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Biochimica et Biophysica Acta 1327 (1997) 69–74

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

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

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Received 30 September 1996; revised 14 February 1997; accepted 20 February 1997

Abstract

In previous works, other authors characterized a compartment (LCT) of A431 carcinoma cells in which markers of transferrin endocytose had accumulated during a long chase period. This compartment, was essentially formed by large stationary vacuoles. A few small vesicles budded from these vacuoles, rapidly saltated along microtubules and eventually fused with other vacuoles, causing an intracellular transport of the marker bound to the limiting membrane (M. De Brabander, R. Nuygens, H. Geerts, C.R. Hopkins, Cell. Mot. Cytoskel. 9 (1988) 30). In the present paper, we derived the fluorescence recovery after photobleaching (FRAP) of a fluorescent marker of LCT. We assumed that the rate of the intracellular transport of the marker was controlled by the fission–fusion process between vesicles and vacuoles. We showed that the concentration of a bleached fluorescent marker was a decreasing exponential function of the time elapsed from the beginning of the recovery phase. The rate constant of this exponential was equal to the product of the vesicle surface by the number of vesicles which fused with a unit of vacuole surface during one second. If a fraction of the marker spontaneously reactivated itself with a much higher rate constant of reaction than the rate constant of the transport process, the fractional FRAP of the marker was the sum of the fractional FRAP of both processes occurring separately. In a companion paper (F. Azizi, P. Wahl, Biochim. Biophys. Acta 1327 (1997) 75–88), our FRAP experiments will be described and analysed with the mathematical expressions derived in the present paper. © 1997 Elsevier Science B.V.

Keywords: Fluorescent recovery after photobleaching (FRAP), theory; Intracellular transport; Carrier vesicle; Reactivation; Bleached molecule; Transferrin; Endocytosis

1. Introduction

FRAP is a technique by which the transport of fluorescent molecules can be measured within small samples of a few micrometers. The technique has already been used to study the translational diffusion of fluorescently labelled proteins, of ligand-receptor complexes and of fluorescent lipids incorporated with the plasma membrane of eukaryotic cells. The FRAP has also been applied to the study of the diffusion of fluorescent macromolecules injected into the cytoplasm of these cells [1–3].

In living cells, macromolecules are most often transported by vesicles which bud from vacuolar or

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PII S0005-2736(97)00045-X

tubular membranous organelles, saltate along microtubules and fuse with other membranous organelles.

Biochemical experiments involving the destruction of cells allowed much progress to be made in the understanding of these processes [4–7]. There is, however, a need for non-invasive techniques providing the means to study the dynamics of these intracellular transports in living cells.

We investigated the possibility of using FRAP for this purpose. We chose to study the late compartment of transferrin endocytosis (LCT) in epidermoid A431 carcinoma cells.

The endocytosis of transferrin (Tf) by A431 cells has already been carefully studied by several authors. The cellular sites of gold conjugates of an Anti-Transferrin Receptor (ATR-gold) and of a Horse Radish Peroxidase conjugate of Transferrin (Tf-HRP) were identified at different stages of endocytosis by electron microscopy (EM). During a long chase period, the internalized Tf-HRP and ATR-gold were seen accumulating in a late endosome compartment (LCT), made essentially of vacuoles called Multivesicular Bodies (MVB) [8,9].

The diameter of these MVB varied from 1 to 2 μm. ATR-gold was located near the limiting membrane of each vacuole, while Tf-HRP was partly situated on that limiting membrane, and partly located in the vacuole lumen [8]. It was concluded that the transferrin receptors are situated in the limiting membrane of the MVBs and that Tf-HRP is partly bound to these transferrin receptors and partly free in the vacuole lumen.

