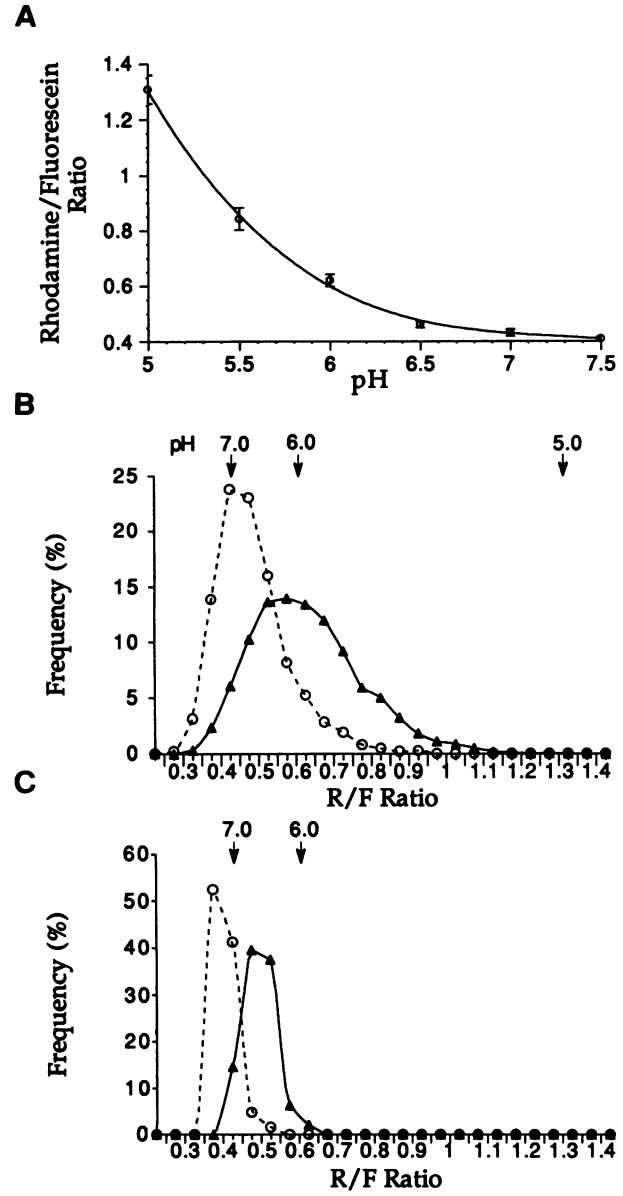

Figure 2. pH of Tf-labeled compartments in control versus bafilomycin A_1-treated cells. (A) F-R-Tf emission ratio pH calibration curve. Cells were incubated for 30 min with 20 μg/ml of F-R-Tf, fixed, and equilibrated with a range of pH buffers. Cells were imaged by confocal microscopy, and fluorescence ratios were calculated for each endosome as described in MATERIALS AND METHODS. Indicated values are means ± SD of the average endosome rhodamine to fluorescein fluorescence ratio for six fields. (B) Frequency distributions of punctate endosome rhodamine/fluorescein emission ratios for TRVb-1 cells in the presence of 0.25 μM bafilomycin A_1 (○) or an equivalent volume of DMSO (▲). (C) Frequency distributions of pericentriolar recycling compartment endosome rhodamine/fluorescein emission ratios in the presence of 0.25 μM bafilomycin A_1 (○) or an equivalent volume of DMSO (▲). Cells were incubated with bafilomycin A_1 (or equivalent DMSO) for 30 min and then for another 10 min in the presence of 20 μg/ml of F-R-Tf at which point imaging was conducted for another 10 min (all at 37°C). Corresponding pH values calculated from the calibration curve are indicated at the top of each frequency distribution. Note that indicated pH values are not linearly related to the R/F ratios due to the nonlinear pH dependence of fluorescein fluorescence.

tively, and the median pHs of the pericentriolar recycling compartment in control versus bafilomycin A_1-treated cells were ~6.4 and >7.0, respectively. (This last measurement is estimated, because the relatively low pKa of fluorescein gives F-R-Tf a poor sensitivity above pH 7.0, as can be seen in the pH calibration curve shown in Figure 2A). Thus, in vivo measurement of endosomal pH in CHO cells confirmed that brief treatment with a low concentration of the vacuolar H^+-ATPase inhibitor bafilomycin A_1 impairs endosomal acidification. The elevation in pH within Tf-labeled compartments in bafilomycin A_1-treated cells is similar to that observed in acidification-defective mutant cells of the End1 and End2 complementation groups (Yamashiro and Maxfield, 1987; Presley *et al.*, 1993).

Since it was first shown that endosomes are acidic organelles (Tycko and Maxfield, 1982), the role of pH in Tf-mediated iron delivery has been extensively characterized (e.g., van Renswoude *et al.*, 1982; Dautry-Varsat *et al.*, 1983; Klausner *et al.*, 1983). Diferric Tf is internalized into endosomes where the acidic pH promotes the release of iron from Tf. The resultant apo-Tf remains bound to and recycles with the TR back to the plasma membrane. Upon encountering the neutral pH at the plasma membrane, apo-Tf rapidly dissociates. A block in endosome acidification will induce a persistent cycling of Tf, because diferric Tf returned to the cell surface remains receptor bound. This persistent cycling of diferric Tf is detected as a decrease in the amount of Tf released from cells (e.g., Stein and Sussman, 1986). We have used this persistent cycling of diferric Tf induced by endosome alkalinization as an assay to examine the concentration dependence of bafilomycin A_1. As shown in Figure 3A, endosome alkalinization by bafilomycin A_1 reduced release of Tf in a concentration dependent manner to one-third of that released from control cells. When the chase period was extended to 4 h, ~90% of the Tf appeared in the chase media of both control and bafilomycin A_1-treated cells, indicating that the Tf is released slowly rather than being irretrievably trapped within the cell. Because this assay would also detect a reduction in release of Tf because of slowed externalization of TR, it also optimizes the concentration of bafilomycin A_1 for examining this potential effect of endosome alkalinization. In two experiments an effective concentration of bafilomycin A_1 was within the range of 0.125–0.5 μM. This effective concentration is consistent with previous studies (Umata *et al.*, 1990; Yoshida *et al.*, 1990; Yoshimori *et al.*, 1991). We have used 0.25 or 0.5 μM in all experiments.

