Cell-cycle-dependent binding kinetics for the early endosomal tethering factor EEA1

Trygve Bergeland¹, Linda Haugen¹, Ole J.B. Landsverk¹, Harald Stenmark² & Oddmund Bakke^{1,3*}

¹Centre for Immune Regulation, Department of Molecular Biosciences, University of Oslo, Oslo, Norway, ²Department of Biochemistry, Institute for Cancer Research, The Norwegian Radium Hospital, Oslo, Norway, and ³The Gade Institute, University of Bergen, Bergen, Norway

Early endosomal antigen 1 (EEA1) is a cytosolic protein that specifically binds to early endosomal membranes where it has a crucial role in the tethering process leading to homotypic endosome fusion. Green fluorescent protein-tagged EEA1 (EEA1-GFP) was bound to the endosomal membrane throughout the cell cycle, and measurements using fluorescent recovery after photobleaching showed two fractions: one rapidly exchanging with the cytosolic pool, and the other with a long half-life. The exchange consists of a release and binding process, and we have separated these two by using GFP and photoactivable GFP. The release rate was identical to the exchange rate, showing that the dissociation characteristics determine the cycling of this molecule. During mitosis, we found that the dissociation rate was markedly accelerated and, in addition, the long-lived fraction was markedly reduced. This indicates that a fusion arrest in mitosis is not the result of EEA1 not binding to early endosomes, but rather due to the marked shift in membrane-binding characteristics. This might be a general mechanism to fine-tune and control tethering and fusion of early endosomes.

Keywords: cell cycle; EEA1; FRAP; fusion; photoactivation

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INTRODUCTION

In the fusion process between early endosomes, the early endosomal antigen 1 (EEA1) has been found to operate as an important docking/tethering factor. EEA1 binds to the membranes that contain the small GTPase Rab5 and phosphatidylinositol-3-phosphate (PtdIns3P). The carboxy-terminal FYVE domain of EEA1 binds specifically to PtdIns3P, and the Rab5-binding regions of EEA1 are located in two areas, one close to the FYVE domain and

the other at the far amino terminus (Simonsen *et al*, 1998). The effect of Rab5 with respect to binding of EEA1 is considered to be dual, as Rab5 *in vitro* has also been shown to recruit the phosphoinositide kinase (PI3-kinase), Vps34, through the effector protein p150 (Christoforidis *et al*, 1999), thereby enhancing production of PtdIns3P (Shin *et al*, 2005).

During interphase, the early endosome is a dynamic structure that undergoes heterotypic fusion with incoming vesicles, as well as homotypic fusion. At the start of mitosis, there is an arrest of some endocytic processes such as fusion of endosomal vesicles and recycling of membrane from endosomes back to the cell surface (Tuomikoski *et al*, 1989; Boucrot & Kirchhausen, 2007). Contrary to other organelles such as the Golgi apparatus and the nuclear envelope, which break down and fragment during mitosis (Gant & Wilson, 1997; Colanzi *et al*, 2003), endosomes remain distinct and do not fragment. EEA1 is located on the endosomes during both interphase and mitosis, and the presence of EEA1 alone is not sufficient to distinguish a fusing from a non-fusing endosome (Bergeland *et al*, 2001).

In the present study, we measured the kinetic properties of the C terminus of EEA1 (residues 1257–1411, here termed CtEEA1), which contains adjacent Rab5 and PtdIns3P-interacting domains, represented by fusion proteins with either green fluorescent protein (GFP) or photoactivable GFP (PAGFP) (McBride *et al*, 1999; Patterson & Lippincott-Schwartz, 2002). The kinetic properties of CtEEA1 were compared with full-length EEA1, and also with the PtdIns3P-interacting part (FYVE domain). Single endosomes are small and dynamic, and can be studied over time by three-dimensional scanning (Rink *et al*, 2005); however, as they are rapidly moving in all dimensions, it is difficult to obtain precise single-organelle fluorescence measurements. To make the system more stable for measuring fluorescent intensity, we transfected the cells with the major histocompatibility complex class II-associated invariant chain (CD74), which is fusogenic and enlarges the early endosomes in a PI3-kinase- and Rab5-independent manner, without blocking the maturation process (Romagnoli *et al*, 1993; Stang & Bakke, 1997; Engering *et al*, 1998; Nordeng *et al*, 2002). By using fluorescent recovery after photobleaching (FRAP) on single endosomes, we found that CtEEA1-GFP is highly dynamic and cycles rapidly between specific membranes and the cytosol.