Video microscopy (VM) of the ATR-gold complex showed that large vacuoles formed the greatest part of the late endosome compartment, and were stationary [10]. A few small vesicles continuously pinched off from these vacuoles, migrated by linear saltations through the cytoplasm in the retrograde and anterograde directions with high speed ($\sim 1\ \mu m\ s^{-1}$), and finally fused with other large vacuoles. This fission–fusion process yielded an exchange of ATR-gold between large vacuoles and produced an intracellular transport of the ATR-gold complex.

The vesicular motions and the fission–fusion processes were found to be completely stopped as the cells were treated with nocodazole, a compound depolymerizing microtubules, or with sodium azide, a metabolic inhibitor. These results showed that the membranous activity revealed by the VM observations, was dependent on the cellular energy supply and the microtubular integrity.

In this paper, we present a theory of the FRAP of a fluorescent marker bound to LCT. We derived a mathematical expression based on a model in which the fluorescence recovery was obtained by the simultaneous action of an intravacuolar process (for example a spontaneous reactivation of the bleached molecules) and a membranous transport by carrier vesicles. These vesicles were assumed to bud from stationary vacuoles, saltate and fuse with other stationary vacuoles.

In a companion paper [11], we present results of FRAP experiments on a fluorescent conjugate of transferrin (the lissamine rhodamine sulfochloride (Tf-LRSC)), endocyted in A431 cells, and we interpret these experiments with the mathematical expressions derived in the present paper.

2. Theory

2.1. Variation of the number of bleached markers per vacuole during the recovery phase, brought about by the fission–fusion processes

We assume that a fluorescent marker (i.e. Tf-LRSC) reached the late endosome compartment of transferrin. From previously published results on other transferrin conjugates [8,9], we expect that Tf-LRSC is bound to the transferrin receptors included in the limiting membrane of the MVBs. In this paper for simplification, we neglect the possibility that a part of Tf-LRSC is situated in the vacuole lumen. We return to this possibility in the discussion of the companion paper [11].

Photobleaching generates a gradient in the volumetric concentration of the membranous fluorescent vacuole marker, made of two components, that is to say a gradient in the membranous concentration and a gradient in the number of fluorescent molecules per vacuole in the membrane [12].

We assume that the time resolution of our FRAP experiments is significantly longer than the homogenization time of the marker concentration in the vacuole limiting membrane [12]. According to that

assumption which will be experimentally justified in the companion paper [11], the diffusion of the marker in that vacuole limiting membrane only influences the fluorescence intensity at the initial point of the recovery. On the other hand, the kinetics of the FRAP proceeds from the variation of the number of Tf-LRSC vacuolar molecules present at a given time in the zone illuminated by the interrogation beam.

The mechanism of transport considered here explains the fluorescence recovery as follows:

Small vesicles which bud from stationary vacuoles in the illuminated zone, carry away previously bleached marker molecules. These vesicles saltate and fuse with other vacuoles in the non-illuminated zone. Inversely, vesicles budding from vacuoles in the non-illuminated zone bring fluorescent markers into the illuminated zone where they fuse with other vacuoles. The balance of this mechanism will be an increase in the number of fluorescent markers into the illuminated zone, and consequently a recovery of the fluorescence lost by the previous photobleaching.

Our calculations are based on the following assumptions resulting from the EM and the VM observations [8–10]:

(1) The whole quantity of limiting membranes of the mobile vesicles, and consequently of Tf-LRSC contained in them, is very small compared to the quantity of limiting membranes and markers belonging to the whole set of stationary vacuoles.

(2) The velocity of the vesicle saltation is high, so that the rate of the marker transport is controlled by the processes of fission–fusion of these vesicles with the stationary vacuoles.

(3) Furthermore, if the volume of the illuminated zone is a small fraction of the cell volume (one of the usual requirements of FRAP experiments), one may assume that the number of mobile vesicles and stationary vacuoles in the non-illuminated area is much larger than their number in the illuminated area.

As a consequence of these assumptions, the concentration of the fluorescent marker in the membrane of the carrier vesicles is equal to the prebleaching concentration in the whole limiting membranes of the compartment.

