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# Fluorescence recovery after photobleaching (FRAP) of a fluorescent transferrin internalized in the late transferrin endocytic compartment of living A431 cells: Experiments

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**Abstract**

In this work, we verified that transferrin fluorescently labelled with lissamine rhodamine sulfochloride (Tf-LRSC) is internalized in epidermoid A431 carcinoma cells through the specific endocytic pathway of transferrin. The FRAP of this fluorescent marker internalized in the late compartment of transferrin endocytosis (LCT) was measured in living A431 cells. These experiments showed the presence of an active intracellular transport of Tf-LRSC which can be interpreted by a mechanism involving carrier vesicles budding from stationary vacuoles, saltating along microtubules and fusing with other stationary vacuoles, according to previous video-microscopy observations of a membranous traffic dynamics in these cells, revealed by a gold complex of an Anti-Transferrin Receptor (ATR) (M. De Brabander, R. Nuygens, H. Geerst, C.R. Hopkins, Cell. Motil. Cystoskel. 9 (1988) 30). When the A431 cells were treated with nocodazole or metabolic inhibitors, there remained a residual FRAP which was ascribed to the spontaneous reactivation of the bleached molecules. According to a theoretical result obtained in the companion paper (P. Wahl, F. Azizi, Biochim. Biophys. Acta 1327 (1997) 69–74), we derived the fractional FRAP characterizing the transport process of Tf-LRSC by subtracting the fractional FRAP of the nocodazole-treated cells from the fractional FRAP of the non-treated cells. This FRAP of transport was fitted to a formula derived in that companion paper and based on the mechanism outlined above. From the time constant value determined by this fit, the number of vesicles which fused with a unit of vacuole surface was calculated to be 0.15 $\mu m^{-2}$ $s^{-1}$. The rate value of the fusion of vesicles with vacuoles was divided by two in cells treated by $AlF_4^-$, and increased to 20% in cells treated with Brefeldin A. These results correspond to an homotypic fusion process regulated by an heterotrimeric G-protein. Our work suggests that FRAP can be used to bring information on the transport of membrane components in living eukaryotic cells. © 1997 Elsevier Science B.V.

*Keywords:* Lissamine rhodamine conjugate; Transferrin; Kinetics; Endocytosis; Intracellular transport; Carrier vesicle; Nocodazole; Metabolic inhibitor; Aluminum fluoride; Brefelding A; Reactivation; Bleached molecule; Fluorescence recovery after photobleaching (FRAP)

## 1. Introduction

As mentioned in a companion paper [1], the video microscopy observations show that there is an active

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transport of membranes and of an Antitransferrin Receptor-gold complex (ATR-gold) between the stationary vacuoles forming the late compartment of transferrin endocytosis in A431 cells (LCT) [2].

We investigated the possibility of using the FRAP technique for obtaining information on this transport.

Transferrin conjugated to Lissamine Rhodamine Sulfochloride (Tf-LRSC) can be used as a fluorescent marker of transferrin endocytosis [3–6]. In the present work, we measured the FRAP of Tf-LRSC internalized in the late endosomal system of A431 cells. We found an important FRAP of Tf-LRSC when the cells were surrounded by their culture medium. This FRAP was considerably reduced when the cells were treated with nocodazole or with metabolic inhibitors. These experimental data were analysed according to the theory presented in a companion paper [1].

When we added aluminum fluoride ($AlF_4^-$) or Brefelding A (BFA) to the medium surrounding the labelled cells, we noticed a change of FRAP consistent with the general action of these compounds on the homotypic membranous transport in living cells [7].

## 2. Materials and methods

### 2.1. Reagents

Human apotransferrin was purchased from Sigma or Calbiochem. Lissamine Rhodamine Sulfochloride (LRSC) was obtained from Kodak or Molecular Probe. Sodium azide ($NaN_3$) from Merck and sodium fluoride (NaF) from Prolabo. All other proteins and compounds as well as the culture media were from Sigma.

### 2.2. Cell culture

The human epidermoid A431 carcinoma cells were purchased from the Public Health Laboratory Service (Salisbury, U.K.). A431 cells were cultured as monolayers in plastic flasks at 37°C in a humidified atmosphere with 5% $CO_2$ in DME (Dulbecco's Modified Eagle's Medium) without phenol red, completed with Hepes buffer and sodium bicarbonate at a final concentration of 10 mM plus 10% of calf serum, 1% Insulin, 100 mM oxaloacetic acid and 50 mM pyruvic acid. When islets of cells covered 70% of the culture flask surface, $2 \cdot 10^5$ to $4 \cdot 10^5$ cells were plated on circular glass coverslips placed in Petri dishes and subcultured during 2 or 3 days. Viability tests and mycoplasma controls were regularly performed. Before FRAP, microfluorimetric measurements and confocal microscopy observations, the coverslips with adherent cells were washed two times with DME and then incubated in DME at 37°C during 60 min in the $CO_2$ incubator (preincubation period).

### 2.3. Preparation of diferric transferrin

In order to prepare diferric transferrin (Tf) and Tf labelled with LRSC (Tf-LRSC) we followed a modified protocol provided by Dr. A. Dautry-Varsat. 50 mg of apotransferrin were incubated in 0.5 ml of PBS pH 7.4 (phosphate buffer 10 mM, NaCl 0.14 M) during 10 min at 37°C. A solution of 1.35 mg of ammonium ferric citrate in 0.6 ml PBS (pH 7.4) was slowly added to the apotransferrin solution.

The solution was then incubated for 3 h at room temperature and shaded from light, dialysed over night in a cold room against 1 liter of PBS and dialysed a second time under the same conditions during 6 h. Tf. was over 90% saturated with iron, as shown by measuring the ratio of absorbance $A_{465}/A_{480}$ [8].

### 2.4. Preparation of the fluorescent Tf conjugate

10 mg of LRSC was dissolved in a solvent containing 0.4 ml of fresh dioxane and 0.1 ml of dimethyl formamide. The solution was centrifuged and the LRSC concentration determined by absorbance measures at 565 nm ($\epsilon = 73\,000$ l $M^{-1}$) [9]. Tf was dissolved in bicarbonate 1 M (pH 9), to which five aliquots of a volume containing a weight of LRSC equal to 5% of the protein weight was slowly added. The whole process lasted 50 min, at room temperature in the dark. The reaction was stopped by the addition of $NH_4Cl$ at a final concentration of 50 mM.