¹Centre for Immune Regulation, Department of Molecular Biosciences, University of Oslo, PO Box 1041, Blindernveien 31, Oslo 0316, Norway

²Department of Biochemistry, Institute for Cancer Research, The Norwegian Radium Hospital, Oslo 0310, Norway

³The Gade Institute, University of Bergen, N-5021 Bergen, Norway

*Corresponding author. Tel: +47 22 851 479;

E-mail: oddmund.bakke@imbv.uio.no

By combining FRAP and photoactivation, we found that the CtEEA1 off-rate is the regulating step in the CtEEA1 on/off kinetics.

Release and binding of CtEEA1 to early endosomes were studied under two given conditions. First, we established how CtEEA1 cycles between endosomal membranes and the cytosol during interphase. Second, we studied whether the cycling of CtEEA1 was influenced by the increased fusion induced by overexpressing Rab5. These measurements were repeated using mitotic cells in which endosomal fusion was arrested. The on/off cycling of CtEEA1-GFP/PAGFP under these different conditions was significantly changed, and we suggest that the on/off rates together with the highly different immobile fraction of CtEEA1 are decisive for the function of EEA1 as a tethering/fusion molecule.

RESULTS AND DISCUSSION

Membrane to cytosol cycling of EEA1 on single organelles

To study the on/off cycling of EEA1 at a single-organelle level, we stably transfected EEA1-GFP or CtEEA1-GFP together with CD74 into Madin–Darby canine kidney strain II (MDCK) cells. The CD74-induced endosomes are well suited for single-organelle FRAP (Fig 1A). The intensity was normalized, subjected to nonlinear regression and plotted as a function of time (Fig 1B). The cycling of CtEEA1-GFP is a dynamic process with an exchange half-life ($t_{e1/2}$) of 4.78 ± 0.15 s in which 10.8% of the coat was not rapidly exchanged, giving an immobile fraction of 0.108 ± 0.002 . The full-length version of EEA1-GFP had a $t_{e1/2}$ of 14.68 ± 0.35 s and an immobile fraction of 0.127 ± 0.002 (Table 1). The full-length version of EEA1 contains an additional Rab5 interaction site at the amino N terminus and extensive coiled-coil regions that are involved in dimerization of the molecule (Callaghan *et al*, 1999). Both these elements could have an effect on kinetics (see below). The diameter of randomly selected EEA1/CtEEA1-positive endosomes ranged from 0.5 to 5 μ m, and we found that the recovery times were independent of vesicle size (data not shown).

The bleaching laser (405 nm) was used at full effect within a small area, and this might cause radiation damage and influence the CtEEA1-binding characteristics. However, as shown in Fig 1C,D, a second FRAP gave a recovery curve for the mobile fraction that was identical to the first. We also observed complete recovery after the second bleach (Fig 1E), indicating that the binding sites for the mobile fraction are not altered by photo-damage. The small negative fraction shown corresponds to the half-life of the immobile fraction (see below).

The immobile fraction is defined by the difference between initial and recovered intensity. This fraction consists of bleached CtEEA1-GFP and, to assure that the bleaching laser did not influence this fraction, we used a second complementary method. First, we bleached all cytosolic fluorescent CtEEA1-GFP by long-term exposure of one area, followed by measurement of the remaining fluorescence on the endosomes in the unbleached area (Fig 1F,G). The fluorescence intensity in the cytosol, outside the bleached area, rapidly decreased to a basal level indicating a rapid movement of these free molecules in the cell. The fluorescence loss in photobleaching (FLIP) was then measured on single endosomes positive for CtEEA1-GFP and, after an initial loss of fluorescence intensity, a fraction remained stable. The remaining immobile fraction corresponded to the FRAP-immobile fraction above, corroborating the validity of these results.

