Fusion Accessibility of Endocytic Compartments along the Recycling and Lysosomal Endocytic Pathways in Intact Cells

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Abstract. A fluorescence assay developed for the quantitation of intracellular fusion of sequentially formed endocytic compartments (Salzman, N. H., and F. R. Maxfield. 1988. *J. Cell Biol.* 106:1083–1091) has been used to measure the time course of endosome fusion accessibility along the recycling and degradative endocytic pathways. Transferrin (Tf) was used to label the recycling pathway, and alpha$_2$-macroglobulin (α_2M) was used to label the lysosomal degradative pathway. Along the degradative pathway, accessibility of vesicles containing α_2M to fusion with subsequently formed endocytic vesicles decreased with apparent first order kinetics. The $t_{1/2}$ for the loss of fusion accessibility was ~8 min. The behavior of Tf is more complex. Initially the fusion accessibility of Tf decayed rapidly ($t_{1/2} < 3$ min), but a constant level of fusion accessibility was then observed for 10 min. This suggests that Tf moves through one fusion accessible endosome rapidly and then enters a second fusion accessible compartment on the recycling pathway. At 18°C, fusion of antifluorescein antibodies (AFA) containing vesicles with F-α_2M was observed when the interval between additions was 10 min. However, if the interval was increased to 1 h, no fusion with incoming vesicles was observed. These results identify the site of F-α_2M accumulation at 18°C as a prelysosomal late endosome that no longer fuses with newly formed endosomes since no delivery to lysosomes is observed at this temperature.

RECEPTOR-MEDIATED endocytosis is a process by which cells internalize extracellular proteins, including peptide hormones and growth factors (e.g., insulin and epidermal growth factor), and nutritional carrier proteins (e.g., transferrin [Tf][1] and low density lipoprotein [LDL]). Cell surface receptors bind ligands, cluster, and are internalized into endocytic vesicles that rapidly acidify (for reviews, see Goldstein et al., 1985; Yamashiro and Maxfield, 1988). From this point, ligands and receptors are routed to several different destinations.

Several of these ligands (e.g., alpha$_2$-macroglobulin [α_2M] and LDL) are delivered to lysosomes and degraded while their receptors are recycled with high efficiency (Goldstein et al., 1985). For many of these ligands it has been shown that the low pH in the endosome causes ligand–receptor dissociation (Harford et al., 1983). However, beyond this dissociation step, the mechanisms for efficiently separating ligands and receptors are poorly characterized.

Tf is a ligand that does not dissociate from its receptor as a result of exposure to low pH. Rather, the diferric transferrin loses its iron at low pH, and the apotransferrin remains receptor-bound at acidic pH. The transferrin–receptor complex returns to the cell surface where the apotransferrin dissociates at the neutral extracellular pH (Klausner et al., 1983; Dautry-Varsat et al., 1983). These properties make Tf a useful marker for the route taken by recycling receptors after separation from ligands destined for lysosomes.

The separation of lysosomally directed ligands from recycling components has been studied by several methods. By subcellular fractionation, Stoorvogel et al. (1987), have shown that coincubated asialoglycoprotein (ASGP) and Tf separate from each other with a $t_{1/2}$ of ~2 min. Using light microscopy and EM it has been shown that when α_2M and Tf are coincubated, they are initially seen together in small vesicles distributed throughout the cytoplasm of Chinese hamster ovary (CHO) cells (Yamashiro et al., 1984). At later times, the α_2M is seen accumulating in large endosomes, while Tf is segregated to morphologically distinct recycling endosomes (Yamashiro et al., 1984).

In studies of TRVb-1 cells, a CHO cell line lacking endogenous Tf receptor but expressing the transfected human Tf receptor (McGraw et al., 1987), we have used Tf to monitor the receptor recycling pathway and α_2M to follow the route to lysosomes. A schematic model of the endocytic compartments containing these ligands is shown in Fig. 1. The existence of these compartments in various cell types, including CHO cells, has been established morphologically (Geuze et al., 1983; Yamashiro et al., 1984) and biochemically

1. *Abbreviations used in this paper*: AFA, antifluorescein antibodies; α_2M, alpha$_2$-macroglobulin; ASGP, asialoglycoprotein; CURL, compartment of uncoupling receptor and ligand; F, fluorescein isothiocyanate; LDL, low density lipoprotein; Tf, transferrin.

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(Schmid et al. 1988; Mueller and Hubbard, 1986), but the relationships between these compartments and their precise role in carrying out differential sorting is not known.

In the model shown in Fig. 1, a pivotal role is played by a "sorting endosome." Similar compartments to the sorting endosome have been described in various models of endocytosis as CURL (compartment of uncoupling receptor and ligand) (Gueze et al., 1984), early endosomes (Schmid et al., 1988), and receptosomes (Pastan and Willingham, 1985). At present there is no standard nomenclature for the different organelles involved in endocytosis. The terminology used in this paper is consistent with previous work from this laboratory (e.g., Yamashiro and Maxfield, 1987). The sorting endosome has two important characteristics in this model. The acidic pH dissociates α_2M and other acid-sensitive ligands from their receptors, and the recycling membrane components are removed from the sorting endosome by the recycling endosomes that bud off from the sorting endosome.

Despite its importance for understanding endocytic processes, little is known of the properties of the sorting endosome. It is likely that vesicle fusions are an essential part of the endocytic process, but the extent and sites of fusion are not known.