The fluorescent protein was purified with a fine G-25 Sephadex column ($0.5 \times 20$ cm) equilibrated with PBS (pH 7.4) at 4°C. Tf-LRSC was stored in aliquots of 20 $\mu$l at $-20$°C. The average number of LRSC residues per protein molecule was equal to 3,

as determined by the absorbance of Tf-LRSC solution at 570 nm and 280 nm. The purity of Tf-LRSC was controlled by $C_{18}$ thin-layer chromatography (RD18-F254s plates from Merck). The elution solvent was made of nine volumes of acetonitrile and one volume of water.

### 2.5. FRAP apparatus

We previously described the FRAP apparatus [10]. The emission wavelength was 514.5 nm, provided by an Argon ion laser (2016-04 Spectra Physics). For the experiments we chose an output power of 80 mW and a bleaching time of 20 ms. The beam was attenuated $6 \cdot 10^3$ times during the prebleaching and the recovery phases. The microscope was equipped with a neofluar air objective of magnitude 40 and a numerical aperture NA = 0.75, providing a laser beam Gaussian radius $W = 1.8\ \mu$m in the observation plane.

The fluorescence beam detected by the photomultiplier was limited by the microscope photometer diaphragm [10], the optical projection of which in the object space was $S_0 = 8\ \mu$m. We calculated the laser beam persistence length and the optical sectioning characteristic length by the following expressions [11]:

$$Z_1 = n\Pi W^2/\lambda$$

$$Z_e = S_0(1+\cos\alpha)/\left(4-(1+\cos\alpha)^2\right)^{1/2}$$

where $n$ is the refractive index of the objective medium, $\lambda$ the wavelength of the laser beam and where $\alpha$ is defined as follows:

$$\alpha = \sin^{-1}(\mathrm{NA}/n)$$

Numerical calculations taking the parameters of our experimental set up into account yield:

$$Z_1 = 20.4\ \mu\mathrm{m}$$

$$Z_e = 11.9\ \mu\mathrm{m}$$

Those values are significantly larger than the half thickness of the A431 cells ($\sim 7.5\ \mu$m). This justifies the use of Eq. (16) of a companion paper [1], for calculating the bleached fluorescence of each marker species. The interval between two time points was 0.33 s and the total time of exploration of a FRAP experiment was 200 s.

### 2.6. Preparation of cells for FRAP measurements

After the preincubation period in DME, as described above, the coverslips partly (about 70%) overlaid with adherent cells were washed two times at 37°C with DME, then incubated 60 min in DME containing 500 nM of Tf-LRSC (labelled cells) or without Tf-LRSC (non-labelled cells) for autofluorescence measurements. The cells were then washed and post-incubated during 60 min (chase period) in DME without any added compound or in DME containing one or two active compounds (3 $\mu$M nocodazole, 5 mM NaF + 100 $\mu$M $AlCl_3$, or 7 or 20 $\mu$g/ml of BFA) as described later. The coverslips were then washed four times, put in a Dvorak Chamber [12] thermostated at 37°C and filled with the post-incubation medium and the whole set was placed on the microscope stage of the FRAP apparatus. In some experiments, the cells were washed with PBS after the preincubation. The incubation with TF-LRSC was made as above except that PBS replaced DMF. The post-incubation was carried out in PBS without any addition, or in PBS containing metabolic inhibitors (2 mM NaF + 20 mM $NaN_3$). All the incubations and the post-incubations were performed at 37°C in a humidified atmosphere with 5% $CO_2$.

### 2.7. FRAP measures

A single FRAP measurement was performed on a given cell and was repeated during one hour on different cells of the same coverslip. Then another coverslip newly post-incubated was measured. Labelled and non-labelled cells were alternately measured. Strong fluctuations of intensity affected each FRAP curve of a single cell. These curves could not be analysed by a continuous function. Therefore curves obtained from a number of labelled cells submitted to one of the treatments described above were summed and corrected for the autofluorescence by subtracting from the sum of curves of labelled cells a sum of FRAP measured on non-labelled cells submitted to the same treatment, except that the incubation medium did not contain any Tf-LRSC. A multiplicative factor was used to take account of the difference between the number of cells measured in each series of experiment, when required.

The fractional FRAP was then computed by the following relation [13]:

$$y(t) = (F(t) - F(0))/(F(-) - F(0)) \quad (1)$$

where $F(\text{-})$ and $F(t)$ were the corrected fluorescence sums of the labelled cells during the prebleaching period and at a time $t$ after bleaching, respectively.

The exponential function defined in a companion paper [1] was fitted to the experimental fractional FRAP by the least-squares method. For that purpose we used a SX386 IBM microcomputer.

### 2.8. Microfluorimetry

Measurements of the binding of Tf-LRSC to A431 cell surface, of the intracellular accumulation of Tf-LRSC and of the chase of internalized Tf-LRSC, were performed by microfluorimetry, with the FRAP apparatus having its bleaching shutter kept permanently closed. The Photon count was measured during one second. The cells were thermostated at 4°C in order to block all physiological processes able to modify the location of Tf-LRSC. The interrogation beam power was 33 $\mu$W.

### 2.9. Confocal microscopy

Confocal microscopy studies were performed with a Bio-Rad MRC-600 microscope controlled by a computer system. We used a krypton laser and a block of filters YHS composed of an interference filter of excitation centered at 568 nm, a dichroic mirror and a barrier filter which rejected light of wavelength smaller than 585 nm on the detecting side of the apparatus.

Cells cultured on a coverslip were preincubated, incubated and post-incubated under the same conditions as for the FRAP experiments. The cells were then fixed by immersion during 60 min at 4°C in a newly prepared solution of 1% of *p*-formaldehyde. The coverslip bearing the cells was put on a microscope slide. A drop of PBS/glycerol (1:1, v/v) containing 1% DABCO (Sigma, St. Louis, MO, USA) was laid down on the cells that were then covered with a second coverslip. Both coverslips being sealed with a mixture of vaseline and lanoline and the preparation was placed on the microscope stage.

## 3. Results

### 3.1. The binding of Tf-LRSC on the surface of the A431 cells

These measurements were performed with microfluorimetry as described in Section 2. The cells subcultured on coverslips were incubated during 90 min at 4°C in DME containing various concentrations of Tf-LRSC. The cells were washed at 4°C and their fluorescence measured at the same temperature. At 4°C the endocytosis was inhibited and Tf-LRSC was bound to the cell surface. These microfluorimetric measurements were repeated on 100 Tf-LRSC labelled cells, then we computed the average of the fluorescence intensity. The mean autofluorescence was also determined on non-labelled cells, under the same conditions. The mean fluorescence of Tf-LRSC bound to a cell was obtained by the difference between the mean fluorescence of labelled and non-labelled cells.

