Measuring activity of endocytosis-regulating factors in T-lymphocytes by flow cytometry

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Abstract Elucidation of the mechanisms regulating membrane traffic of lymphocyte receptors is of great interest to manipulate the immune response, as well as for accurately delivering drugs and nanoprobe to cells. Aiming to detect and characterize regulators of endocytosis and intracellular traffic, we have modified the FACS-based endocytosis assay to measure and quantify the activity of putative endocytic regulators as EGFP chimeras. To study the activity of putative endocytosis regulators, we transfected Jurkat T-lymphocytes with EGFP-tagged constructs of the regulators to be tested. Cells were then incubated with a α CD3^{APC} antibody, and were allowed to internalize the label. After acid-washing the cells, APC fluorescence was measured by flow cytometry in cells gated for EGFP⁺, as well as in their EGFP[−] (transfection-resistant) counterparts that were taken as internal controls. This approach facilitated intra- and inter-assay normalization of endocytic rates/loads by comparison with the internal control. We have used this assay to test the regulatory activity of polarity kinase EMK1, and here we substantiate a role for EMK1 in the control of receptor internalization in T-lymphocytes. The method here presented gives quantitative measures of internalization, and will facilitate the

development of tools to modulate endocytic rates or the intracellular fate of internalized materials.

Keywords Endocytosis regulators · Nanomedicine internalization · Intracellular trafficking · Internally-controlled FACS-based endocytosis assay · Endocytosis quantification

Introduction

Lymphocytes are very plastic cells that respond to multiple stimuli, in the form of chemical gradients or co-stimulatory molecules, produced by the environment or by neighbour cells. To accomplish this, lymphocytes rely in a complex set of membrane receptors whose expression and intracellular traffic is exquisitely regulated by a number of mechanisms that control their export to the plasma membrane, their exposition to the extracellular milieu, as well as their internalization and posterior intracellular fate (Grigorian et al. 2009). It is therefore of the outmost importance to develop methods that could easily quantify the amount of receptor effectively expressed in the plasma membrane, as well as their dynamics or equilibrium among receptor internalization, degradation or recycling back to the membrane and through endosomal bodies.

First attempts to measure endocytic rates took advantage of the temperature-dependence of plasma membrane fluidity to specifically stain a receptor with a radioactively-labelled ligand (Klausner et al. 1983) or antibody

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(Mellman et al. 1984) at 4 °C, and then promoting internalization of the complex by a temperature shift to 37 °C. Later on, the green fluorescent protein (GFP) and its derivatives were widely used for studying receptor-mediated endocytosis. Thus, GFP has been used for labelling ligands, that retained receptor-binding activity (Medina-Kauwe et al. 2000; Medina-Kauwe and Chen 2002), receptors (Tarasova et al. 1997; Kallal et al. 1998; Gerceker et al. 2000), and components of the constitutive endocytic machinery, as the clathrin light chain (Banerjee et al. 2010), in internalization assays measured by flow cytometry that were, furthermore, amenable to automation (Ghosh et al. 2000). In a further development, transfection of a GFP-Nef construct (Nef is the product of the *nef* gene of HIV that down-regulates cell surface expression of CD4 in T-lymphocytes) allowed to determine the effect of Nef expression on the internalization rates of CD4 (Greenberg et al. 1997).

Our laboratory is interested in studying the mechanisms that regulate internalization and residence of membrane receptors and especially in those proteins that could modulate endocytic rates or total endocytic loads. Our aim for this work has been a double one, on the one hand to standardize current internalization assays and, on the other, to adapt the method to the measure of the functional activity of putative endocytic modulators, with the final objective of facilitating the development of tools to modify the intracellular fate of internalized materials. The assay here presented targeted the CD3 component of the T cell receptor (Smith-Garvin et al. 2009), and relied in the transfection of Jurkat T-lymphocytes with EGFP-tagged putative regulators. Endocytosis was measured by flow cytometry as internalization of a fluorescently labelled anti-CD3^{APC} antibody, simultaneously in the two viable cell populations present in the same test tube: cells expressing the EGFP-chimera (target cells), and transfection-resistant cells (internal control). This approach greatly facilitates intra- and inter-assay normalization of endocytic rates/loads by direct comparison with the internal controls.

