A novel assay reveals preferential binding between Rabs, kinesins, and specific endosomal subpopulations

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Identifying the proteins that regulate vesicle trafficking is a fundamental problem in cell biology. In this paper, we introduce a new assay that involves the expression of an FKBP12-rapamycin–binding domain–tagged candidate vesicle-binding protein, which can be inducibly linked to dynein or kinesin. Vesicles can be labeled by any convenient method. If the candidate protein binds the labeled vesicles, addition of the linker drug results in a predictable, highly distinctive change in vesicle localization. This assay generates robust and easily interpretable results that provide direct experimental evidence of binding between a candidate protein and the vesicle population of interest. We used this approach to compare the binding of Kinesin-3 family members with different endosomal populations. We found that KIF13A and KIF13B bind preferentially to early endosomes and that KIF1A and KIF1Bβ bind preferentially to late endosomes and lysosomes. This assay may have broad utility for identifying the trafficking proteins that bind to different vesicle populations.

Introduction

Organelles in the endomembrane system receive and dispatch vesicles in a highly regulated manner, which is mediated by a complex set of trafficking proteins: motors, SNAREs, tethers, Rabs, coat proteins, cargo adaptors, and others (Vale, 2003; Spang, 2008; Wickner and Schekman, 2008). Identifying the trafficking proteins that associate with different vesicle populations is an essential step in understanding the mechanisms that regulate vesicle trafficking. To accomplish this task, specific vesicle populations can be enriched by subcellular fractionation, immuno-isolation, or fluorescence sorting, and then the proteins present can be identified (Franzusoff et al., 1992; Takamori et al., 2006; Duclos et al., 2011; Zhang et al., 2011; Rhee et al., 2013). Two-color fluorescence microscopy is often used to confirm that a candidate protein binds the relevant vesicle population in vivo, in the appropriate biological context. However, this approach cannot always provide a definitive answer, as visual colocalization is often difficult to evaluate. Alternate approaches such as immuno-EM can be tedious.

Recently, we developed a new strategy to determine which members of a set of candidate kinesins associate with dendritically polarized vesicles in neurons (Jenkins et al., 2012). This “split kinesin” assay involves the expression of separate constructs encoding a kinesin tail fused to the FKBP12-rapamycin–binding (FRB) domain and a kinesin motor domain fused to the FKBP12 domain. These two domains can be inducibly assembled by adding a membrane permeant rapamycin analogue (Belshaw et al., 1996; Kapitein et al., 2010b; Robinson et al., 2010). The kinesin tail contains the cargo-binding domain and can bind vesicles, but it is incapable of influencing their movement because it lacks a motor domain. The kinesin motor domain is constitutively active and translocates into the axon but cannot move vesicles because it lacks a cargo-binding domain. Linking these two components together leads to a dramatic increase in vesicles entering the axon if and only if the expressed kinesin tail binds to the vesicles in question.

This assay offers several advantages. First, the induced change in vesicle trafficking is rapid and unmistakable. Second, the assay allows an unbiased evaluation of interactions between a given vesicle population and all relevant members of a family of trafficking proteins. Finally, the assay works well even if two different kinesins mediate the transport of a given organelle. Despite these advantages, the split-kinesin assay has limitations that restrict its applicability. First, the assay depends on the
A set of candidate trafficking proteins associates with a given vesicle population. Candidate proteins were tagged with FRB so that they could be linked to a constitutively active motor by rapamycin analogue-induced heterodimerization. We hypothesized that attaching dynein to vesicles would result in their accumulation near the MTOC. Previous work has shown that the localization of mitochondria or peroxisomes can be changed by adding a homotypic tether or by directly cross-linking constitutively active motors to integral membrane proteins present in these organelles (Sengupta et al., 2009; Kapitein et al., 2010a,b; van Spronsen et al., 2013). It is less clear whether linking motors to trafficking proteins that associate only transiently with the cytoplasmic surface of vesicles can produce a distinctive change in vesicle distribution.