To investigate the stability of the immobile fraction, we carried out FRAP on single endosomes and measured the recovery for up to 10 min. This fraction was also exchanged, but with a relatively long half-life of 91.21 ± 38.78 s and, after 7 min, there was a 94% recovery of the immobile fraction (Table 1). The relatively large standard error in half-life is presumably due to fusion and fission of small vesicles into and out from the bleached enlarged endosome during the measuring period. We also observed this in rapid four-dimensional imaging of these vesicles (F. Skjeldal and O.B., unpublished data). The CtEEA1 coat thus contains two mobile fractions: one that cycles rapidly, and the other that cycles slowly between the membrane and cytosol. As the half-life of the more stable fraction is almost 20-times longer, we assign this as the 'immobile fraction' and the rapid exchangeable fraction as the 'mobile fraction', as used previously (Lippincott-Schwartz *et al*, 2001).

In the above FRAP experiments, we measured the rate for the GFP fusion molecules to be exchanged. To measure specifically the off rates, we replaced the GFP with PAGFP for CtEEA1 (CtEEA1-PAGFP) and transfected the construct into CD74-over-expressing cells. Single enlarged endosomes were selected by using bright-field microscopy and photoactivated by the 405 nm laser (Fig 1H). The loss in intensity was normalized, and nonlinear regression was carried out and plotted as a function of time (Fig 1I). We observed a residence half-life ($t_{r1/2}$) of 4.78 ± 0.57 s and an immobile fraction of 0.084 ± 0.008 (Table 2), showing that the $t_{r1/2}$ measured after photoactivation is almost identical to the $t_{e1/2}$ measured by FRAP. This indicates that the on rate is much faster than the off rate, and shows that the residence time of CtEEA1 on the endosomal membrane is determined by the off rate.

Rab5 slows down the cycling of EEA1

Overexpression of both GTPase-defective Rab5_{Q79L} (Rab5GTP) and Rab5 wild type (Rab5wt) have been found to increase the recruitment of EEA1 and enhance homotypic early endosome fusion, resulting in an increase in endosomal size (Gorvel *et al*, 1991; Stenmark *et al*, 1994; Li *et al*, 1995; Simonsen *et al*, 1998). We subcloned these two constructs into the inducible expression vector, pMep4, and stably coexpressed them with either CtEEA1-GFP or EEA1-GFP in MDCK cells. Single endosomes positive for EEA1-GFP or CtEEA1-GFP in all these four cell lines were selected for FRAP and the exchange rates were measured. In cells expressing Rab5GTP, we observed a less rapid exchange and a higher immobile fraction for both EEA1-GFP and CtEEA1-GFP as compared with the CD74-transfected cells (Fig 2A; Table 1). From this, we conclude that Rab5GTP increases the binding of both EEA1 and CtEEA1 to early endosomes. This might lead to increased homotypic tethering of endosomes, resulting in the observed higher rate of fusion and enlargement of endosomes. When FRAP was carried out on vesicles positive for CtEEA1-GFP or EEA1-GFP in cells expressing Rab5wt, we also found a slower exchange and a higher immobile fraction than in the control cells (Fig 2A; Table 1). However, the effects were intermediate to those of Rab5GTP and the control CD74 endosomes. As Rab5wt is able to hydrolyse its bound GTP, the stronger effect of Rab5GTP indicates that the observed effect is due to the active GTP form of the molecule. Conversely, the differences in kinetics between EEA1-GFP and CtEEA1-GFP did not increase in the presence of Rab5wt or Rab5GTP when compared with the CD74-expressing