We also determined the fluorescence of A431 cells incubated in DME containing 1 $\mu$M of Tf-LRSC plus 100 $\mu$M of Tf-Fe, during 90 min at 4°C. The average fluorescence measured under these conditions was equal to the average autofluorescence of the cells. This result shows that the fluorescence of Tf-LRSC comes exclusively from molecules bound to the Tf specific binding sites on the A431 plasma membrane.

The variation of the Tf-LRSC fluorescence bound to the cell surface was plotted as a function of the Tf-LRSC concentration in the incubation medium

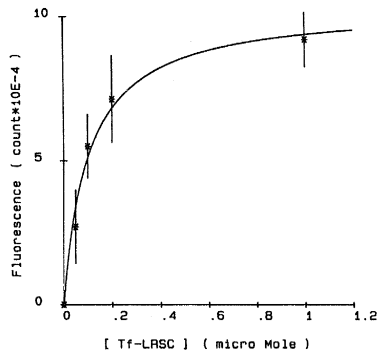



Fig. 1. Binding of Tf-LRSC to the surface of A431 cells. Cells were incubated 90 min at 4°C in DMF containing Tf-LRSC at various concentrations. The fluoresence was measured by microfluorimetry.

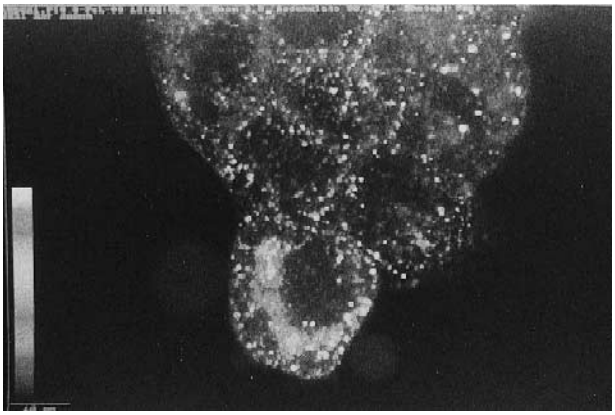
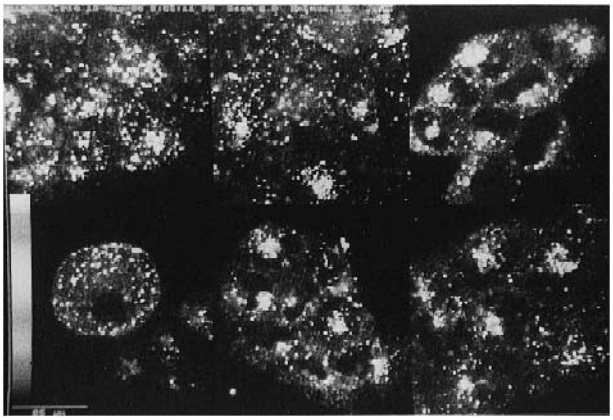


Fig. 2.



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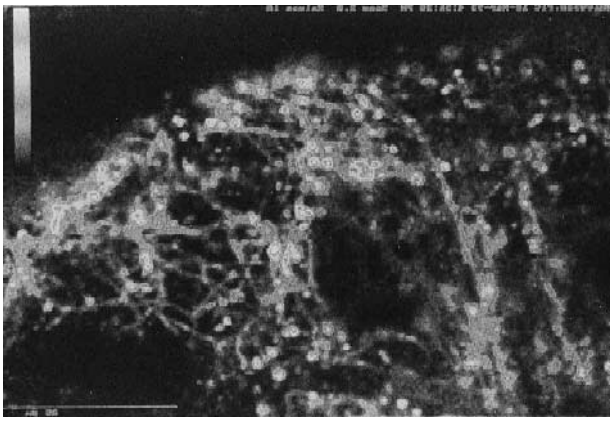

Fig. 2.

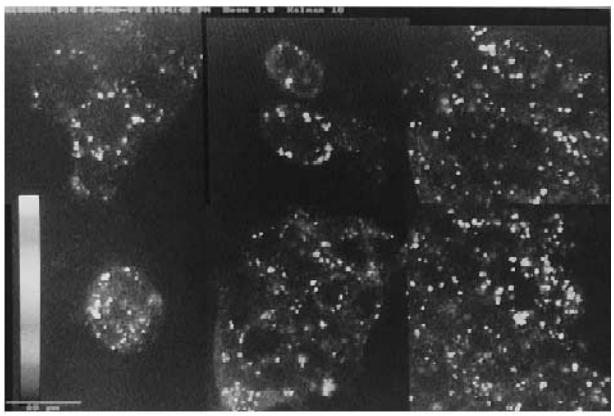

Fig. 2. Confocal microscopy image of A431 cells having endocyted Tf-LRSC. Cells were incubated in DME containing 500 nM of Tf-LRSC during 60 min at 37°C, then post-incubated during 60 min at 37°C in: (1, top left) DME; (2, top right) DME + $AlF_4^-$; (3, bottom left) DME + BFA; (4, bottom right) DME + nocodazole. Finally cells were immersed in a solution of 1% of *p*-formaldehyde during 60 min at 4°C and observed with the confocal microscope.

(Fig. 1). From this curve, the Tf-LRSC binding to the Tf-receptors of the plasma membrane appeared to be saturable. We determined an approximate value of the dissociation constant $K_d$ equal to 80 nM. This value was somewhat higher than the values of the binding of $^{125}$I-Tf-Fe to several cell types in which $K_d$ varied from 2 nM to 30 nM [13,14]. Labelling Tf-Fe with LRSC might cause a decrease in its affinity for the Tf-receptors.

### *3.2. Internalization of Tf-LRSC in A431 cells; confocal microscopy*

A431 cells were incubated during 60 min at 37°C in DME containing 500 nM of Tf-LRSC and immediately fixed or post-incubated during 60 min at 37°C in DME and then fixed. The cells were then examined with the confocal microscope at different cross-sections. Tf-LRSC fluorescence appeared punctate, a typical image of fluorescent compounds internalized in intracellular vacuoles.

By confocal microscopy we also investigated A431 cells treated with compounds known to influence the morphology and the dynamic properties of membrane organelles. In these cases, cells were post-incubated during 60 min in DME containing 5 mM NaF + 100 $\mu$M $AlCl_3$ ($AlF_4^-$) or BFA (7 or 20 $\mu$g/ml) or nocodazole (3 $\mu$M).

