# Intracellular Fusion of Sequentially Formed Endocytic Compartments

**Nita H. Salzman*‡ and Frederick R. Maxfield***

*Department of Pathology, Columbia University College of Physicians and Surgeons, New York, New York 10032; and
‡Department of Pharmacology, New York University School of Medicine, New York, New York 10017

***Abstract.*** A polyclonal anti-fluorescein antibody (AFA) which quenches fluorescein fluorescence has been used to distinguish between two models of intracellular vesicle traffic. These models address the question of whether sequentially endocytosed probes will mix intracellularly or whether they are carried through the cell in a sequential, isolated manner. Using transferrin (Tf) as a recycling receptor marker, we incubated Chinese hamster ovary (CHO) cells with fluorescein-Tf (F-Tf) which is rapidly endocytosed. After the F-Tf was completely cleared from the surface, AFA was added to the incubation medium and entered endocytic compartments by fluid phase endocytosis. Fusion of a vesicle containing AFA with the compartment containing F-Tf results in binding of AFA to fluorescein and the quenching of fluorescein fluorescence. When AFA was added to the culture medium 2 min after clearance of F-Tf from the surface, time dependent fluorescence quenching occurred. After 20 min, 67% saturation of F-Tf with AFA was observed. When the interval between F-Tf clearance and AFA addition was increased to 5 min only 41% saturation of F-Tf was found. These data indicate that there are some compartments which are accessible for mixing with subsequently endocytosed molecules, but the efficiency of mixing falls off rapidly as the interval between pulses is increased. In CHO cells Tf swiftly segregates to a collection of vesicles or tubules in the para-Golgi region, and at steady state most of the F-Tf is in this compartment. Using digital image analysis to quantify quenching in this region, we have found that F-Tf/AFA mixing is occurring either within this compartment or before transferrin enters it.

THE process of receptor mediated endocytosis has been a topic of concerted investigation (10, 20). Briefly, cell surface receptors bind ligands, cluster, and are internalized into endocytic vesicles. These vesicles then rapidly acidify (20, 22, 25, 26). In some cases the pH change causes a conformational change in the receptor allowing the dissociation of the ligand (6). These ligands are sorted from their receptors and degraded in a lysosome while the receptors can recycle. After the point of ligand dissociation, the spatial and temporal movement of recycling receptors through a series of endocytic vesicles and organelles is poorly characterized.

Cellular receptor traffic is carried on by a large collection of membrane bound endocytic vesicles which fuse, separate, and sort themselves. In the process they shuttle membrane proteins to their destinations. They are involved in the sorting of a receptor from its ligand and one receptor from another. The mechanism, time course, and spatial progression of such vesicle traffic is poorly defined in comparison with similar vesicle traffic involved with membrane protein biosynthesis and delivery to the cell surface (9).

The ability of endocytic compartments to separate to segregate recycling molecules from those which are degraded is well documented (5, 10, 24). An equally important property of vesicular organelles is their ability to fuse with each other. As a starting point for studying vesicle fusions on the endocytic and receptor recycling pathways, we have designed experiments to distinguish between two simple models for receptor traffic (Fig. 1). In one model (Fig. 1 *b*) membrane components within a single vesicle are able to mix with those contained in previously internalized vesicles by fusion. Alternatively (Fig. 1 *c*) the components within a vesicle may be carried along the endocytic recycling pathway in an isolated "conveyor belt" fashion. The property of vesicle fusion would have important consequences. A fusion organelle could carry out sorting functions in addition to the segregation of molecules directed to lysosomes from those which are recycled back to the plasma membrane. Possible functions include the regulation of receptor traffic and distribution, such as the control of the expression of receptors at the cell surface or the targeting of membrane proteins to other destinations (storage, degradation, etc.). If no vesicle fusion occurs, it is difficult to envision differential sorting steps.

To study vesicles on the recycling pathway we have used fluorescein isothiocyanate-labeled transferrin (F-Tf)[1]. Tf is a useful marker since it accompanies its receptor through the recycling pathway rather than dissociating and being delivered to lysosomes (4, 14, 27). In Chinese hamster ovary

1. *Abbreviations used in this paper*: AFA, anti-fluorescein antibody; F, fluorescein isothiocyanate; Rh, tetramethylrhodamine isothiocyanate; Tf, transferrin.

(CHO) cells F-Tf is segregated to a collection of small vesicles and tubules in the para-Golgi region starting within 5 min after internalization (31). At steady state, most of the F-Tf in a CHO cell is in this compartment. Although neither the structure nor the function of the para-Golgi compartment is well understood, it appears that it is an important part of the recycling pathway and may be a site for the mixing, sorting, and storage of membrane proteins. The relationship, if any, between this recycling compartment and a *trans*Golgi reticulum involved in transport of newly synthesized proteins is under investigation (9, 30).

Vesicle fusion events have been studied recently using assays which were either cell-free or involved subcellular fractionation (1, 3, 12). The development of an optical microscopy assay for vesicle fusion would provide a clearer view of the process in intact cells and avoid artifacts introduced by cellular fractionation. Observation of fusion by optical microscopy required making the fusion event observable even though the vesicle may be well below the resolution limit of the microscope. To do this the cells are sequentially pulsed with two probes. The second probe is designed to have an observable effect on the first probe only if it should come into close molecular contact (i.e., if vesicle fusion takes place). In this study we used F-Tf as our initial probe. This probe is chased by a polyclonal antifluorescein antibody (AFA) which is capable of quenching fluorescein fluorescence. If vesicles containing this antibody fuse with those containing F-Tf, the antibody binds to the fluorescein and quenches the fluorescein fluorescence. By measuring the extent of quenching with microspectrofluorometry, it is possible to quantify the rate and extent of mixing. Digital image analysis allows the determination of quenching within specific compartments. In this paper, we show that extensive mixing of sequentially internalized molecules does occur at or before the para-Golgi compartment. This suggests a possible sorting role for this organelle or one occuring even earlier on the endocytic pathway.

## Materials and Methods

### Cell Cultures

CHO cells were grown on plastic tissue culture dishes in F12 medium containing 5% FCS (Gibco, Grand Island, NY), penicillin (100 U/ml) and streptomycin (100 μg/ml), at 34°C in 5% $CO_2$ in a humidified air atmosphere. Cells were passaged using trypsin (Gibco). HeLa cells were grown in DME containing 5% calf serum (Gibco), penicillin (100 U/ml) and streptomycin (100 μg/ml), at 37°C in 5% $CO_2$ in a humidified air atmosphere. Cells were passaged using trypsin-EDTA (Gibco). TRVb-1 cells, a CHO cell line lacking endogenous TF receptor but expressing the transfected human transferrin receptor (19), were grown in F12 medium containing 5% fetal calf serum, 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. All experiments were carried out using Medium 1 (NaCl 150 mM, Hepes 20 mM pH, 7.4, $CaCl_2$ 1 mM, KCl 5 mM, $MgCl_2$ 1 mM, and containing 1 mg/ml ovalbumin and 40 μM desferyl mesylate, an iron chelating agent which binds free iron but does not strip bound iron from transferrin).

### Labeled Transferrins

Human Tf was saturated with iron and radioiodinated using Na[$^{125}$I] (New England Nuclear, Boston, MA) as previously described (31). The specific activity of the [$^{125}$I]Tf ranged from 200–500 cpm/ng. Diferric Tf was conjugated to FITC (F) or tetramethylrhodamine isothiocyanate (Rh) (Research Organics, Cleveland, OH) as previously described (31).