To investigate the spatial and temporal relationships among endocytic compartments, we have developed quantitative assays for vesicle fusion along both the receptor recycling pathway and the lysosomal pathway. These methods will address the questions of whether fusion is occurring along both pathways and whether ligands move from fusion-accessible to fusion-inaccessible compartments. In this paper, we describe a variation of an assay used in our earlier work (Salzman and Maxfield, 1988). F-Tf is used as a marker for the endocytic recycling pathway, and F-α_2M is used as a marker for the lysosomal pathway. These fluorescein-labeled markers are pulsed into cells, and after specific intervals they are chased with polyclonal antifluorescein antibodies (AFA). If vesicle fusion occurs, AFA can bind to the fluorescein and perturb fluorescein fluorescence. AFA not only quenches fluorescein fluorescence but also changes its pH sensitivity. These changes provide a way to quantitatively measure binding of AFA to fluorescein.

Using this method, we find that newly formed endosomes fuse with endosomes containing both α_2M and Tf. However, the time course of the fusion properties of the endosomes containing the two ligands are markedly different. We interpret the data to be consistent with a maturation of sorting endosomes into fusion-inaccessible late endosomes. This maturation occurs with a half time of ~8 min. Tf is apparently removed from the sorting endosome more rapidly ($t_{1/2}$ < 3 min) and is delivered to a second, fusion-accessible compartment on the recycling pathway. The existence of the fusion-inaccessible late endosome is confirmed by studies of cells incubated at 18°C with F-α_2M. Under these conditions, delivery to lysosomes is blocked (Dunn et al., 1980), but F-α_2M moves beyond the sorting endosome into a compartment that does not fuse efficiently with newly formed endosomes.

Materials and Methods

Cell Cultures

TRVb-1 cells, a CHO cell line lacking endogenous Tf receptor but expressing the transfected human Tf receptor (McGraw et al., 1987), were grown in F12 medium containing 5% FCS (Gibco Laboratories, Grand Island, NY) penicillin (100 U/ml), streptomycin (100 μg/ml), and 100 μg/ml G418, at 37°C in 5% CO_2 in a humidified air atmosphere. TRVb-1 cells were passaged using trypsin-EDTA (Gibco Laboratories). All experiments were carried out using medium 1 (NaCl 150 mM, KCl 5 mM, $CaCl_2$ 1 mM, $MgCl_2$ 1 mM, Hepes 20 mM, pH 7.4) supplemented with 1 mg/ml ovalbumin and 40 μM desferyl mesylate, an iron chelating agent that binds free iron but does not strip bound iron from Tf (Klausner et al., 1984).

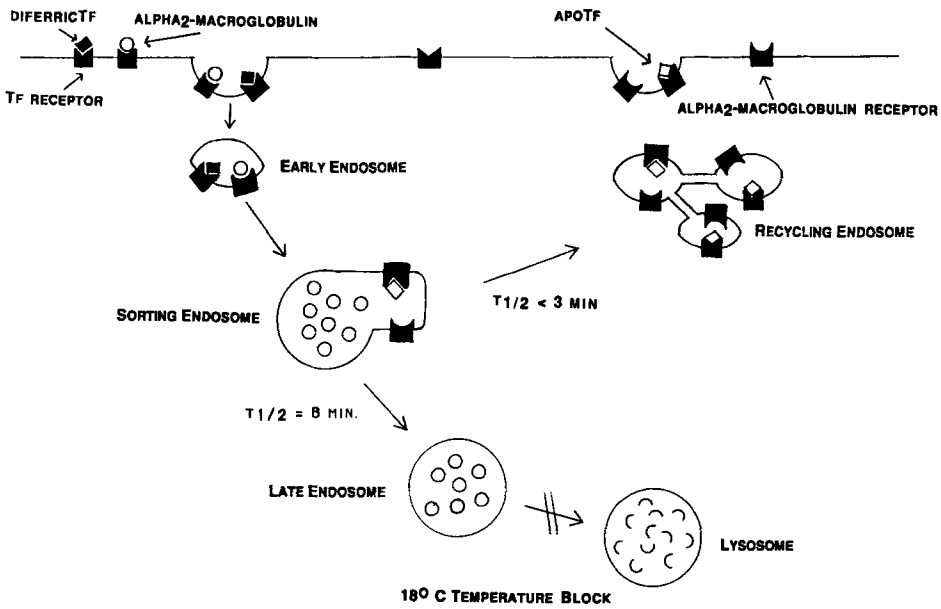

Figure 1. Model of endocytic vesicle traffic in TRVb-1 cells. Receptors are taken up into the cell together in small endocytic vesicles (*Early Endosome*). Sequentially formed vesicles fuse with the semistable sorting endosomes. The sorting endosomes have also been referred to as early endosomes (Schmid et al., 1988), receptosomes (Pastan and Willingham, 1985), or CURL (Gueze et al., 1984). pH-sensitive ligands are released from their receptors. Receptors are separated from their ligands and removed from the sorting endosome by budding off in recycling endosomes. These vesicles fuse with other recycling endosomes and the recycling receptors are returned to the cell surface. Ligand is accumulated in the sorting endosome through several rounds of incoming vesicle fusion and remains there for the lifetime of the compartment. When the sorting endosome no longer has the capacity to accept incoming vesicles, it has matured into a late endosome. The late endosome would be accessible to fusion with Golgi-derived vesicles carrying lysosomal enzymes, eventually forming lysosomes.