The same assay that was used to test concentration dependence was used to assess the time required for endosome alkalinization by bafilomycin A_1. Figure 3B shows that the effect of bafilomycin A_1 was maximal within 30 min of treatment and did not change thereafter for ≥2 h. In all of our receptor trafficking assays,

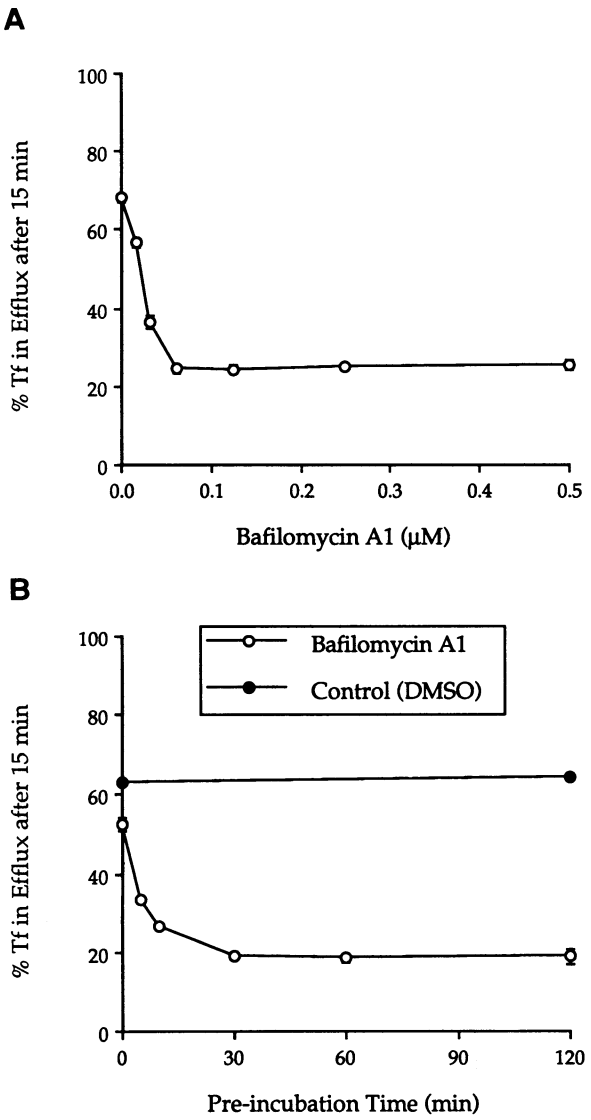

Figure 3. Concentration and time dependence of bafilomycin A_1. (A) Cells were loaded to steady state by incubation for 2 h at 37°C in medium 1 containing ^{125}I-Tf and varying concentrations of bafilomycin A_1. Surface-bound ^{125}I-Tf was removed by mild acid treatment (see MATERIALS AND METHODS), and the cells were incubated for a 15 min chase period at 37°C in the continued presence of the same concentration of bafilomycin A_1 (in medium 1 containing 3 μg/ml unlabeled Tf and 100 μM desferrioxamine to prevent rebinding of dissociated ^{125}I-Tf). After 15 min the chase medium was removed and counted (^{125}I-Tf efflux), the cells were solubilized and counted (^{125}I-Tf cell-associated), and the cell-associated ^{125}I-Tf was expressed as a percentage of the total (cell-associated + efflux) per well. (B) The assay was exactly the same as in A except that the bafilomycin A_1 concentration was kept constant at 0.25 μM, whereas pretreatment time in the presence of bafilomycin A_1 was varied by adding it at different times during the 2 h ^{125}I-Tf labeling period. The difference at pretreatment time 0 between the control and bafilomycin A_1 conditions results from the bafilomycin A_1 present during the 15-min chase period. In both A and B values are means ± SD of four measurements from a representative experiment (the error bars usually fall within the symbols); similar results were obtained in two experiments. Total ^{125}I-Tf binding (100%) in these experiments was 12 000–30 000 cpm, with maximum nonspecific binding of ~100–300 cpm.

unless otherwise noted, cells were pretreated with bafilomycin A_1 for 30 min.

Bafilomycin A_1 Does Not Affect TR Internalization

To determine whether the bafilomycin A_1 block in endosome acidification affects TR internalization, cells were preincubated in serum-free medium containing bafilomycin A_1 before measuring the internalization rate using a previously described assay (McGraw and Maxfield, 1990). The internalization rate constant was not affected by a 1 h preincubation with bafilomycin A_1, demonstrating that there was no direct effect of endosome alkalinization on internalization rate. To test whether slowing of internalization rate might be a secondary effect of endosome alkalinization and therefore take longer to develop, pretreatment with bafilomycin A_1 was extended to 5 h. As shown in Figure 4, even after 5 h bafilomycin A_1 did not significantly alter the internalization rate. These results suggest that normal endosomal acidification is not required to maintain efficient receptor internalization kinetics.

Bafilomycin A_1 Slows TR Externalization

To determine whether endosome alkalinization by bafilomycin A_1 slows the TR externalization rate, the approach to steady-state TR occupancy was measured (Figure 5). After initial binding of ^{125}I-Tf to TR on the cell surface, the rate of increase in cell-associated label to a maximum value (steady state) is a function of the TR externalization rate constant, as described in MATERIALS AND METHODS. This assay measures externalization of unoccupied receptors, rather than the release of ^{125}I-Tf from cells, and hence is not affected by the persistent recycling of diferric Tf induced by endosome alkalinization. The data in Figure 5 were fit to an expression for Tf accumulation (MATERIALS AND METHODS) and showed that bafilomycin A_1 slowed the receptor externalization rate to one-half of control (0.035 ± 0.002 versus 0.069 ± 0.009 min^{-1}, respectively, mean ± SD, N = 2 or $t_{1/2}$ = 20 versus 10 min, respectively).