As proof of principle, we asked whether linking an FRB-tagged Rab to an unregulated, constitutively active kinesin or dynein produces a distinctive change in the localization of the vesicles that bind that Rab. One potential advantage of directing vesicles toward the minus ends of microtubules is that they are likely to form a single aggregate near the cell center, which should provide a strong signal. We adapted the strategy developed by Kapitein et al., 2010a,b, who investigated dynein-driven peroxisome transport by inducibly linking BicD2, a dynein cargo adaptor, to peroxisomes (Dienstbier and Li, 2009; Kardon and Vale, 2009). Using this approach, we were able to identify the Rabs and kinesins that bind to different endosomal populations, based on drug-induced mislocalization of vesicles when FRB-tagged candidate proteins were linked to active motors. This new assay generates robust and easily interpretable results and, in contrast to conventional, two-color localization experiments, provides direct experimental evidence that a candidate protein binds to a particular vesicle population.

**Results and discussion**

**Linking Rabs to active motors results in mislocalization of endosomes**

We set out to develop an assay in which inducible, motor-driven vesicle mislocalization serves as a readout to indicate which of a set of candidate trafficking proteins associates with a given vesicle population. Candidate proteins were tagged with FRB so that they could be linked to a constitutively active motor by rapamycin analogue-induced heterodimerization. We hypothesized that attaching dynein to vesicles would result in their accumulation near the MTOC. Previous work has shown that the localization of mitochondria or peroxisomes can be changed by adding a homotypic tether or by directly cross-linking constitutively active motors to integral membrane proteins present in these organelles (Sengupta et al., 2009; Kapitein et al., 2010a,b; van Spronsen et al., 2013). It is less clear whether linking motors to trafficking proteins that associate only transiently with the cytoplasmic surface of vesicles can produce a distinctive change in vesicle distribution.

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association with active motors was never observed in control cells and because this mislocalization requires that the FRB-tagged proteins bind to the labeled vesicles, we interpret positive results as strong evidence of vesicle binding, even if observed in only a fraction of the cells.

Evaluating the selectivity of Rab binding to early and late endosomes

We next asked whether the binding of different FRB-Rabs shows the expected selectivity for different endosome populations. We used two approaches to identify early endosomes: immunostaining to localize EEA1 and uptake of Tf555 or LysoTracker red. LysoTracker red was used to visualize late endosomes and lysosomes.

In drug-treated cells, the localization of GFP-Rab5 vesicles was profoundly altered. Vesicles were found almost exclusively at the cell center, presumably because they were driven toward the MTOC by dynein. Virtually all of the BicD2 was also concentrated at the cell center, which suggests that molecules of BicD2 that dimerize with FRB-Rab5 remain attached to endosomal vesicles. A comparable experiment was conducted using a kinesin motor domain that was fused to FKBP (Vale et al., 1996; Friedman and Vale, 1999; Jacobson et al., 2006; Cai et al., 2009; Nakata et al., 2011). Endosomes were successfully misdirected to the cell periphery (Fig. S1), but the resulting change was not as obvious as that which resulted from linking vesicles to dynein.

In the experiment just described (and in those that follow), only about half of the transfected cells exhibited the profound changes in vesicle localization illustrated in Figs. 1 and S1. In the other cells, the distribution of endosomes and of BicD2 was no different than in controls. This is not surprising, because the assay depends on the coexpression of multiple proteins at levels appropriate to induce vesicle movement. Because the pattern of vesicle mislocalization induced by association with active motors was never observed in control cells and because this mislocalization requires that the FRB-tagged proteins bind to the labeled vesicles, we interpret positive results as strong evidence of vesicle binding, even if observed in only a fraction of the cells.

Figure 2. Rab7 interacts primarily with late endosomes. (A) A schematic showing the components of the assay. GFP-BicD2FKBP and FRB-3myc-Rab7 were coexpressed. Vesicles were labeled by anti-EEA1 staining or by uptake of Tf555 or LysoTracker red. (B) Representative images showing the distribution of each vesicle population and the distribution of BicD2. In controls, EEA1- and Tf555-labeled vesicles were found throughout the cell, whereas LysoTracker red vesicles were more concentrated in the perinuclear region. BicD2 had a soluble distribution. In cells treated with the linker drug, the distribution of EEA1- and Tf555-labeled vesicles was largely unchanged. Vesicles labeled with LysoTracker red (LysoRed) were massively misdirected to the cell center. BicD2 also accumulated in the cell center. The yellow lines outline the cell boundaries. Bar, 30 µm.
We next used this assay to investigate the kinesins that are present on different endosomal populations, using multiple approaches to label early and late endosomes (Figs. 3 and S2). We focused on motors of the Kinesin-3 family. Although some of these motors have been previously implicated in endosomal transport (Matsushita et al., 2004; Hoepfner et al., 2005; Blatner et al., 2007; Delevoye et al., 2009; Huckaba et al., 2011; Kanai et al., 2014), there has been no systematic study of whether different Kinesin-3’s bind preferentially to different endosomes.