Materials and methods

Materials and reagents

For the receptor-mediated endocytosis assay we used an APC-conjugated anti-CD3 (E-subunit) antibody (herein α CD3^{APC}), tested for flow cytometry (Pharmingen-BD

Biosciences, Ref: 555335, San Jose, CA, USA). As secondary antibody we used an Alexa 647-conjugated goat-anti mouse IgG, from Molecular Probes-Life Technologies (Eugene, OR, USA). Human pure fibronectin was from Sigma-Aldrich (St Louis, MO, USA) and the antifade reagent (ProLong Gold Antifade with DAPI) was from Molecular Probes-Life Technologies (Eugene, OR, USA).

RPMI 1640/Glutamax-1, heat inactivated fetal bovine serum (FBS) and sodium pyruvate were from Gibco-Life Technologies (Eugene, OR, USA); penicillin-streptomycin mix was from Biological Industries (Kibbutz Beit Haemek, Israel); propidium iodide (in solution), para-formaldehyde (PFA), Triton X-100, bovine serum albumin (BSA) and general laboratory reagents were from Sigma-Aldrich (St Louis, MO, USA); the parental plasmid pEGFP-N1 was from CLONTECH Laboratories Inc. (Mountain View, CA, USA). Preparation of the EGFP-derived plasmids used in this work (EGFP-EMK1wt and EGFP-EMK1ELKLless) will be described elsewhere (Beltran-Sastre et al., manuscript in preparation). For electroporation, we used a Gene Pulser II apparatus (Bio-Rad, Hercules, CA, USA). Fibronectin-coated coverslips were prepared in our laboratory by dropping 50 μ l of a solution of fibronectin from human plasma (100 μ g/ml in PBS) on the surface of a glass coverslip, and then incubating the coverslip, upside-down inside a 6-well plate, at 37 °C for one hour.

Cell culture and transfections

Jurkat T leukaemia cells (clone E6.1 from ECACC) were cultured in RPMI 1640/Glutamax-1, 10 % FBS, 50 U/ml penicillin, 50 μ g/ml streptomycin, 1 % (v/v) sodium pyruvate at 37 °C and 5 % CO₂. For each transfection, 50 μ g of plasmid (in 500 μ l of RPMI medium without antibiotics) were added to 10⁷ Jurkat cells and electroporated at 280 V and 975 μ F using a Gene Pulser II apparatus (BioRad). Cells were maintained for 5 min on ice, prior to being transferred to a culture flask with complete medium.

Transfection efficiency was determined by analysing cytometric data. For every EGFP-EMK1 transfection, we scored the percentage of cells at the R3 (transfected cells) and R4 (transfection-resistant cells, i.e. cells that did not acquire the EGFP-EMK1 construct) gates at the FL4 (APC) versus FL1 (EGFP) cytogram, at the initial $t = 0$. This yielded a value of 45.6 ± 6.6 % for transfection efficiency.

Immunocytochemical analysis

For the immunocytochemical detection of internalized $\alpha\text{CD3}^{\text{APC}}$, cells were seeded, after completion of the internalization assay, on human fibronectin-coated coverslips for 30 min, fixed with 4 % PFA, permeabilized with 1 % Triton X-100 in PBS for 20 min at room temperature, and blocked with 1 % BSA, 0.2 % Triton X-100 in PBS for 30 min at room temperature. Cells were then washed, incubated with an Alexa 647-conjugated anti mouse secondary antibody for 1 h at room temperature and mounted with ProLong Gold Antifade reagent with DAPI.

FACS-based endocytosis assay

As the basis for our work we used the antibody mediated endocytosis assay, with acid-wash and fluorescently-labelled antibodies described by Chambers et al. (1993), and the use of GFP constructs in internalization assays from Greenberg et al. (1997). In our determinations, 10^7 Jurkat cells were transfected by electroporation with EGFP or EGFP-tagged forms of the wild type or mutated human polarity kinase EMK1/Par1b (Espinosa and Navarro 1998). 4 h post-transfection, cells were washed in PBS and taken up in 500 μl of ice-cold RPMI, 2 % BSA, 10 mM HEPES pH 7.4, to which 100 μl of $\alpha\text{CD3}^{\text{APC}}$ antibody (10 μl antibody/ 10^6 cells) were added. Cells were incubated for 1 h at 4 °C, washed twice with 5 ml of ice-cold RPMI, 0.2 % BSA, 10 mM HEPES pH 7.4 to eliminate excess antibody, resuspended in 1 ml of RPMI, 0.2 % BSA, 10 mM HEPES pH 7.4 and re-incubated at 37 °C to allow internalisation of label. At the stated times, 2×50 μl -aliquots of cells were extracted, and added either to 2 ml of ice-cold PBS (total antibody loading), or to 2 ml of ice-cold PBS pH 2.0 (acid-wash to remove surface-bound antibody to measure loading of internal endosomal compartments). Cells were maintained in the acid-wash for 45 s before being neutralized with 10 ml of ice-cold PBS, 0.05 % sodium azide. The two aliquots were then centrifuged and taken up in 500 μl of ice-cold PBS, 2.5 $\mu\text{g}/\text{ml}$ propidium iodide. Total fluorescence of live cells was measured by flow cytometry in a FACScalibur apparatus (BD Biosciences, San Jose, CA, USA) using argon (488 nm, for EGFP) and HeNe (633 nm, for APC) lasers, and expressed as the geometric mean (GeoMean) of fluorescence intensity. For each time-point, the GeoMean values of EGFP/APC fluorescence intensity