Labeled Ligands

Human Tf was saturated with iron (Yamashiro et al., 1984). Diferric Tf and α_2M were radioiodinated using Na[^{125}I] (New England Nuclear, Boston, MA) as previously described (Yamashiro et al., 1984). The specific activity of the [^{125}I]Tf and the [^{125}I]α_2M ranged from 200–500 cpm/ng. Diferric Tf and α_2M were conjugated to fluorescein isothiocyanate (F) or TRITC (Research Organics Inc., Cleveland, OH) as previously described (Yamashiro et al., 1984).

Preparation of AFA

Rabbit AFA was prepared as previously described (Salzman and Maxfield, 1988). The IgG was extracted from the serum by performing three successive 30% ammonium sulfate precipitations. The pellet was dissolved and dialyzed exhaustively against PBS.

Characterization of AFA Effect on the pH Dependence of Fluorescein Fluorescence

Buffers were prepared in 100 mM NaCl for pH 6 (sodium phosphate 25 mM, Tris-Maleate 25 mM), pH 7 (sodium phosphate 50 mM), and pH 8 (Tris 50 mM). Excitation spectra of F-Tf or F-α_2M in the presence or absence of AFA were taken using a fluorometer (AMINCO 8000C; SLM Instruments, Inc., Urbana, IL). The spectra in the presence of AFA showed a significant decrease in fluorescein fluorescence, and the fluorescein fluorescence was nearly pH independent (Fig. 2). Fluorescein fluorescence of native fluorescein is highly pH dependent at 495 nm excitation. The decreased pH dependence of fluorescein fluorescence when bound to AFA has been used to develop an assay for the binding of AFA to fluorescein that would be suitable for use on cells. As described below, we were able to calculate the fraction of fluorescein saturated by AFA. We define R, as the ratio of fluorescence intensity of AFA-treated fluorescein at one pH to the fluorescence intensity of the AFA-treated fluorescein at a second pH. The fluorescence intensity of the experimental sample at each pH is equal to the fluorescence intensity of AFA bound fluorescein plus the fluorescence intensity of AFA unbound fluorescein:

$$R = \frac{(f_b)\,(I_{bx}) + (f_u)\,(I_{ux})}{(f_b)\,(I_{by}) + (f_u)\,(I_{uy})}; \tag{1}$$

where f_b = fraction bound, f_u = fraction unbound, I = fluorescence intensity at pH = x, y, of antibody bound (b) or unbound (u) fluorescein.

For each sample, the fraction of unbound fluorescein plus the fraction of bound fluorescein is equal to 1:

$$f_b + f_u = 1, \tag{2}$$

substituting for f_u and rearranging:

$$R = \frac{(f_b)\,(I_{bx}/I_{uy}) + R_u - (f_b)\,(R_u)}{(f_b)\,(I_{by}/I_{uy}) + 1 - f_b}, \tag{3}$$

where $R_u = I_{ux}/I_{uy}$.

Rearranging Eq. 3 in terms of f_b:

$$f_b = \frac{R_u - R}{(R)\,(I_{by}/I_{uy}) - R - (I_{bx}/I_{uy}) + R_u}. \tag{4}$$

The fraction of AFA bound fluorescein (f_b) can also be described as the fraction of fluorescein saturated by AFA, the fractional saturation. This value will be referred to as the fractional saturation in the Results and Discussion sections.

The pH dependence profiles were slightly different for F-Tf and F-α_2M, so separate values of I_b/I_u were used depending on the ligand used. The ratio R_u was obtained by measuring the fluorescence intensities of ligand internalized by cells after equilibrating all compartments to different pH values. I_b/I_u values could not be obtained by measurements on cells at 4°C because the values proved to be too close to the cellular autofluorescence for reliability. This was as expected because of the fluorescence quenching ability of the AFA. The I_b/I_u values were obtained using fluoresceinated ligand in solution with or without added AFA.

^{125}I-Tf Release

Cells were grown to near confluence in 6-well plastic tissue culture dishes. Kinetic recycling experiments were carried out at 37°C. The cells were rinsed 3 times with medium 1 and incubated with 2 μg/ml [^{125}I]Tf for 5 min. Parallel competition experiments were performed in the presence of 1 mg/ml diferric Tf. The cells were then rinsed with medium 1, followed by a mild acid/neutral rinse used to strip the surface of uninternalized Tf (Salzman and Maxfield, 1988). Cells were returned to medium 1 for 2–60 min. The extracellular medium was collected to determine the amount of exocytosed [^{125}I]Tf. Cell-associated radioactivity was measured by dissolving the cells in 0.1 N NaOH. Radioactivity was measured using a minigamma counter (1275; LKB Instruments, Inc., Gaithersburg, MD). The nonspecific binding ranged from 5–10% of the total binding.