A second assay was also used to measure the effect of bafilomycin A_1 on receptor externalization rate. Cells were incubated with ^{125}I-Tf to achieve steady-state TR occupancy with bafilomycin A_1 present during the final 30 min of incubation. Surface-bound ^{125}I-Tf was removed, and cells were allowed to externalize ^{125}I-Tf for varying times in the continued presence of bafilomycin A_1. At the end of the efflux period, the ^{125}I-Tf in the efflux medium, surface-bound, or internal was quantitated (Figure 6A). In the absence of bafilomycin A_1, ^{125}I-apo-Tf was externalized to the plasma membrane and rapidly released into the medium. The loss of internal label to the medium approximated a single first order kinetic process (Figure 6B). As expected from the approach to steady-state results, the bafilomycin A_1 in-

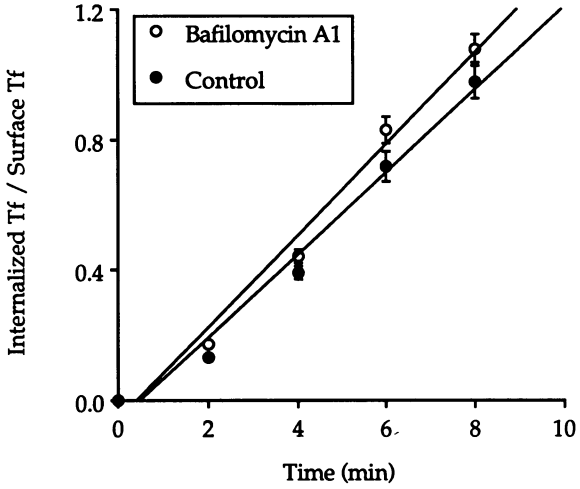

Figure 4. Effect of bafilomycin A_1 on ^{125}I-Tf internalization rate. Cells were pretreated for 5 h in medium 1 containing 0.25 μM bafilomycin A_1 (or equivalent DMSO). To measure the internalization rate constant, k_i, surface TR were quantified in one plate of cells by binding for 2 h at 4°C in the presence of ^{125}I-Tf. TR internalized vs. time was measured in parallel plates of cells by incubating for varying times at 37°C in the presence of ^{125}I-Tf (in medium 1 containing bafilomycin A_1 or equivalent DMSO), placing the plate on ice, and removing surface-bound ^{125}I-Tf by treatment for 2 min at 4°C in 0.5 M NaCl, 0.5 M acetic acid pH 2.0 then washing, solubilizing, and counting for internal ^{125}I-Tf. The internalized ^{125}I-Tf plotted as a ratio of the steady-state surface-bound ^{125}I-Tf (mean ± SD, N = 4) is linear with a slope corresponding to the internalization rate constant, k_i. The same experiment performed after 1 h pretreatment with bafilomycin A_1 also detected no significant effect on k_i.

hibition of endosomal acidification slowed externalization of diferric ^{125}I-Tf to the plasma membrane. Recycling of diferric ^{125}I-Tf was indicated by increased surface-bound ^{125}I-Tf during the chase period relative to control (Figure 6A). This persistent recycling of diferric ^{125}I-Tf complicated quantitation of receptor recycling rate from the loss of cell-associated label, because a fraction of the surface-bound ^{125}I-Tf was reinternalized during the efflux period. Under these conditions the TR externalization rate constant cannot be calculated directly from the slope of the loss of cell-associated label. Rather, the intracellular label must be corrected for the label reinternalized during the chase period. The ^{125}I-Tf reinternalized during time t is: $Tf_{reinternalized} = k_i \cdot \int [Tf_s(t)]dt$, where k_i is the internalization rate constant and $Tf_s(t)$ is the surface-bound diferric ^{125}I-Tf at each time t (MATERIALS AND METHODS). Hence, the ^{125}I-Tf that has remained internal since time 0 can be calculated from the measured value of internal ^{125}I-Tf at time t minus the ^{125}I-Tf that has been reinternalized at time t, using two experimentally determined values: the internalization rate constant and the area beneath the curve of surface-bound Tf at time t (MATERIALS AND METHODS). The corrected values for the internal ^{125}I-Tf in the presence of bafilomycin A_1 are shown in Figure 6B. The TR externalization rate derived from this analysis was 0.091 ± 0.018 min^{-1} in the absence of bafilomycin A_1 and 0.032 ± 0.001 min^{-1} in the presence of bafilomycin A_1 (mean ± SD, N = 2). These values are within measurement error of the values obtained based on approach to steady-state TR occupancy. Thus, the ^{125}I-Tf externalization assay and the approach to steady-state TR occupancy assay independently indicate that bafilomycin A_1 slows TR externalization. These results demonstrate, using a specific inhibitor of the vacuolar proton pump, that endosome acidification is required for efficient trafficking of TR from the endocytic pathway back to the plasma membrane.

Slowing of TR Externalization Rate by Bafilomycin A_1 Requires TR Cytoplasmic Tail

In CHO cells fluorescent lipid (C_6-NBD-sphingomyelin) internalized from the plasma membrane traffics through the same intracellular compartments and at the same rates as Tf(Mayor *et al.*, 1993). We have demonstrated in an acidification-defective End2 mutant cell line, 12-4, that although TR are recycled at one-half of the wild-type rate, fluorescent lipid internalized from the plasma membrane is recycled back to the cell surface at the wild-type rate (Presley *et al.*, 1993). The observation

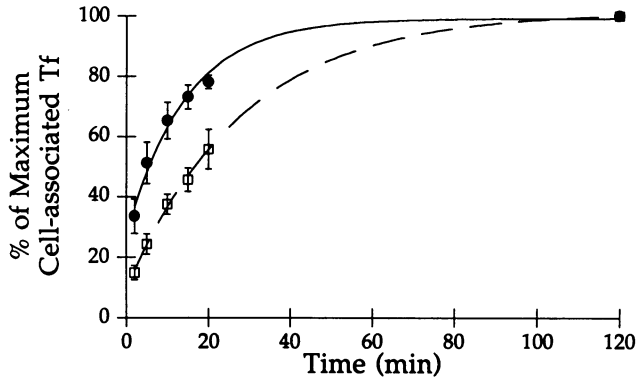

Figure 5. Effect of bafilomycin A_1 on exocytic rate constant, k_e, assessed using rate of approach to steady state binding by ^{125}I-Tf at 37°C. Cells were preincubated for 15 min in medium 1 containing 0.5 μM bafilomycin A_1 (□) or equivalent DMSO (●) before incubation for varying times out to steady state at 2 h in medium 1 containing bafilomycin A_1 (or equivalent DMSO) and ^{125}I-Tf. An alternative method that gave the same result was to vary the preincubation time between 1 and 3 h such that the total bafilomycin A_1 treatment time at the end of the varied labeling period was 3 h for all plates. At the end of the labeling period, the cells were washed three times rapidly with medium 2 and solubilized; the cell-associated counts were expressed as a percentage of the maximum steady-state value at 2 h. The figure summarizes two experiments (mean ± SEM). The maximum value (100%, steady-state binding) of ^{125}I-Tf in the experiments was 60 000–115 000 cpm, with maximum nonspecific binding of <900 cpm. The difference in y-intercept between conditions is consistent with a bafilomycin A_1-induced shift of TR from surface to intracellular pools, as would be expected to result from slowing of k_e.