Fig 1 | Rapid on/off cycling of CtEEA1-GFP/PAGFP. (A) Single endosomes positive for CtEEA1-GFP were bleached and the fluorescence intensities were monitored over time. (B) The measured intensities were normalized to the starting values (set to 1) and plotted against time. (C) Sequential bleaching was carried out on endosomes positive for CtEEA1-GFP and were monitored over time. A representative experiment is presented in (D), in which MFI is plotted against time. (E) To compare the first (upside down filled triangle) with the second (filled circle) recovery, time 0 was set to the first image acquisition after each bleach and normalized to the value before each bleach (set to 1). (F) Cells were subjected to FLIP, and the fluorescence on CtEEA1-GFP-positive endosomes outside the bleached area was monitored. (H) CtEEA1-PAGFP on single endosomes was activated by a rapid 405 nm pulse and the loss of fluorescence was monitored over time. (I) The measured fluorescence was normalized and plotted against time. The arrows indicate representative enlarged endosomes where fluorescent intensity was measured. Scale bars, 10 μ m. EEA1, early endosomal antigen 1; FLIP, fluorescence loss in photobleaching; GFP, green fluorescent protein; MFI, mean fluorescent intensity; PAGFP, photoactivable GFP.

Table 1|FRAP of single endosomes positive for CtEEA1-GFP, EEA1-GFP or 2 × FYVE-GFP

	<i>N</i>	<i>F</i> (0)	<i>F</i> (∞)	<i>t</i> _{e1/2} (s)	Immobile fraction
CtEEA1-GFP					
<i>Interphase</i>					
CD74, first bleach	19	0.361 ± 0.008	0.931 ± 0.001	4.778 ± 0.145	0.108 ± 0.002
CD74, second bleach	9	0.516 ± 0.008	1.012 ± 0.001	4.864 ± 0.181	−0.025 ± 0.002
CD74, immobile fraction	7	0.905 ± 0.008	0.994 ± 0.008	91.21 ± 38.78	0.062 ± 0.086
CD74, G1 phase	6	0.373 ± 0.013	0.929 ± 0.003	4.501 ± 0.270	0.114 ± 0.005
CD74, S phase	5	0.260 ± 0.015	0.916 ± 0.004	4.415 ± 0.271	0.113 ± 0.005
CD74, G2 phase	9	0.298 ± 0.012	0.917 ± 0.003	4.873 ± 0.249	0.119 ± 0.005
Rab5wt	10	0.319 ± 0.010	0.899 ± 0.002	8.999 ± 0.389	0.148 ± 0.003
Rab5GTP	17	0.275 ± 0.012	0.881 ± 0.005	18.31 ± 1.103	0.165 ± 0.007
<i>Metaphase</i>					
CD74	8	0.389 ± 0.018	0.972 ± 0.001	1.824 ± 0.108	0.0463 ± 0.002
Rab5wt	12	0.446 ± 0.013	0.973 ± 0.001	2.716 ± 0.141	0.0486 ± 0.003
Rab5GTP	10	0.369 ± 0.015	0.945 ± 0.001	3.047 ± 0.160	0.0873 ± 0.002
EEA1-GFP					
<i>Interphase</i>					
CD74	27	0.428 ± 0.004	0.927 ± 0.001	14.680 ± 0.349	0.127 ± 0.002
Rab5wt	12	0.498 ± 0.008	0.918 ± 0.003	19.910 ± 1.060	0.163 ± 0.006
Rab5GTP	18	0.465 ± 0.006	0.885 ± 0.003	24.800 ± 1.142	0.215 ± 0.006
<i>Metaphase</i>					
CD74	22	0.473 ± 0.007	0.975 ± 0.001	7.175 ± 0.225	0.048 ± 0.002
Rab5wt	10	0.460 ± 0.009	0.967 ± 0.002	8.869 ± 0.397	0.061 ± 0.004
Rab5GTP	12	0.457 ± 0.006	0.966 ± 0.001	9.840 ± 0.281	0.063 ± 0.002
2 × FYVE-GFP					
<i>Interphase</i>					
CD74	10	0.386 ± 0.022	0.728 ± 0.004	0.704 ± 0.118	0.442 ± 0.007
Rab5GTP	10	0.392 ± 0.026	0.637 ± 0.005	0.810 ± 0.228	0.596 ± 0.009
<i>Metaphase</i>					
CD74	7	0.442 ± 0.029	0.795 ± 0.005	0.341 ± 0.083	0.367 ± 0.008
Rab5GTP	10	0.437 ± 0.021	0.719 ± 0.003	0.404 ± 0.085	0.498 ± 0.006

