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Rab11-FIP1A regulates early trafficking into the recycling endosomes

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Abstract

The Rab11 family of small GTPases, along with the Rab11-family interacting proteins (Rab11-FIPs), are critical regulators of intracellular vesicle trafficking and recycling. We have identified a point mutation of Threonine-197 site to an Alanine in Rab11-FIP1A, which causes a dramatic dominant negative phenotype when expressed in HeLa cells. The normally perinuclear distribution of GFP-Rab11-FIP1A was condensed into a membranous cisternum with almost no GFP-Rab11-FIP1A(T197A) remaining outside of this central locus. Also, this condensed GFP-FIP1A(T197A) altered the distribution of proteins in the Rab11a recycling pathway including endogenous Rab11a, Rab11-FIP1C, and transferrin receptor (CD71). Furthermore, this condensed GFP-FIP1A(T197A)-containing structure exhibited little movement in live HeLa cells. Expression of GFP-FIP1A(T197A) caused a strong blockade of transferrin recycling. Treatment of cells expressing GFP-FIP1A(T197A) with nocodazole did not disperse the Rab11a-containing recycling system. We also found that Rab5 and EEA1 were accumulated in membranes by GFP-Rab11-FIP1A but Rab4 was unaffected, suggesting that a direct pathway may exist from early endosomes into the Rab11a-containing recycling system. Our study of a potent inhibitory trafficking mutation in Rab11-FIP1A shows that Rab11-FIP1A associates with and regulates trafficking at an early step in the process of membrane recycling.

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INTRODUCTION

Rab small GTPases are essential proteins in intracellular vesicle trafficking. The Rab protein family contains more than 40 mammalian Rabs, and each exists in both a GDP bound state and a GTP bound state [1]. In the GTP bound state, a Rab is considered active and is capable of binding effector proteins to do downstream work. When a Rab is bound to GDP, it is inactive and thus unavailable for protein transport. It is thought that Rab proteins not only define sub-populations of endosomal membranes, but also participate in trafficking through these compartments [2]. Thus, Rab4 and Rab5 are important in early endosome transport, Rab7 is involved in late endosome transport, and Rab11a participates in recycling endosome transport. In some contexts, Rab protein may overlap at key transition points in trafficking, as for Rab4 and Rab5 at the transition between early and sorting endosomes or for Rab4 and Rab11a between sorting and recycling endosomes [3].

The Rab11 family of Rab proteins are essential regulators of endosomal trafficking and specifically, trafficking through recycling endosomes [4, 5]. This family consists of Rab11a, Rab11b, and Rab25 [6]. Previous work identified a family of effectors, which are capable of binding the Rab11 family and acting as effectors in endosomal trafficking. This family of effectors, designated as the Rab11 Family Interacting Proteins (Rab11-FIPs) includes Rab11-FIP1 with multiple splice isoforms, Rab11-FIP2, Rab11-FIP3, Rab11-FIP4, and Rab11-FIP5 [7, 8]. All members of the Rab11-FIPs bind the Rab11 family members through a conserved carboxyl-terminal amphipathic alpha-helical domain [7, 9]. Beyond that, there is great variety in the Rab11-FIP protein structure. Rab11-FIP1C, Rab11-FIP1B, Rab11-FIP2, and Rab11-FIP5 contain amino-terminal C2 domains [10]. Rab11-FIP3 and Rab11-FIP4 contain ERM (ezrin-radixin-moesin) domains and 2 EF-hand motifs for interaction with Arf5 or Arf6 [11]. Rab11-FIP2 interacts with both Rab11 family members and MYO5A and MYO5B [12], which are also Rab11 interacting proteins [13, 14]. While there is variety in protein structure, the Rab11-FIP proteins are generally flexible. This has been shown through crystal structure of the carboxyl terminus of Rab11-FIP2 [15] and is readily seen by the lack of domain structure in the Rab11-FIP1 isoforms Rab11-FIP1A and Rab11-FIP1B.