^{125}I-α_2M Internalization and Degradation at 18°C and 37°C

TRVb-1 cells were grown to near confluence in 6-well tissue culture plates. The cells were rinsed 3 times with Hams F-12 medium (supplemented with 1 mg/ml BSA) and incubated with [^{125}I]α_2M (10 μg/ml) for 10 min at 37°C. Parallel competition experiments were done in the presence of 2 mg/ml unlabelled α_2M. The cells were rinsed with F-12 + Na_2EDTA (5 mM) to prevent further binding of α_2M. Cells were returned to F-12 + Na_2EDTA for 5–120 min. Extracellular medium was collected, and 10% TCA precipitations were performed to determine the amount of intact versus degraded protein released. The cells were solubilized in 0.1 N NaOH, and 10% TCA precipitations were performed on the cell-associated counts. For the 18°C experiments, the cells were rinsed and equilibrated at 18°C for 30 min before incubation with [^{125}I]α_2M for 30 min. The cells were rinsed with F-12

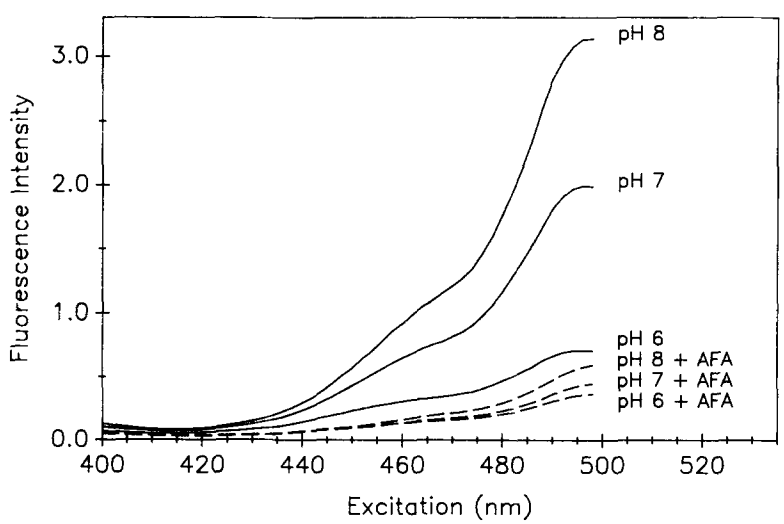

Figure 2. Effects of AFA on the pH sensitivity of fluorescein. F-α_2M (5 μg/ml) was added to buffers of pH = 6, 7, and 8, with (– –) and without (–) AFA (2 mg/ml). Fluorescence was measured using a fluorometer (excitation scanned from 400–500 nm, emission = 520).

+ Na_2EDTA and left in this medium at 18°C for 0–240 min. Analysis of release and degradation was performed as in the 37°C experiments.

Fluorescence Measurements of Endosome Fusion

No. 2 class coverslips (Thomas Scientific, Philadelphia, PA) were cut to 10 mm × 30 mm, cleaned using a solution of Nochromix (Godax Laboratories, Inc., New York) (10 g Nochromix/100 ml concentrated H_2SO_4), and rinsed for 2 h in distilled H_2O. The coverslips were treated with poly-*d*-lysine (100 μg/ml in distilled H_2O for 30 min, rinsed, and dried). TRVb-1 cells were grown to near confluence on these coverslips in 6-well tissue culture plates.

All experiments were carried out at 37°C. The cells were rinsed 3 times in medium 1, and incubated in medium 1 plus F-Tf (30 μg/ml) for 5 min. Beginning with a mild acid/neutral wash, the cells were rinsed and incubated for 2, 3, 4, 5, and 10 min. The cells were then incubated in the presence of AFA (5 mg/ml) for 10 min. The cells were rinsed 3 times with medium 1, fixed in 3.7% formaldehyde in medium 1 for 5 min, rinsed and left in medium 1. Each coverslip was placed in a coverslip holder similar to that described by Ohkuma and Poole (1978), rinsed with saline and placed in the pH 8 buffer. For experiments with cells, the pH buffers were supplemented with 20 mM methylamine and 20 mM ammonium acetate to cause equilibration of all endosomal compartments with the buffer pH. The cells were allowed to equilibrate to pH 8 (for 2 min), and the fluorescence intensity at 495 nm excitation was obtained. Measurements were then made on the same coverslip after reequilibration to pH 7 and pH 6. Measurements were corrected for autofluorescence and other sources of background signal. The correction factor was obtained by measuring and averaging the fluorescence signal of 6 coverslips of cells without added ligands. The actual cellular autofluorescence was close to half of the total background signal and ranged from 10–25% of the total fluorescence measured. R_u was obtained for Eq. 4 by taking measurements of cells that had endocytosed fluorescent ligand in the absence of AFA. The R_u values obtained in cells were the same (within 5%) as values obtained for F-Tf and F-α_2M in solution. This indicates that the fluorescence properties of the ligands are similar under these two conditions. The binding of AFA to fluorescein was quantified using Eq. 4.

A similar experiment was performed using F-α_2M. In this experiment, the cells were rinsed with F-12 medium, and incubated with F-α_2M (150 μg/ml) in F-12 in the 5% CO_2 incubator for 10 min, the cells were rinsed with F-12 containing 5 mM Na_2EDTA to prevent rebinding of F-α_2M, for 2, 5, or 10 min. The cells were then incubated in the presence or absence of AFA (5 mg/ml) in F-12 + 5 mM Na_2EDTA for 10 min. The cells were carefully rinsed and fixed with 3.7% formaldehyde. The fluorescence measurements were performed as for F-Tf.

Intracellular Fusion at 18°C

Cells grown on polylysine-coated coverslips were rinsed in F-12 medium at room temperature, and equilibrated in 18°C F-12 medium for 30 min (10 mM glucose and 20 mM Hepes were added to the medium which was bicarbonate free). F-α_2M (150 μg/ml) was then added for 60 min. The cells were rinsed with F-12 medium containing 5 mM Na_2EDTA. The cells remained in this medium for either 10 or 60 min, and were then incubated in the presence or absence of AFA (5 mg/ml) for 60 min. The cells were gently rinsed 3 times with F-12, fixed in medium 1 + 3.7% formaldehyde for 5 min at room temperature, and left in medium 1. The fluorescence measurements were performed as for the 37°C experiments.