In all cases the fluorescence was localized in vacuoles more of less scattered in the cytoplasm. The vacuoles of $AlF_4^-$-treated cells were more strongly aggregated in the perinuclear area than those of non-treated cells (Fig. 2). The same effect had previously been observed with NRK cells in which the compartment containing Tf had been labelled by immunofluorescence [7]. In cells treated with BFA, a network of fluorescent tubules could be seen (Fig. 2). This agrees with previous fluorescence microscopy observations in other cell types and particularly in NRK cells [7]. In cells treated with nocodazole, the fluorescent vacuoles were quite dispersed throughout the cytoplasm. The fluorescent images of cells treated with $AlF_4^-$ or BFA, then incubated during 60 min at 37°C with $AlF_4^-$ + nocodazole or BFA + nocodazole, were similar to those of cells treated with nocodazole alone (Fig. 2). These observations suggest that the aggregation of vacuoles in $AlF_4^-$-treated cells as well as the tubular structure in BFA-treated cells, required the presence of microtubules.

### *3.3. Internalization of Tf-LRSC in A431 cells; microfluorimetry*

The A431 cells were incubated at 37°C in DME with 500 nM of Tf-LRSC and extensively washed at 4°C. Some of these cells were then incubated in PBS containing 50 $\mu$g/ml of trypsin during 60 min at 4°C. The trypsin treatment of the cells did no change their average fluorescence. This means that the fluorescence of the surface bound Tf-LRSC was negligible.

We also compared the fluorescence of cells incubated in DME containing 500 nM of Tf-LRSC in presence and in absence of 50 $\mu$M of Tf-Fe at 37°C during 60 min. We found that the average fluorescence of cells incubated in presence of Tf-Fe was less than 12% of the fluorescence measured in cells incubated in absence of the non-labelled protein. This result meant that over 88% of Tf-LRSC was internalized by means of Tf-receptor.

### *3.4. Chase of Tf-LRSC internalized in A431 cells*

A431 cells were incubated in DME containing 500 nM of Tf-LRSC during 60 min at 37°C, extensively washed at 4°C and post-incubated at 37°C during various time intervals in DME containing 50 $\mu$M of Tf-Fe, in order to facilitate the dissociation of Tf-LRSC from the cell surface receptors [15]. The cells were then washed with DME and their fluorescence measured at 4°C by microfluorimetry.

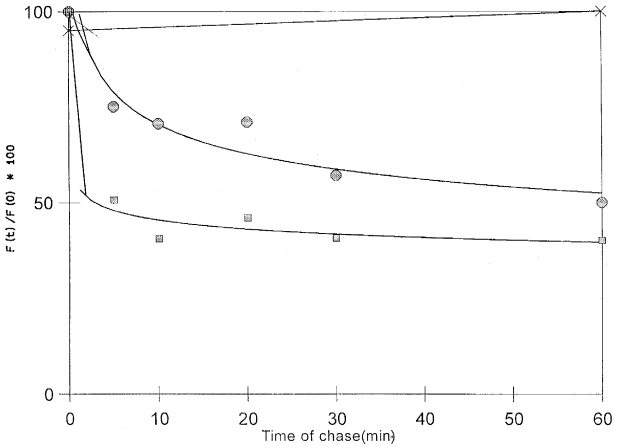


Fig. 3. Kinetics of chase of Tf-LRSC internalized in A431 cells. $F(t)/F(0)$ is the relative fluorescence at the time $t$ of chase. Fluorescence of Tf-LRSC was measured on cells after the following treatments. □, Incubation in DME containing 500 nM of Tf-LRSC during 60 min at 37°C and post-incubation in DME containing 50 $\mu$M of Tf-Fe during various times at 37°C. ○, Incubation in PBS containing 500 nM of Tf-LRSC during 60 min at 37°C and post-incubation in PBS containing 50 $\mu$M of monensin plus 50 $\mu$M of TF-Fe. ×, Incubation in PBS containing 500 nM of Tf-LRSC during 40 min plus incubation in the same medium containing metabolic inhibitions (2 mM of NaF + 20 mM of $NaN_3$) during 20 min and post-incubation in PBS containing the same concentration of inhibitors during 0 or 60 min at 37°C.

The kinetics of Tf-LRSC chase was plotted on Fig. 3. Each point on the curve represented the average value of two experiments. It can be seen on Fig. 3 that the Tf-LRSC fluorescence rapidly decreased during about 10 min of chase, with $t_{1/2} = 3$ min. After those 10 min of chase, the fluorescence was reduced to 45% of its initial value. Then the fluorescence remained equal to 40% of its initial value after 50 min of chase. Even after 120 min of chase in DME containing 100 $\mu$M of Tf-Fe, we could not detect any significant change of the Tf-LRSC fluorescence of cells. Similar chase profiles have been measured for $^{125}$I-Tf [15–19].

The rapid decrease of Tf-LRSC fluorescence during the first minutes of chase must be ascribed to the

exocytosis of the internalized Tf-LRSC. It has been shown by other authors that the iron contained in ferrotransferrin bound to its receptors dissociates itself from the protein at the acid pH of endosomes [18,19]. While the apotransferrin remains associated with its receptors, and the complex is recycled back to the cell surface where the apotransferrin is released in the extracellular medium. Thus our microfluorimetric measurements show that Tf-LRSC has the same behaviour as Tf. In order to confirm this conclusion, we performed the experiments of chase in presence of metabolic inhibitors or monensin.

Cells were incubated at 37°C in PBS containing 500 nM of Tf-LRSC during 40 min, then further incubated for 20 min in the same medium containing the following metabolic inhibitors: 2 mM NaF + 20 mM $NaN_3$. The cells were washed extensively with PBS at 4°C, then post-incubated in PBS containing the same concentration of metabolic inhibitors plus 50 $\mu$M of Tf-Fe, during 60 min at 37°C. The cells were again extensively washed in PBS at 4°C and their fluorescence measured by microfluorimetry at the same temperature. The fluorescence intensity did not decrease during the 60 min period of chase (see Fig. 3). This result agreed with the work of Podbilewicz and Mellman [20] who showed that Tf exocytosis was blocked by metabolic inhibitors.

We also studied the effect of monensin on Tf-LRSC exocytosis. Cells were incubated in PBS containing 500 nM of Tf-LRSC at 37°C during 60 min, then post-incubated in PBS with 50 $\mu$M of monensin and 50 $\mu$M of Tf-Fe during various time intervals. The cells were then washed and their fluorescence was measured as described above. As it can be seen on Fig. 3, the rate of Tf-LRSC exocytosis in monensin-treated cells was significantly slower than in non-treated cells. These results agreed with the measurement of $^{125}$I-Tf exocytosis by Ciechanover et al. (1983) [19] who showed that monensin neutralizes the endosome pH and therefore inhibits the formation of apotransferrin. The ferrotransferrin-receptor complex is then recycled back to the cell surface and reendocytosed. Therefore monensin induces a decrease of the exocytosis rate of $^{125}$I-Tf exocytosis.