### Preparation of Anti-Fluorescein Antibody (AFA)

Fluorescein isothiocyanate was conjugated to Keyhole Limpet hemocyanin (18, 23). Rabbits were injected with 100 μg F-hemocyanin and boosted monthly with 50 μg of the labeled protein. After 3 mo the rabbit serum showed significant ability to quench fluorescein fluorescence. 30 ml of serum was fractionated using a solution of 50% saturated ammonium sulfate. The pellet was dissolved in 10 ml of 0.01 M phosphate and dialyzed for 17 h against 0.01 M phosphate, pH, 8.0. This fraction was run over a gradient ion-exchange column using a starting buffer of 0.01 M phosphate, pH 8.0, and a final buffer of 0.3 M phosphate, pH 8.0 (8), to separate out the IgG. The column fractions were assayed for protein content and fluorescein quenching. Those with quenching activity were pooled, concentrated fivefold to a concentration of 20 mg/ml using a minicon concentrator (Amicon), and dialyzed for 17 h against medium 1 before use. The ability of the antibody to quench fluorescein fluorescence was measured against a standard curve derived by adding aliquots of F-Tf to PBS (NaCl 136.9 mM, KCl 2.7 mM, $Na_2HPO_4$ heptahydrate 8.1 mM, $KH_2PO_4$ 1.5 mM, $CaCl_2$ 1 mM, pH, 7.4). The same amounts of F-Tf were added by aliquot to a 1:10 dilution of antibody in PBS. Measurements were made using a Perkin-Elmer 650-10S fluorescence spectrophotometer (excitation wavelength = 495 nm, emission wavelength = 520 nm).

### Stripping Tf from the Cell Surface

All experiments were done at room temperature. Cells were grown to confluence in 6-well plastic tissue culture dishes (diameter of well = 35-mm). The cells were rinsed with medium 1 and incubated with [$^{125}$I]Tf at 2 μg/ml for 10 min. Parallel competition experiments were done in the presence of 1 mg/ml diferric transferrin. The cells were rinsed 3× with medium 1, and subjected to a 30 s rinse with mild acid wash medium (2-[*N*-morpholino] ethanesulfonic acid; MES; 50 mM, sucrose 280 mM, pH, 5.0) followed by return to neutral pH.

To test the effectiveness of this mild acid wash procedure for removing surface Tf, it was followed by a more stringent acid wash. Cells were incubated for 30 s in 1 ml of mild acid medium, which was removed and saved. The cells were then rinsed with 1 ml of medium 1 (pH 7.4). This wash was removed and added to the saved acid wash. The neutral medium 1 at room temperature was replaced by medium 1 at 4°C. The cells were rinsed 4× with ice cold medium 1 and subjected to a harsh acid wash (acetic acid 0.2 N, NaCl 0.5 N, pH, 2.7) followed by return to neutral pH at 4°C (13, 17) to determine whether acid releasable counts remained bound to the cells after a room temperature mild acid wash. No specific counts were released by the harsh acid wash followed by rinse with neutral medium 1.

### [$^{125}$I]Tf Release

Cells were grown to confluence in 6-well tissue culture dishes. Kinetic recycling experiments were carried out at 23°C. The cells were rinsed 3× with medium 1 and incubated with 2 μg/ml [$^{125}$I]Tf for 10 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. Cells were returned to medium 1 for 5–60 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 1 N NaOH. Radioactivity was measured using a Beckman 4,000 gamma counter. The nonspecific binding ranged from 5–10% of the total binding.

### F-Tf Release

Cells were grown to confluence in coverslip-bottom dishes. These dishes consist of glass coverslips, coated with polylysine, attached to the lower surface of 35-mm tissue culture dishes which have had holes punched out on the bottom. Experiments were carried out at 23°C. The cells were rinsed 3× with medium 1, and incubated with F-Tf for 10 min. The cells were then rinsed with medium 1, followed by a mild acid/neutral rinse. F-Tf exocytosis was allowed for the same times as used for [$^{125}$I]Tf. The cells were fixed for 5 min in 3.7% formaldehyde in PBS, and rinsed with PBS. Cell-associated fluorescence was determined using the microscope spectrofluorometer. Measurements of fluorescence of confluent fields of cells were taken and averaged for each dish of cells.

### Effects of AFA Binding on Tf Kinetics of Recycling

Cells were grown to confluence in 6-well tissue culture dishes. The cells were incubated in medium 1 with 2 μg/ml of [$^{125}$I]F-Tf for 15 min at 23°C.

