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Kinetics of Endosome Acidification in Mutant and Wild-type Chinese Hamster Ovary Cells

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Abstract. Acidification of endocytic compartments is necessary for the proper sorting and processing of many ligands and their receptors. Robbins and coworkers have obtained Chinese hamster ovary (CHO) cell mutants that are pleiotropically defective in endocytosis and deficient in ATP-dependent acidification of endosomes isolated by density centrifugation (Robbins, A. R., S. S. Peng, and J. L. Marshall. 1983. *J. Cell Biol.* 96:1064–1071; Robbins, A. R., C. Oliver, J. L. Bateman, S. S. Krag, C. J. Galloway, and I. Mellman. 1984. *J. Cell Biol.* 99:1296–1308). In this and the following paper (Yamashiro, D. J., and F. R. Maxfield. 1987. *J. Cell Biol.* 105:2723–2733) we describe detailed studies of endosome acidification in the mutant and wild-type CHO cells. Here we describe a new microspectrofluorometry method based on changes in fluorescein fluorescence when all cellular compartments are equilibrated to the same pH value. Using this method we measured the pH of endocytic compartments during the first minutes of endocytosis. We found in wild-type CHO cells that after 3 min, fluorescein-labeled dextran (F-Dex) was in endosomes having an average pH of 6.3. By 10 min, both F-Dex and fluorescein-labeled α_2-macroglobulin (F-α_2M) had reached acidic endosomes having an average pH of 6.0 or below. In contrast, endosome acidification in the CHO mutants DTG 1-5-4 and DTF 1-5-1 was markedly slowed. The average endosomal pH after 5 min was 6.7 in both mutant cell lines. At least 15 min was required for F-Dex and F-α_2M to reach an average pH of 6.0 in DTG 1-5-4. Acidification of early endocytic compartments is defective in the CHO mutants DTG 1-5-4 and DTF 1-5-1, but pH regulation of later compartments on both the recycling pathway and lysosomal pathway is nearly normal. The properties of the mutant cells suggest that proper functioning of pH regulatory mechanisms in early endocytic compartments is critical for many pH-mediated processes of endocytosis.

Ligands and receptors that are internalized by receptor-mediated endocytosis pass through several distinct, acidic intracellular compartments (11, 13). For example, asialoglycoproteins, the serum protease inhibitor α_2-macroglobulin (α_2M),[1] and the iron transport protein transferrin (Tf) can be found in large endosomes that have a pH of 5.0–5.6 (26, 27, 29, 34). Many of these ligands, including α_2M and the asialoglycoproteins, are eventually degraded in lysosomes, organelles that have a pH of 4.8–5.2 (16, 27). In Chinese hamster ovary (CHO) cells, Tf also passes through recycling endosomes comprised of small vesicles and tubules near the Golgi complex that are only mildly acidic (pH 6.4) (reference 34). Very little, however, is known about the acidification that occurs during the first few minutes after endocytosis. Therefore, it is not clear what role acidification of the earliest endosomes has in the functioning of various endocytic processes.

Several groups have obtained CHO mutants pleiotropically defective in endocytosis by selecting cells that were resistant to diphtheria toxin (2, 9, 14, 21). These mutants are also resistant to other toxins and certain viruses that require an acidic pH for toxicity or infectivity. The CHO mutants DTF 1-5-1 and DTG 1-5-4 obtained by Robbins and coworkers are defective in the uptake of lysosomal enzymes via the mannose-6-phosphate receptor (21, 22) and have a diminished capacity to obtain iron from Tf (7; Robbins, A., personal communication). Genetic complementation has shown that DTG 1-5-4 is in the complementation group "End1" and DTF 1-5-1 in another group "End2" (23). The characteristics of the CHO mutants suggest that they are defective in delivery of ligands to an appropriate acidic environment. In support of this idea, endosomes isolated by density centrifugation from the CHO mutants were deficient in ATP-dependent acidification, whereas lysosomes isolated by the same tech-

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1. *Abbreviations used in this paper*: α_2M, α_2-macroglobulin; AA/MA, ammonium acetate/methylamine; BCECF-AM, tetraacetoxymethyl ester of 2′,7′-bis(carboxyethyl)-5,6-carboxyfluorescein; F-α_2M, fluorescein-labeled α_2-macroglobulin; F-Dex, fluorescein isothiocyanate dextran; F-Tf, fluorescein-labeled transferrin; Tf, transferrin; WTB, wild-type CHO cells.

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nique were unaffected (22). The endosomal pH in these mutants has not been measured in living cells.

In CHO cells, at least three distinct types of endosomes can be identified (31, 34). These include small vesicles and tubules near the surface of the cell (early endosomes), larger endosomes with diameters of ~150–250 nm (large endosomes), and a collection of small vesicles and tubules near the Golgi complex (recyclng endosomes), which contains recycling Tf after segregation from molecules such as α_2M which are delivered to lysosomes. The phenotype of the CHO mutants are consistent with a defect in the acidification of an endocytic compartment. One of the possible sites where the defect could occur is in the large, prelysosomal endosomes. However, when we measured the pH of large endosomes, as distinguished by light microscopy, we found that the mean pH was nearly the same in the mutants DTG 1-5-4 and DTF 1-5-1 as in the wild-type cells (32). This suggested that the defect might be at an earlier step in the endocytic pathway.

To directly demonstrate a defect in early endosome acidification in vivo, appropriate measurement methods had to be developed. Previous measurements of endosome pH have been obtained using the pH-sensitive probe fluorescein by comparing the ratio of fluorescent intensities at two excitation wavelengths, typically 450 and 490 nm, to a pH calibration curve (16, 26). The major problems in making such measurements have been the relatively weak fluorescent signal obtained after brief incubations and the interference from fluorescent molecules remaining outside the cell (11). Extracellular fluorescence is a serious problem because fluorescein fluorescence is significantly reduced at the pH values normally encountered in endocytic compartments.