were measured in the populations of acid-washed EGFP-transfected and transfection-resistant cells, and expressed as percentage of the GeoMean of total fluorescence at $t = 0$. FACS profiles were analyzed with the CellQuest Pro software (BD Biosciences).

Confocal microscopy

Fluorescently-labeled cells were imaged with a Leica TSC SL spectral confocal microscope (400–850 nm), using argon (488 nm) and HeNe (543 nm) lasers, and a HCX PL Apo 63 $\times$ 1.40 oil objective. Single confocal images were taken at a medium focal plane of cells, and subsequently analyzed using the LCS Lite software (Leica, Wetzlar, Germany).

Results and discussion

We are interested in studying the mechanisms that regulate endocytosis and intracellular traffic in T-lymphocytes, and specially their functional integration with the components of the polarity complex. In this work we have tested EMK1/Par1b, one of the human forms of polarity kinase EMK1/Par1 (Navarro et al. 1999), as a putative regulator of endocytosis. EMK1 has been previously associated to the process of receptor internalization, by being shown to co-localize with AP1/2 vesicular structures (Matenia et al. 2005), and to interact “in vitro” with the AP2 endocytic complex (Schmitt-Ulms et al. 2009). Furthermore, we have demonstrated its binding to the $\sigma 2$ subunit of the adaptor-related protein complex-2 (AP2S1/AP17) after a two hybrid cloning experiment (Beltran-Sastre et al. submitted).

In this work, we have measured the effect of overexpressing the putative endocytosis regulator EMK1 on the internalization rate of a fluorescently labelled ligand of the CD3 receptor of T-lymphocytes (a $\alpha\text{CD3}^{\text{APC}}$ -specific antibody). We firstly transfected Jurkat T-leukemia cells with EGFP-EMK1. This originated two cell populations: cells that acquired the plasmid and expressed the EGFP-EMK1 construct (herein called transfected cells), and cells that did not (herein called transfection-resistant cells), and preliminary flow cytometry analysis of Jurkat cells electroporated with the EGFP-EMK1 construct showed that only 45.6 ± 6.6 % of cells ($n = 7$) acquired (and

Fig. 1 Flow cytometry gatings for measuring the effect of putative endocytic regulators on internalization rates in T-lymphocytes. Jurkat cells were transiently transfected with pEGFP-EMK1, saturated with $\alpha\text{CD3}^{\text{APC}}$ and washed. Total APC fluorescence at $t = 0$ was measured by flow cytometry (initial 100 % loading). Cells were then incubated at 37 °C to allow internalisation of the $\alpha\text{CD3}^{\text{APC}}$ -CD3 complex. Aliquots of cells were extracted, submitted to an acid-wash to remove surface-bound antibody, and APC fluorescence was quantified. GeoMean values of fluorescence intensity were expressed as percentage of the initial value. **a** Cells were first gated by side scatter versus forward scatter (*left*), and then by propidium iodide fluorescence versus forward scatter to select those alive (*right*). **b** Events acquired in the FL4/FL1 channels were gated into control and transfected cells (R4 and R3). **c** Representative profiles of initial $\alpha\text{CD3}^{\text{APC}}$ loadings for control (*upper left panel*) and EGFP-EMK1 transfected cells (*upper right panel*), and their variations after acid-wash at $t = 0$ (*central panels*) and at $t = 240$ min, endpoint of the experiment (*lower panels*)

expressed) the EGFP-chimera, while the other 49.9 ± 6.6 % were transfection-resistant (see “[Materials and methods](#)”). Figure 1 shows a representative experiment in which EGFP-EMK1-electroporated Jurkat cells were first gated by side scatter versus forward scatter (Fig. 1a, left), then by propidium iodide fluorescence versus forward scatter to select those alive (Fig. 1a, right), and finally by FL4 (APC fluorescence) versus FL1 (EGFP fluorescence). The windows R4 and R3 included transfection-resistant (internal control), and EGFP-EMK1-transfected cells, respectively, that in this particular experiment accounted for 58 and 35.43 % of the input cells.