Each data set was fitted by nonlinear regression and *F*(0), *F*(∞) and *t*_{1/2} were calculated with their respective standard error of mean. EEA1, early endosomal antigen 1; FRAP, fluorescent recovery after photobleaching; GFP, green fluorescent protein; Wt, wild type.

cells. This indicates that the Rab5-interacting site at the N terminus of EEA1-GFP is not the determining factor for the differences in kinetics between EEA1-GFP and CtEEA1-GFP. It has been reported that both EEA1 and CtEEA1 have the capacity to make dimers; however, the coiled-coil region of EEA1 makes the dimerization more stable (Callaghan *et al*, 1999). Thus, the slower on/off cycling we measured for EEA1-GFP compared with CtEEA1-GFP can probably be explained by a higher number of dimers for EEA1-GFP. As the differences in kinetics of these two constructs were not determined by Rab5, our data are in line with earlier reports in which the N-terminal part of EEA1 has been proposed to be directed outward from the membrane, facilitating tethering with adjacent Rab5-positive vesicles (Dumas *et al*, 2001).

The observed decrease in the on/off rate of CtEEA1-GFP following Rab5 expression could be a result of slower off rate and/or an increased on rate. By using the CtEEA1-PAGFP to measure the off rate by photoactivation, we found a *t*_{1/2} similar to the *t*_{e1/2} (Fig 2B; Table 2) for both the Rab5wt and Rab5GTP cell lines. From this, we conclude that the significantly slower CtEEA1 exchange rates induced by excess Rab5wt or Rab5GTP are the result of prolonged off rates.

Cycling of EEA1 is accelerated in mitosis

As endosomal fusion and fission are strongly inhibited or arrested in mitosis, we wanted to study the binding kinetics and carried out FRAP of EEA1-GFP and CtEEA1-GFP on CD74-induced

Table 2|Photoactivation of single endosomes positive for CtEEA1-PAGFP

	<i>N</i>	<i>F</i> ₁	<i>F</i> (0)	<i>F</i> (∞)	<i>t</i> _{1/2} (s)	Immobile fraction
CtEEA1-PAGFP						
Interphase						
CD74	15	0.447 ± 0.034	0.999 ± 0.021	0.493 ± 0.005	4.781 ± 0.570	0.084 ± 0.008
Rab5wt	8	0.655 ± 0.036	0.981 ± 0.016	0.693 ± 0.005	8.648 ± 1.368	0.116 ± 0.014
Rab5GTP	10	0.613 ± 0.020	0.960 ± 0.014	0.675 ± 0.007	17.25 ± 2.741	0.178 ± 0.019
Metaphase						
CD74	14	0.543 ± 0.040	1.016 ± 0.032	0.568 ± 0.005	2.069 ± 0.442	0.053 ± 0.011
Rab5wt	6	0.565 ± 0.066	1.010 ± 0.048	0.582 ± 0.009	2.977 ± 0.993	0.038 ± 0.020
Rab5GTP	8	0.522 ± 0.043	0.996 ± 0.037	0.582 ± 0.008	4.262 ± 1.078	0.126 ± 0.016

Each data set was fitted by nonlinear regression, and *F*(0), *F*(∞) and *t*_{1/2} were calculated with their respective standard error of mean (s.e.m.). *F*₁ represents mean fluorescence (± s.e.m.) in the activated area before activation. EEA1, early endosomal antigen 1; PAGFP, photoactivable GFP; Wt, wild type.