In this paper, we describe a method for measuring pH during the first 10 min of endocytosis. Using this method, we find that in wild-type CHO cells F-Dex rapidly (3 min) enters an acidic compartment, which has an average pH of 6.3. By 10 min, both F-Dex and fluorescein-labeled α_2M (F-α_2M) are in compartments having an average pH of 6.0 or below. In contrast, the initial pH of endosomes in DTG and DTF cells is near 6.7. Upon further incubation in the mutants both F-Dex and F-α_2M are delivered to more acidic endocytic compartments with an average pH of 6.0 or below. Our results demonstrate that the mutant cells are defective in acidification of an early endocytic compartment. Normal acidification of the compartment is apparently necessary for several sorting processes in endocytosis.

Materials and Methods

Cells

All cell lines were grown in McCoy's 5A (modified) medium containing 5% FBS in a humidified incubator in 5% CO_2 at 34°C. Wild-type CHO cells (WTB) and diphtheria toxin resistant mutants, DTF 1-5-1 and DTG 1-5-4, were a gift from Dr. April Robbins of the National Institutes of Health. The isolation and characterization of these cells has been published (7, 21, 22, 25). Mutant cell lines were retested for resistance to diphtheria toxin by incubating cells with diphtheria toxin (100 ng/ml) for 24 h. The majority of mutant cells survived toxin treatment, while we estimate that >95% of WTB cells were killed. Inhibition of protein synthesis by diphtheria toxin was also determined. WTB was more sensitive to diphtheria toxin than the mutant cell lines, with an EC_{50} (dose of toxin that inhibits protein synthesis by 50%) of 80 ng/ml for WTB, 200 ng/ml for DTF 1-5-1, and 440 ng/ml for DTG 1-5-4. Robbins et al. (22) have found similar EC_{50} values for DTF 1-5-1 and DTG 1-5-4. For fluorescence microscopy, cells were grown on 35-mm coverslip bottom dishes as previously described (27).

Materials

Diphtheria toxin was a gift of Dr. William Habig, Food and Drug Administration, Bethesda, MD. α_2M was purified from human plasma and labeled with fluorescein (F-α_2M) as previously described (12, 30). The α_2M was prepared by a procedure that cleaves the internal thioester and converts the α_2M to the high affinity form (28). Human Tf (Sigma Chemical Co., St. Louis, MO) was loaded with iron, further purified on a Sephacryl S-300 column (Pharmacia Fine Chemicals, Piscataway, NJ), and labeled with fluorescein (F-Tf) or radiodinated (^{125}I-Tf) as previously described (34). [^{59}Fe]Tf was prepared by the nitrilotriacetic acid method (7). $^{59}FeCl_3$ and $Na^{125}I$ were obtained from New England Nuclear (Boston, MA). Fluorescein isothiocyanate dextran (F-Dex), average mol wt 40,500, was obtained from Sigma Chemical Co. and extensively dialyzed against PBS (137 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4). The tetraacetoxymethyl ester of 2,7'-bis(carboxyethyl)-5,6-carboxyfluorescein (BCECF-AM) was obtained from Molecular Probes Inc. (Junction City, Oregon).

Fluorescence Microscopy

Fluorescence experiments were conducted on a Leitz Diavert fluorescence microscope system (E. Leitz, Inc., Rockleigh, NJ), which has been previously described in detail (11). The microscope is equipped with interchangeable 450- and 490-nm excitation filters, an image intensification video system, and a Leitz MPV spectrofluorometer.

Null Point Method. The pH of F-Dex containing compartments was determined by a null point method. Cells were incubated with F-Dex (50 mg/ml) in PBS containing $CaCl_2$ (1 mM) for 2 or 5 min at 34°C. Cells were rinsed with medium 1 (150 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 10 mM glucose, and 20 mM Hepes, pH 7.4) and then reincubated for 1, 5, or 10 min at 34°C. The cells were then chilled to 4°C in medium 1 and the dish transferred immediately to the fluorescence microscope. Intensity measurements at 490-nm excitation were made as previously described (33). After an initial intensity measurement, the cells were rinsed with ice cold buffer containing 150 mM NaCl, 1 mM $CaCl_2$, and 20 mM 2-(*N*-morpholino)ethanesulfonic acid (MES), adjusted to pH 6.8, 6.4, or 6.0 with NaOH. Intensity readings were made from the same field after this change in the extracellular pH. A mixture of ammonium acetate (AA; final concentration 20 mM) and methylamine (MA; final concentration 20 mM) was then added to equilibrate the pH of the intracellular compartments with the extracellular pH. A final set of intensity readings were made from the same field. The temperature of the cells was below 16°C over the time course of the experiments. The percent change in intensity was determined by:

$$\Delta\text{Intensity} = 100 \times \frac{(I^{int} - I^{ext})}{I^{ext}},$$

where I^{ext} is the intensity just before AA/MA addition (extracellular pH at the test value) and the I^{int} is the intensity 90 s after addition of AA/MA (intracellular compartments also at the test value). A representative experiment is shown in Fig. 4. The cellular autofluorescence of the three cells was ~25–30% of the initial fluorescence intensity in the F-Dex experiments and 45–55% in the F-α_2M and F-Tf experiments.

Cytoplasmic pH. To measure the cytoplasmic pH, cells were incubated with 5 μg/ml BCECF-AM (20) for 30 min at 34°C in medium 1. Cells were rinsed with medium 1 and then reincubated for 2 min at 34°C. The cells were chilled to 4°C and the fluorescence intensity was measured at 450- and 490-nm excitation. Alteration of the extracellular and intracellular pH was conducted as described above. To determine the pH from the I_{450}/I_{490}, an "in cell" pH calibration curve was obtained by use of a series of high K^+ pH buffers (150 mM KCl, 1 mM $CaCl_2$, 10 mM MES) and the K^+/H^+ ionophore nigericin (20 μM).