### *3.5. FRAP of Tf-LRSC endocytosed in A431 cells surrounded by their culture medium*

FRAP was measured on 173 A431 single cells as described in Section 2. After having internalized Tf-LRSC, these cells were post-incubated during 60 min in DME without added compound.

Each FRAP measurement recorded on a single cell presented strong fluctuations of fluorescence intensity and there was considerable variation between the FRAP of different cells. A strong decrease of these random fluctuations was found in the curve representing the sum of the 173 individual measurements. This sum of FRAP curves was corrected for cell autofluorescence as described in Section 2. The resulting corrected Tf-LRSC FRAP curve was drawn in Fig. 4 and the corresponding fractional fluorescence was represented in Fig. 5. We reported some avarage parameters of FRAP in Table 1.

Table 1
Prebleaching fluorescence and bleached fraction of FRAP measures on Tf-LRSC endocyted in A431 cells

| Surrounding medium | $\langle F(\text{-})\rangle \pm \sigma_N$ ($N$) | $\langle F_A(\text{-})\rangle \pm \sigma_N$ ($N$) | $P$ (%) |
|---|---|---|---|
| DME[a] | 1780 ± 43 (173) | 241 ± 12 (72) | 44 |
| DME + $AlF_4^-$[b] | 1952 ± 59 (121) | 313 ± 12 (60) | 55 |
| DME + BFA[c] | 1620 ± 27 (149) | 305 ± 11 (34) | 42 |
| DME + nocodazole[d] | 2179 ± 118 (75) | 345 ± 27 (35) | 48 |

$\langle F(\text{-})\rangle$, mean prebleaching Tf-LRSC fluorescence per cell, taken over $N$ cells. $\langle F_A(\text{-})\rangle$, mean prebleaching autofluorescence. $\sigma_N$, standard error of the mean fluorescence. $P$ (%), amount in per cent of bleached fluorescence at the end of the bleaching phase of labelled cells. The mean fluorescences are expressed in number of photoelectrons detected per 0.33 s (the interval between two consecutive time points in a FRAP record).

[a] Dulbecco's Modified Eagle's Medium (DME).

[b] DME + 5 mM NaF + 100 $\mu$M $AlCl_3$.

[c] DME + 7 $\mu$g/ml BFA.

[d] DME + 3 $\mu$M nocodazole.

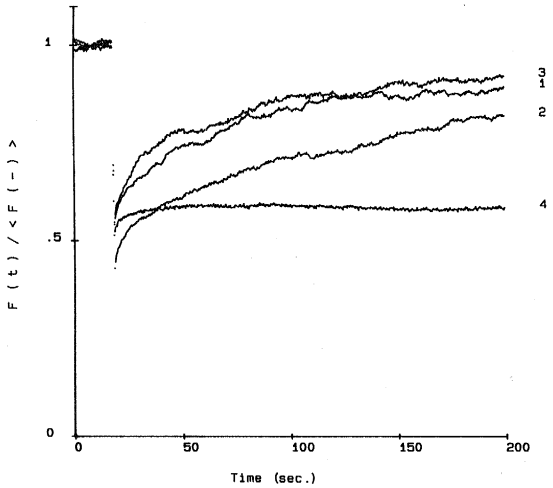


Fig. 4. FRAP of Tf-LRSC internalized in A431 cells surrounded by the following media: (1) DME; (2) DME + $AlF_4^-$; (3) DME + BFA; (4) DME + nocodazole. The meaning of these abreviations are given in the legend of Table 2.

### *3.6. FRAP of Tf-LRSC endocytosed in A431 cells post-incubated with the culture medium containing nocodazole or metabolic inhibitors*

When Tf-LRSC labelled cells were post-incubated in DME containing 3 $\mu$M of nocodazole, the fluctuations affecting the FRAP of a single cell surrounded by the post-incubation medium were much smaller than the fluctuations of the FRAP of non-treated cells. As before, the FRAP curves of individual cells were summed and this sum was corrected for the cell autofluorescence. The resulting corrected FRAP curve was drawn on Fig. 4 and the corresponding fractional FRAP on Fig. 5.

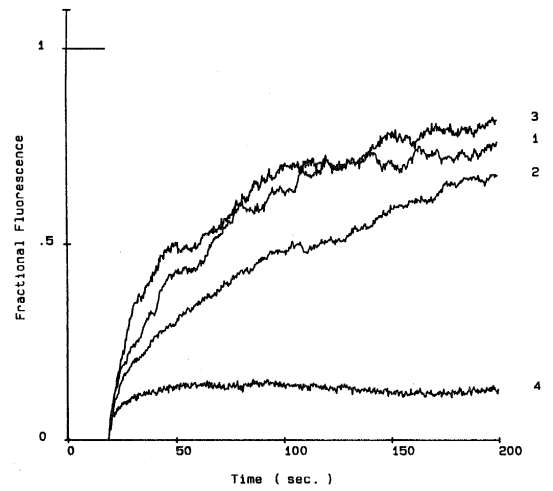


Fig. 5. Fractional FRAP of Tf-LRSC internalized in A431 cells. These curves were calculated from the curves of Fig. 4 by applying Eq. (1). The numerical index of each curve refer to the same media as in Fig. 4.

If we substituted DME by PBS during the incubation, the post-incubation and the FRAP measurements, the sum of the FRAP of individual cells did not change (data not shown). If in the post-incubation and measurement media, metabolic inhibitors (2 mM NaF + 20 mM $NaN_3$) were added, the sum of the FRAP curves of single cells was similar to the sum of the FRAP of nocodazole-treated cells (data not shown).

As shown on Fig. 4 and Fig. 5, nocodazole (as well as metabolic inhibitors) bring about an important decrease of the fluorescence recovery of Tf-LRSC endocytosed in A431 cells. This result suggests that an active intracellular Tf-LRSC transport depending on the energy supply and on the microtubule integrity of cells contributes to the FRAP of the cells surrounded by their normal culture medium.

There remains, however, in cells treated by nocodazole (or metabolic inhibitors), a FRAP which we must attribute to some intravacuolar processes.

We further investigated the nocodazole-treated cells and found that their fractional FRAP was the same at 17°C and 37°C. These results indicate that the residual FRAP in nocodazole- (or metabolic inhibitors)-treated cells comes from a reversal of the photochemical bleaching of Tf-LRSC as will be discussed later in this work.