The fact that only over half of the culture incorporated the EGFP chimeric construct prompted us to use these transfection-resistant cells, i.e. those that did not acquire or express the EGFP-chimera, as internal controls for the endocytosis experiments. This was a significant improvement of the technique, not only because transfectants and controls suffered exactly the same manipulations, but also because it greatly facilitated the inter-assay analysis of results.

For developing the method, we first performed a number of controls:

1. a saturation control with different amounts of $\alpha\text{CD3}^{\text{APC}}$ antibody allowed us to choose the minimum amount required for saturating cells ($10 \mu\text{l}$ antibody/ 10^6 cells),
2. we confirmed that the acid-wash at pH 2 actually eliminated surface label (thus leaving only internalized label available for quantification), and

3. non-electroporated Jurkat cells were incubated with the $\alpha\text{CD3}^{\text{APC}}$ antibody, analysed by flow cytometry and finally gated at the FL1 and FL4 channels to define their upper limits of green and red autofluorescence. Thus allowed the definition of the EGFP transfected/transfection-resistant and $\alpha\text{CD3}^{\text{APC}+}/\alpha\text{CD3}^{\text{APC}-}$ cell subpopulations. These gates are visible in Fig. 1b (R4 for transfection-resistant cells, R3 for EGFP-EMK1 transfected cells), and were used all thorough the experiments here described.

For the endocytosis assays, we electroporated Jurkat cells with the EGFP-EMK1, and allowed to express the construct for 4 h. Cells were then saturated with $\alpha\text{CD3}^{\text{APC}}$ at 4 °C for 1 h, washed to eliminate non-bound antibody, and returned at 37 °C to allow a time-course of internalization of the $\alpha\text{CD3}^{\text{APC}}$ -CD3 complex. At $t = 0$, two aliquots of cells were extracted, one of them was submitted to the acid-wash and its APC-fluorescence was measured to determine the initial antibody loading. At stated times ($t = 60$; $t = 120$; $t = 180$; and $t = 240$ min), new aliquots of cells were extracted acid-washed and treated as described (see “[Materials and methods](#)” for a more detailed description of the procedure).

Internalization of the $\alpha\text{CD3}^{\text{APC}}$ -CD3 complex was quantified by first gating side scatter versus forward scatter (Fig. 1a, left), and then by propidium iodide fluorescence versus forward scatter to select alive cells (Fig. 1a, right). Events were acquired in the FL4/FL1 channels (Fig. 1b), gated into transfection-resistant and transfected cells after acid-washing (Fig. 1b, R4 and R3 respectively), and the GeoMeans of the two populations were expressed as percentage of the GeoMean of the initial total fluorescence of non acid-washed cells at $t = 0$. Figure 1c shows the FACS profiles obtained for the initial APC loadings of control, transfection-resistant, (upper left panel) and EGFP-EMK1 transfected cells (upper right panel), and their variations after acid-washing at $t = 0$ (central panels) and at $t = 240$, the end-point of the experiment (lower panels). In these, it can be clearly seen the shift in fluorescence intensity due to the acid-wash elimination of membrane-bound antibody (allowing only internalized material to be measured), as well its decrease at the $t = 240$ min time-point, presumably due to lysosomal degradation of the CD3^{APC} .

Results were plotted as the percentage of acid-resistant fluorescence versus internalization time. Figure 2 evidences that after 60 min of incubation, label accumulation was higher in cells expressing EGFP-EMK1 (61.21 ± 3.1 % of the initial label; Fig. 2a, solid line) than in transfection-resistant controls (43.76 ± 10.36 %; Fig. 2a, dotted line), or in cells expressing the mutated EGFP-EMK1^{ELKLless} (49.45 ± 2.38 %; Fig. 2b, solid line) or EGFP (51.47 %; Fig. 2b, interrupted line). EGFP-EMK1^{ELKLless} is an EMK1 form that lacks the entire C terminal domain so that it is unable to be targeted to

the plasma membrane and remains mostly cytoplasmic (Moravcevic et al. 2010), thus demonstrating that correct membrane-targeting of EMK1 is required for its internalizing function.