Reacidification and Endosomes. Cells were incubated with F-α_2M (100 μg/ml) for 10 min at 34°C, rinsed in medium 1, and the fluorescence intensity at 490-nm excitation was measured with a microscope spectrophotometer as described (33). The percent reacidification (RA) was determined from:

$$\text{RA} = 100 \times \frac{(I_{7min} - I_{0min})}{(I_{3min} - I_{0min})},$$

where I_{0min} is the initial fluorescence intensity, I_{3min} the fluorescence inten-

sity after the addition of monensin, and I_{7min} is the fluorescence intensity 4 min after monensin is removed (see Fig. 8).

Uptake of ^{59}Fe and ^{125}I-Tf. Cells on 35-mm dishes were incubated with ^{125}I-Tf (2 μg/ml) and [^{59}Fe]Tf (50 μg/ml) in McCoy's 5A medium containing Hepes (20 mM) and ovalbumin (1 mg/ml) for 2 h at 34°C. Cells were then rinsed and solubilized in 1 N NaOH. Nonspecific binding was determined by the addition of diferric Tf (1 mg/ml). Since ^{59}Fe released from Tf remains cell-associated while ^{125}I-apo-Tf is released at the cell surface, the ^{59}Fe/^{125}I ratio was used as an indicator of the efficiency of stripping iron from Tf within the cell (5).

Results

pH Measurement by Whole Cell Null Point Method

We have developed a method that has enabled us to examine the pH of endocytic compartments as early as 3 min after internalization. The method measures pH by determining whether the pH of the endocytic compartments is above or below the pH of the extracellular medium. The basis of the method is illustrated in Fig. 1, and in this example tests whether the average pH of the intracellular compartments is above or below the "test pH" of 6.4. To monitor changes in pH, we measure the fluorescence intensity at 490-nm excitation using a microscope spectrophotometer. The fluorescence of fluorescein is highly pH dependent at 490-nm excitation, with the fluorescence intensity increasing sharply with increasing pH.

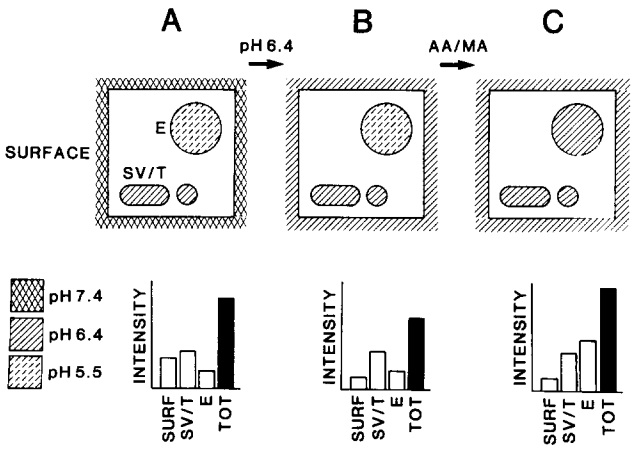

Figure 1. Illustration of null point method of determining the pH of intracellular compartments. In this schematic model of a cell, fluorescein-labeled ligand, as indicated by the lined patterns, is found on the cell surface, in a large endosome (*E*), and in a small vesicle and tubule (*SV/T*). The different lined patterns each correspond to a different pH. In *A* the cell surface is at pH 7.4, the large endosome at pH 5.5, and the SV/T at pH 6.4. The separate contributions of the cell surface (*SURF*), large endosome (*E*), and the SV/T to the total fluorescence intensity are shown in the bar graphs. When the fluorescence intensities of the three compartments are added together they give the total fluorescence intensity (*TOT*), which is measured in our experiments. When the pH of the extracellular medium is changed to the reference pH of 6.4 (*B*), the fluorescence from the cell surface fluorescein decreases. Since a nonpermeant buffer is used, there is no change in the fluorescence from either the large endosome or SV/T. Therefore, the decrease in the total fluorescence intensity corresponds to the decrease in the intensity of the cell surface fluorescence. When AA/MA is added (*C*) the pH of the large endosome increases from pH 5.5 to 6.4. The resulting increase in fluorescence intensity shows that the average pH of all the intracellular compartments was below pH 6.4.

In cells that have been incubated with a fluorescein-labeled probe, fluorescein will be found on the cell surface and heterogeneously distributed among intracellular compartments of different pH. In the example shown in Fig. 1, the total fluorescence intensity is comprised of fluorescence contributed by fluorescein-labeled ligand found on the cell surface at pH 7.4, in a large endosome at pH 5.5, and in a small vesicle and tubule compartment at pH 6.4 (Fig. 1 *A*). (These values are shown for illustrative purposes only. The method does not depend on the pH values or nature of the compartments.) Since all labeled endocytic compartments will contribute to the fluorescence measurement, this method will determine whether the average pH of all the endocytic compartments in the cell is above or below the test pH.

The first step is to eliminate the effect that cell surface fluorescein will have on the later measurements. This is done by lowering the extracellular pH to the test pH, which in this case is pH 6.4 (Fig. 1 *B*), using the membrane impermeant buffer MES (3). Lowering the pH from 7.4 to 6.4 decreases the fluorescence of the fluorescein on the cell surface. However, since we used a nonpermeant buffer, the pH of the intracellular compartments is not altered (see below). Therefore, the decrease in the total fluorescence intensity caused by lowering the pH of the extracellular medium is due solely to the decrease in the fluorescence contributed by the cell surface fluorescein.

To determine if the average pH of the intracellular compartments is above or below the test pH we measure the change that occurs in the fluorescence intensity when the pH of the intracellular compartments is equilibrated with the test pH of the extracellular medium. The pH gradient can be collapsed by using a membrane-permeant weak acid, such as acetic acid, and a weak base, such as ammonium (10, 17, 18). In our experiments we used a mixture of ammonium acetate and the weak base methylamine (AA/MA) to equilibrate the pH of the intracellular compartments. When AA/MA is added (Fig. 1 *C*), the pH of the large endosomes rises from pH 5.5 to 6.4, causing an increase in the fluorescence intensity. The fluorescence intensity from the small vesicle and tubule does not change since it is already at pH 6.4. By summing the changes in the fluorescence intensities of the different intracellular compartments, it can be seen that the total cell fluorescence increases. The observation that the total cell fluorescence increases upon AA/MA addition indicates that the average pH of the intracellular compartments is <pH 6.4.