Results

We have studied intracellular vesicle fusion along the endocytic pathway, encompassing both the receptor recycling and lysosomal pathways. The goal of this study has been to obtain the time course of sequential endosome fusion, thus determining the transit time of a receptor or ligand through a fusion-accessible compartment and the lifetime of the fusion-accessible compartment.

We measured fusion using a variation of an earlier spectrofluorometric assay, modified for use in the fluorometer. Fluorescein-labeled ligands are taken up by cells and chased by AFA. If vesicle fusion occurs, AFA binds to fluorescein and changes its fluorescent properties in a measurable manner.

Characterization of AFA

Antifluorescein IgG was prepared from the serum of rabbits inoculated with fluorescein-labeled hemocyanin. When fluorescein-labeled ligands are saturated by AFA, the fluorescein fluorescence is quenched, and the remaining fluorescence becomes nearly pH independent, unlike native fluorescein which is strongly pH-dependent at excitation of 495 nm (Fig. 2). At pH 5 (data not shown), AFA saturated F-α_2M fluoresces with greater intensity than F-α_2M alone, with an excitation spectrum close to that seen with AFA saturated F-α_2M at pH 6, 7, and 8. This shows that AFA binds to fluorescein even at the lowest pH values found in endosomes.

This characteristic of AFA can be exploited for the measurement of the fraction of fluorescein saturated by AFA (fractional saturation) and the quantitation of intracellular fusion of endosomes. The pH dependence of fluorescein fluorescence will depend on the fractional saturation by AFA. The quantitative relationship between pH dependence and fractional saturation is described in Materials and Methods. In Fig. 3, we illustrate how the ratio of fluorescein fluorescence intensity at pH 8 and 6 will vary as a function of saturation by AFA. From this type of curve (or Eq. 4, Materials and Methods), we can calculate the fractional saturation of fluorescein by AFA from the ratio of fluorescence intensities at two pH values.

For measurements on cells, fluorescein-labeled ligands were endocytosed and chased by AFA. The cells were then fixed. Fluorescence intensity measurements were then made on the same cells after they were sequentially equilibrated to pH 6, 7, and 8. The fractional saturation was then calculated from the ratio of fluorescence intensities. The fractional saturation was essentially the same when calculated from different pairs of pH values. Since measurements were made on the same coverslip equilibrated to different pH values, the fractional saturation measured was not affected by the absolute amount of fluorescein within the cells. The use of this

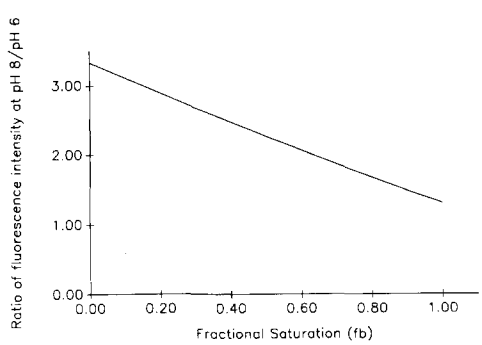

Figure 3. The pH dependence of fluorescein fluorescence as a function of increasing AFA saturation. The ratio of fluorescence intensities at pH 8 and 6 was calculated from Eq. 3 (see Materials and Methods) using fluorescence intensities at 495 nm. As fluorescein is progressively saturated by AFA, the pH dependence decreases. Using this curve, the amount of AFA saturation of fluorescein can be determined from the experimentally measured pH dependence of fluorescein fluorescence.

type of a ratio method was very helpful in obtaining reproducible values for the kinetics of vesicle fusion.

The method we have developed measures the saturation of fluorescein by AFA. We have used this to quantitatively estimate the extent of fusion of fluorescein-containing endosomes with subsequently formed endosomes containing AFA. In our earlier work (Salzman and Maxfield, 1988), we showed that AFA binding to fluorescein was independent of the pH of intracellular compartments. We also showed that AFA/F-Tf binding was not occurring at the cell surface in significant enough amounts to be detected by our method (Salzman and Maxfield, 1988). We have used high concentrations of AFA (5 mg/ml) to try to ensure that fusion of an antibody-containing endosome will deliver enough AFA to saturate the fluorescein. Measurements of fractional saturation do not increase when the concentration of AFA is increased beyond this level. If, despite this, the AFA is insufficient to saturate the fluorescein, we may be underestimating the amount of endosome fusion.

Time Course of Vesicle Fusion Along the Lysosomal Pathway

TRVb-1 cells were allowed to bind and internalize F-α_2M for 10 min at 37°C and followed by a rinse with medium containing Na_2EDTA that released α_2M bound to the surface and prevented rebinding. The cells remained in the medium for intervals of 2, 5, and 10 min, before incubation in the presence or absence of AFA for 10 min. Incubations with AFA were limited to 10 min to minimize the observation of later fusion events, such as endosome–lysosome fusion. Cell-associated fluorescence was measured on fixed cells in buffers of pH 8, 7, and 6. The fractional saturation of F-α_2M with AFA was calculated and the results are shown in Fig. 4. With a 2-min interval between F-α_2M and AFA, 56% of the F-α_2M was saturated by AFA. As the interval increased, the fractional saturation fell off. The loss of accessibility of F-α_2M to AFA appeared to show first order kinetics. As shown in Fig. 4, the data could be fit to a single exponential curve (fractional saturation = $0.67 \exp[-0.091t(\text{min})]$). The rate constant for the loss of fusion-accessibility would correspond to a $t_{1/2}$ of ~8 min for the process.