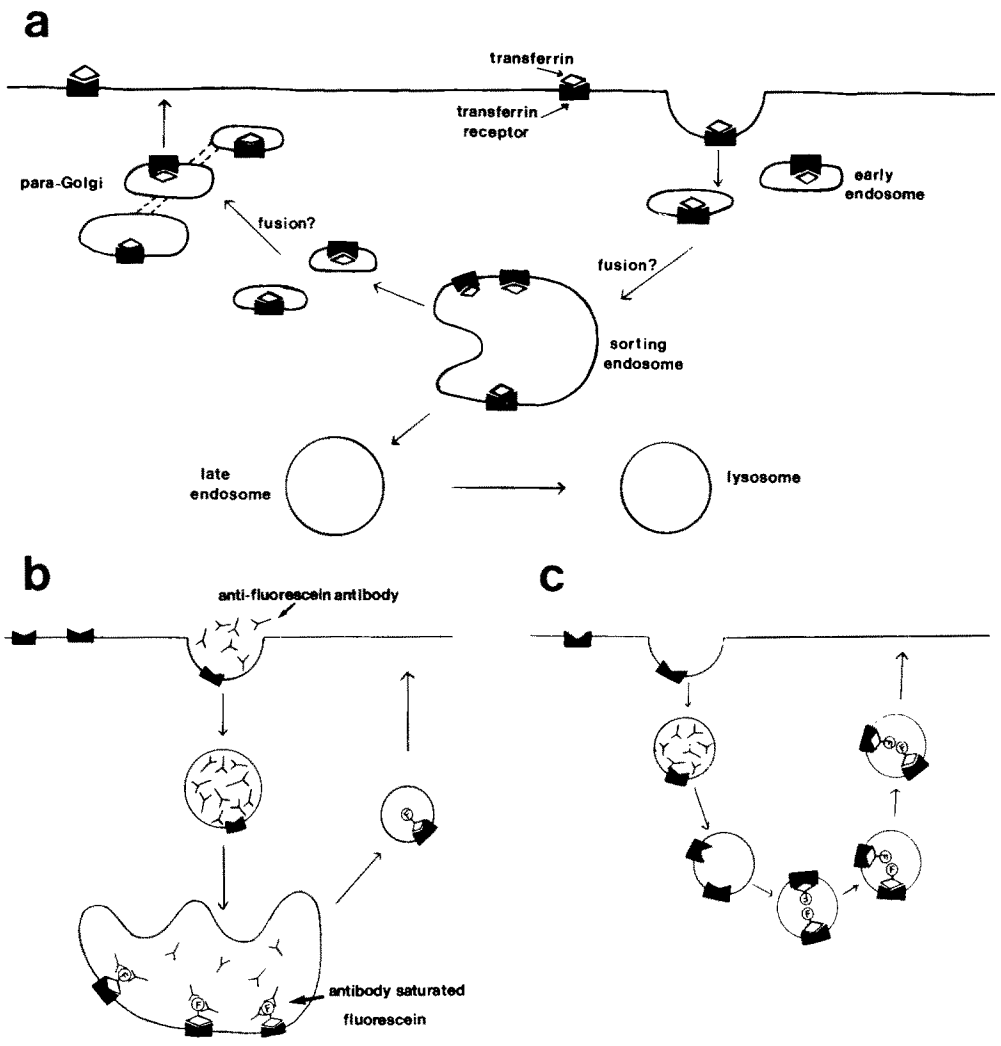


*Figure 1.* Model of vesicle traffic in receptor recycling in CHO cells. (*a*) Sequentially formed endocytic vesicles may fuse at various points on the recycling pathway. Two possible sites of fusion are the sorting endosome, and the para-Golgi compartment containing recycling receptors. Either of these possibilities would allow the mixing of vesicle contents along the route of receptor recycling. Alternatively, fusion of sequentially formed endosomes may not occur anywhere on the recycling pathway. Panels *b* and *c* show the rationale of experiments designed to distinguish between these models. (*b*) Fusion model. Mixing of F-Tf with pinocytosed AFA, added after clearance of F-Tf from the surface, causes quenching of fluorescein fluorescence in an intermediate compartment. (*c*) Nonfusion model. The brief interval between uptake of F-Tf and AFA prevents mixing, and there is no quenching of fluorescence.

The cells were rinsed for 2 min, and reincubated either in the presence or absence of AFA (8 mg/ml) for 0, 5, 10, and 20 minutes. Cells were rinsed, dissolved in 1 N NaOH, and counted to determine cell-associated radioactivity.

## *Intracellular Mixing of Sequentially Endocytosed Molecules*

Cells were grown to confluence in coverslip bottom dishes, rinsed at room temperature with medium 1, and incubated with a fluorescent probe. Cells were incubated with F-Tf (30 μg/ml), or a 1:1 mixture of F-Tf (20 μg/ml) and Rh-Tf (20 μg/ml), for 10 min. The cells were rinsed for either 2 or 5 min; a rinse which began with a mild acid/neutral rinse. The cells were then incubated in either medium 1 or medium 1 containing AFA (at a protein concentration of 8 mg/ml) which would enter cells by pinocytosis. The cells were incubated in these solutions for 5, 10, and 20 min, rinsed, fixed using 3.7% formaldehyde in PBS, and placed in PBS. Methylamine (30 mM) was added to collapse any remaining pH gradients, removing the pH effect on fluorescence intensity (21).

The cell associated fluorescence was analyzed using two different methods, microscope spectrofluorometry or digital image processing. Using the microscope spectrofluorometer, the fluorescence intensities of confluent fields of cells were measured. 15 fields (70 μm wide containing ~10 cells/field) were measured per dish. The fluorescence intensities were averaged for each dish. Fluorescence intensities of dishes treated with AFA were compared with those untreated to determine the quenching by the AFA as a measure of the amount of mixing. In experiments using both F-TF and Rh-Tf, fluorescein fluorescence and rhodamine fluorescence were measured for each field of cells, and F/Rh ratios were determined for both AFA-treated and untreated cells. The averaged F/Rh ratios for AFA-treated cells were compared with ratios for untreated cells for quantitative determination of quenching. As a control for any nonspecific effect of IgG, certain dishes were treated with IgG purified from preimmune serum.

We tested whether acidic endosomal pH interfered with AFA binding to F-Tf. Cells were incubated in medium 1 with 20 μg/ml F-Tf for 10 min at 23°C. The cells were rinsed for 2 min beginning with a mild acid rinse, and reincubated in either the presence or absence of AFA for 15 min, as in the mixing experiments. Before formaldehyde fixation, methylamine (30 mM) was added to one half of the dishes to collapse pH gradients before fixation. There was no difference in the amount of quenching between methylamine treated and untreated cells. Thus, the acidic pH of the endocytic vesicle does not interfere with AFA binding and quenching.

## *Microscope Spectrofluorometer*

A Leitz Diavert fluorescence microscope was equipped with interchangeable fluorescein and rhodamine excitation filters. A 520-nm bandpass filter was used to block rhodamine fluorescence while taking fluorescein measurements. Intensity measurements were made using a Leitz MPV microscope spectrofluorometer as previously described in detail (25).

## *Digital Image Processing*

Images of the cells were recorded via the DAGE/MTI 65 MKII SIT camera using a Panasonic (Tokyo, Japan) NV8030 video tape recorder. The video tape recorder signal was synchronized through a Fortel CCDHPS time base corrector. The signal was passed from the time base corrector to the image processor. The images were digitized into a 512 × 512 matrix of pixels (picture elements) using a Gould IP8500 image processor driven by a DEC

Micro VAXII computer. To improve the signal to noise ratio for digitized images, the pixel intensities of 4 video frames were averaged. The digitized images were further processed by a background correction. For each pixel, the background intensity was taken as the median intensity in a 40 pixel × 40 pixel region (13.2 × 13.2 μm) centered at that pixel, and the background fluorescence was subtracted from each pixel. This region size was chosen because it gave a reasonable level of background fluorescence without decreasing the F-Tf fluorescence from the para-Golgi region. To measure the para-Golgi fluorescence, a box of uniform size was centered over the area of high intensity para-Golgi fluorescence, and an intensity histogram analysis was performed for that area. This was repeated for each cell in the recorded field, for both the fluorescein and rhodamine image.

## Results

The main question addressed in this work is whether sequentially formed endosomes can fuse, thereby allowing the mixing of their contents (Fig. 1). To detect fusion at the resolution of the light microscope, cells were first incubated with F-Tf to prelabel endocytic compartments on the receptor recycling pathway; followed by incubation with anti-fluorescein antibody (AFA). If a vesicle containing AFA mixes with one containing F-Tf, the AFA will bind to the fluorescein and quench its fluorescence (Fig. 1 *b*). If mixing does not occur, there will be no decrease in fluorescein fluorescence (Fig. 1 *c*).

### *Characterization of Anti-fluorescein Antibodies*

Anti-fluorescein IgG was prepared from the serum of rabbits inoculated with F-hemocyanin. The fluorescein quenching ability of each AFA preparation was determined by titration with F-Tf (Fig. 2). The quenching efficiency of the AFA was obtained by comparing the fluorescence intensity of F-Tf solutions in the presence or absence of AFA. In solution, at saturating concentrations of antibody, only 60–80% of the fluorescence is quenched. Hence the measurement of AFA quenching of F-Tf in cells would underestimate the actual amount of mixing. It is, therefore, necessary to correct the measured fluorescence quenching by dividing by a factor, termed the quenching efficiency, which is the fractional quenching of fluorescein fluorescence at saturating AFA concentrations. This correction gives a better estimate of the amount of AFA/F-Tf mixing. As expected, the antibody had no effect on rhodamine fluorescence (data not shown).

### *Kinetics of Tf Recycling and Release*

The kinetics of F-Tf release from cells was compared with parallel experiments with [$^{125}$I]Tf to determine whether fluorescein labeling altered the behavior of transferrin in cells. The kinetics of Tf loss from CHO cells was found to be the same for [$^{125}$I]Tf and F-Tf (Fig. 3). This was also the case for TRVb-1 and HeLa cells (data not shown). The $t_{1/2}$ for Tf loss was ~15 min for CHO cells at 23°C. To establish intracellular mixing by quenching of fluorescence, the quenching must be shown to exceed the amount lost due to F-Tf recycling. At times beyond 20 min, the amount of fluorescence remaining was too near cellular autofluorescence levels to extract meaningful data using the light microscope. This placed limitations on the time courses used in these experiments. Experiments were carried out at 23°C rather than 37°C to slow down recycling and exocytosis of F-Tf.