Let $\gamma(-)$ be the value of this concentration, $\gamma(\tilde{\rho}_o,t)$ the fluorescent marker concentration in the limiting membrane of a vacuole, the center of which is situated at the point $M(\tilde{\rho}_o)$ at a time t after bleaching, and s, the limiting membrane surface of a mobile vesicle.

The number $n(\tilde{\rho}_o,t)$ of fluorescent markers situated in the limiting membrane of a given vacuole obeys the following differential equation:

$$\mathrm{d}n(\tilde{\rho}_o,t)/\mathrm{d}t = k_f s\gamma(-) - k_p s\gamma(\tilde{\rho}_o,t) \quad (1)$$

where k_f and k_p are respectively the number of vesicles which fuse with and pinch off from a vacuole during one second.

Since the quantity of membranes in the vesicular and vacuolar subcompartments are necessarily in thermodynamic equilibrium, that is to say in a steady state, one can write:

$$k_f = k_p \quad (2)$$

Let us define the number of bleached molecules present in a given vacuole at a time t of the recovery (or after bleaching) as follows:

$$\Delta n(\tilde{\rho}_o,t) = n(-) - n(\tilde{\rho}_o,t) \quad (3)$$

where $n(-)$ is the number of fluorescent markers contained in the limiting membrane of a vacuole, during the prebleaching phase.

One may write:

$$n(-) = S\gamma(-) \quad (4)$$

$$n(\tilde{\rho}_o,t) = S\gamma(\tilde{\rho}_o,t) \quad (5)$$

where S is the limiting membrane surface of a vacuole.

According to Eqs. (2)–(5), Eq. (1) may be written as:

$$\mathrm{d}\Delta n(\tilde{\rho}_o,t)/\mathrm{d}t = -h_1\Delta n(\tilde{\rho}_o,t) \quad (6)$$

where:

$$h_1 = (s/S)k_f \quad (7)$$

If we admit that the number of vesicles which fuse with a vacuole, per second, is proportional to the surface of the vacuole limiting membrane, we may write:

$$k_f = \tau_f S \quad (8)$$

where τ_f is the number of vesicles that fuse with a unit of vacuole surface in one second. From Eqs. (7) and (8) one obtains:

$$\tau_f = h_1/s \quad (9)$$

2.2. Variation of the number of bleached marker molecules per vacuole resulting from the simultaneous action of the transport and the spontaneous reactivation processes

We now assume that only a fraction of the vacuolar Tf-LRSC can be exchanged with another vacuole by the fission–fusion process described above. This assumption will be discussed in the companion paper [11].

In that paper we will see that the marker incorporated with cells treated by nocodazole partially recovers its fluorescence after photobleaching. This result shows that a fraction of the bleached molecules is spontaneously reactivatable, while another fraction cannot reactivate itself and remains bleached.

We also assume that the reactivation reaction is of first order. Its rate constant h_2 is the same for the exchangeable and non-exchangeable markers.

Consequently, there are four kinds of marker molecules defined in Table 1. To each kind we assign an index, $j = 1$ to 4.

In the prebleaching phase of the FRAP experiment, one vacuole contains $n_j(-)$ marker molecules of kind j. The molar fraction of these marker molecules is then:

$$a_j = n_j(-)/n(-) \tag{10}$$

where $n(-)$ is the total number of marker molecules per vacuole before bleaching and is defined by the following equation:

$$n(-) = \sum_{j=1}^{4} n_j(-) \tag{11}$$

One has obviously:

$$\sum_{j=1}^{4} a_j = 1 \tag{12}$$

Table 1
Rate constants of marker molecules

Molecular species	j	k_j
Exchangeable and reactivatable	1	$h_1 + h_2$
Exchangeable and non-reactivatable	2	h_1
Non-exchangeable and reactivatable	3	h_2
Non-exchangeable and non-reactivatable	4	0

During the recovery phase of the FRAP experiment, the number of bleached markers per vacuole obeys a differential equation specific to each marker kind, which may be written as follows:

$$\mathrm{d}\,\Delta n_j(\tilde{\rho}_o,t)/\mathrm{d}t = -k_j \Delta n_j(\tilde{\rho}_o,t) \tag{13}$$

The values of the rate constants k_j as functions of h_1 and h_2 are also given in Table 1.