Time Course of Fusion along the Recycling Pathway

To learn whether fusion occurs along the recycling pathway with comparable kinetics, similar experiments were performed using F-Tf. TRVb-1 cells were allowed to bind and internalize F-Tf for 5 min at 37°C, followed by a mild acid/neutral rinse that was used to strip remaining surface-bound Tf. Intervals of 2–10 min were allowed before the addition of AFA. The cells were incubated in the presence or absence of AFA for 10 min, rinsed, and fixed. We have previously shown that fusion of endosomes containing AFA with previously endocytosed F-Tf plateaued within 10 min in TRVb-1 cells (Salzman and Maxfield, 1988). Cell-associated fluorescence at pH 6, 7, and 8 was measured as in the F-α_2M experiments. The fractional saturation at each time point is shown in Fig. 5.

There is significant fusion along this pathway as well. With

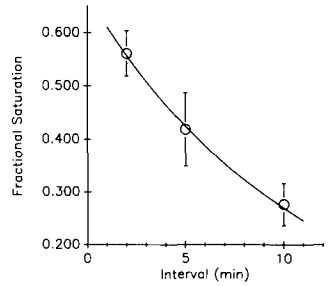

Figure 4. Loss of fusion accessibility along the lysosomal pathway. Cells were incubated with F-12 containing 150 μg/ml F-α_2M for 10 min at 37°C. The cells were rinsed free of ligand and left in medium for the times indicated and then incubated in the presence of AFA (5 mg/ml) for 10 min, rinsed, and fixed. Fluorescence intensity was measured at pH 6, 7, and 8, as described in Materials and Methods. The fluorescence intensity values were corrected for autofluorescence and the pH ratio values were calculated. Fractional saturation was calculated from eq. 4. The points shown are the means of 30 coverslips/time point. The curve through the average points is the single exponential curve fit to the data (fractional saturation = $0.67 \exp[-0.091t(\text{min})]$). The error bars represent the standard error of the mean.

a 2-min interval, there is 80% saturation of F-Tf with AFA. The kinetics of loss of fusion accessibility differs significantly from that of F-α_2M. Unlike the α_2M pathway, fusion along the Tf pathway is biphasic. For F-Tf, there is an immediate, rapid drop in fractional saturation that is completed in 3 min followed by a nearly constant amount of saturation for the next 10 min. The data are consistent with movement of Tf out of a fusion-accessible compartment with a $t_{1/2}$ of <3 min, but the Tf then moves into a second compartment that stays accessible to AFA. The F-Tf in the second compartment remains accessible to fusion independent of the time it entered the compartment.

At intervals longer than 10 min (15 min after the start of the pulse of F-Tf) the fluorescence has dropped off to levels below the limits of accurate measurement. Although the fractional saturation of cell-associated Tf remains constant for intervals as long as 10 min, the amount of F-Tf in this compartment does not remain constant since both saturated and unsaturated F-Tf are leaving the cell. AFA-saturated F-Tf leaves the cell with the same kinetics as F-Tf (Salzman and Maxfield, 1988). Thus, there is a gradual loss of Tf from the cell and a decline in the amount of F-Tf accessible to AFA.

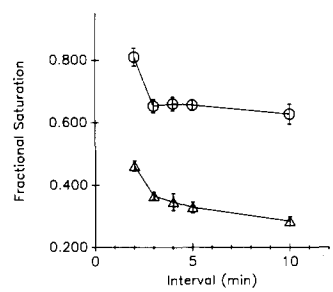

Figure 5. Vesicle fusion along the recycling pathway. Cells were incubated with medium 1 containing 30 μg/ml F-Tf for 5 min. The cells were rinsed free of ligand and left in medium for the times indicated before incubation in the presence of AFA (5 mg/ml) for 10 min. Fluorescence intensity measurements were made as discussed in Materials and Methods. Fluorescence intensities were corrected for autofluorescence, and pH ratios were calculated. The fractional saturation was calculated using Eq. 4 and plotted (0). The lower curve (Δ) represents the fractional saturation relative to the total fluorescein fluorescence in the cells before addition of AFA. The values are the products of the fractional saturation and the fraction of the initial total Tf remaining at each time point. The values are the mean of 30 coverslips/time point. The error bars represent the standard error of the mean.

Using [^{125}I]Tf washout kinetics (Fig. 6), we can correct the fractional saturation for the loss of Tf from the cell due to Tf recycling (Fig. 5). The behavior of Tf is clearly different from α_2M. Whereas, α_2M moves to an intracellular fusion-inaccessible compartment, the Tf inside the cell remains equally fusion accessible from 3–10 min after endocytosis. During this time, Tf is being returned to the cell surface and released. Of course, release to the extracellular medium makes the Tf fusion-inaccessible in our assay.