To investigate intracellular mixing, it was necessary to establish that mixing did not occur extracellularly. It was especially important to prevent F-Tf from binding AFA at the cell surface and entering the cell. This could happen if apo-F-Tf was able to bind additional iron at the cell surface and reinternalize, if diferric Tf remained bound to the cell surface at the point when AFA was added to the incubation medium, or if Tf was not stripped of all of its iron on one cycle through the cell. To ensure that recycled apo-Tf could not pick up iron and rebind, the iron chelator desferyl mesylate (40 μM) was added to all incubation solutions. To ensure that mono- or diferric-Tf would not be on the surface when AFA was added, a low pH wash was used.

Iron can be removed from Tf by lowering its surrounding pH to 5.0, and apo-Tf will dissociate from the Tf receptor when the pH is raised back to pH 7.0 (4, 27). We found that a mild acid rinse with a solution that contained only impermeant ions was effective in stripping receptor-bound Tf at room temperature. By this method, the necessity of chilling the cells to 4°C was removed, and potential artifacts caused by changing the temperature were avoided (28, 29). A test of the effectiveness of this method showed that no [$^{125}$I]Tf remained bound to the cell surface after acid/neutral rinse (Fig. 4). This experiment also demonstrated that no [$^{125}$I]Tf was bound to the surface at later times. Additional acid/neutral rinses produced no more release of [$^{125}$I]Tf than a

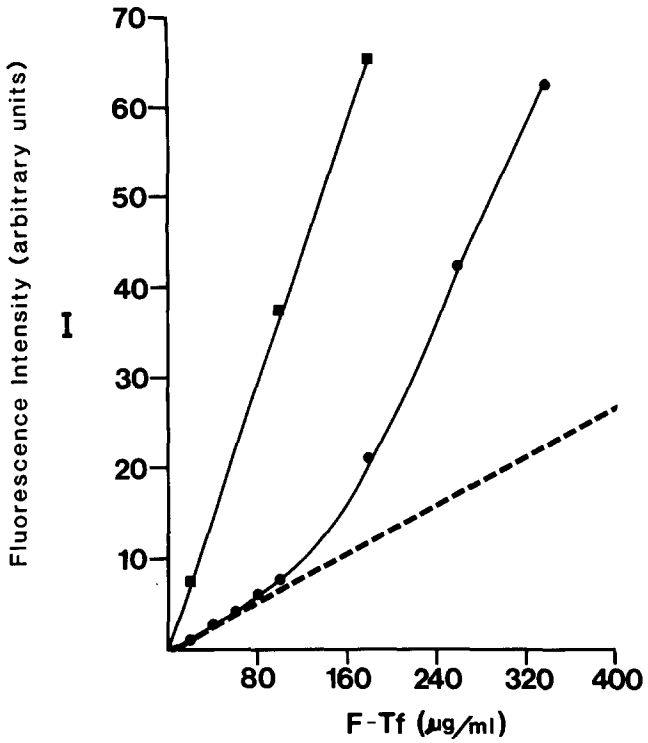


*Figure 2.* Characterization of AFA. Aliquots of F-Tf were added to 1 ml of PBS with (●) or without (■) AFA (2 mg/ml). Fluorescence was measured using a fluorometer (excitation = 495 nm, emission = 520 nm). The solid line represents the F-Tf fluorescence in the absence of AFA. The dashed line shows a linear extrapolation of the data at low F-Tf concentration in the presence of AFA. The ratio of the difference in the slopes of these lines to the slope of the line showing fluorescence in the absence of AFA was used to determine the efficiency of quenching for each lot of AFA.

medium 1 (pH 7.4) rinse. Therefore, radioactivity released by the additional acid/neutral rinses reflected the loss of [$^{125}$I]Tf from the cell in the time required to rinse the cells, not the release of [$^{125}$I]Tf bound to the cell surface.

In addition, it was important to determine whether AFA binding affected the kinetics of F-Tf recycling in CHO cells. [$^{125}$I]F-Tf was endocytosed and chased by AFA using the same time course employed in the mixing experiments. The time course of [$^{125}$I]F-Tf recycling and release from the cells was identical whether it was chased in the presence or absence of AFA. Thus, within the time course used in the following mixing experiments, AFA binding does not interfere with the recycling kinetics of Tf or redirect Tf to lysosomes.

### *Intracellular Mixing of Endosomal Contents*

CHO cells were allowed to bind and internalize F-Tf for 10 min at 23°C followed by an acid/neutral rinse. The rinse created an interval of 2 min, and was followed by incubation with AFA for 5, 10, and 20 min. Cell-associated fluorescence with or without AFA treatment was measured. The fractional quenching is the fluorescence loss attributed to the presence of AFA divided by the fluorescence in the absence of AFA. The amount of mixing (fractional saturation of F-Tf with AFA) was calculated from the fractional quenching, corrected for the quenching efficiency of the antibody as described above. The time dependence of mixing is shown in Fig. 5. The fractional saturation values shown in the figure provide a minimum estimate of the extent to which vesicles containing F-Tf had fused with subsequently endocytosed AFA. After 20 min, there was ~67% mixing. When the experiment was repeated allowing a 5-min interval between F-Tf and AFA, only 41% mixing was observed at 20 min. This reduced quenching may either reflect that only a certain percentage of F-Tf was in a compartment which was still accessible to AFA or that the amount of AFA which was able to reach this compartment was insufficient to fully saturate the F-Tf contained in the compartment. Both of these possibilities would result in a reduction in mixing.

The mixing we measured was probably an underestimate of the actual extent of mixing. Full saturation of F-Tf with AFA could only occur when there is a molar excess of AFA. If fusion events do not deliver sufficient AFA, less than maximal quenching will occur in compartments that have fused. It is also possible that steric hindrance within the endosome would prevent full saturation of F-Tf by AFA. The assay

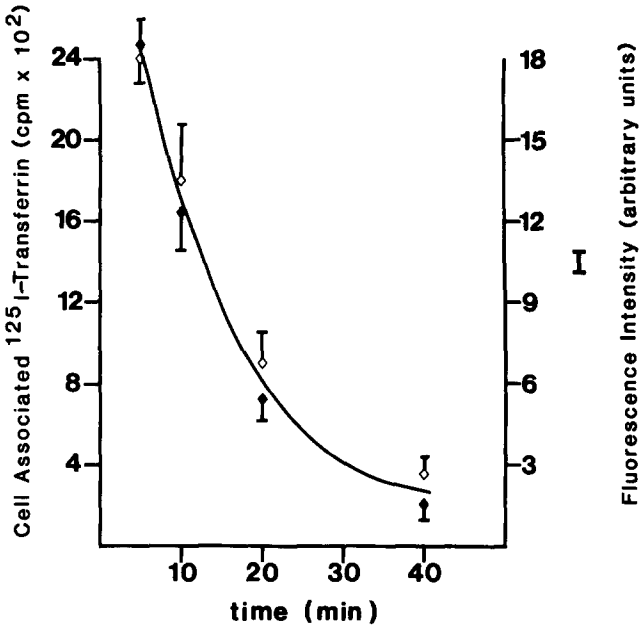


*Figure 3.* [$^{125}$I]Tf and F-Tf release from CHO cells. (♦) [$^{125}$I]Tf release. CHO cells were incubated with [$^{125}$I]Tf (2 μg/ml) for 10 min as described in Materials and Methods and allowed to release [$^{125}$I]Tf for the times indicated at 23°C. Cells associated radioactivity was determined. The values shown have been corrected for nonspecific cell-associated $^{125}$I by addition of 500-fold excess of unlabeled diferric transferrin. (◇) F-Tf release. CHO cells were incubated with 30 μg/ml F-Tf for 10 min, and Tf exocytosis was allowed for the times indicated at 23°C. The cells were rinsed, fixed in 3.7% formaldehyde in PBS, and left in PBS. Cell associated fluorescence was determined using the microscope spectrofluorometer. Fluorescence measurements were made of 15 fields. The numbers reflect the means of 3 dishes/time point. The error bars give the SD of the mean.