The solution of Eq. (13) is:

$$\Delta n_j(\tilde{\rho}_o,t) = \Delta n_j(\tilde{\rho}_o,0)\exp(-k_j t) \tag{14}$$

where $\Delta n_j(\tilde{\rho}_o,0)$ is the number of bleached marker of species j present in a vacuole, the center of which is defined by $\tilde{\rho}_o$ at the end of the bleaching phase. According to our preceding work, we can write the following expression [12]:

$$\Delta n_j(\tilde{\rho}_o,0) = n_j(-)/4\pi R^2 \int \exp\left[-KI(\tilde{\rho}_o + \tilde{u})/I_0\right] \times \sigma(\tilde{u},R)\,\mathrm{d}^3\tilde{u} \tag{15}$$

where K is the bleaching parameter defined by Axelrod et al. [1] which we assume to be identical for the four marker kinds. $I(\tilde{\rho})$ and I_0 are the intensities of the laser beam during the bleaching phase at the point $M(\tilde{\rho})$ of the sample and at a point situated on the optical axis, respectively; $\sigma(\tilde{u},R)$ is a function of the vector $\tilde{u}$ and the scalar R such as [12]:

1. $\sigma(\tilde{u},R) = 0$ for $|\tilde{u}| \neq R$
2. for all functions $f(\tilde{u})$
   $\int f(\tilde{u})\sigma(\tilde{u},R)\,\mathrm{d}^3\tilde{u} = R^2 \int f(R,\theta,\varphi)\sin\theta\,\mathrm{d}\theta\,\mathrm{d}\varphi$

where $f(|\tilde{u}|,\theta,\varphi)$ is the expression of $f(\tilde{u})$ in the polar coordinates of $\tilde{u}$ [12]. R is the radius of the vacuole assumed to be spherical.

2.3. FRAP due to the simultaneous actions of the transport and of the spontaneous reactivation processes

We assume that the three-dimensional FRAP sample (defined as a A431 cell in the companion paper) [11] may be considered as planar. This would be the case if the half-thickness of the sample was small compared to two critical lengths, namely the laser beam persistence length, and the optical sectioning characteristic length which depends on the detection

system of the fluorescent light [13]. In the companion paper [11] we will verify that our experiments fulfill these conditions.

During the recovery phase of a FRAP experiment, the bleached fluorescence intensity of the marker j is then given as a function of the time t after bleaching by the following formula:

$$\Delta F_j(t) = (qe/A)\int \Delta C_j(\tilde{\rho},t)\, I(\tilde{\rho})\, \mathrm{d}^2\tilde{\rho} \tag{16}$$

where q is a factor which takes account of the absorbtion of the laser beam in the sample, of the fluorescence quantum yield (assumed to be the same for the four marker kinds), and of the photon loss in the detection system of the FRAP apparatus; e, is the thickness of the sample; A, the attenuation factor of the laser beam in the recovery phase, $\Delta C_j(\tilde{\rho},t)$ is the volumetric concentration of the bleached marker at a point $M(\tilde{\rho})$ of the observation plane.

If the marker is included in the limiting membrane, one has [12]:

$$\Delta C_j(\tilde{\rho},t) = N/(4\pi R^2)\int \Delta n_j(\tilde{\rho}+\tilde{u},t)\,\sigma(\tilde{u},R)\,\mathrm{d}^3\tilde{u} \tag{17}$$

where N is the number of vacuoles per unit volume.