Fusion at 18°C

It has been shown that lowering the cell temperature to 18°C will block delivery of ligands to lysosomes (Dunn et al., 1980). The temperature block could be occurring in a number of places. (*a*) Ligands could be trapped before entering the fusion compartment (in this case no fusion would occur at 18°C), even with short intervals between F-α_2M and AFA. (*b*) The temperature block could be at the transition from fusion-accessible to fusion-inaccessible endosome, in which case the lifetime ($t_{1/2}$) of fusion capability would be prolonged indefinitely at 18°C. (*c*) The block could be after the fusion compartment but before the lysosome. This would identify a fusion-inaccessible late endosome, the existence of which, as an organelle separate from sorting endosomes, has not yet been well defined. If this were so, after a long enough interval fusion would no longer be evident.

We confirmed that [^{125}I]α_2M accumulated in the cells at 18°C, but was not degraded for up to 4 h. Vesicle fusion experiments were carried out as described in the Materials and Methods section.

We found that with a 10-min interval there was an extent of fusion comparable to that seen with a 2-min interval at 37°C (Table I; Fig. 4). Therefore, the temperature block does not occur before the fusion-accessible compartment. Fusion is not prolonged indefinitely since with an interval of 60 min no detectable fusion was found (Table I). Therefore, the temperature block occurs with F-α_2M in a fusion-inaccessible late endosome.

Discussion

It seems evident that fusion of endosomes is an essential feature of sorting along endocytic pathways. There have been several recent descriptions of in vitro methods for observing fusion (Braell, 1987; Gruenberg and Howell, 1986; Diaz et al., 1988). Fusion in intact cells has been directly observed by optical microscopy (Deng and Storrie, 1988; Pastan and Willingham, 1985; Kielian and Cohn, 1980), and it has been measured by flow cytometry of cell homogenates (Murphy, 1985). The methods used previously for observing fusion in intact cells have been most useful for analyzing fusion late on the endocytic pathway. In this work, we describe a method for quantitative analysis of endosome fusion in intact cells that is suitable for kinetic analysis of fusion during the first few minutes following internalization. Using a related method, we showed that vesicle fusion occurs along the receptor recycling pathway, but the organelle where fusion takes place was not ascertained (Salzman and Maxfield, 1988). In addition to demonstrating that fusion of sequentially formed endosomes occurred, our earlier work showed that fusion with F-Tf containing compartments continued for 5–10 min after

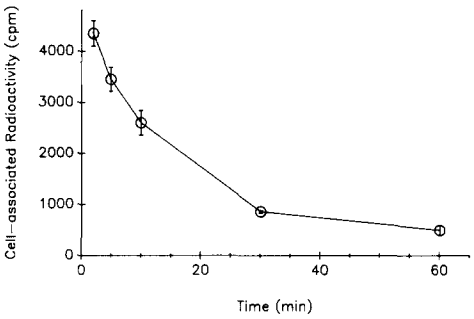

Figure 6. [^{125}I]Tf release from TRVb-1 cells. TRVb-1 cells were grown to near confluence in 6-well tissue culture dishes. The cells were rinsed with medium 1 and incubated with [^{125}I]Tf (2 μg/ml) for 5 min at 37°C, rinsed, and allowed to release [^{125}I]Tf for the times indicated. Cell-associated radioactivity was determined. The values have been corrected for nonspecific ^{125}I determined by competition experiments in which 500-fold excess of unlabeled diferric transferrin was added to the cells. The numbers reflect the means of 4 dishes/time point. The error bars give the standard deviation of the mean.

the Tf had been internalized. In this paper, we have examined the kinetics of loss of fusion accessibility along the recycling and lysosomal branches of the endocytic pathway. Pinocytosed AFA should enter all endocytic compartments along both the recycling and degradative pathways, and this allows us to measure fusion accessibility in vesicles on both pathways. In support of this, we have immunolocalized pinocytosed AFA, and we find it both in endosomes throughout the cell and in the para-Golgi recycling endosomes (data not shown). The data obtained show that the ability to fuse with subsequently formed endosomes decays differently along the two pathways.

Using F-α_2M as a lysosomal pathway marker, we find that with a 2-min interval between probes, at least 56% of the F-α_2M was accessible to AFA. As the interval increased, the accessibility of F-α_2M decreased with apparent first order kinetics. By fitting the data to a single exponential, the rate constant for loss of accessibility of F-α_2M to AFA is 0.091 min^{-1} yielding an approximate $t_{1/2}$ of 8 min.

The kinetic data are consistent with the stochastic maturation of a single type of compartment. We know that α_2M is taken into CHO cells together with Tf (Yamashiro et al., 1984). It is concentrated in an intermediate sorting endosome which shows the presence but not a concentration of Tf (Yamashiro et al., 1984). This is the most likely point of ligand-receptor sorting. The sorting endosome may have the same function as the CURL compartment described in liver parenchymal cells. The CURL compartment accumulates

Table I. Fractional Saturation of F-α_2M with AFA at 18°C

Incubation (min)	Interval (min)	Fractional saturation
60	10	0.62 ± 0.06
60	60	0.00 ± 0.04

Fusion along the lysosomal pathway was measured at 18°C. At this temperature, ligand delivery to lysosomes is blocked. Cells were preincubated in F-12 for 30 min at 18°C, and incubated with F-α_2M (150 μg/ml) for 60 min. Cells were rinsed with F-12 containing 5 mM Na_2EDTA for 10 or 60 min, and incubated with F-12 + Na_2EDTA in the presence or absence of AFA (5 mg/ml) for 60 min. The fractional saturation was determined as described in Materials and Methods. Measurements were made on 6–20 coverslips/time point. The errors given are the SEM.

ligand in its vesicular portion while sorting away the recycling receptors into tubular extensions (Geuze et al., 1983, 1984).