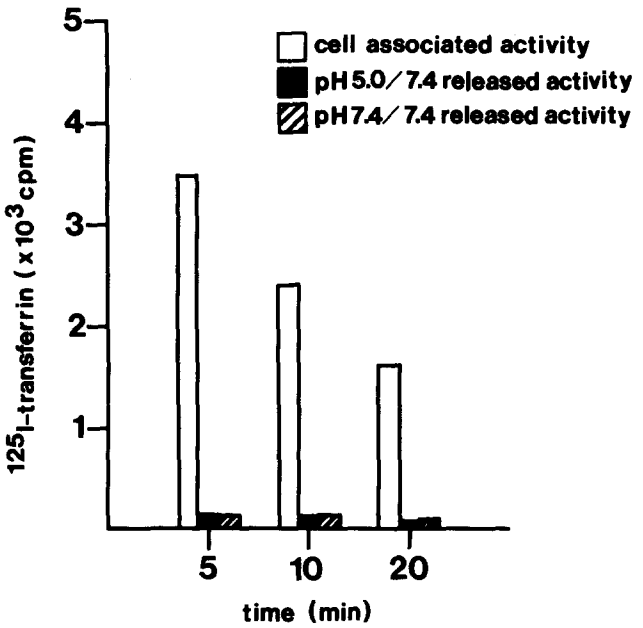


*Figure 4.* Stripping Tf from the cell surface. CHO cells were grown to confluence in 6-well plates. Cells were rinsed with medium 1 and incubated in medium 1 containing 2 μg/ml [$^{125}$I]Tf for 10 min. The cells were rinsed with medium 1, followed by a mild acid/neutral rinse as described in Materials and Methods. Tf exocytosis was allowed for the times indicated. Cells were rinsed with medium 1 followed by a second mild acid/neutral rinse. (■) The amount of [$^{125}$I]Tf released by the acid/neutral treatment was determined. (□) Cells were solubilized in Lowry buffer and cell associated [$^{125}$I]Tf was determined. (▨) Parallel experiments were done in which the second acid wash was replaced by a medium 1 rinse to account for [$^{125}$I]Tf exocytosed during the time required for rinsing. The same amounts of radioactivity were released with an acid/neutral wash as with a medium 1 wash, demonstrating that no [$^{125}$I]Tf was bound to receptors on the surface. The values shown here have been corrected for non-specific cell associated $^{125}$I by addition of 500-fold excess of unlabeled diferric transferrin to parallel competition experiments.

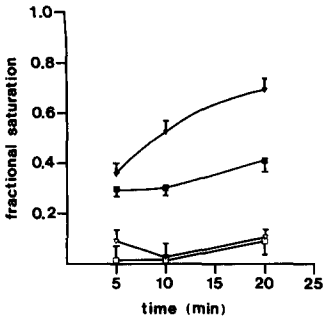


*Figure 5.* Intracellular mixing of endosomal contents. CHO cells were grown to confluence in coverslip bottom dishes, rinsed with medium 1 at room temperature, and incubated in medium 1 containing 30 μg/ml F-Tf for 10 min. The cells were rinsed for 2 min (▽,▼) or 5 min (□,■). This rinse includes a mild acid/neutral rinse. The cells were then incubated in either medium 1, medium 1 containing AFA, or medium 1 containing pre-immune IgG (▽,□) for the times indicated. The cells were rinsed, fixed, and placed in PBS. Methylamine was added to collapse pH gradients. Fluorescence intensity measurements were made using the microscope spectrofluorometer. Fractional saturation was determined from the quenching of fluorescein by the AFA after correction for the quenching efficiency of the antibody. Fluorescence intensity measurements were made on 15 fields of cells/dish. The numbers reflect the mean of 12 dishes/time point. The error bars represent the SEM.

would then indicate that there has been less vesicle fusion than has actually occurred.

To control for cell-to-cell variation in these experiments, Rh-Tf and F-Tf were co-internalized in some experiments. AFA quenches fluorescein fluorescence but has no effect on rhodamine fluorescence. Therefore, by measuring both fluorescein and rhodamine fluorescence for each field of cells, we quantified the amount of mixing by the decrease in the ratio of F/Rh fluorescence. This ratio method provided an internal standard for Tf uptake. Similar results were obtained using this method or using the absolute loss of fluorescein fluorescence.

Since, at steady state, most of the F-Tf is localized in the para-Golgi region of the CHO cell (31), the F/Rh ratio method was used together with digital image processing to determine if quenching was observable in recycling endosomes in the para-Golgi region. As shown in Fig. 6, the para-Golgi is progressively quenched over time, and it contains quenched F-Tf. The time course of quenching measured in this region was parallel to that measured in the whole cell. However, the extent of quenching is somewhat less than was found in the whole cells by photometry. This experimental method may be more useful in determining the time course rather than the absolute extent of quenching. Unlike the pH determinations for which this method has been used frequently (31), these experiments do not provide a method for internal calibration of each experiment. Additionally, these experiments involve the coincubation of F-Tf and Rh-Tf. The competition for the Tf receptor causes a decrease in fluorescence intensity of both the rhodamine and fluorescein signals, making the system more sensitive to any systematic errors in performing background subtractions. Nevertheless, the quenching observed in this experiment does show that mixing follows the same time course within the para-Golgi as it does in the whole cell and that mixing has taken place either within the para-Golgi compartment or before Tf delivery into the para-Golgi.

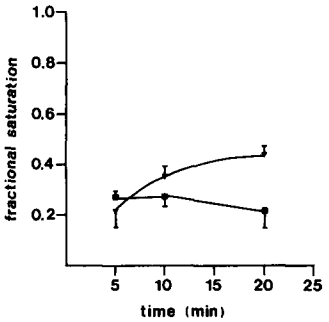


*Figure 6.* Intracellular mixing in the para-Golgi compartment. CHO cells were grown to confluence in coverslip bottom dishes, rinsed with medium 1 at room temperature and incubated in medium 1 containing a 1:1 mixture of F-Tf and Rh-Tf for 10 min. The cells were rinsed for 2 min (▼) or 5 min (■). The rinse began with a mild acid/neutral rinse. The cells were then incubated in either medium 1 or medium 1 containing AFA, for the times indicated. The cells were rinsed, fixed, and placed in PBS with methylamine. The cells were observed by video image intensification fluorescence microscopy and videotaped. The fluorescein and rhodamine images were digitized. The intensity of the concentrated para-Golgi fluorescence was measured for each cell, for both its fluorescein and rhodamine image, and a F/Rh ratio was calculated. The fractional saturation was determined from the quenching of the fluorescein fluorescence in the presence of the AFA. Fractional saturation was measured for 20–40 cells/time point. The numbers shown are the mean of these measurements. The error bars represent the SEM.