endosomes during mitosis. Cells in metaphase were selected for FRAP at the microscope table by incubation with Hoechst stain. Compared with interphase endosomes, the kinetics were markedly altered. For CtEEA1-GFP, the *t*_{e1/2} was reduced from 4.8 to 1.8 s, and the immobile fraction from 0.11 to 0.05 (Fig 2C; Table 1); similar effects were seen for the full-length version of EEA1 (Table 1), and also after photoactivation of CtEEA1-PAGFP-positive endosomes (Fig 2D; Table 2). The CtEEA1-GFP off rate is more rapid in mitosis than in interphase and the immobile fraction is reduced, indicating that the binding kinetics are a crucial factor for the function of EEA1 in homotypic endosomal tethering.

On the basis of the marked differences in EEA1-binding kinetics between interphase and mitosis, we speculated whether there could also be differences within the different phases of interphase. By using Hoechst DNA stain and flow cytometry we found a distribution of 45% in G1 phase, 20.8% in S phase and 34.1% in G2/M phase from the DNA histograms. The live monolayer cells were also stained by Hoechst, and the different phases were determined by DNA stain intensity as described in the Methods. When comparing the *t*_{e1/2} between G1, S and G2 phase, we found no differences in the endosomal exchange rate for CtEEA1 during the various stages of interphase (Table 1). The immobile fractions were also the same throughout interphase. The marked alteration in coat kinetics for CtEEA1 is then seen only when the cells enter the mitotic phase of the cell cycle.

To investigate whether Rab5 has a similar effect on EEA1-binding kinetics in mitosis as in interphase, we carried out FRAP on endosomal EEA1-GFP or CtEEA1-GFP in mitotic cells overexpressing Rab5wt. Interestingly, as previously described for CD74, we observed a similar ‘mitotic shift’ (shorter *t*_{e1/2} and reduced stable fraction) in the on/off cycling of both EEA1-GFP and CtEEA1-GFP in cells expressing Rab5wt (Fig 2C). A similar shift was also found when we photoactivated metaphase endosomes containing CtEEA1-PAGFP and compared them with interphase endosomes (Fig 2D). By using overexpressed Rab5GTP, FRAP measurements showed a more pronounced effect for both EEA1-GFP and CtEEA1-GFP. The *t*_{e1/2} was reduced from 18.3 s in interphase to 3.0 s for CtEEA1-GFP in mitosis (Fig 2C; Table 1), again corroborated by photoactivation of endosomes in cells containing CtEEA1-PAGFP (Fig 2D; Table 2). The qualitatively

different EEA1-binding parameters in interphase and mitosis might thus explain directly the lack of endosomal fusion observed during mitosis, even in cells overexpressing Rab5GTP.

Rab5 controls the kinetics of 2 × FYVE-GFP

The reduced cycling observed for EEA1 following Rab5wt or Rab5GTP overexpression might be caused by direct interactions between Rab5 and EEA1. However, active Rab5 has also been found to recruit the PI3-kinase, Vps34 (Christoforidis *et al*, 1999), and as EEA1 interacts with PtdIns3P, the reduced cycling of EEA1 when Rab5wt or Rab5GTP is overexpressed could be the outcome of increased PI3-kinase activity. The FYVE domain of EEA1 interacts with PtdIns3P and to examine the contribution of this binding site alone, we transfected MDCK cells with the PtdIns3P-interacting construct 2 × FYVE-GFP and carried out FRAP on enlarged endosomes in cells expressing Rab5GTP or CD74. We observed a decrease in the on/off cycling for 2 × FYVE-GFP in cells overexpressing Rab5GTP when compared with the cells overexpressing CD74 (Fig 3A; Table 1). We suggest that this change is caused by a higher concentration of PtdIns3P in the Rab5GTP-expressing cells when compared with control cells. However, this effect is much smaller than that observed for the EEA1 constructs, indicating that Rab5GTP is the main factor for determining EEA1 binding when Rab5GTP is overexpressed.