### *3.7. FRAP of the intracellular transport of Tf-LRSC endocytosed in A431 cells*

Coming back to non-treated cells, we concluded that two independent processes contributed to their FRAP, namely an active intracellular transport of the organelles containing Tf-LRSC and an intravacuolar process of Tf-LRSC molecules. This last process was very probably a spontaneous reactivation of the bleached marker molecules. The intravacuolar process was the only one which contributed to the FRAP of cells treated by nocodazole. We approximated the FRAP of nocodazole-treated cells by an exponential function, the parameters of which are given in Table 2. The time constant is equal to 3.5 s, a much shorter

value than that of the time constant of the transport process (see below). We obtained the fractional FRAP of the intracellular transport of Tf-LRSC by subtracting the fractional FRAP measured in nocodazole-treated cells from the fractional FRAP of the cells surrounded by DME without nocodazole (see the companion paper [1]). The resulting curve is drawn in Fig. 6.

As discussed in the companion paper [1] and later in this paper, we assumed that the active transport of Tf-LRSC proceeded from a mechanism involving carrier vesicles budding from stationary vacuoles, saltating rapidly and finally fusing with other vacuoles.

We then fitted the function defined by Eq. (29) of the companion paper [1] to the experimental FRAP of transport, and determined the values of $L_1$ and $t_1 = 1/h_1$ given in Table 2.

### 3.8. FRAP of Tf-LRSC labelled A431 cells post-incubated in DME containing $AlF_4^-$

In order to obtain more information on the origin of the FRAP of Tf-LRSC internalized in A431 cells, we examined the effect of two compounds ($AlF_4^-$ and BFA) known to modify the fission–fusion processes of membranous organelles.

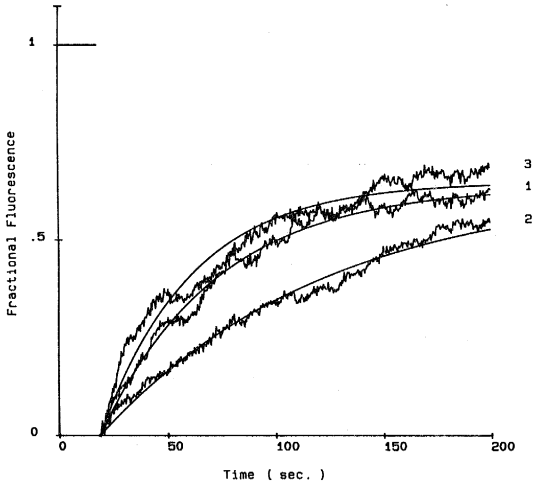


Fig. 6. Fractional FRAP of the active transport of Tf-LRSC internalized in A431 cells. The fluctuating curves 1, 2, 3 were obtained by subtracting the curve 4 from the curves 1, 2, 3 of Fig. 5, respectively. The smooth curves represents the function $y(t) = L_1(1-\exp(-t_1/t))$ derived in a companion paper (Eq. (29) of [1]), the fitted parameters $L_1$ and $t_1$ having the values given in Table 2 of the present paper.

Table 2
Tf-LRSC internalized in A431 cells: parameters of the exponential function fitted to the experimental fractional FRAP of the transport of Tf-LRSC and of the spontaneous reactivation of the bleached markers

| Surrounding medium | $t_1$ (s) | $L_1$ | $\tau_f$ ($s^{-1}$ $\mu m^{-2}$) |
|---|---|---|---|
| DME | 54 (a) | 0.64[a] | 0.15[a] |
| DME + $AlF_4^-$ | 104 (a) | 0.64[a] | 0.07[a] |
| DME + BFA | 42 (a) | 0.65[a] | 0.19[a] |
| DME + nocodazole | 3.5 (b) | 0.11[b] | – |

The fitted function is $y(t) = L_1(1-\exp(-t/t_1))$ obtained in the companion paper [1] for the active transport of the marker by vesicles between stationary vacuoles (Eq. (29) of this paper [1]). The compositions of the surrounding media are given in Table 1. $t_1$, time constant of the exponential function. $L_1$, fraction of the transported fluorescence[a], or of the reactivatable fluorescence[b]. $\tau_f$, number of vesicles which fuse during 1 s with 1 $\mu m^2$ of vacuole surface, assuming that the vesicle is spherical with a diameter of 0.2 $\mu$m and computed by applying Eq. (9) of the companion paper [1].

[a]Fractional FRAP of transport of Tf-LRSC.

[b]Fractional FRAP of the spontaneous reactivation of bleached molecules.

A series of 121 single FRAP measurements was performed on individual labelled cells post-incubated with DME containing 100 $\mu$M $AlCl_3$ and 5 mM NaF. It has been shown that aluminum fluoride molecules of various stoichiometry were formed in this mixture and that the most biologically active of them was probably $AlF_4^-$ [21].

The sum of these individual FRAP measurements corrected for cell autofluorescence is drawn in Fig. 4, and the corresponding fractional FRAP is given in Fig. 5 where both curves can be compared with the FRAP sum curves of non-treated cells. One can see that the rate of fluorescence recovery decreases when the cells have been treated by $AlF_4^-$. When labelled cells were post-incubated with 1 mM of deferroxamine or 5 mM of NaF plus 1 mM of deferroxamine, the FRAP was identical to that of non-treated cells (data not shown). These experiments showed that $Al^{3+}$ ions were necessary, and NaF alone not sufficient, to induce the change in FRAP. Deferroxamine chelates any trace of $Al^{3+}$ [46] which might be present as an

impurity in the different reagents or might have been extracted by NaF from the glassware [47].

Finally we measured the FRAP of A431 cells post-incubated with DME containing 100 $\mu$M $AlCl_3$ plus 5 mM NaF, and then post-incubated a second time in the same medium in which was added 3 $\mu$M of nocodazole. The fractional FRAP of these cells was identical to the fractional FRAP of cells submitted to a single post-incubation in DME containing 3 $\mu$M of nocodazole alone. Consequently, we took the sum of both fractional FRAP curves obtained on cells treated with nocodazole (in presence and in absence of $AlF_4^-$) as the reference curve $y_{No}$ [1]. This curve was represented in Fig. 3 and was used for computing the fractional FRAP of transport in cells surrounded by DME according to Eq. (28) of the companion paper [1]; this fractional FRAP was drawn in Fig. 6. The same curve $y_{No}$ was used to determine the FRAP (drawn in the same figure) of transport in $AlF_4^-$-treated cells. The function defined by Eq. (29) of the companion paper [1] was fitted to this fractional FRAP of transport to provide the parameters of the fission–fusion process given in Table 2.