Recent investigations have focused on the characterization of an increasingly complex network of recycling compartments that manifest a dynamic structure ranging from discrete vesicles to tubule vesicular elements and polymorphic tubules. Rab11-FIP proteins define discrete subdomains within endosomal recycling pathways [16]. Mutations in the actin motor MYO5B or motorless tail constructs of MYO5B inhibit trafficking through the recycling system [17, 18]. We and others have also reported that specific mutations in Rab11-FIPs can also potently inhibit trafficking of cargoes through the recycling system [10, 19, 20]. Two mutations in Rab11-FIP2 are known that cause a disruption of endosomal trafficking. One mutation truncates the amino-terminal 128 amino acids including the C2 domain, Rab11-FIP2(129-512). This truncation mutation blocks trafficking through the plasma membrane recycling system and accumulates Rab11a in a collapsed membrane cisternum [7, 10, 20]. The second mutation, Rab11-FIP2(SARG), causes a similar blockade in trafficking at a somewhat later stage in recycling by changing two amino acids (Serine 227 to Alanine and Arginine 413 to Glycine) [20]. Constructs of Rab11-FIP1C and Rab11-

FIP5 lacking amino-terminal C2-domains also demonstrate significant inhibition of recycling endosome trafficking and accumulation of Rab11a [8, 21, 22].

While the Rab11-FIP1 gene encodes multiple splice isoforms [23], these protein products have very distinct localizations and likely distinct functions [16, 24]. Rab11-FIP1A, Rab11-FIP1B, and Rab11-FIP1C reside on different membranes within the endosomal system indicating that they may interact with Rab11a at different points in recycling. Rab11-FIP1C can also interact with Rab14 at a site overlapping with the Rab11a binding site [25, 26] and Rab11-FIP1C has been implicated in HIV trafficking in combination with Rab14 [27].

Our previous studies demonstrated that transferrin enters a Rab11-FIP1A-containing compartment early in the process of recycling [16]. While we have observed Rab11-FIP1A at different points in the cell, its function within the hierarchy of membrane recycling remains unclear. Although Rab11-FIP1A lacks an amino terminal C2-domain, our recent studies have shown that the protein associates with membranes enriched in phosphatidylserine [24]. The current work seeks to elucidate a specific role for Rab11-FIP1A in recycling through identification of potential interactors and characterization of a novel Rab11-FIP1A mutant that blocks trafficking. In the course of evaluating the protein domains in Rab11-FIP1A, we identified a point mutant (T197A), which caused a strong inhibition of trafficking of transferrin through the Rab11a-containing recycling system. Rab11-FIP1A(T197A) caused accumulation of both Rab11a and Rab5 in a collapsed perinuclear membrane cisternum without affecting the distribution of Rab4. The results suggest that Rab11-FIP1A defines an early point in endosomal trafficking into the recycling system and may define a direct pathway from Rab5a-containing early endosomes into Rab11a-containing recycling membranes.

RESULTS

Identification of a novel inhibitory mutant of Rab11-FIP1A

Previous work has identified Rab11-FIP1A as a Rab11a binding partner that resides on recycling endosomes and participates in plasma membrane recycling [16, 19]. To understand further the role of Rab11-FIP1A, we performed a split ubiquitin yeast two-hybrid screen to identify novel binding partners of Rab11-FIP1A and identified 14-3-3 γ as a potential binding partner. Because 14-3-3 often associates with consensus binding sites on target proteins, we manually scanned Rab11-FIP1A for these consensus sites and found one such sequence at amino acids 193-199 (RESVTTP). Furthermore, the threonine at amino acid 197 is predicted to be phosphorylated. While we have found no direct evidence of phosphorylation at T197 of Rab11-FIP1A (Supplementary Figure 1), mutation of threonine-197 to alanine to inactivate the putative 14-3-3 binding site elicited a dramatic change in the subcellular localization of GFP-Rab11-FIP1A (Figure 1). By structured illumination microscopy (SIM), we observed the expected tubular network of GFP-Rab11-FIP1A in the perinuclear area and throughout the cell (Figure 1A). In contrast, GFP-Rab11-FIP1A(T197A) was dramatically redistributed into a condensed tubular focus in the perinuclear region. Furthermore, live cell imaging of transfected HeLa cells demonstrated that, while GFP-Rab11-FIP1A exhibited expected movement along extended tubular structures and in vesicles throughout the cell, GFP-Rab11-FIP1A demonstrated almost no movement and there were few vesicles

observed outside the collapsed membranous cisternum (Figure 1A). Quantification of the distribution patterns showed that GFP-Rab11-FIP1A was distributed predominantly within diffuse tubulovesicular elements (Figure 1B). In contrast, GFP-Rab11-FIP1A(T197A) was distributed into one or two condensed puncta as well as in multiple large puncta (Figure 1B).