To estimate the contribution of PtdIns3P binding alone in metaphase, we used 2 × FYVE-GFP and carried out FRAP on enlarged endosomes in cells expressing CD74 or Rab5GTP. Comparing the kinetic data from mitotic endosomes with those from interphase, we observed a shorter half-life in the mitotic cells both by expressing CD74 and Rab5GTP (Fig 3B; Table 1). These differences in on/off cycling could be due to changes in the amount of PtdIns3P on endosomes in mitosis, and to determine the amount of PtdIns3P, we used *in situ* labelling of fixed cells with a 2 × FYVE-biotin-glutathione-S-transferase (GST) probe (Gillooly *et al*, 2000; Fig 4A). We discovered a 37% reduction in the total PtdIns3P in metaphase cells when compared with interphase cells (Fig 4B). This indicates that the mitotic shifts in kinetics for 2 × FYVE-GFP are a result of less PtdIns3P on endosomes in mitosis. These shifts in kinetics were, however, much smaller than for EEA1. As the mitotic shift is much

Fig 2 | FRAP and photoactivation of CtEEA1-GFP/PAGFP. Endosomes positive for CtEEA1-GFP/PAGFP in cells expressing CD74, Rab5wt or Rab5GTP were subject to (A) FRAP in interphase, (B) photoactivation in interphase, (C) FRAP in mitotic metaphase and (D) photoactivation in mitotic metaphase. EEA1, early endosomal antigen 1; FRAP, fluorescent recovery after photobleaching; GFP, green fluorescent protein; PAGFP, photoactivable green fluorescent protein.

more significant for EEA1 than for $2 \times$ FYVE, we suggest that the Rab5-interacting domains of EEA1 are fundamental for its accelerated kinetics in mitosis.

The definition of organelles has been based on the presence of specific organelle markers. Rab5 and EEA1 have been used as markers for early endosomes. As we have shown here, the presence of a marker on an organelle does not indicate its function, as this also depends on its binding kinetics. EEA1 is present on endosomes both in a fusion-active and a fusion-inactive state; however, the capacity for tethering seems to be regulated by the dissociation kinetics of EEA1. Regulating the binding parameters, such as speed of exchange and the immobile

fraction, might be important for the control of vesicular transport in a cell, in addition to the presence of the correct docking factors such as the SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor) molecules (Jahn & Scheller, 2006). There is very little information concerning the binding kinetics of the many tethering and docking factors involved in membrane transport. This information might, however, be crucial to understanding their function. Similar data from many membrane-binding proteins at different stages of activity would create a better basis to understand to what extent the cell is regulating these parameters to control and/or fine-tune the various functions within organelle and membrane transport. For EEA1, our

Fig 3 | FRAP of 2 × FYVE-GFP. Endosomes positive for 2 × FYVE-GFP in cells expressing CD74 or Rab5GTP were subject to FRAP in (A) interphase and (B) mitotic metaphase. FRAP, fluorescent recovery after photobleaching; GFP, green fluorescent protein.

experiments show that the residence time on early endosomes is controlled by the molecules off rate; however, whether this is a general mechanism for other coat proteins remains to be seen.

METHODS

Constructs. Complementary DNAs encoding CD74 (Bakke & Dobberstein, 1990), Rab5wt (Chavrier *et al*, 1990) and Rab5Q79L (Stenmark *et al*, 1994) have been described previously and were subcloned into the pMep4 vector (Invitrogen, Carlsbad, CA, USA) as *KpnI*–*Bam*HI, *Hind*III–*Xho*I and *Hind*III–*Xho*I fragments, respectively. EEA1-GFP, CtEEA1-GFP, 2 × FYVE-GFP and 2 × FYVE-biotin-GST have been previously described (McBride *et al*, 1999; Gillooly *et al*, 2000; Lawe *et al*, 2002), and CtEEA1-GFP acted as an individual template for two consecutive Quikchange (Stratagene, La Jolla, CA, USA) reactions (converting S65T and T203H in CtEGFP) to generate CtEEA1-PAGFP.