We generated kinesin tails that included the cargo-binding domain but lacked the motor domain and the first coiled coil. These tails were fused to an N-terminal FRB-3myc. Each of these tails was then expressed together with FLAG-BicD2-FKBP to determine whether they bound to different endosomal populations (Fig. 3 A). We used the same three labels of endogenous vesicles as before. In controls, EEA1- and Tf555-labeled vesicles were found throughout the cell, whereas LysoTracker red vesicles were concentrated in the perinuclear region. In cells treated with the linker drug, EEA1- and Tf555-labeled vesicles were moved toward the cell center only in cells expressing FRB-3myc-KIF13Atail or FRB-3myc-KIF13Btail. Vesicles labeled with LysoTracker red moved to the cell center only in cells expressing FRB-3myc-KIF1F1Atail or FRB-3myc-KIF1Btail. The control cell expressed FRB-3myc-KIF1Atail and was not exposed to the linker drug. The yellow lines outline the cell boundaries. Bars: (EEA1) 25 μm; (Tf555 and LysoTracker red) 30 μm.

Identifying the motor proteins associated with different endosomal populations

We next used this assay to investigate the kinesins that are present on different endosomal populations, using multiple approaches to label early and late endosomes (Figs. 3 and S2). We focused on motors of the Kinesin-3 family. Although some of these motors have been previously implicated in endosomal transport (Matsushita et al., 2004; Hoepfner et al., 2005; Blatner et al., 2007; Delevoye et al., 2009; Huckaba et al., 2011; Kanai et al., 2014), there has been no systematic study of whether different Kinesin-3’s bind preferentially to different endosomes.

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In cells expressing KIF1A\textsubscript{tail} or KIF1B\textsubscript{tail} and treated with the linker drug, there was no change in distribution of early endosomes labeled with either Tfn555 or EEA1, but there was a profound redistribution of the late endosome marker LysoTracker red. Labeled endosomes formed a compact cluster in the center of the cell; few vesicles could be detected elsewhere. In cells expressing KIF13A\textsubscript{tail} or KIF13B\textsubscript{tail} and treated with linker drug, there was no effect on the distribution of late endosomes/lysosomes, but the addition of linker drug caused EEA1 and Tfn555 vesicles to aggregate in the cell center. This shows that different Kinesin-3 family members bind different endosomal populations; KIF1A and KIF1B\textsubscript{tail} bind to late endosomes, whereas KIF13A and KIF13B bind early endosomes. These results were confirmed using a series of GFP-tagged proteins to label different endosomal populations (Fig. S3).

An individual vesicle can bind different kinesins

The results just described indicate that each endosomal population is capable of binding two different Kinesin-3 family members. This raises the question of whether different kinesins are found on individual endosomes or whether there are subpopulations of early and late endosomes that bind different kinesins. This assay can address this question by using one kinesin tail to label vesicles, then determining whether other FRB-tagged kinesins bind to the same vesicles. Consistent with the results described earlier (Figs. 3 and S3), there was no misdirection of GFP-KIF1A\textsubscript{tail}–labeled vesicles in cells expressing FRB-KIF13A\textsubscript{tail} or FRB-KIF13B\textsubscript{tail}. These results show that a single vesicle can bind to both KIF1A and KIF1B\textsubscript{tail}.