Additional studies were done using a cell line which has recently been developed, TRVb-1 cells (19). This is a CHO cell line selected for the lack of endogenous Tf receptor into which the human Tf receptor has been stably transfected. These cells possess greater numbers of Tf receptors than wild type CHO, which allowed us to use a shorter pulse of F-Tf (2–4 min). We tested the possibility that this shorter incubation would increase the synchrony of Tf movement through its recycling pathway, and increase the resolution of our experiments at longer AFA chase times by beginning the antibody chase before a large percentage of the Tf was already being exocytosed. We found that these cells also show fusion of endosomes in our mixing assay (Table I). No significant differences in the time course of mixing were found between the short or long incubations. With short (2 min) or long (10 min) incubation times, the maximum saturation was ~70%. Previous work from this laboratory (19) has shown that Tf receptors have a longer recycling time in TRVb-1 cells than in wild-type CHO cells, giving rise to the possibility that Tf movement to inaccessible compartments would be slower in TRVb-1 cells. This possibility was investigated by performing mixing experiments using either a 2-min or a 5-min interval between F-Tf removal and AFA chase, similar to those done for CHO cells. The quenching did not fall off to the same extent as it did in CHO cells, and was only reduced by 15% after a 10-min chase with AFA in this experiment (data not shown). Therefore a large part of the F-Tf remains accessible to AFA quenching in TRVb-1 cells after 5 min. The slow movement to an inaccessible compartment is consistent with the observation that a similar extent of quenching was seen after a 2-min or 10-min incubation with F-Tf (Table I).

The calculation of fractional saturation used for Fig. 5 and Table I demonstrates the percentage of mixing relative to the remaining cell fluorescence at each time point. Alternatively, we can calculate the amount of quenching relative to the total fluorescein fluorescence in the cell before addition of AFA. This value can be calculated by taking the product of the fractional saturation and the fraction of the initial total cellular fluorescence remaining at each time point. As shown by the

*Table I. Percent Saturation of F-Tf with Subsequently Endocytosed AFA*

| Cell type | Incubation | Interval | Chase period | | |
|---|---|---|---|---|---|
| | *min* | *min* | *min* | | |
| | | | 5 | 10 | 20 |
| CHO | 10 | 2 | 37 ± 3 (28) | 51 ± 4 (30) | 67 ± 4 (21) |
| CHO | 10 | 5 | 24 ± 2 (24) | 25 ± 2 (18) | 41 ± 4 (12) |
| HeLa | 10 | 2 | 3 ± 2 (3) | 22 ± 2 (16) | 42 ± 2 (23) |
| TRVb-1 | 10 | 2 | 21 ± 2 (18) | 50 ± 5 (37) | 73 ± 4 (39) |
| TRVb-1 | 2 | 2 | 24 ± 4 (20) | 49 ± 3 (36) | 66 ± 3 (35) |

Mixing of endosomal contents in HeLa, TRVb-1, and CHO cells was measured. Similar experiments were performed using each cell type. Cells were incubated with F-Tf (and in the case of HeLa cells Rh-Tf as well) for 10 min at 23°C. TRVb-1 cells were incubated for either 2 or 10 min as indicated. The cells were rinsed for 2 or 5 min as indicated, beginning with a mild acid/neutral rinse. The cells were then incubated in either medium 1 or medium 1 containing AFA, for the times shown. The saturation of fluorescein by subsequently internalized AFA was determined by microscope spectrofluorometry as described in Materials and Methods. Measurements were made of 15 fields/dish. The numbers reflect the averages of 12 dishes/time point. The errors given are the SEM.

The values contained in parentheses represent the amount of quenching relative to the total fluorescein fluorescence in the cell before addition of AFA. These values are calculated by taking the product of the fractional saturation and the fraction of the initial total cellular fluorescence remaining at each time point.

values in parentheses in Table I, a large percentage of F-Tf in CHO cells is quenched, and the quenching plateaus between 5 and 10 min. The amount of quenched F-Tf falls off at 20 min as a result of exocytosis of quenched and unquenched F-Tf. In TRVb-1 cells, the maximum quenching plateaus later, between 10 and 20 min. These results are consistent with the earlier findings on the increased recycling time in TRVb-1 cells. The increasing fractional saturation vs. time (Fig. 5) at the later time points may be partly due to the increasing loss of unquenched F-Tf.

Intracellular vesicle fusion has been recently studied by Ajioka and Kaplan (1) in HeLa cells using a method which detects the presence of horseradish peroxidase and [$^{125}$I]Tf in the same vesicle. As judged by light microscopy, HeLa cells do not appear to possess a compartment of similar morphology to the para-Golgi vesicles and tubules found in CHO cells (Fig. 7). In HeLa cells most of the fluorescence remains in discrete vesicles, whereas in CHO cells, the fluorescence from the dense network of small vesicles or tubules is not resolvable as separate vesicles. We investigated vesicle fusion in HeLa cells using the fluorescence quenching method and found, in agreement with Ajioka and Kaplan, that no intracellular mixing occurred at short chase times. After a 5-min incubation with a mixture of F-Tf and Rh-Tf, a 2-min acid/neutral rinse, followed by a 5-min chase with AFA, we found no quenching and, therefore, no mixing. However, at longer chase times (10 and 20 min) the percent saturation reached 42%, and nearly 25% of the initial cellular F-Tf was quenched (Table I).

## Discussion

In this paper we investigated one property of vesicle behavior, by asking whether sequentially internalized vesicles fuse, allowing the mixing of their contents (Fig. 1 *b*) or whether they move through the endocytic pathway without fusing with each other (Fig. 1 *c*). Our results demonstrated that mixing does occur intracellularly in CHO and HeLa cells. We found that F-Tf was accessible to quenching by subsequently endocytosed AFA. When a 2-min interval was allowed between the probes, up to 67% of the Tf was accessible to antibody. A 5-min interval causes a falling off of accessibility, and only 41% mixing occurred. These data are consistent with the model shown schematically in Fig. 1, *a* and *b*. Mixing can occur efficiently within an intermediate compartment, but fusion between compartments formed several minutes apart is not as efficient.

The location of the vesicles where fusion takes place has not been defined, and the morphological identity of the mixing compartment is not known. Our schematic model (Fig. 1 *a*) shows two possible locations where fusion could be occurring. One possibility is that the mixing we observe is taking place in the sorting endosome, which is also involved in the sorting of ligands from their receptors (alpha$_2$-macroglobulin, LDL, etc.). An alternate possibility is that vesicle fusion is occurring at the para-Golgi compartment which contains recycling receptors after they have been segregated from ligands being delivered to lysosomes. It is not known whether this compartment is a stable connected structure (similar to GERL or transreticular Golgi [9, 30]) or a collection of closely packed separate vesicles. However, the para-Golgi is a potential site for receptor pooling, since Tf is collected there within 5 min after endocytosis (31). By restricting the measurement of fluorescence intensity to the para-Golgi compartment in each cell, we found that the time course of quenching was nearly the same as that measured by whole cell photometry, although the extent of quenching appeared to be somewhat less. Thus, much of the Tf/AFA mixing is occurring either within the para-Golgi compartment or before Tf entrance into the para-Golgi. The time dependence of quenching suggests that the para-Golgi is a major site of mixing since nearly all F-Tf would be cleared from the earlier compartment after a 5-min chase, yet quenching plateaus between 5 and 10 min. Our results do not discount, however, that mixing may also be occurring in the earlier sorting endosome as well.