### *3.9. FRAP of Tf-LRSC labelled A431 cells post-incubated in Brefeldin A*

In a last series of experiments, Tf-LRSC labelled and non-labelled A431 cells were post-incubated in DME containing 7 $\mu$g/ml of Brefeldin A (BFA). We measured the FRAP of 149 of these labelled cells. The sum of these FRAP curves, corrected for cell autofluorescence, is given in Fig. 4, and the corresponding fractional FRAP in Fig. 5.

As seen above, the morphology and the cytoplasmic distribution of the fluorescent vacuoles of labelled cells treated with BFA and then with nocodazole, were the same as those of cells treated with nocodazole alone or of cells treated with $AlF_4^-$ and nocodazole. We then obtained the FRAP of the transport process of BFA-treated cells by applying the subtraction defined in Eq. (28) of the companion paper [1] in which $y_{No}(t)$ was the experimental function determined above. The fractional FRAP of transport resulting from this subtraction is drawn in Fig. 6. The exponential function expressed by Eq. (29) of the companion paper [1] was fitted to the experimental fractional FRAP. The values of both parameters of the fission–fusion process of the endosomal system in BFA-treated cells were then obtained (see Table 2).

## 4. Discussion

In eukaryotic cells, carrier vesicles transfer various kinds of macromolecules between stationary organelles [22–25]. Our aim in this work was to determine how the FRAP technique could provide information on the dynamics of the intracellular transports. We chose to investigate the late endocytic compartment of transferrin in A431 cells (LCT), defined in both introductions of a companion paper [1] and the present paper. We took the conjugate of transferrin with Lissamine Rhodamine Sulfochoride (Tf-LRSC) as a fluorescent marker of the LCT compartment.

With the microfluorimetry technique, we found that Tf-LRSC was internalized by transferrin receptors, as already reported by others [4–6].

After 1 h of incubation in the culture medium (DME) containing Tf-LRSC, A431 cells rapidly exocytosed a fraction of the fluorescent markers, while they were very slowly eliminating another fraction of them. By measuring the exocytosis kinetics of transferrin labelled with radioactive iodine, other authors observed that a fraction of the endocytosed marker slowly recycled itself [15,17]. By confocal microscopy, we found that the slowly recycling Tf-LRSC fraction was included in vacuoles dispersed throughout the cytoplasm of A431 cells, and sometimes aggregated preferentially in the perinuclear area. Similar microscopy images had been previously seen in various cell lines having endocytosed either fluorescein-labelled transferrin or non-labelled transferrin revealed by immunofluorescence [26,27]. The vacuoles accumulating Tf-LRSC during the slow recycling process of endocytosis were identified as forming the late endocytic compartment of transferrin (LCT) [28,29].

Ultrastructural studies of A431 cells, labelled with Tf-LRSC or ATR-gold complexes, showed that the LCT compartment was made of MVB [30,31].

The FRAP experiments described in the present work precisely concerned Tf-LRSC internalized in

the LCT of the A431 cells. These conditions were realized by incubating the cells during 1 h in DME containing Tf-LRSC. The labelled cells were then post-incubated (chased) during 1 h in DME without any marker, and the FRAP measurements were performed in the post-incubation medium.

In the following discussion, we call FRAP the sum of 100 to 200 FRAP measurements performed on single labelled cells identically prepared, this sum being corrected for cell autofluorescence.

We found that the FRAP of cells surrounded by DME alone was relatively large. When we added nocodazole or metabolic inhibitors in the chase and measurement media, the FRAP was considerably reduced, which showed that the FRAP of Tf-LRSC in non-treated cells contained an important contribution of an active intracellular transport. There remained, however, a residual FRAP in nocodazole- or metabolic inhibitors-treated cells, which must be ascribed to some intravacuolar processes. These intravacuolar processes may include the following possibilities: the marker diffusion of translation and rotation [32,33], the spontaneous reversal of the photochemical reaction [33–35] or some chemical exchange reactions [36]. We found that the FRAP of nocodazole-treated cells was independent from temperature. Consequently the FRAP could not be attributed to any of the above mentioned mechanism except the spontaneous reactivation of the bleached molecules, generally called a reversible photochemical reaction. Other authors measured the FRAP of fluorescent protein conjugates bound to the cytoplasmic side of the plasmic membrane or adsorbed on glass in a deoxygenated environment [34,35]. They found this type of reactivation of bleached molecules with a time constant of the order of a second. We found that the time constant of the FRAP measured in nocodazole-treated cells was equal to 3.5 s.

In the FRAP of cells surrounded by DME without nocodazole, we distinguished two contributions: one proceeding from an intracellular transport and the other proceeding from a spontaneous reactivation of the bleached marker, reactivation which brought about the FRAP of nocodazole-treated cells. In a companion paper [1] we established that the fractional FRAP due to the contribution of two simultaneous processes was the sum of the fractional FRAP of each process occurring separately, provided that the values of the characteristic time of these processes were very different from each other [1]. By subtracting the fractional FRAP of nocodazole-treated cells from the fractional FRAP of the non-treated cells, we obtained a curve to which we could fit an exponential function of time constant equal to 52 s. This value was definitely larger than the time constant value of the FRAP of nocodazole-treated cells. Consequently, the curve derived by this subtraction represented the fractional FRAP of the intracellular transport process of Tf-LRSC [1].

Our interpretation of the FRAP of transport was grounded on the VM observations of ATR-gold in A431 cells. We ascribed the intracellular Tf-LRSC active transport to a mechanism involving successively the following three steps: budding of small vesicles from stationary vacuoles (MVB), rapid saltations of these vesicles along microtubules, and fusion with other distant stationary vacuoles. Assuming that the membranous exchange between vesicles and vacuoles was in a steady state (see the companion paper [1]), we ascertained that the formation rate of vesicles by fission was equal to the rate of their fusion with vacuoles. We assumed then that the travelling velocity of the vesicles was high compared to the rate of vesicles fusion, and we showed that the mathematical expression of the FRAP of transport was an exponential function with a time constant inversely proportional to the rate of the vesicle fusion with the vacuoles [1]. According to the VM observations, the average velocity of the vesicles is about 1 $\mu$m $s^{-1}$ [2] so that the average length of time spent by the vesicles to cross the laser beam (of radius 1.8 $\mu$m) is 1.8 s, a negligible time compared to the experimental value of the characteristic time of the FRAP of Tf-LRSC transport measured here ($\sim$ 52 s). This short time of 1.8 s justifies our considering the FRAP of transport as controlled by the fission–fusion processes. Knowing that the vesicular diameter is equal to 0.2 $\mu$m [2], we applied the Eq. (9) of the companion paper [1] and we calculated the number of vesicles which fused per unit of vacuole surface; we found it equal to 0.15 $\mu m^{-2}$ $s^{-1}$. According to the EM observations, the average diameter of a MVB vacuole is equal to 1.5 $\mu$m [30,31]. By applying Eq. (7) of the companion paper [1], we found that there were 60 vesicles which fused with one vacuole during one minute.