Using the assay to define the vesicle-binding domains of trafficking proteins

In principle, this assay could also be used to define the vesicle-binding domains of trafficking proteins. To evaluate this possibility, we attempted to define the domain of KIF13B that binds early endosomes (Fig. 5). The KIF13B tail contains a forhead-associated (FHA) domain (residues 423–557) that binds centaurin-\(\alpha\) (Tong et al., 2010) and a membrane-associated guanylate kinase (MAGUK) binding stalk (residues 607–831) that interacts with homologues of Drosophila melanogaster discs-large (hDlg; Hanada et al., 2000). Previous studies have implicated the interaction with centaurin-\(\alpha\) and the interaction with hDlg as important for the binding of KIF13B to vesicles (Tong et al., 2010; Kanai et al., 2014). We generated an FRB-tagged fragment of the KIF13B tail that contains the FHA domain and the MAGUK binding stalk (KIF13B\textsubscript{442–831}). A second FRB-tagged construct contained the remainder of the tail (KIF13B\textsubscript{832–1,826}). These constructs were each coexpressed with transferrin receptor (TfR)-GFP and FLAG-BicD2\textsuperscript{594-FKBP} (Fig. 5 A). In control cells, TfR-GFP vesicles were distributed throughout the cell (Fig. 5 B). In cells that expressed KIF13B\textsubscript{442–831} and were treated with the linker drug, there was no change in the localization of TfR-GFP vesicles. In cells expressing KIF13B\textsubscript{832–1,826}, addition of the linker drug resulted in a pronounced redistribution of TfR-GFP to the cell center. This experiment shows that residues 832–1,826 are sufficient to mediate binding of KIF13B tail to early endosomes and that neither the FHA domain nor the MAGUK binding stalk mediates this interaction.

Advantages and limitations of this new assay

The motor-driven vesicle mislocalization assay described here offers a new approach to evaluate protein–vesicle association that has several advantages compared with other methods.
could also arise if the FRB-tagged protein cycles off the vesicle membrane quickly or if the FRB epitope becomes inaccessible for linking to FKBP. Despite these potential caveats, the conditions needed to obtain a positive result were not difficult to achieve.

We believe this assay will have broad application for investigating interactions between trafficking proteins and different vesicle populations. Once a set of FRB-tagged constructs has been prepared, they can be screened against a large number of vesicle populations in a remarkably short period of time. This assay may be particularly useful for evaluating the binding of trafficking proteins that are members of large families, such as Rabs, where any given vesicle is likely to bind a limited number of family members. If different family members bind different vesicles in this assay, this establishes that the FRB-tagged proteins are capable of binding and translocating vesicles but do not bind indiscriminately. This assay can also be adapted to explore other aspects of protein–vesicle interactions. By using one GFP-tagged Kinesin-3 to label vesicles, it was possible to establish that a second Kinesin-3 family member also bound to the same vesicle. The assay can also be used to identify the regions of trafficking proteins that mediate vesicle binding.

The role of Kinesin-3 family members in endosomal transport

We show that early and late endosomes bind different members of the Kinesin-3 family with high specificity. KIF13A and KIF13B, two closely related Kinesin-3s, bind early endosomes. KIF1A and KIF1B, two other Kinesin-3s that are also closely related to each other, bind late endosomes and lysosomes. These conclusions are based on consistent results obtained with all

Because binding of a candidate protein causes the vesicles of interest to move to a well-defined end point in the cell, positive interactions result in an unambiguous change in vesicle distribution that is easily interpretable. This makes it possible to evaluate the results by eye, thus allowing rapid screening of multiple combinations of candidate proteins and vesicle populations. Vesicles can be labeled by any convenient method, including the expression of fluorescent proteins, labeling with fluorescent dyes, or immunostaining.

Although the FRB-tagged candidate proteins are overexpressed, we saw little evidence that this led to nonspecific binding. For example, KIF1A and KIF1Bβ never associated with early endosomes and KIF13A and KIF13B never bound late endosomes or lysosomes. Vesicles may change their identity over time, shedding some trafficking proteins and gaining others. Thus, it is possible that an FRB-tagged protein could bind an unlabeled vesicle and induce a change in its localization; if the identity of that vesicle then changed, it could become labeled after it had been misdirected. The mislocalization of some LysoTracker red vesicles that occurred in cells expressing FRB-Rab5 may be an example of this (Fig. S2).