By using test buffers of different pH we can calculate the average pH of the labeled endocytic compartments. The average pH is the pH at which no change in fluorescence intensity is seen upon addition of the weak acid/weak base mixture, i.e., the null point (see Fig. 5 *A*). Because this method relies only on the change in fluorescence intensity after addition of AA/MA, this technique compensates for both autofluorescence and cell surface fluorescein.

Alteration of Cytoplasmic pH

To ensure that the intracellular pH was varying as expected after the buffer changes and the addition of AA/MA, we measured the cytoplasmic pH using the dye BCECF (20).

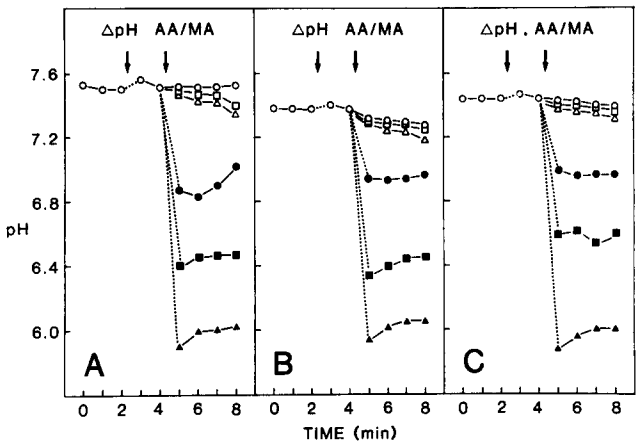

Figure 2. Alteration of cytoplasmic pH. WTB (*A*), DTG 1-5-4 (*B*), and DTF 1-5-1 (*C*) cells were loaded with BCECF and the cytoplasmic pH was determined as described in Materials and Methods. After 2 min, the pH of the extracellular medium was changed to pH 6.8 (○), pH 6.4 (□), or pH 6.0 (△) (see Materials and Methods). Since the cytoplasmic pH before the addition of AA/MA was not significantly different amongst the various conditions, only the pH 6.8 points are shown before 5 min. After 4 min, AA/MA (20 mM/20 mM final concentration) was added (*solid symbols*), to equilibrate the cytoplasmic pH with the extracellular pH. Open symbols indicate parallel incubations with no addition of AA/MA.

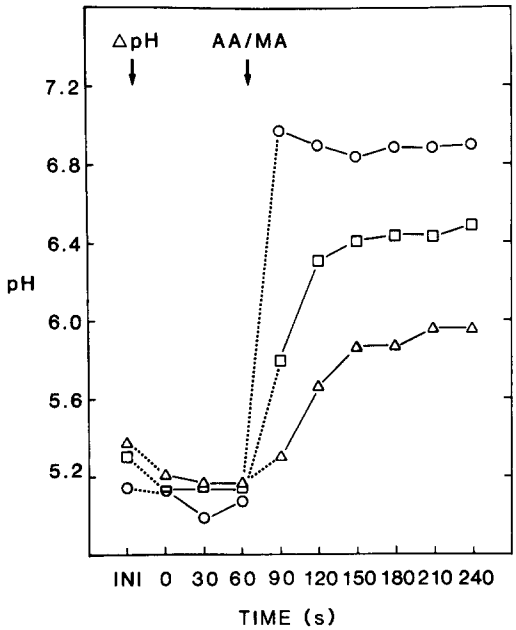

Figure 3. Alteration of lysosomal pH. WTB cells were incubated with F-Dex (5 mg/ml) for 2–3 h at 34°C, rinsed, and then reincubated for an additional 30–60 min at 34°C. The lysosomal pH was determined from the I_{450}/I_{490} by comparison with an "in solution" pH calibration curve (26, 27). After an initial pH measurement was made (*INI*), the pH of the extracellular medium was changed to pH 6.8 (○), pH 6.4 (□), or pH 6.0 (△) as described in Materials and Methods. After 60 s, AA/MA (20 mM/20 mM final concentration) was added. These results are representative of three experiments.

WTB, DTG 1-5-4, and DTF 1-5-1 cells were loaded with dye by incubating cells with the membrane permeant ester, BCECF-AM. Cytoplasmic esterases cleave off the ester, trapping the dye in its indicator form within the cell. The cytoplasmic pH was determined from the fluorescence intensities at 490- and 450-nm excitation as measured with a microscope spectrophotometer (Fig. 2). When the extracellular medium was changed to pH 6.8, 6.4, or 6.0 there was only a small, gradual decrease in the cytoplasmic pH of the three cell types. However, if AA/MA was added, the cytoplasmic pH rapidly decreased to approximately the pH of the extracellular medium. These results demonstrate that the use of the nonpermeant buffer MES does not significantly alter the intracellular pH when the extracellular pH is in the range of 6.0–7.4. Addition of a permeant weak acid/weak base mixture such as AA/MA rapidly equilibrates the pH of the cytoplasm with that of the external pH.

Alteration of Lysosomal pH

To check whether this method could equilibrate the pH of acidic intracellular compartments with the extracellular pH, we tested whether the pH of lysosomes could be raised to the test pH when AA/MA was added. WTB cells were incubated with F-Dex for 2–3 h, rinsed, and then reincubated without F-Dex for 30–90 min, to ensure that the probe would be located primarily in lysosomes. The pH of lysosomes (Fig. 3) was determined from the ratio of fluorescence intensities at 450- and 490-nm excitation. When the pH of the extracellular medium was changed to pH 6.8, 6.4, or 6.0, there was little change in the lysosomal pH. However, when AA/MA was added, there was a rapid rise in the lysosomal pH for all three test pHs. The pH of lysosomes 90 s after AA/MA addition was 6.9 ± 0.1, 6.4 ± 0.1, and 5.8 ± 0.1 (mean ± SD, n = 3) for the test pH values of 6.8, 6.4, and 6.0 respectively.