We interpret the measurement of the loss of fusion accessibility of AFA to α_2M as the measure of the lifetime of the sorting endosome, or more explicitly the $t_{1/2}$ for the maturation of the sorting endosome from a fusion-accessible to a fusion-inaccessible compartment. The interpretation that the sorting endosome has a finite lifetime of fusion accessibility is consistent with several in vitro studies on early endosome fusion. The in vitro studies consistently show that there is a window for endosome–endosome fusion of endosomes that are 5–15 min old, and that fusion competence decays as ligands move into later compartments (Diaz et al., 1988; Braell, 1987; Gruenberg and Howell, 1987).

The existence of a fusion-inaccessible late endosome was confirmed by lowering the temperature to 18°C. Since fusion occurred with a short interval (10 min) between F-α_2M and AFA, F-α_2M is not being trapped before its entrance into the sorting endosome. The absence of fusion after long intervals (60 min) shows that the sorting endosome lifetime is not indefinitely prolonged at 18°C. At 18°C, F-α_2M is collected in a compartment that is not accessible to fusion with incoming endosomes, and is therefore not the sorting endosome. Since no degradation occurs, this compartment is not a lysosome. By this assay, we have defined a late endosomal compartment to which lysosomally directed ligands are delivered. Mueller and Hubbard (1986), using perfused rat liver, have identified a compartment at 16°C that accumulates endocytosed ASGP but also retains the ASGP receptor. They also identify a later prelysosomal compartment that contains ASGP but lacks the ASGP receptor. Wolkoff et al. (1984), also using rat hepatocytes, have defined two endocytic steps blocked by lowering the temperature to 18°C; ligand-receptor dissociation and the delivery of ligands to lysosomes. The system in rat liver is not directly comparable to that in CHO cells, and the methods and receptor used in these two systems are different. Although we may be identifying a similar compartment, our experiments do not address whether the compartment retains any receptors. In CHO cells, the fusion inaccessible late endosome may be the immediate precursor to the lysosome. Although the late endosomal compartment we measure at 18°C is no longer accessible to fusion with newly formed endosomes, it is likely accessible to fusion with a Golgi-derived prelysosome compartment carrying lysosomal enzymes (Griffiths et al., 1988), which together would form a lysosome.

Fusion accessibility along the recycling pathway is significantly different from that along the degradative pathway. When a 2-min lag is allowed between F-Tf and AFA, up to 80% of the F-Tf was accessible to AFA. When the interval was increased to 3 min, the F-Tf accessibility dropped rapidly to 65% and remained at this point for as long as a 10-min interval between probes. It appears that there are two phases to the fusion process along the TF pathway, an initial rapid phase followed by a later constant phase. The Tf moves through the first fusion compartment with a $t_{1/2}$ of <3 min. This is consistent with the time course of Tf separation from ASGP (Stoorvogel et al., 1987) or from α_2M (Yamashiro et al., 1984). Tf then moves into a later compartment that appears to remain fusion competent for as long as it is measurable by our methods.

We know that immediately after endocytosis of F-Tf, we can see a pattern of punctate fluorescence in TRVb-1 cells (Dunn, McGraw, and Maxfield, 1989). In both wild type CHO cells and TRVb-1 cells, F-Tf is then swiftly segregated into a collection of small vesicles and tubules (recycling endosomes) nearly devoid of α_2M, located in the para-Golgi region of the cell (Yamashiro et al., 1984; Dunn, McGraw and Maxfield, 1989). At steady state, most of the F-Tf in wild type CHO cells is in the para-Golgi region (Yamashiro et al., 1984). An interpretation of the F-Tf fusion data is that F-Tf moves into the sorting endosome, the first site of vesicle fusion, and is swiftly sorted away from the other acid-released ligands and transported via recycling endosomes into the para-Golgi region of the cell. The recycling endosomes are apparently also fusion accessible, perhaps indefinitely. We would expect the AFA, which catches up with the F-Tf, to pass through the same set of organelles. It is also possible that some early endosomes are bypassing the sorting endosome and fusing directly with recycling endosomes. Although we think this is unlikely since very little α_2M is seen in the para-Golgi recycling endosomes (Yamashiro and Maxfield, 1984), our experiments do not entirely rule out this possibility.

There are several possible explanations for the time independence of fusion accessibility of the recycling endosomes. One possibility is that vesicles containing AFA fuse with the recycling endosomes containing Tf randomly, irrespective of the age of the particular recycling endosome. An alternative possibility is that the recycling endosomes form an interconnected network, with all parts of the network accessible to fusion with incoming vesicles. These possibilities would not be distinguished by our experiments.

Our knowledge of the behavior of Tf and α_2M in concert with the data presented in this paper allowed us to develop a model for endocytosis and receptor sorting (Fig. 1). Tf and α_2M are cointernalized into the sorting endosome via multiple fusion events. Iron is released from Tf and α_2M dissociates from its receptor. The α_2M receptor and Tf with its receptor are sorted away from the dissociated ligands, delivered to recycling endosomes, and eventually returned to the cell surface. Ligands, such as α_2M, are accumulated in the sorting endosome through several fusion events. The endosome then matures into a late endosome inaccessible to fusion with incoming endocytic vesicles. This late endosome should be accessible to fusion with Golgi-derived vesicles bearing lysosomal enzymes, together leading to the generation of a lysosome (Griffiths et al., 1988).

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