It has recently been shown, using cell-free fusion assays, that newly formed endocytic compartments may fuse with each other. More mature vesicles, however, are not able to fuse. This work has been done both in CHO cells and BHK cells (3, 12). These in vitro data may be related to our finding that fusion falls off as the interval between probes is increased. However, it is not clear at this time whether studies using reconstituted systems are measuring the same types of fusion as we observe in the intact cell. A recent study in whole cells, using an enzyme/substrate assay demonstrates that more mature vesicles are not completely accessible to fusion with newly formed vesicles (22).

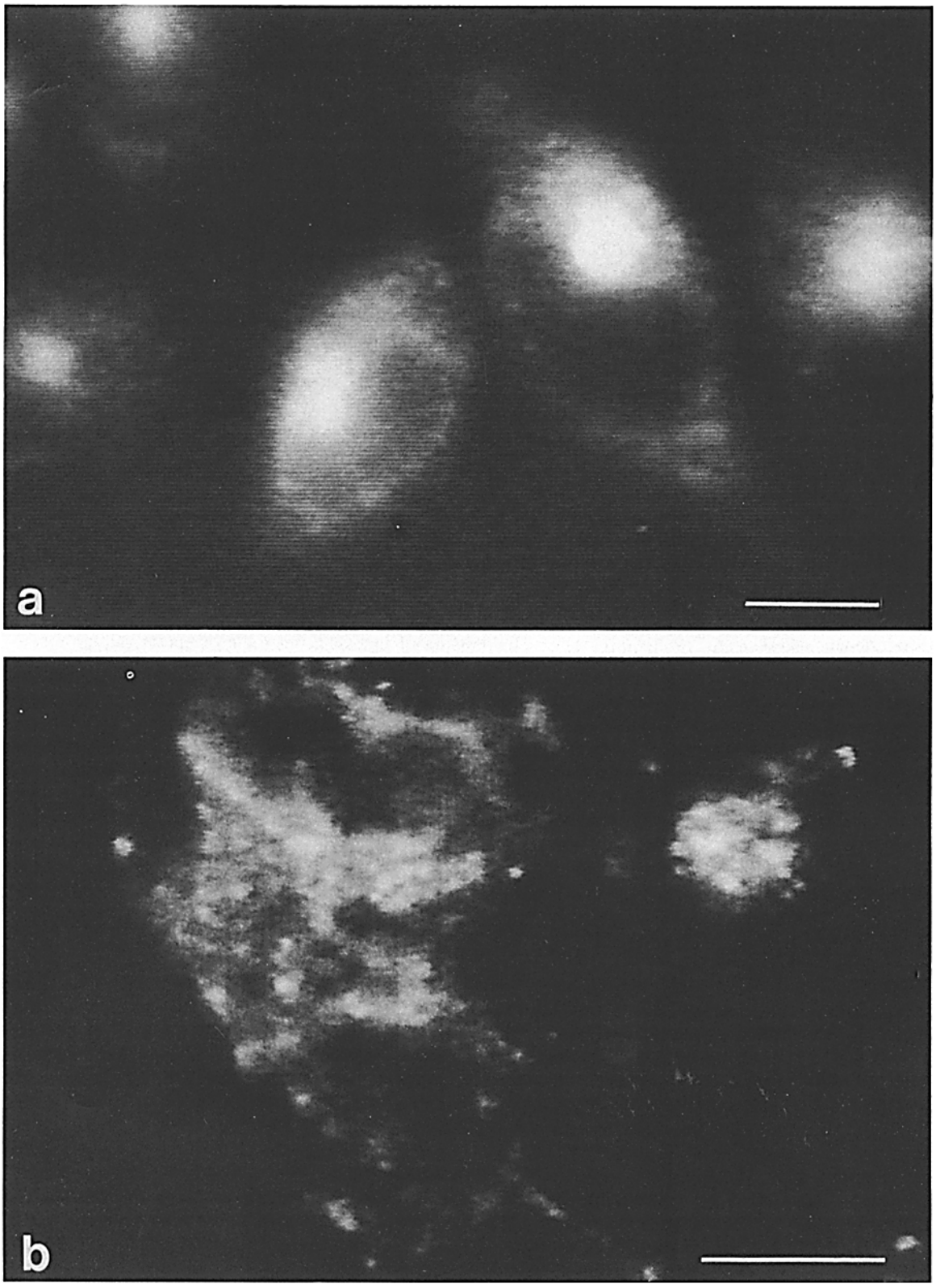


*Figure 7.* (*a*) Fluorescein fluorescence from CHO cells after 10-min uptake of F-Tf, showing Tf concentrated in the para-Golgi region. (*b*) Fluorescein fluorescence from HeLa cells after 10-min uptake of F-Tf, showing a pattern of dispersed vesicular fluorescence. Cells were grown on coverslip bottom dishes, rinsed with medium 1 at room temperature, and incubated with medium 1 containing F-Tf (30 μg/ml). Cells were rinsed with medium 1, fixed, and placed in PBS. Methylamine (30 mM) was added to collapse pH gradients. Images were obtained by fluorescence microscopy with a DAGE/MTI 65 MK II SIT video camera. Bar, 10 μm.

A predominantly whole cell assay has been developed by Ajioka and Kaplan, using HeLa cells, to investigate the question of intracellular mixing (1). This assay also eventually involves fractionating the cell to determine whether fusion has occurred. Using short pulses of their two probes, the authors found no mixing of successively endocytosed Tf in HeLa cells. In agreement with this we found, at the time course used by Ajioka and Kaplan, that there was no mixing in HeLa cells. At later times, we find that considerable quenching becomes evident. It may be that the mixing and sorting compartment appears later on the Tf pathway in HeLa cells than in CHO cells. It must be noted that in HeLa cells Tf is not localized to a compartment morphologically similar to that found in CHO cells. In HeLa cells, the Tf containing compartments appear vesicular and distributed throughout the cytoplasm by fluorescence microscopy, but in CHO cells the compartment is concentrated near the Golgi region and appears tubulovesicular. This difference in cell morphology may reflect a significant difference in the Tf recycling pathway between HeLa and CHO cells.

We have developed a method which should be useful in investigating other questions about vesicle and receptor traffic within cells. This technique should make it possible to look at other receptor/ligand pathways through the cell, such as the pathway taken by alpha$_2$-macroglobulin leading to degradation in lysosomes. F-alpha$_2$-macroglobulin gives a punctate pattern of fluorescence when taken up by CHO cells and is rapidly segregated from transferrin. In combination with the Tf experiments, this should clarify whether the earlier (sorting endosomes) or later compartment is more significantly involved in mixing.

It is clear that segregation (sorting) of ligands from receptors takes place in a sorting endosome. Our results indicate that fusion and mixing events can take place at this point or later along the receptor recycling pathway. It is possible that the para-Golgi or other endosomal compartments could function as differential sorting chambers, by pooling receptors and regulating the return of molecules to the cell surface or to other organelles.

We thank Dr. Timothy McGraw and D. J. Yamashiro for their helpful discussions, and A. Ryan for technical assistance. We thank Dr. Pierre Henkart of the National Institutes of Health (NIH) for his gift of AFA used in preliminary studies, and Yvel Calderon and Dr. David Ennulat for assistance in the preparation of the manuscript.

This work was supported by NIH grant No. DK27083 (FRM) and MSTP grant No. 5T32GM07308 from the NIH.