These results demonstrate that the addition of the weak acid/weak base mixture, AA/MA, rapidly equilibrates the pH of an endocytic compartment with the test pH. Therefore, we should be able to determine whether the pH of endocytic compartments is above or below a particular test pH by measuring the change in fluorescence intensity after the addition of AA/MA.

Kinetics of F-Dex Acidification

Using the method described above, we first examined the changes in fluorescence intensity when F-Dex containing compartments were equilibrated to the test pH of 6.4 (Fig. 4). WTB cells were incubated with F-Dex for 5 min, rinsed to remove uninternalized F-Dex, and then reincubated ("chased") for either 1 or 5 min (Fig. 4 *A*). The cells were then chilled to 4°C to inhibit movement of ligand and immediately examined using the microscope spectrophotometer. When the pH of the extracellular medium was changed to pH 6.4 there was a rapid decrease in the fluorescence intensity, due to the decrease in fluorescence from the F-Dex remaining on the cell surface. After the initial decrease, there was a gradual decrease in the fluorescence intensity with time (−5%, from 60 to 150 s). However, if AA/MA was added, there was a rapid increase in the fluorescence intensity for both the 1- and 5-min chase, indicating that the average pH of the F-Dex compartments was already <pH 6.4. The larger increase in the fluorescence intensity for the 5-min chase (+37%, 90 s after AA/MA addition) relative to the 1-min chase (+8%) suggests that the F-Dex after the

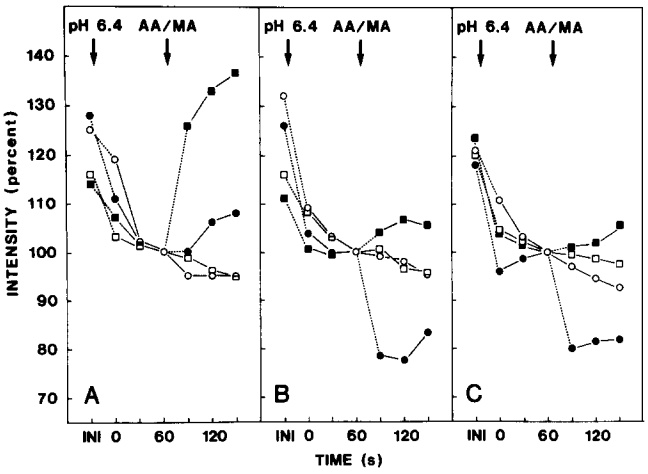

Figure 4. Change in fluorescence intensity of endosomes containing F-Dex at pH 6.4. WTB (*A*), DTG 1-5-4 (*B*), and DTF 1-5-1 (*C*) cells were incubated with F-Dex for 5 min at 34°C, rinsed, and then reincubated for either 1 min (○) or 5 min (□) as described in Materials and Methods. After an initial intensity measurement (*INI*) was made, the pH of the extracellular medium was changed to pH 6.4. After 60 s, AA/MA (20 mM/20 mM final concentration) was added (*solid symbols*). Open symbols indicate parallel incubations with no addition of AA/MA. The fluorescence intensity is expressed as the percent of the intensity at the 60-s time point.

5-min chase is in a more acidic compartment compared with the 1-min chase. In cells not incubated with a fluorescein-labeled ligand, addition of AA/MA did not cause a significant change in cellular autofluorescence (data not shown).

We also examined the pH of F-Dex compartments in DTG 1-5-4 (Fig. 4 *B*) and DTF 1-5-1 (Fig. 4 *C*) cells. As in wild-type cells, after the external pH was lowered to pH 6.4, there was an initial rapid decrease in the fluorescence intensity due to cell surface F-Dex, followed by a gradual decrease in fluorescence intensity with time (−3% to −7%, from 60 to 150 s). However, with a 5-min pulse and 1-min chase, the addition of AA/MA caused a rapid decrease in fluorescence intensity for both DTG 1-5-4 (−16%, 90 s after AA/MA addition) and DTF 1-5-1 (−18%). The decrease in fluorescence intensities indicates that the average pH of the endocytic compartments in the mutants is higher than pH 6.4, in contrast to wild-type cells. With a 5-min chase, there was a small increase in fluorescence upon AA/MA addition in DTG 1-5-4 (+5%) and DTF 1-5-1 (+3%), indicating that F-Dex was in a more acidic compartment, relative to the 1-min chase.

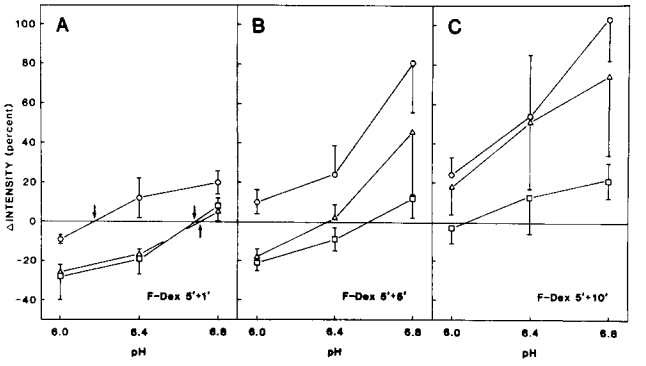

Figure 5. Time course of acidification of endosomes containing F-Dex. WTB (○), DTG 1-5-4 (□), and DTF 1-5-1 (△) cells were incubated with F-Dex (50 mg/ml) for 5 min at 34°C, rinsed, and then reincubated for 1 min (*A*), 5 min (*B*), or 10 min (*C*) as described in Materials and Methods. The change in fluorescence intensity upon addition of AA/MA was determined as described in Materials and Methods. Each point represents the mean ± SD of four to six experiments. The point corresponding to no change in intensity, as indicated by the arrows in *A*, is taken as the average pH of the intracellular compartments and is summarized in Table I.