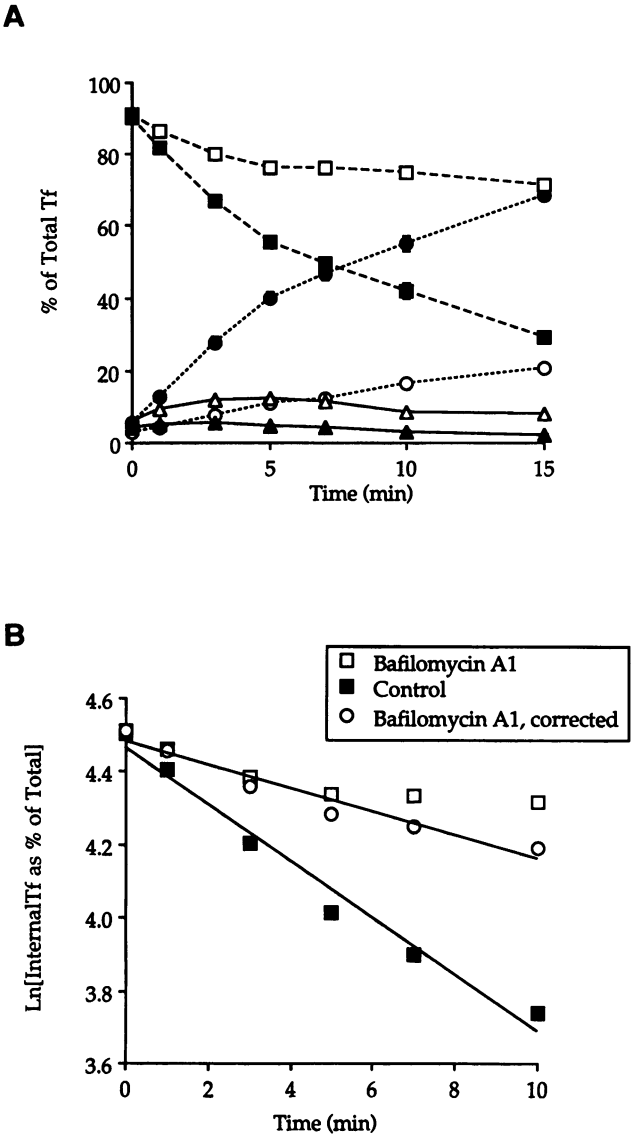

Figure 6. Effect of bafilomycin A_1 on exocytic rate constant, k_e, assessed using efflux of ^{125}I-Tf. Representative results of the ^{125}I-Tf efflux assay used to measure k_e are shown (MATERIALS AND METHODS). Cells were labeled to steady state by incubation for 2 h at 37°C in medium 1 containing ^{125}I-Tf, during the last 30 min of which bafilomycin A_1 (or equivalent DMSO) was added. Surface-bound ^{125}I-Tf was then removed by mild acid treatment, and the cells were allowed varying chase times to externalize their intracellular ^{125}I-Tf. After the chase period the ^{125}I-Tf was measured as in the efflux medium, bound to the surface of the cells, or intracellular. (A) ^{125}I-Tf in the efflux (circles), surface-bound (triangles), and internal (squares) are expressed as a percentage of the total per well for control cells (closed symbols) and bafilomycin A_1-treated cells (open symbols), mean ± SD, N = 4, error bars usually fall within the symbols, total/well ~30 000–40 000 cpm, with maximum nonspecific binding of <600 cpm. (B) The logarithm of the ^{125}I-Tf intracellular (percentage of total) is plotted vs. time. For control cells, the slope of this line corresponds to $-k_e$. For bafilomycin A_1-treated cells, this curve must be corrected for ^{125}I-Tf reinternalized from the surface during the chase period, as described in MATERIALS AND METHODS, and the slope of this corrected curve corresponds to $-k_e$.

that in endosome acidification-defective mutants membrane lipid is recycled normally but TR recycling is slowed, suggests that alkalinization of endosomes does not inhibit vesicular traffic but, rather, slows TR recycling resulting in the retention of TR relative to bulk membrane flow.

Because the cytoplasmic domain mediates sequestration of TR in plasma membrane clathrin-coated pits, we next examined the role of the cytoplasmic domain of the TR in bafilomycin A_1-induced slowing of TR recycling. For these studies a cell line was used, TRVbΔ3-59, that expresses only a TR construct lacking 57 of the 61 amino acid cytoplasmic domain. The Δ3-59 TR is internalized at a rate only 25% that of the wild-type receptor or 0.033 ± 0.001 versus 0.135 ± 0.025 min^{-1}, respectively (Figure 7 and Table 1), consistent with previous studies that found inefficient internalization of cytoplasmic domain-deleted receptors (Rothenberger *et al.*, 1987; Jing *et al.*, 1990). Although the Δ3-59 TR is internalized slowly, once internalized it is recycled at a rate indistinguishable from the wild-type rate (Table 1). This result is in agreement with other reports (Jing *et al.*, 1990) and is consistent with the proposal that efficient internalization requires a cytoplasmic determinant, whereas efficient recycling occurs via a bulk flow process (e.g., Dunn *et al.*, 1989; Mayor *et al.*, 1993). The Δ3-59 TR is trafficked through the same intracellular compartments as the wild-type TR. The morphology of fluorescent Tf-labeled intracellular compartments in TRVbΔ3-59 is indistinguishable from wild-type with most of internal Tf concentrated in the pericentriolar recycling compartment. The pericentriolar recycling compartment in TRVbΔ3-59 cells (measured using the confocal fluorescein-rhodamine Tf technique as in Figure 2) has a pH of ~6.15 (mean ± 0.15, n = 42). In this experiment the recycling compartment in cells expressing the wild-type TR had a pH of ~6.35 (mean ± 0.15, n = 50). As is the case for the recycling compartment labeled by the wild-type TR, the pH of the recycling compartment containing the Δ3-59TR is sensitive to bafilomycin A_1, being raised to a pH of >7.0 after a 40-min incubation with 0.5 μM bafilomycin A_1. These findings are consistent with the previous observations that internalization-defective TR deliver iron to cells at a rate approximately proportional to the rate at which they mediate Tf internalization (McGraw and Maxfield, 1990; McGraw *et al.*, 1991).