As with any assay, negative results must be interpreted with caution. If the FKBP- and FRB-tagged constructs are not expressed at sufficiently high levels, too few motors will become linked to the vesicles to move them to the intended destination. If a significant fraction of the FKBP-tagged motor becomes linked to FRB-tagged proteins that are not associated with vesicles—either because the expression level of the FRB-tagged protein is too high or because there is a large pool of unbound FRB protein—this could also result in too few vesicle-bound motors to produce effective movements. False negatives

Figure 5. Identifying the domain of KIF13B that binds to endosomes. (A) As shown in the schematic, different regions of KIF13B tail tagged with FRB were coexpressed with tdFim-BicD2Δ104FKBP and TfR-GFP. (B) In control cells, TfR-GFP was distributed throughout the cell. After treatment with the linker drug TfR-GFP, vesicles were moved to the cell center in cells expressing FRB-3myc-KIF13B832–1,826 but not FRB-3myc-KIF13B442–831. The yellow lines outline the cell boundaries. Bar, 30 µm.
seven of the markers we used to label these two endosomal populations (Figs. 3 and S3).

Although the work described here is the first systematic investigation of the interaction of Kinesin-3s with different endosomal populations, our findings are consistent with previous studies that implicated one or another Kinesin-3 in different aspects of endosomal trafficking. Kinesin-73, a Drosophila KIF13 homologue, binds Rab5 vesicles in S2 cells (Huckaba et al., 2011), KIF13A plays a role in tubule formation at sorting endosomes (Delevoye et al., 2009), and KIF13B has been implicated in endocytosis in hepatocytes (Kanai et al., 2014). In contrast, KIF18B binds lysosomes in COS7 cells (Matsushita et al., 2004). KIF16B, a Kinesin-3 we did not examine in this study, is also involved in endocytic trafficking (Hoepfner et al., 2005; Blatner et al., 2007). The new assay we developed allowed a systematic approach to this question, which revealed a pattern of Kinesin-3–endosome binding that appears to be consistent over a range of cell types and species but that was not apparent from earlier studies that examined one kinesin and its interaction with a single population of vesicles.

Conclusions

In the experiments presented here, we introduce a new method to identify protein–vesicle interactions in intact cells. Using an inducible dimerization system to link candidate vesicle-binding proteins to activated motors, the association of a candidate protein with a labeled vesicle is transduced into a predictable, highly distinctive change in vesicle localization. This output provides direct experimental evidence of vesicle binding, in contrast to approaches such as two-color colocalization, which are essentially correlative. Using this approach to investigate trafficking proteins that bind early and late endosomes, we show that the assay has a high specificity, a wide range of utility, and a readout that is easily interpretable.

Materials and methods

Constructs

All constructs were cloned into the pCAG expression vector (Niwa et al., 1991). This expression system consists of a cytomegalovirus–immediate early enhancer combined with a chicken β-actin promoter and has been shown to express at steady levels. We generated the following constructs: KIF5C559-tdTM-FKBP (Friedman and Vale, 1999; Jacobson et al., 2006), tdTM-Bicaudal594-tdTM-FKBP, and FLAG-Bicaudal594-FKBP (Kapitein et al., 2010a). FRB-tagged Rab proteins were prepared by inserting the FRB-3myc sequence at their N termini. FRB-tagged kinesin tails were engineered by removing the N-terminal motor domain and the first coiled-coiled domain, replacing them with an FRB-3myc domain. Details about linkers, accession numbers, and tags for each of these constructs can be found in Table 1. The following cDNAs were used to label specific vesicle populations: mouse Rab5a [GFP-Rab5a; GenBank accession number NM_025887] or Rab7 [GFP-Rab7; GenBank accession number NM_00107730] tagged with EGFP at their C terminus (TR-GFP; Burack et al., 2000; Silverman et al., 2001), human low density lipoprotein receptor tagged by insertion of EGFP downstream of the signal sequence [GFP-LDLR; GenBank accession number NM_00107730] tagged with EGFP at its C terminus (TR-GFP; Silverman et al., 2005); human Lamp1 tagged with a C-terminal GFP [LAMP1-GFP; GenBank accession number NM_00107730] tagged with EGFP at its C terminus (TR-GFP; Silverman et al., 2005).