The above results demonstrate that the acidification of an early endocytic compartment is altered in the mutants, with the pH below 6.4 in wild-type cells, but above pH 6.4 in the mutants. To determine more precisely the average pH of the F-Dex compartments, test buffers of pH 6.0 and 6.8 were also used. The change in fluorescence intensity 90 s after addition of AA/MA was determined and plotted against the pH of the test buffer (Fig. 5). From these graphs we can calculate an average pH value (Table I) as the pH where no change in intensity would be observed upon addition of AA/MA. In WTB cells, F-Dex entered an acidic compartment as soon as we could measure, having an average pH of 6.3 at 3 min. Acidification to an average pH of <6.0 occurs by 10 min.

In contrast, the average endosomal pH at early times was significantly higher in the mutants, with an average pH of 6.7 after a 5-min pulse and 1-min chase (Table I). With a longer chase period of 5 min, F-Dex was in a more acidic compartment of pH 6.4–6.5, but was still at least 0.5 pH units more neutral than wild-type cells. After a 10-min chase the average pH in DTF 1-5-1 was comparable to that found in WTB (both <pH 6.0, Fig. 5 *C*). This progressive acidification was somewhat slower in DTG 1-5-4, reaching a pH of 6.1 after a 10-min chase. These results demonstrate that there is an initial defect in acidification in both DTG 1-5-4 and DTF 1-5-1. However, with time, F-Dex reaches progressively more acidic compartments in both mutants.

Kinetics of F-α₂M Acidification

In addition to determining the kinetics of acidification of endocytic compartments labeled with a fluid phase marker, we examined acidification of α_2M, a ligand internalized by receptor-mediated endocytosis. The results with F-α_2M (Fig. 6, Table I) were similar to those found with F-Dex. In

Table I. pH of Endocytic Compartments by Whole Cell Null Point Method

Ligand	Condition	pH WTB	DTG 1-5-4	DTF 1-5-1
F-Dex	2 min/1 min*	6.3 ± 0.1	ND	ND
	5 min/1 min	6.2 ± 0.1	6.7 ± 0.1	6.7 ± 0.1
	5 min/5 min	<6.0	6.5 ± 0.2	6.4 ± 0.1
	5 min/10 min	<6.0	6.1 ± 0.3	<6.0
F-α_2M	9 min/1 min	6.0 ± 0.1	6.8 ± 0.3	6.4 ± 0.2
	9 min/6 min	<6.0	6.4 ± 0.2	6.0 ± 0.3
F-Tf	5 min/1 min	6.2 ± 0.1	6.7 ± 0.1	6.3 ± 0.2
	18 min/2 min	6.5 ± 0.1	6.6 ± 0.1	6.5 ± 0.1

The average pH of F-Dex, F-α_2M, and F-Tf endosomes was calculated from Fig. 5–7 by determining the pH at which there was no change in the intensity (0 percent line). The error limits were estimated from the standard deviations in Figs. 5–7.

* The graph of the F-Dex, 2 min/1 min time point for WTB is not shown.

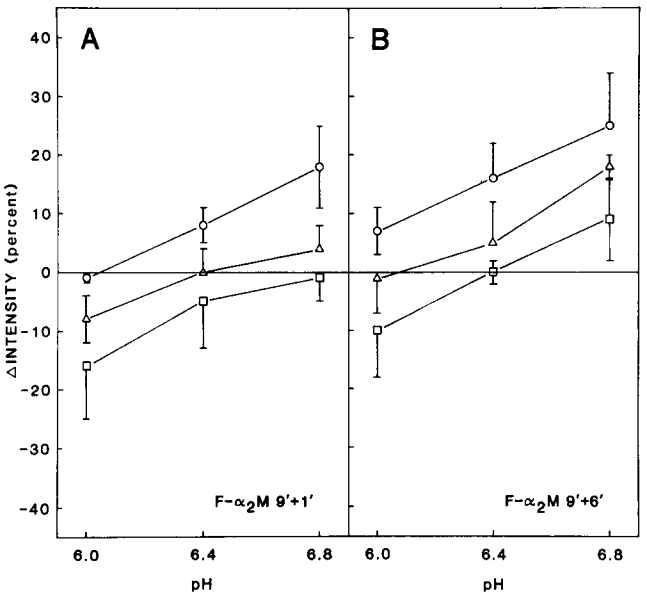

Figure 6. Time course of acidification of endosomes containing F-α_2M. WTB (○), DTG 1-5-4 (□), and DTF 1-5-1 (△) cells were incubated with F-α_2M (100–200 μg/ml) in medium 1 containing 5 mg/ml BSA for 9 min at 34°C, rinsed, and then reincubated for either 1 min (*A*) or 6 min (*B*) at 34°C. The change in fluorescence intensity upon addition of AA/MA was determined as described in Materials and Methods. Each point represents the mean ± SD of four to six experiments.

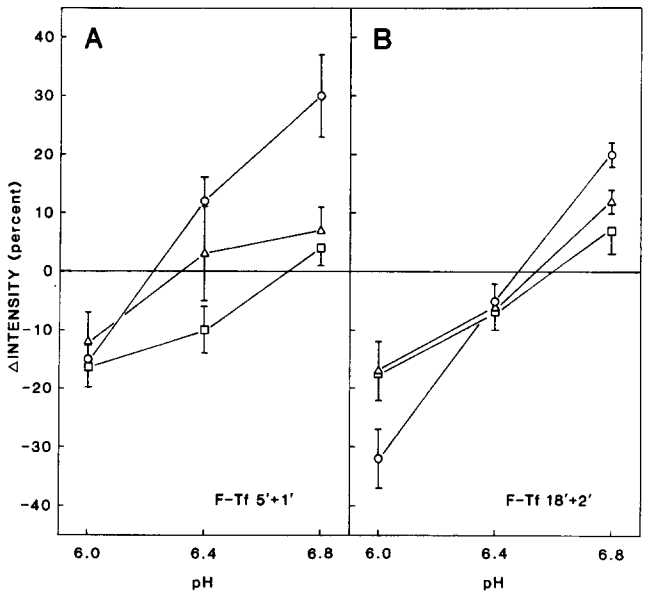

Figure 7. Time course of acidification of endosomes containing F-Tf. WTB (○), DTG 1-5-4 (□), and DTF 1-5-1 (△) cells were incubated with F-Tf (100 μg/ml) in medium 1 containing 5 mg/ml ovalbumin at 34°C for either 5 min (*A*) or 18 min (*B*). Cells were then rinsed and reincubated in medium 1 for either 1 min (after the 5-min pulse) or 2 min (after the 18-min pulse). The change in fluorescence intensity was determined as described in Materials and Methods. Each point represents the mean ± SD of four to six experiments.