Notably, recycling of the Δ3-59 TR is not significantly slowed by bafilomycin A_1 treatment (Figure 8 and Table 1), even though the pH of the recycling compartment is alkalinized. The values in Figure 8B for bafilomycin A_1-treated cells have been corrected for ^{125}I-Tf reinternalized during the chase period using the measured Δ3-59 TR internalization rate constant (Table 1) and the area beneath the curve of surface-bound Tf (Figure 8A) as described for Figure 6. Thus, although the wild-type TR is temporarily retained during recycling in the pres-

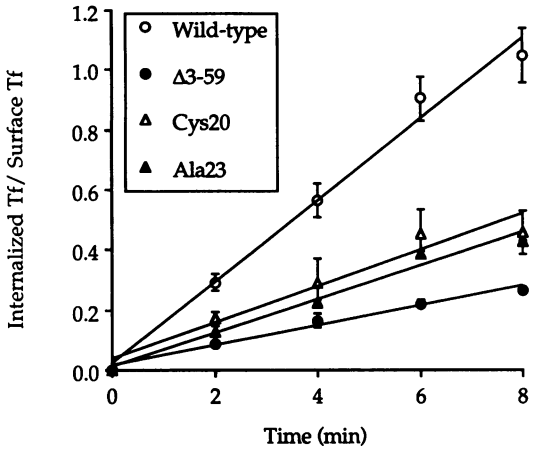

Figure 7. Internalization rate constant, k_i, of wild-type and mutant constructs of the human TR. The assay was identical to that shown in Figure 4 except that the preincubation in medium 1 was for 15 min. Values plotted are means ± SEM from multiple experiments, wild-type, N = 5, each mutant, N = 2. The slope of each line corresponds to k_i for that receptor.

ence of bafilomycin A_1, the Δ3-59 TR continues to recycle with the kinetics of bulk membrane flow. This result suggests that intracellular retention of the wild-type TR is mediated by the cytoplasmic domain. The finding that the cytoplasmic domain of the TR is required for the bafilomycin A_1-induced slowing of TR recycling suggests that intravesicular pH can influence interactions between the cytoplasmic domain of the TR and some cytoplasmic component.

Bafilomycin A_1 Slowing of TR Externalization Requires Intact TR Internalization Signal

The only functional region that has been identified within the cytoplasmic domain of the TR is the YTRF internalization motif, which mediates efficient internalization (Collawn *et al.*, 1990). To delineate further the sequences within the cytoplasmic domain that are required for slowed TR recycling under conditions of endosome alkalinization, the trafficking of TR with mutations in the internalization motif was examined. The effect of bafilomycin A_1 on TR externalization rate was measured in two cell lines that express TR containing single amino acid substitutions that disrupt internalization: cysteine for tyrosine at position 20 (C20 TR) or alanine for phenylalanine at position 23 (A23 TR). Both of these mutant TRs are internalized slowly compared to the wild-type TR (McGraw and Maxfield, 1990; McGraw *et al.*, 1991), though not as slowly as the Δ3-59 TR (Figure 7 and Table 1). Furthermore, as is the case for the Δ3-59 TR, the C20 and A23 TRs are externalized at rates not significantly different from the wild-type TR (McGraw and Maxfield, 1990; McGraw *et al.*, 1991). Strikingly, both of these substitutions abrogate much of the effect of bafilomycin A_1 in slowing TR externalization (Figure 9). As in their effect on receptor internalization, these substitutions do not block the effect of bafilomycin A_1 as completely as deletion of the cytoplasmic tail. Thus, two different single amino acid substitutions that disrupt recognition of the cytoplasmic tail for sequestration in plasma membrane clathrin-coated pits also disrupt the slowed externalization of TR induced by bafilomycin A_1. These results indicate that the region of the cytoplasmic tail required for bafilomycin A_1 to slow receptor externalization is the internalization motif or at least shares critical features with this motif. This finding suggests that inhibition of endosome acidification leads to intracellular retention of receptors by causing recognition of this motif by an interacting factor.

AP-2 Localization Is Not Grossly Altered by Bafilomycin A_1

Because an intact TR internalization motif is necessary for slowed recycling in the presence of bafilomycin A_1, it seems likely that a protein recognizing this sequence

Table 1. Endocytic rate constants of TR constructs

	Internalization		Externalization		
			Rate constant (k_e, min^{-1})[b]		
TR	Rate constant (k_i, min^{-1})[a]	% of Wild-type	Control	Bafilomycin	% of Control
Wild-type	0.135 ± 0.025	100	0.091 ± 0.018	0.032 ± 0.001	35
Δ3-59	0.033 ± 0.001	24	0.097 ± 0.008	0.087 ± 0.011	90
Cys20	0.060 ± 0.014	44	0.105 ± 0.011	0.073 ± 0.001	70
Ala23	0.056 ± 0.001	41	0.102 ± 0.011	0.087 ± 0.004	85

[a] Values are means ± SD, wild-type N = 5, each mutant N = 2, from assays summarized in Figure 7.
[b] Values are means ± SD, N = 2, from assays represented in Figures 6, 8, and 9.