FRB-tagged constructs included a 3myc epitope tag so that expressing cells could be identified by immunostaining; in preliminary experiments, we established that the great majority of cells that expressed both of the fluorescent constructs also expressed the FRB-tagged protein, based on anti-mycc immunostaining.

Cell culture

Rat embryonic fibroblast cells (Heidemann et al., 1999) were grown at 37°C in Dulbecco's modified Eagle's medium (Gibco) containing 10% fetal bovine serum, 4.5 g/liter d-glucose, 548 mg/liter d-glutamine, 110 mg/liter sodium pyruvate, 0.1 g/liter streptomycin, and 100 U/ml penicillin. 1 or 2 d before transfection, cells were trypsinized, and cells were replated on glass coverslips.

Vesicle binding assay

cDNAs encoding each of the components for the vesicle binding assay were expressed by transfection with FuGENE 6 (Promega). Transfected components included the putative vesicle binding protein tagged with FRB, either KIF5C559-tdTM-FKBP, tdTM-Bicaudal594-tdTM-FKBP, or FLAG-Bicaudal594-FKBP, and in some cases a GFP-tagged protein that served to label the vesicles of interest. After expression for ~48 h, heterodimerization of the FRB-tagged and FKBP-tagged proteins was induced by treating cells with 100 nM AP21967, a rapamycin analogue (Muthuswamy et al., 1999; Kapitein et al., 2010a). Kinesin tails were designed by removal of the N-terminal motor domain and the first coiled-coiled domain, replacing them with an FRB-3myc domain. Details about linkers, accession numbers, and tags for each of these constructs can be found in Table 1. The following cDNAs were used to label specific vesicle populations: mouse Rab5a [GFP-Rab5a; GenBank accession number NM_025887] or Rab7 [GFP-Rab7; GenBank accession number NM_00107730] tagged with EGFP at their C terminus (TR-GFP; Burack et al., 2000; Silverman et al., 2001), human low density lipoprotein receptor tagged by insertion of EGFP downstream of the signal sequence [GFP-LDLR; GenBank accession number NM_00107730] tagged with EGFP at its C terminus (TR-GFP; Silverman et al., 2005); human Lamp1 tagged with a C-terminal GFP [LAMP1-GFP; GenBank accession number NM_00107730] tagged with EGFP at its C terminus (TR-GFP; Silverman et al., 2005).

Table 1. Constructs used in the induced dimerization assay

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Accession numbers were obtained from GenBank.

*Includes epitope tag or label (FLAG, myc, GFP, or tdTM), heterodimerization domain (FRB or FKBP), amino acid linker sequence (using single letter code), and trafficking protein (amino acids included).
Fluor 55-tagged human transferrin before fixation (Molecular Probes). In some experiments, EEA1-positive endosomes were labeled after fixation by immunostaining with the rabbit monoclonal anti-EEA1 (C45B10; Cell Signaling Technology). Myc-tagged proteins were detected by immunostaining with the mouse monoclonal anti–c-myc 9E10 (M4439; Sigma-Aldrich).

**Microscopy**

Cells were imaged using an epifluorescence microscope (AxioObserver.Z1; Carl Zeiss) equipped with an LCI Plan Apochromat 40×/1.3 NA or LCI Plan Apochromat 63×/1.4 NA objective. Images of cells were acquired using a camera (AxioCam MRm; Carl Zeiss) and AxioVision software (Carl Zeiss).

**Online supplemental material**

Fig. S1 shows that linking constitutively active Kinesin-1 to Rab7 endosomes results in their accumulation in the periphery of the cell. Fig. S2 shows that linking FRB-Rab5 to dynein results in the accumulation of early endosomes at the cell center. Fig. S3 shows that different Kinesin-3 family members bind early and late endosomes. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201408056/DC1.

We thank Barbara Smoody for her outstanding technical assistance, Dr. Stefanie Kaech for advice on imaging, and Drs. Fried Robinson, Bruce Schnapp, and Vladimir Gelfand for their comments on the manuscript. This work was supported by National Institutes of Health grant MH056179. H. Decker was the recipient of the McDonald Fellowship from the Multiple Sclerosis International Federation. The authors declare no competing financial interests.

**Submitted:** 12 August 2014  
**Accepted:** 26 December 2014

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