WTB cells, F-α_2M after a 9-min pulse and 1-min chase was in a compartment with an average pH of 6.0. The average pH of the F-α_2M compartment at this time is significantly more neutral in DTG 1-5-4 (pH 6.8) and DTF 1-5-1 (pH 6.4). With a longer chase period (6 min), F-α_2M was in a more acidic compartment, but the average pH in the mutants was still significantly more neutral than in wild-type cells (Fig. 6 *B*).

Kinetics of F-Tf Acidification

Since Tf remains associated with its receptor, it can be used to label the receptor recycling pathway. After a 5-min pulse and 1-min chase, F-Tf in WTB cells was found in endosomes having an average pH of 6.2 (Fig. 7, Table I). With a longer pulse of 18 min, when Tf is distributed throughout the recycling endosomal compartments, F-Tf was at a slightly more neutral pH of 6.5. Under these conditions, most of the F-Tf is in the *para*-Golgi recycling endosomes, which have a pH of 6.4 when measured by digital image analysis (34).

Robbins and co-workers have found that there is a decrease in the release of iron from transferrin in DTF 1-5-1 (7) and DTG 1-5-4 (Robbins, A., personal communication). We have also examined the release of iron from Tf and found that the mutants were much less efficient at stripping iron from Tf as indicated by a markedly smaller $^{59}Fe/^{125}I$-Tf ratio (27% and 46% of WTB for DTG 1-5-4 and DTF 1-5-1, respectively). These results suggest that the majority of Tf does not pass through a compartment of pH <6.0 in the mutants. (A pH of <6.0 is needed to dissociate both iron atoms from transferrin [1, 8, 19]). We found that the average pH of F-Tf endosomes after 5 min (Table I) was significantly higher in DTF 1-5-4 (pH 6.7) than in WTB (pH 6.2). This result is consistent with the observation that as Tf recycles back to the cell surface it fails to pass through an endocytic compartment sufficiently acidic to dissociate the iron. With a longer pulse (18 min), the pH in DTG 1-5-4 and WTB was similar. With DTF 1-5-4, the pH of F-Tf endosomes was 6.3 at early times, and 6.5 at late times (Table I).

Reacidification of Endocytic Compartments

The pH measurements described above showed that at early times the average endosomal pH was significantly higher in the mutant cells. We next determined whether these compartments had retained the ability to reacidify after their pH gradient had been collapsed with the ionophore monensin. We have previously shown that endosomes rapidly reacidify to near their previous level after monensin is removed (10). This acidification requires ATP (33). Cells were incubated with F-α_2M for 10 min, and the fluorescence intensity at 490 nm was measured (Fig. 8). The effect of monensin was reversible in the wild-type and mutant cells as indicated by the decrease in fluorescence intensity after monensin was removed. The reacidification in the mutants and wild-type cells was similar, with an average percent reacidification after 4 min of 71 ± 20 for WTB, 82 ± 2 or DTG 1-5-4, and 80 ± 17 for DTF 1-5-1 (mean ± SD, n = 5). This suggests that the altered endosomal pH in the mutants is due to the acidification mechanism being regulated to a higher pH. We also found that lysosomes labeled with F-Dex reacidified to a similar extent in all three cell types (77 ± 20, 65 ± 8, and 86 ± 1 for WTB, DTG 1-5-4, and DTF 1-5-1, respectively; mean ± SD, n = 3).

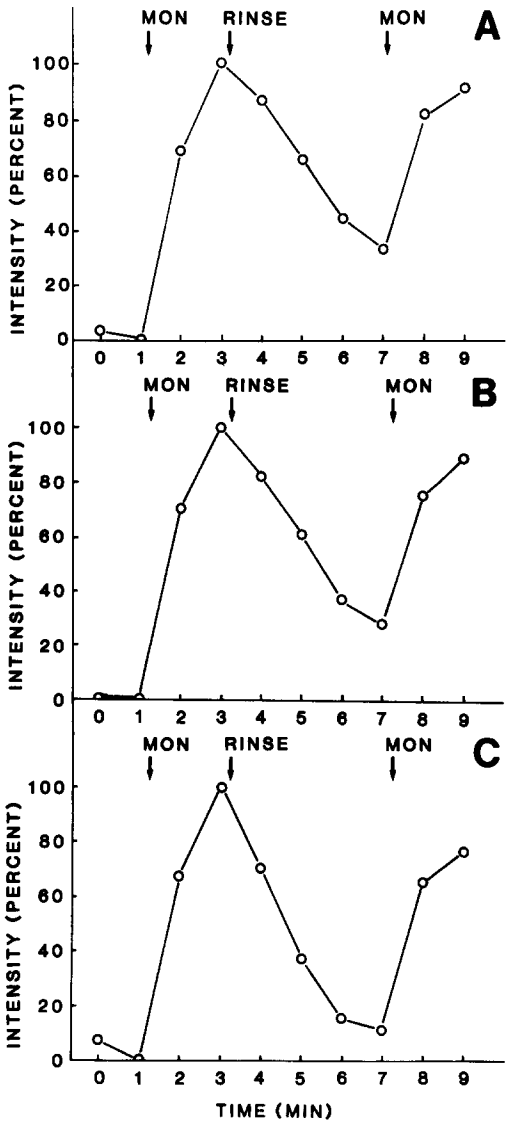

Figure 8. Reacidification of endosomes containing F-α_2M. WTB (*A*), DTF 1-5-1 (*B*), and DTG 1-5-4 (*C*) cells were incubated with F-α_2M for 10 min. The fluorescence intensity at 490-nm excitation was measured as described (33). Monensin (10 μM) was added after 1 min, collapsing the pH gradient as indicated by the increase in fluorescence intensity. Removal of monensin at 3 min caused a decrease in fluorescence intensity indicating reacidification. Monensin (10 μM) was added at 7 min, confirming endosome reacidification. This figure is a representive example of five reacidification experiments.