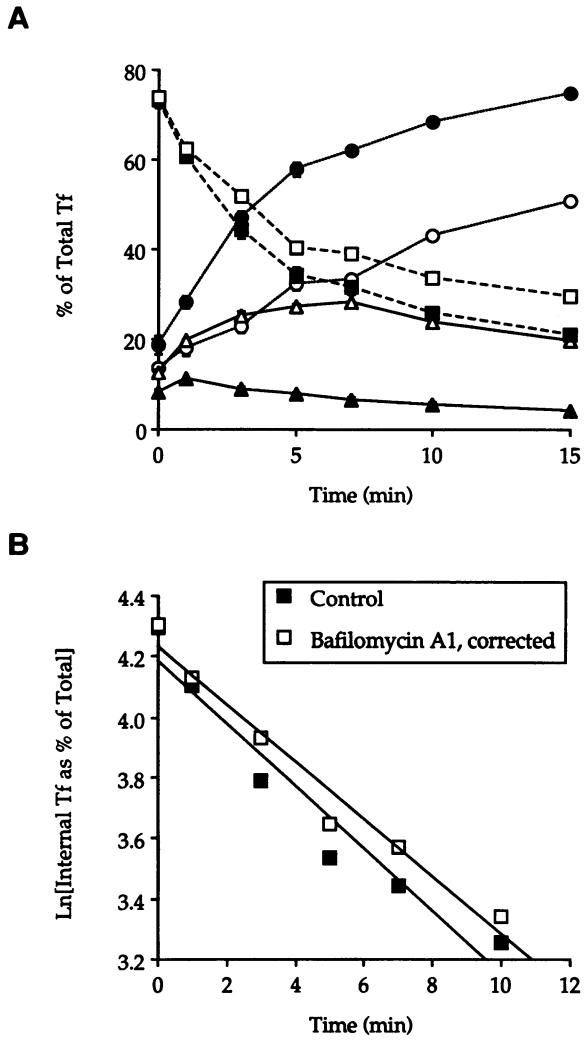

Figure 8. Effect of bafilomycin A_1 on exocytic rate constant, k_e, of deletion mutant TR lacking a cytoplasmic tail. Representative results are presented of the same assay shown in Figure 6 except done using the TRVbΔ3-59 cell line, which expresses TR lacking a cytoplasmic tail domain. (A) ^{125}I-Tf in the efflux (circles), surface-bound (triangles), and internal (squares) are expressed as a percentage of the total per well for control cells (closed symbols) and bafilomycin A_1-treated cells (open symbols), mean ± SD, N = 4, error bars usually fall within the symbols, total/well ~5000–7000 cpm, with maximum nonspecific binding of <400 cpm. The greater increase in surface-bound Tf caused by bafilomycin A_1 for the Δ3-59 TR compared with wild-type (Figure 6A) is in agreement with the slower rate of internalization of the Δ3-59 TR (Figure 7). (B) The bafilomycin A_1 data have been corrected for reinternalization of ^{125}I-Tf during the chase period, as described for Figure 6B and in MATERIALS AND METHODS.

mediates the effect. The plasma membrane clathrin-associated protein complex, AP-2, is believed to recognize this motif and thereby mediate sequestration in clathrin-coated pits (Pearse, 1988; Glickman *et al.*, 1989). Slowed externalization of receptors by a mechanism utilizing the receptor's internalization motif might be explained by abnormal AP-2 binding inside the cell. For instance, if endosomal acidification were to play a role in destabilizing TR/AP-2 complexes, then bafilomycin A_1 might inhibit destabilization of these complexes and thereby cause intracellular retention of receptors. We have used fluorescence microscopy to determine whether bafilomycin A_1-induced endosome alkalinization causes increased association of AP-2 complexes with Tf-containing endosomes in the recycling compartment. Fluorescence microscopy revealed no gross alteration in the immunolocalization of AP-2 induced by bafilomycin A_1 treatment. Notably, AP-2 complexes were not detectable in the recycling compartment of cells in the presence or absence of bafilomycin A_1 (Figure 10). This result suggests that AP-2 is not the factor responsible for the slowed externalization of TR in response to endosome alkalinization, although with this analysis we cannot rule out subtle changes in AP-2 distribution.

DISCUSSION

We have shown that the specific inhibitor of the vacuolar proton pump, bafilomycin A_1, increases endosome pH and markedly slows the rate at which internalized TRs are recycled back to the plasma membrane. The finding that endosome acidification is needed to maintain rapid trafficking of receptors from endosomes back to the plasma membrane is consistent with previous results obtained using less specific reagents to neutralize endosome pH (e.g., Basu *et al.*, 1981; Klausner *et al.*, 1983; Schwartz *et al.*, 1984), as well as a recent study of acidification-defective End1 and End2 mutants (Johnson *et al.*, 1993). The current study represents a significant extension of previous studies in that it provides the first information concerning the mechanism by which perturbed acidification interferes with receptor recycling. The mechanism requires the cytoplasmic domain of the receptor and, more specifically, is dependent upon at least two amino acids that are also critical for

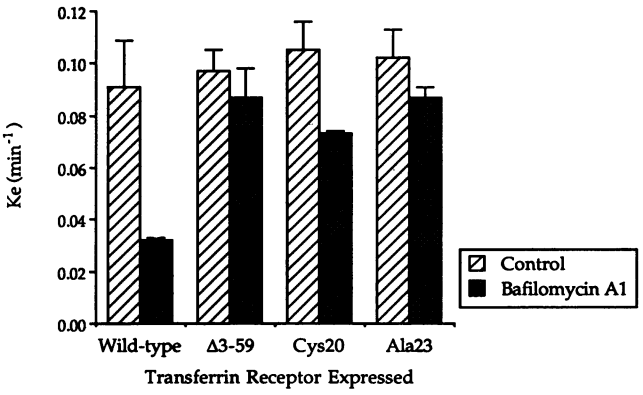

Figure 9. Effect of bafilomycin A_1 on exocytic rate constant, k_e, of wild-type vs. mutant constructs of the human TR. Each value is the mean of k_e ± SD measured in two experiments identical to those shown in Figures 6 and 8.