Cells and reagents. MDCK cells stably transfected with CD74, Rab5wt or Rab5Q79L under the control of the metallothionein promoter in the pMep4 vector were used. The expression of CD74, Rab5wt or Rab5Q79L was induced by adding 15 μM CdCl₂. The cells were stably transfected with CtEEA1-GFP/PAGFP, EEA1-GFP or 2 × FYVE-GFP and grown in complete medium: DMEM (Bio Whittaker, Walkersville, MD, USA) supplemented with 9% fetal bovine serum (PAA Laboratories, Pasching, Austria), 2 mM glutamine (Bio Whittaker), 25 U/ml penicillin (Bio Whittaker) and 25 μg/ml streptomycin (Bio Whittaker) in 6% CO₂ in a 37 °C incubator. For selection of cells in metaphase and nuclear staining, the cells were incubated with Hoechst (Sigma-Aldrich, St Louis, MO, USA) to a concentration of 5 μg/ml.

Confocal microscopy. MDCK cells were grown on chambered cover glass (MatTek Corporation, Ashland, MA, USA) containing DMEM. Before microscopy was carried out, the medium was changed to DMEM lacking phenol red and sodium bicarbonate, and supplemented with 25 mM HEPES (Gibco, Paisley, UK) and

9% fetal bovine serum. Constant temperature was set to 37 °C by an incubator enclosing the microscope stage.

Confocal images were obtained using an Olympus FluoView 1000 inverted microscope equipped with a PlanApo × 60/1.10 oil objective and a SIM scanner. For FLIP, FRAP and photoactivation applications, GFP/PAGFP molecules were bleached and activated using a 405 nm laser diode. The data obtained from FRAP and photoactivation were normalized and corrected for bleaching (Pelkmans *et al*, 2001), and fitted by nonlinear regression to a function that assumes a single diffusion coefficient (Yguerabide *et al*, 1982):

$$F(t) = \frac{F(0) + F(\infty)(t/t_{1/2})}{1 + (t/t_{1/2})}$$

The values for $F(0)$, $F(\infty)$ and $t_{1/2}$ were calculated using Prism4 (GraphPad Software) and used to calculate the immobile fractions (Lippincott-Schwartz *et al*, 2001; Lippincott-Schwartz & Patterson, 2003).

For *in situ* labelling of PtdIns3P, MDCK cells were grown on coverslips and fixed in 3% paraformaldehyde overnight at 4 °C, stained with Hoechst and biotinylated-GST-2 × FYVE (20 μg/ml) followed by Cy3-streptavidin (2 μg/ml; Jackson Immunoresearch, West Grove, PA, USA). Confocal images were acquired using an open pinhole. Total fluorescent intensity of cells was measured using ImageJ (NIH). Cells were grouped, according to nuclear stain, into interphase ($N=104$) or metaphase ($N=20$). Cells in other stages of the cell cycle were not included in the analysis.

Determination of cell-cycle phases. Single-cell suspensions were prepared by treating the cells with trypsin and resuspended in PBS. The cells (1×10^6) were pelleted and fixed in 70% ice-cold ethanol at 4 °C for 30 min. The cells were washed twice in PBS, diluted in PBS and then Hoechst was added. Flow cytometry was carried out on a FACS DiVa (Becton Dickinson, San Jose, CA, USA) equipped with a 10 mW UV laser spanning from 351 to 364 nm for excitation of Hoechst. For microscopy, live monolayer

Fig 4 | PtdIns3P in interphase and metaphase. Non-transfected MDCK cells were fixed and stained with a specific probe directed against PtdIns3P ($2 \times$ FYVE-biotin-GST). The arrow indicates a representative cell in metaphase. **(B)** Relative signals for total labelling of interphase cells were compared with those of metaphase cells ($\pm$ s.e.m.). Statistical analysis showed a significant difference; $P < 0.0001$ (t -test). GST, glutathione- S -transferase; MDCK, Madin-Darby canine kidney strain II; PtdIns3P, phosphatidylinositol-3-phosphate.

cells were stained by Hoechst and the intensity measurements showed whether the cells were in G1, S or G2 phase.

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