Discussion

In a previous study using weak bases, we have shown that ligands enter an acidic compartment within 5 min after internalization (10). However, it has been technically difficult to accurately measure the pH of endosomes at early times. In this paper we have described a null point method for determining the average pH of endocytic compartments at early times. With this method we can easily account for autofluorescence and cell surface fluorescein, both of which can be significant problems in ratio methods of pH determination. Because we can correct for cell surface fluorescein, we are able to incubate cells with a relatively high concentration of ligand, thus providing a signal that is sufficient to measure the pH of early endocytic compartments.

The key to the method presented here is that it relies only upon the change in fluorescence that occurs after the addition of AA/MA. Therefore, both autofluorescence and cell surface ligand are constants in the intensity measurements and will not affect whether the fluorescence increases or decreases at a particular test pH. If the autofluorescence and cell surface fluorescein is a large percentage of the signal, the percentage increase or decrease that occurs after addition of AA/MA will be smaller. However, the null point (i.e., the pH at which no change in fluorescence intensity would occur) and therefore the calculated pH will not be affected.

Since the method relies upon the change in fluorescence intensity that occurs after the addition of AA/MA, loss of cell-associated fluorescein will cause the calculated pH to underestimate the acidity of the compartment. In our experiments, we chilled the cells to decrease the exocytosis of ligand. In the F-Dex experiments, the fluorescence decreased by ~5% if AA/MA was not added (Fig. 4). We estimate that this could introduce an error of ~0.1 pH units.

A requirement of our method is that the weak base/weak acid mixture equilibrate the pH of the intracellular compartment with the extracellular pH. We found that AA/MA rapidly equilibrated the cytoplasmic pH and lysosomal pH with the extracellular pH to within 0.2 pH units.

Since we are using ammonia and methylamine as weak bases to collapse the pH gradient, we are unable to use test buffers that are more acidic than pH 6.0. At pH values below 6.0 the effective concentration of the weak base is extremely low since little of the unprotonated base is present to cross the cell membranes and collapse the pH gradient (18). Weak bases with lower pK_a's could be used to directly determine the null point average pH with test buffers below pH 6.0. We were unable to use the ionophore monensin to collapse the pH gradient due to its relatively poor activity at low temperatures and at pH values below 6.0.

The accuracy of the null point method can be assessed by comparison with measurements based on the I_{450}/I_{490} intensity ratio. The ratio technique has been used in many studies (4, 16, 26, 27, 29, 34) and is useful for measuring pH at longer incubation times. In WTB cells incubated with F-Tf for 18 min followed by a 2-min chase, the average endosomal pH determined by the ratio method is 6.3–6.4 (34). Using the null point method, we obtained a pH of 6.5 under the same conditions.

We have found in wild-type cells that there was a very rapid acidification of endocytic compartments containing F-Dex; acidification to pH 6.3 occurred as early as 3 min. With a 5-min pulse, both F-Dex and F-Tf were in endosomes with an average pH of 6.2. Rapid acidification of endosomes to pH $\leq$6.2 has also been observed in baby hamster kidney cells by Kielian et al. (6), using the fusion requirements of Semliki Forest virus and in BALB/c 3T3 cells by Sipe and Murphy (24) using F-Tf. The slight alkalinization of Tf to pH 6.5 at later times is also reported by Sipe and Murphy who found a gradual alkalinization of Tf compartments after the initial acidification (24). This is consistent with the maintenance of a higher pH in the *para*-Golgi recycling endosomes than in the early endosomes. In contrast, F-Dex and F-α_2M move to endocytic compartments that are more acidic, having an average pH of <6.0 as measured by the null

point method. Similar lowering of the average endosomal pH has been found in BALB/c 3T3 cells (15).

In the mutants DTG 1-5-4 and DTF 1-5-1 we found that acidification of endocytic compartments containing F-Dex or F-α_2M was retarded. DTG 1-5-4 was more severely affected, with F-Dex in a pH 6.5 compartment after 10 min. In DTF 1-5-1 the time course for acidification was also slowed, but by 15 min the pH was similar to that of WTB. These results are consistent with those of Robbins et al. (22) who found that endosomes containing F-Dex isolated by density centrifugation from the mutants were defective in ATP-dependent acidification. They also found that DTG 1-5-4 was the more severely affected of the two mutants.

A pH of 6.2–6.3, which is reached in the first 3 min of internalization in WTB, is sufficient for dissociation of ligands such as α_2M and low density lipoprotein from their receptors. This pH is also close to the pH required (pH <6.0) for the release of iron from Tf and the dissociation of lysosomal enzymes from their receptors. Since the method described here determines a pH that is an average of the acidity of the many individual organelles, it is likely that some of the endosomes will have a pH <6.2. The more neutral pH of 6.7–6.8 for the early compartment in the mutants is consistent with the increased resistance to toxins. A more complete analysis of the phenotype of the mutants is presented in the following paper (32).

Our results show for both wild-type and mutant cells that ligands are found in progressively more acidic compartments. A possible explanation for this observation is that ligands move from a more neutral compartment to a more acidic one. Alternatively, ligands may remain in the same compartment, but the compartment further acidifies. In the following paper, we examine the pH of morphologically distinct endosomes to address these possibilities (32). We found that the mutants were defective in the acidification of early endosomes comprising small vesicles and tubules. The acidification of later, larger endosomes was only partially impaired in the mutants. Our work with the CHO wild-type and mutant cells suggests that the pH regulatory mechanisms of the early endosomes are critical for the proper functioning of many events in endocytosis.

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