Papers reporting measurements of the rate of vesicular fusion with stationary organelles in vivo, are scarce. Using quantitative fluorescence microscopy, Dunn et al. (1989) [37] measured the fluorescence increase with time of a single sorting endosome in CHO cells having internalized the Rhodamine conjugate of transferrin. These authors found that there were more than four endocytic vesicles which fused per minute with one stationary endosome. According to our FRAP measurements, the number of vesicles which fused per minute with one MVB of A431 cells was then 15-times higher than the lower limit determined by Dunn et al. (1989) [37]. We must, however, observe that the cell lines and the endocytic compartments involved in both works, ours and theirs, are different.

When $AlF_4^-$ was present in the medium surrounding the labelled A431 cells, the time constant of the FRAP of Tf-LRSC transport was multiplied by two, relatively to its value for non-treated cells. If the change of the spatial distribution of vacuoles, induced by $AlF_4^-$ in the cell, influenced the value of the time constant of the transport FRAP, this change should also significantly modify the value of the apparent fraction of the transported Tf-LRSC, $L_1$. Since this was not the case, we concluded that $AlF_4^-$ caused a 50% decrease in $\tau_f$, the number of vesicles which fused with the vacuoles in one second per unit of vacuole surface. $AlF_4^-$ specifically replaces GTP in the nucleotide site of heterotrimeric G-proteins [21] which intervene in the regulation of fission–fusion processes, during the transfer of components between organelles of the endocytosis and exocytosis pathways [22,37]. $AlF_4^-$ inhibits the transport between organelles in vivo and in vitro [38–40]. Our results agree with this general effect of $AlF_4^-$.

We found that BFA did not modify the apparent fraction $L_1$ of Tf-LRSC transported in A431 cells. On the other hand, the time constant of the BFA-treated cells was 20% lower than the time constant of the non-treated cells. This result must then be ascribed to an increase in $\tau_f$, by 20%. BFA is a macrocyclic lactone synthetized by a variety of fungi. This compound does not significantly modify the intracellular homotypic membranous traffic, implying fusion between like organelles (as for example fusion between endosomes or between elements of the endoplasmic reticulum). On the other hand, BFA inhibits the membranous heterotypic traffic involving transport between different homotypic organelles [7,25]. Our FRAP measurements, therefore, indicate that the fission–fusion processes between vesicles and stationary vacuoles are homotypic, which we expected.

With fluorescence videomicroscopy, Hopkins et al. (1990) observed Hep2 carcinoma cells having previously been incubated for more than one hour in a culture medium containing transferrin conjugated to Texas Red [41]. In these cells, they saw fluorescent MVB (vacuoles) moving very slowly by saltation, which suggests that saltation of MVB might also contribute to the FRAP of Tf-LRSC internalized in A431 cells. On the other hand, VM of A431 cells let appear that the velocity of endosomes containing ATR-gold and moving by saltations varied according to the size of these organelles: the larger they were, the slower they moved [2]. Therefore, it may be expected that the velocity of the large tubular endosomes induced by BFA should be considerably reduced if not null. On the contrary, as above mentioned, our FRAP measurements indicated a small increase in the transport rate when the A431 cells were treated by BFA. We concluded that the saltations of MVB cannot play an important role for the transport of transferrin in the LCT of A431 cells.

The present state of our experimental studies does not allow us to give an exact meaning of the fraction $L_1$ of the transported Tf-LRSC. We may, however, remark that we assumed Tf-LRSC to be bound to the limiting membrane of the vacuoles. The EM studies of Tf-HRP suggest that some Tf-LRSC is situated in the vacuole lumen [30,31]. The rate of transport of the Tf-LRSC molecules is expected to be different according as they are situated in the limiting membrane or in the lumen of the vacuoles. This heterogeneity of rate might explain why only a fraction of the marker appeared to be transported.

On the other hand, when A431 cells were incubated for 60 min with ATR-gold or Tf-HRP and then transferred 60 min in a medium without ligand, the observations of these cells by EM showed that only a part of the ligands was located in MVB, the rest being situated in lysosome-like vacuoles [30]. Therefore one may identify the Tf-LRSC fraction non-transported within the A431 cells to the fraction of ligands included in lysosome-like vacuoles.

By analysing the fluorescent microscopy images of

CHO and Hep2 cells, Maxfield et al. showed that the complex of FITC-transferrin with the Tf-Receptor was first internalized in sorting endosomes and then in the recycling compartment from which the ligand is exocytosed into the extracellular medium [37,48,49]. In a recent paper [50], Ghosh and Maxfield found that the ligand is partially transported backwards from the recycling compartment to the sorting endosomes, this mechanism producing a delay in exocytosis. The LCT of A431 cells may be identical to the recycling compartment, and a part of the Tf-LRSC transport measured by us may be ascribed to the non-vectorial exchange between the sorting endosomes and the recycling compartment. According to Ghosh and Maxfield, this non-vectorial exchange may be used by the cell in order to store proteins of sorting endosomes into the recycling compartment, allowing the sorting endosomes to mature into prelysosomal endosomes [50].

In conclusion, our FRAP measurements show that there is an active intracellular transport of Tf-LRSC in A431 cells, a long time after the ligand entered these cells. This transport can be satisfactorily described by a mechanism involving three steps: small carrier vesicles saltating rapidly along the microtubules, then budding from and fusing with stationary endosomes. This description corresponds to the VM observations of ATR-gold complexes internalized in cells of the same line [2]. The number of vesicles which fuse per second and per unit of vacuole surface can be deduced from the analysis of the FRAP data. This parameter cannot be obtained easily in vivo by other techniques currently used in cell studies. $AlF_4^-$ modifies the FRAP of endocytosed Tf-LRSC in a way which indicates that the transport of Tf in the late endocytosis compartment of A431 cells is probably controlled by an heterotrimeric G-protein. The action of BFA on the FRAP shows that the transport is homotypic, as expected.

The present work suggests that FRAP could be an interesting non-invasive technique to get information on membranous transport of organelle components within eukaryotic living cells [41–44]. It may be expected that the full efficiency of the technique would be obtained in conjunction with video microscopy and pharmacological agents tested for their effect on vesicular movements along microtubules [45].

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