Received for publication 23 June 1987, and in revised form 16 November 1987.

### *References*

1. Ajioka, R. S., and J. Kaplan. 1986. Intracellular pools of transferrin receptors result from constitutive internalization of unoccupied receptors. *Proc. Natl. Acad. Sci. USA.* 83:6445-6449.
2. Bleil, J. D., and M. S. Bretscher. 1982. Transferrin receptor and its recycling in HeLa cells. *EMBO (Eur. Mol. Biol. Organ.) J.* 1:351-355.
3. Braell, W. A. 1981. Fusion between endocytic vesicles in a cell free system. *Proc. Natl. Acad. Sci. USA.* 84:1137-1141.
4. Dautry-Varsat, A., A. Ciechanover, and H. F. Lodish. 1983. pH and the recycling of transferrin during receptor-mediated endocytosis. *Proc. Natl. Acad. Sci. USA.* 80:2258-2262.
5. Dickson, R. B., J. A. Hanover, M. C. Willingham, and I. Pastan. 1983. Prelysosomal divergence of transferrin and epidermal growth factor during receptor-mediated endocytosis. *Biochemistry.* 22:5667-5674.
6. DiPaola, M., and F. R. Maxfield. 1984. Conformational changes in the receptors for epidermal growth factor and asialoglycoproteins induced by the mildly acidic pH found in endocytic vesicles. *J. Biol. Chem.* 259 9163-9171.
7. Enns, C. A., J. W. Larrick, H. Suomalainen, J. Schroder, and H. H. Sussman. 1983. Co-migration and internalization of transferrin and its receptor in K562 cells. *J. Cell Biol.* 97:579-585.
8. Fahey, J. L. 1967. Chromatographic separation of immunoglobulins. *In* Methods in Immunology and Immunochemistry. Preparation of Antigens and Antibodies. C. A. Williams and M. W. Chase, editors. Academic Press, New York.
9. Farquhar, M. G. 1985. Progress in unraveling pathways of Golgi traffic. *Annu. Rev. Cell Biol.* 1:447-488.
10. Goldstein, J. L., M. S. Brown, R. G. W. Anderson, D. W. Russell, and W. J. Schneider. 1985. Receptor-mediated endocytosis: concepts emerging from the LDL system. *Annu. Rev. Cell Biol.* 1:1-39.
11. Griffiths, G., S. Pfeiffer, K. Simons, and K. Matlin. 1985. Exit of newly synthesized membrane proteins from the trans cisterna of the Golgi complex to the plasma membrane. *J. Cell Biol.* 101:949-964.
12. Gruenberg, J. E., and K. E. Howell. 1986. Reconstitution of vesicle fusions occurring in endocytosis with a cell free system. *EMBO (Eur. Mol. Biol. Organ.) J.* 5:3091-3101.
13. Haigler, H. T., F. R. Maxfield, M. C. Willingham, and I. Pastan. 1980. Dansylcadaverine inhibits internalization of $^{125}$-epidermal growth factor in Balb 3T3 cells. *J. Biol. Chem.* 255:1239-1241.
14. Hanover, C. J. and R. B. Dickson. 1985. Transferrin: receptor-mediated endocytosis and iron delivery. *In* Endocytosis. I. Pastan and M. C. Willingham, editors. Plenum, New York. 131-162.
15. Klausner, R. D., J. van Renswoude, G. Ashwell, C. Kempf, A. N. Schechter, A. Dean, K. R. Bridges. 1983. Receptor-mediated endocytosis of transferrin in K562 cells. *J. Biol. Chem.* 258:4715-4724.
16. Klausner, R. D., J. van Renswoude, C. Kempf, K. Rao, J. L. Bateman, and A. R. Robbins. 1984. Failure to release iron from transferrin in a CHO cell mutant pleiotropically defective in endocytosis. *J. Cell Biol.* 98:1098-1101.
17. Lamb, J. E., F. Rays, J. H. Ward, J. P. Kushner, and J. Kaplan. 1983. Internalization and subcellular localization of transferrin and transferrin receptors in HeLa cells. *J. Biol. Chem.* 258:8751-8757.
18. Lopatin, D. E., and E. W. Voss, Jr. 1971. Fluorescein. Hapten and antibody active-site probe. *Biochemistry.* 10:208-213.
19. McGraw, T. E., L. Greenfield, and F. R. Maxfield. 1987. Functional expression of the human transferrin receptor cDNA in chinese hamster ovary cells deficient in endogenous transferrin receptor. *J. Cell Biol.* 105:207-214.
20. Mellman, I., R. Fuchs, and A. Helenius. 1986. Acidification of the endocytic and exocytic pathways. *Annu. Rev. Biochem.* 55:663-700.
21. Ohkuma, S., and B. Poole. 1978. Fluorescent probe measurement of the intralysosomal pH in living cells and the perturbation of pH by various agents. *Proc. Natl. Acad. Sci. USA.* 75:3327-3331.
22. Roederer, M., R. Bowser, and R. F. Murphy. 1987. Kinetics and temperature dependence of exposure of endocytosed material to proteolytic enzymes and low pH: evidence for a maturation model for the formation of lysosomes. *J. Cell Phys.* 131:200-209.
23. Sklar, L. A., Z. G. Oades, A. J. Jesaitis, R. G. Painter, and C. G. Cochrane. 1981. Fluoresceinated chemotactic peptide and high-affinity antifluorescein antibody as a probe of the temporal characteristics of neutrophil stimulation. *Proc. Natl. Acad. Sci. USA.* 78:7540-7544.
24. Stoorvogel, W., M. J. Geuze, and G. J. Strous. 1987. Sorting of endocytosed transferrin and asialoglycoprotein occurs immediately after internalization in HepG2 cells. *J. Cell Biol.* 104:1261-1268.
25. Tycko, B., C. H. Keith, and F. R. Maxfield. 1983. Rapid acidification of endocytic vesicles containing asialoglycoprotein in cells of a human hepatoma line. *J. Cell Biol.* 97:1762-76.
26. Tycko, B., and F. R. Maxfield. 1982. Rapid acidification of endocytic vesicles containing alpha$_2$-macroglobulin. *Cell.* 28:643-651.
27. van Renswoude, J. K., K. R. Bridges, J. B. Harford, and R. D. Klausner. 1982. Receptor-mediated endocytosis of transferrin and the uptake of iron in K562 cells: identification of a nonlysosomal acidic compartment. *Proc. Natl. Acad. Sci. USA.* 79:6186-6190.
28. Weigel, P. H., and J. A. Oka. 1983. The surface content of asialoglycoprotein receptors on isolated hepatocytes is reversibly modulated by changes in temperature. *J. Biol. Chem.* 258:5089-5094.
29. Wiley, H. S. 1985. Receptors as models for the mechanisms of membrane protein turnover and dynamics. *In* Current Topics in Membranes and Transport. P. A. Knauf and J. S. Cook, editors. Academic Press, New York. 364-412.
30. Willingham, M. C., S. A. Hanover, R. B. Dickerson, and I. Pastan. 1984. Morphological characterization of the pathway of transferrin endocytosis and recycling in human KB cells. *Proc. Natl. Acad. Sci. USA.* 81: 175-179.
31. Yamashiro, D. J., B. Tycko, S. R. Fluss, and F. R. Maxfield. 1984. Segregation of transferrin to a mildly acidic (pH 6.5) para-Golgi compartment in the recycling pathway. *Cell.* 37:789-800.