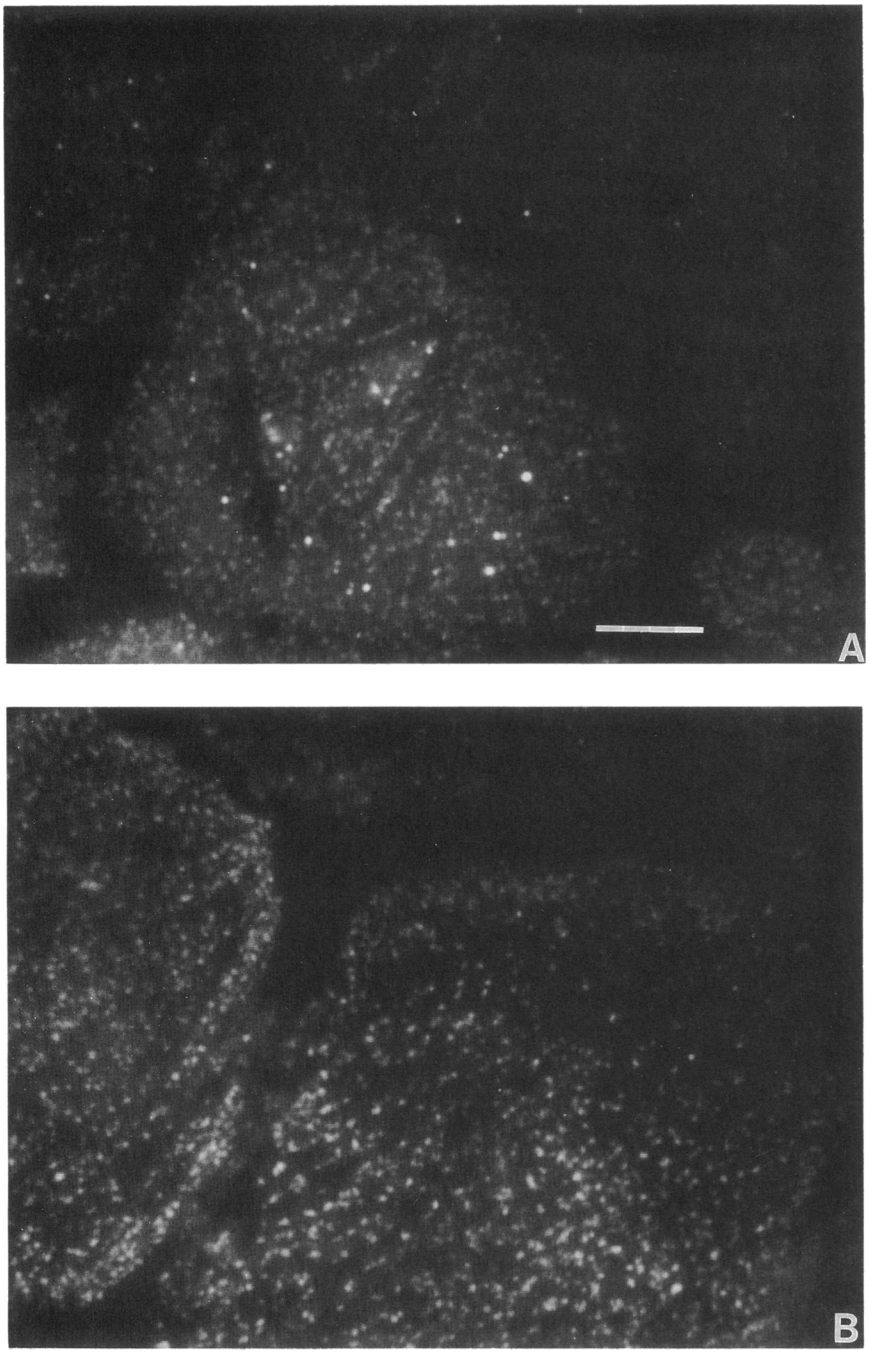

Figure 10. Effect of bafilomycin A_1 on the distribution of α-adaptin. TRVb-1 cells were incubated for 1.5 h in the presence of cy3-Tf with either 0.1% DMSO (A) or 0.25 μM bafilomycin A_1 (B) included during the final 30 min of incubation. The cells were fixed and stained with a mAb against α-adaptin (MATERIALS AND METHODS). The Tf-labeled compartments were as shown in Figure 1. Bafilomycin A_1 treatment did not detectably alter the distribution of the plasma membrane α-adaptin. In both control and bafilomycin A_1-treated cells, there is no apparent α-adaptin staining of the pericentriolar recycling compartment. Bar, 10 μm.

efficient uptake of the receptor via clathrin-coated pits. The simplest interpretation of these results is that the block in acidification of the endosome lumen leads to a cytoplasmic interaction involving the Tyr β-turn motif. These results suggest a novel role for intravesicular acidification: regulation of cytoplasmic interactions involved in the trafficking of membrane proteins.

Despite the involvement of two amino acids within the receptor's internalization motif, the mechanism is not mediated by gross mislocalization of AP-2, the plasma membrane clathrin-associated protein complex believed to bind to receptors by recognition of the internalization motif. Thus, although subtle changes in AP-2 distribution remain a possibility, endosome alkalinization does not dramatically alter the overall distribution of adaptins between cytosol and membranes, as is seen for the Golgi clathrin-associated protein γ-adaptin after treatment with brefel-

din A or GTPγS (Robinson and Kreis, 1992; Wong and Brodsky, 1992).

Basu *et al.* reported that treatment of human fibroblasts with monensin led to trapping of wild-type low density lipoprotein receptors (LDL-R) intracellularly, but that this effect was eliminated in cells expressing LDL-R containing a single amino acid substitution for the tyrosine residue within the cytoplasmic NPVY β-turn internalization motif (Basu *et al.*, 1981). The result was interpreted as being consistent with a lack of internalization of the J.D. mutant receptor (cysteine substituted for the tyrosine of the internalization motif). Because the J.D. mutant receptor has been shown to be internalized, albeit with markedly lower efficiency than wild-type receptors (Davis *et al.*, 1986), the result is also consistent with the finding in this study that the Tyr β-turn motif is necessary for slowed receptor recycling to result from endosome alkalinization. It may also be significant that the macrophage Fc receptor has been reported to recycle with little or no inhibition in the presence of monensin or ammonium chloride (Mellman *et al.*, 1984). This receptor is unusual in that it contains a cytoplasmic clathrin-coated pit localization determinant that is tyrosine-independent (Miettinen *et al.*, 1992). Hence, this result is also consistent with the requirement of a Tyr β-turn motif for slowed receptor recycling to result from endosome alkalinization.

The impetus for these studies was the observation that endosomal acidification mutants of both the End1 and End2 complementation groups have defects in the rates of TR trafficking (Johnson *et al.*, 1993). These findings raised the possibility that the altered trafficking in the mutant cells could be responsible for their acidification defects. We now know that the correlation of endosome alkalinization and slowed TR recycling observed in the End1 and End2 mutant cell lines is reproduced by a specific inhibitor of the vacuolar H^+-ATPase, bafilomycin A_1. This result indicates that the impaired endosome acidification in End2 and End1 mutants is sufficient to account for their slowed recycling of TR. The cumulative body of evidence from studies using weak bases or ionophores, acidification-defective End1 and End2 mutants, and bafilomycin A_1 is now in agreement that blocking endosome acidification causes slowed recycling of internalized receptors back to the plasma membrane. It should be noted that although we discuss blocked endosome acidification in terms of its effect on proton concentration (pH), the relevant effect could also be dissipation of transmembrane proton concentration gradient or transmembrane electrical potential.