As an additional control, we confirmed that internalized $\alpha\text{CD3}^{\text{APC}}$ -CD3 complex was actually present in the inside of cells. For this, cells were fixed and permeabilized, incubated with an Alexa⁶⁴⁷-conjugated anti mouse antibody to label the αCD3 moiety, and studied by confocal microscopy. Addition of the secondary antibody was required because preliminary experiments showed that the remaining fluorescence

Fig. 2 Internalization plots of EGFP-EMK1 transfected cells (EMK1) versus control cells (JK), EGFP-transfected control cells (EGFP) and EGFP-EMK1^{ELKLless}-transfected control cells (ELKL). GeoMean values from internalization experiments were expressed as the mean $\pm$ SD of total, initial loading at $t = 0$ and plotted against internalization time. **a** Plots of α CD3^{APC}-CD3 internalization at the stated times for EGFP-EMK1 (EMK1, $n = 3$), and transfection-resistant Jurkat control cells (JK, $n = 6$). Overexpression of polarity kinase EMK1 increased internal loading of endocytosed α CD3^{APC}-CD3 complex in Jurkat cells. The *dotted line* indicates a value of 60 % of internalization. **b** Plots of α CD3^{APC}-CD3 internalization at the stated times for the different controls: EGFP-EMK1^{ELKLless}-transfected cells (ELKL, $n = 2$), EGFP (EGFP, $n = 1$), and transfection-resistant Jurkat control cells (JK, $n = 6$). The plot for transfection-resistant cells is the same as above. ELKL and EGFP controls showed the same internalization dynamics, which was slightly higher than that of Jurkat cells but lower than that of EMK1-expressing cells. The *dotted line* indicates a value of 60 % of internalization. ELKL and EGFP controls were measured at the same gates established for EMK1. **c** Internalized α CD3^{APC} can still be detected in of Jurkat T-lymphocytes at the end-point of the internalization assay. Cells removed from the end-point time (240 min) in the assay shown in **a** were seeded on coverslips, fixed, permeabilized and immunostained with an Alexa-647 conjugated secondary anti mouse antibody to detect internalised α CD3^{APC}. Shown are single confocal Z-sections of representative pEGFP-EMK1-transfected, and untransfected control cells. The *left panel* shows two EMK1-polarized cells, the *central panel* displays the Alexa-647 labelling of internalised α CD3^{APC} in these cells as well as in a non-polarized control cell at the same focal plane, showing the cortical distribution of α CD3^{APC}, and its polarized location in EMK1 transfectants (*arrows*). The *right panel* demonstrates colocalization of internalised α CD3^{APC} and EGFP-EMK1 exclusively at the uropod/pericentriolar region of EGFP-EMK1 cells (in *yellow*). Scale bar is 8 μ m. (Color figure online)

of intracellular α CD3^{APC} was hardly detected by the confocal microscope. In this sense, we selected the Alexa⁶⁴⁷ fluorochrome because its emission spectrum is very similar to that of APC, thus increasing signal strength. Figure 2c shows that, at the end-point of the assay, α CD3 could be detected at the uroplasm of EMK1-polarized cells (see the central and merge panels); while in transfection-resistant control cells it was symmetrically and homogeneously distributed in endosomal bodies through the cell cytoplasm.

The work here described should be of interest to researchers working in the interface of nanomaterials and lymphoid cells, since endocytosis is one of the most widely used mechanisms by which nanostructures enter to a cell (Bareford and Swaan 2007; Decuzzi and Ferrari 2008; Sahay et al. 2010) and characterization of the intracellular trafficking pathways of nanoconjugates is critical for their use in theranostics (Tekle et al. 2008).

Nevertheless, and despite of intense research efforts on this topic, much work is still required to characterize internalization and trafficking mechanisms to such extend that could be used to specifically target drugs or intracellular probes (Zaki and Tirelli 2010). In this sense, the method here presented will facilitate the development of tools to modulate internalization rates and intracellular fate of internalized materials. Furthermore, this is not restricted to the characterization of EGFP-tagged proteins as endocytic modulators, since replacement of α CD3^{APC} by other labelled nanostructures would increase the span of structures amenable to be studied, thus facilitating the development of this promising research field.

Conclusions

We have modified the FACS-based, receptor-mediated, endocytosis assay to quantitatively measure the effect of putative endocytosis regulators on the internalization rates of the lymphocyte membrane receptor CD3. We overexpressed the putative endocytic regulator EMK1/Par1b as an EGFP-tagged construct, measured its effect on the internalization of α CD3^{APC} in transfected and transfection-resistant (internal control) cells, and showed that the internalized cargo could be followed up in its trafficking through endosomal bodies. This work provides a proof-of-concept that this analytical method is functional. Furthermore we also demonstrate that polarity kinase EMK1 has a role regulating receptor internalization in T-lymphocytes.

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