From Eqs. (14)–(17), one may write:

$$\Delta F_j(t) = \Delta F_j(0)\exp(-k_j t) \tag{18}$$

where

$$\Delta F_j(0) = a_j F(-) P(K,R/w) \tag{19}$$

is the bleached fluorescence at the end of the bleaching phase, $F(-)$ is the prebleaching fluorescence intensity and $P(K,R/w)$ is the fraction of bleached fluorophores given by formula (38) of our preceding work [12].

The bleached fluorescence of the vacuole markers can then be written:

$$\Delta F(t) = \sum_{j=1}^{4} \Delta F_j(t) \tag{20}$$

The corresponding fractional bleached fluorescence is then [2]:

$$X(t) = \Delta F(t)/\Delta F(0) \tag{21}$$

Taking Eqs. (18)–(20) and Table 1 into account, one can write Eq. (21) as follows:

$$X(t) = a_1\exp(-(h_1+h_2)t) + a_2\exp(-h_1 t) + a_3\exp(-h_2 t) + a_4 \tag{22}$$

The experiments of FRAP described in the companion paper [11] show that the rate of the spontaneous reactivation of the bleached molecules is significantly higher than the rate of the fusion–fission process. It means that:

$$h_1 + h_2 \sim h_2$$

Eq. (22) then becomes:

$$X(t) = a_2\exp(-h_1 t) + (a_1+a_3)\exp(-h_2 t) + a_4 \tag{23}$$

The fractional fluorescence is given by the following expression:

$$y(t) = 1 - X(t) \tag{24}$$

Replacing $X(t)$ by its value in Eq. (23) and taking Eq. (12) into account we can write:

$$y(t) = L_1(1-\exp(-h_1 t)) + L_2(1-\exp(-h_2 t)) \tag{25}$$

where:

$$L_1 = a_2 \text{ and } L_2 = a_1 + a_3 \tag{26}$$

When cells are treated by nocodazole, the transport process is stopped, that is to say $h_1 = 0$; Eq. (25) becomes:

$$y_{\mathrm{No}}(t) = L_2(1-\exp(-h_2 t)) \tag{27}$$

According to Eqs. (25) and (27), one may write:

$$y_{\mathrm{T}}(t) = y(t) - y_{\mathrm{No}}(t) \tag{28}$$

where $y_{\mathrm{T}}(t)$ is the fractional fluorescence recovery characterizing the transport process without spontaneous reactivations, and is given by the following equation:

$$y_{\mathrm{T}}(t) = L_1(1-\exp(-h_1 t)) \tag{29}$$

Eq. (28) remains valid whatever the mathematical expression of the fluorescence recovery of the transport and of the intravacuolar process, provided that their characteristic time constants are very different.

3. Conclusion

According to the Video Microscopy work of De Brabander et al. (1988) [10], small vesicles transport the transferrin receptors between stationary vacuoles into the late compartment of transferrin endocytosis of A431 cells.

From these observations, we predicted that fluorescent transferrin molecules contained in this compartment will contribute to the FRAP measured in A431 cells. Assuming that the rate of the marker transport was controlled by the fission–fusion processes of the vesicles with the vacuoles, we showed that the mathematical expression of this contribution was an exponential function. The time constant was inversely proportional to the product of the number of vesicles fusing in one second with a unit of vacuole surface and the whole vesicle surface (Eq. (9)).

In order to apply our mathematical analysis to the experiments of FRAP described in the companion paper [11], we assumed that a fraction of the bleached marker molecules was spontaneously reactivatable, and that the rate of reactivation was significantly higher than the rate of the fission–fusion process. Under these conditions, the fractional FRAP of both processes simultaneously occurring was the sum of the fractional FRAP of each process occurring separately.

In eukaryotic cells, there are couples of membranous organelles linked together by exchanges of components, mediated by shuttle vesicles travelling between each other [4–7].

In the future, one may think of applying the FRAP technique to study the dynamic of these exchanges.

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