What mechanism might account for the intracellular retention of receptors in response to impaired acidification? Aggregation induced by cross-linking with antibodies or other polyvalent ligands diverts receptors to lysosomes (e.g., Anderson *et al.*, 1982; Mellman and Plutner, 1984; Weissman *et al.*, 1986). Thus, one possible mechanism is that blocking endosome acidification would disrupt receptor recycling by resulting in aggregation of receptors. In bafilomycin A_1-treated cells all of the ^{125}I-Tf is released, indicating that it is not being delivered to a terminal endosomal (or lysosomal) compartment. In addition, the Tf labeling pattern of bafilomycin A_1-treated cells is not consistent with significant diversion of Tf to lysosomes. Thus, endosome alkalinization appears to slow receptor recycling with characteristics distinct from receptor aggregation.

The role of a Tyr β-turn cytoplasmic determinant in efficient clathrin-mediated internalization from the plasma membrane has been established in multiple receptor types. Recognition of the Tyr β-turn motif by the AP-2 protein complex is thought to mediate the sequestering of receptors in clathrin-coated pits (Robinson, 1992). One interpretation of our finding that this motif is also involved in slowed recycling of TR would be that endosome acidification normally facilitates efficient receptor recycling by destabilizing an association involving the β-turn motif (presumably AP-2). Thus, it could be that for cells to utilize the AP-2/β-turn mechanism of efficient receptor internalization, it is also necessary, to maintain efficient receptor recycling, for cells to utilize an endosomal mechanism mediating intracellular dissociation from AP-2. Like previously established functions of endosomes, this mechanism could be pH-dependent. As noted above, this interpretation leads to the prediction of significantly increased association of AP-2 with Tf-labeled endosomes in bafilomycin A_1-treated cells, which we have been unable to detect. However, a definitive conclusion will require a more detailed analysis to detect subtle changes in AP-2 distribution.

An alternative interpretation of these results is that endosome acidification normally facilitates efficient receptor recycling by preventing inappropriate interactions involving the β-turn motif. In this regard it is of interest to note that in the polarized epithelial Madin-Darby canine kidney cell type, sorting of several basolateral membrane proteins from the trans-Golgi network (TGN) requires a Tyr-containing cytoplasmic determinant that often, though not always (Hunziker *et al.*, 1991; Matter *et al.*, 1992; Dargemont *et al.*, 1993), can also serve as an internalization motif (Brewer and Roth, 1991; Casanova *et al.*, 1991; Hunziker *et al.*, 1991; Le Bivic *et al.*, 1991; Matter *et al.*, 1992). The retention of the resident protein TGN 38/41 in the TGN also depends upon a similar motif (Humphrey *et al.*, 1993). These findings have led to the suggestion that there may be a family of cytoplasmic proteins, potentially related to the adaptins, each of which recognizes and binds to this type of motif (Matter *et al.*, 1992). It could be through this interaction that the dynamic distribution of membrane proteins among organelles is maintained. Two notable examples consistent with this proposal are the intracellular retention of the insulin-dependent glu-

cose transporter (GLUT4), which is dependent upon a Phe-containing sequence also thought to mediate efficient internalization (Piper *et al.*, 1992), and the retention in the Golgi of the yeast Kex2 protein, which is dependent upon a cytosolic tyrosine-containing sequence similar to the coated-pit localization signal of mammalian cells (Wilcox *et al.*, 1992). If, as these various results suggest, there indeed exists a family of cytoplasmic trafficking proteins that bind to Tyr β-turn motifs, then it may be that impaired endosome acidification leads to the inappropriate recognition of this motif within recycling receptors by one of the members of this family, thereby slowing receptor externalization. Thus, it is possible that acidification of endosomes normally serves to prevent inappropriate binding to receptor cytoplasmic domains by a member of this family.

How might acidification of the endosome lumen modulate a cytoplasmic interaction? Several receptors have been shown to undergo pH-sensitive conformational changes (e.g., DiPaola and Maxfield, 1984; Turkewitz *et al.*, 1988), so one explanation might be that endosome acidification normally induces an overall conformational change in the receptor that alters interactions involving the internalization motif. Because recycling of multiple receptor types is perturbed by endosome alkalinization (e.g., Basu *et al.*, 1981; Klausner *et al.*, 1983; Schwartz *et al.*, 1984), it would be necessary to invoke a conformational change induced by luminal acidic pH in several different receptor types that similarly alters the cytoplasmic β-turn interaction. Because it has been shown that the location of the internalization motif within a receptor's cytoplasmic domain can be changed without altering the rate of receptor recycling (e.g., McGraw *et al.*, 1991), it appears unlikely that a specific conformational change in the receptor is required during recycling. Thus, it is likely that a protein other than the individual recycling receptors is required to transmit the necessary information concerning luminal pH.

Our findings suggest that vacuolar acidification plays a role in regulating sorting interactions. Nearly all vacuolar compartments within the cell are acidified, each to its own characteristic pH. Perhaps a compartment's pH helps to identify or define it, specifying appropriate interactions between its membrane proteins and the various soluble proteins involved in trafficking. For instance, a number of proteins believed important in membrane trafficking (e.g., clathrin, adaptins, rab proteins) are believed to cycle between cytosolic and membrane-bound states. It is possible that luminal pH recruits specific soluble adaptin-like proteins to the cytoplasmic face of the appropriate membrane compartment where they could interact with the Tyr β-turn motifs. Thus, perhaps there is an organelle-specific protein (or protein family) that senses luminal pH and transmits this information, either by conformational change or some other signaling mechanism, to effect proper trafficking interactions on the cytosolic side of the membrane. One intriguing candidate for such a protein could be the recently reported receptor for the AP-2 complex (Peeler *et al.*, 1993). Although this model is currently speculative, it is of interest to note evidence that the association with target membranes of vesicle coat proteins, including Golgi coatomer proteins and TGN adaptins, is regulated (Donaldson *et al.*, 1991a,b; Robinson and Kreis, 1992; Wong and Brodsky, 1992). Moreover, the association of ADP-ribosylation factor, a protein required for Golgi membrane trafficking, can be modulated in vitro by the luminal pH of target vesicles (Zeuzem *et al.*, 1992).

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