Biochemical properties of GFP-Rab11-FIP1A(T197A)

Since Rab11-FIP1 proteins can associate with multiple Rab proteins, we examined the association of wild type and the T197A mutant forms of Rab11-FIP1A with Rab11a, Rab14 and Rab4a in a split-ubiquitin yeast two-hybrid assay (Supplementary Table 2). We found that Rab11a and Rab14 interacted with both wild type Rab11-FIP1A and Rab11-FIP1A(T197A). Rab4a did not show a significant interaction with Rab4a, although Rab11-FIP1A(T197A) did show a weak interaction. These results indicate that the T197A mutation did not alter the interaction of Rab11-FIP1A with Rab11a and Rab14.

Because there is almost no predicted structure in Rab11-FIP1A, we sought to determine the contribution of the single amino acid change to the overall order of the protein. Rab11-FIPs are generally flexible proteins. The amino acid of interest at position 197 is located near the middle of the 612 amino acid protein (Figure 2A). We produced and purified recombinant full-length Rab11-FIP1A and Rab11-FIP1A(T197A) from bacteria. CD spectral analysis was performed to examine changes in protein structure between the wild-type and the mutant protein (T197A). We found that the wild-type protein displayed a relatively flat CD spectrum indicating very little structure or order (Figure 2B). However, the mutant protein (T197A) exhibited a strong peak at 196 nm suggesting the presence of a β -sheet. This analysis indicates that the single amino acid change at T197A did have a dramatic affect on the order of the protein and resulted in a shift to a more structured, less flexible protein.

Expression of GFP-Rab11-FIP1A(T197A) inhibits Transferrin recycling

Because we observed that the condensed perinuclear structure created by overexpressing GFP-Rab11-FIP1A(T197A) was similar to previously characterized mutations in other recycling endosome proteins including the MYO5B motorless tail and Rab11-FIP2(129-512), we sought to characterize trafficking of Transferrin in the presence of GFP-Rab11-FIP1A(T197A). Previous work demonstrated that Rab11-FIP1A participates in the recycling of Transferrin [16]. Using live cell imaging, we loaded Transferrin-Alexa568 into HeLa cells expressing either GFP-Rab11-FIP1A or GFP-Rab11-FIP1A(T197A) and followed recycling of Transferrin-Alexa568 out of the cells over 1 hour. By 40 minutes, nearly all the Transferrin-Alexa568 was trafficked out of the cells containing wild type GFP-Rab11-FIP1A. However, after an hour, Transferrin was retained in the condensed Rab11-FIP1A(T197A)-containing structure (Figure 3, Supplementary Videos 3 and 4). This live cell observation shows that expression of the mutant FIP1A protein potently inhibits Transferrin recycling.

GFP-Rab11-FIP1A(T197A) affects the distribution of endosomal proteins

Because we discovered a dramatic change in the localization of GFP-Rab11-FIP1A(T197A) compared to wild-type, we next sought to examine the localization of proteins that either interact with Rab11-FIP1A or function in the same endosomal pathway. First, we analyzed

Rab11a as a known Rab11-FIP1A binding partner whose localization is affected by overexpression of other endosomal mutant proteins such as MYO5B tail, Rab11-FIP2(SARG), and Rab11-FIP2(129-512). In HeLa cells overexpressing GFP-Rab11-FIP1A or GFP-Rab11-FIP1A(T197A), we observed colocalization with endogenous Rab11a by structured illumination microscopy (Figure 5). While in cells expressing GFP-Rab11-FIP1A wild type, Rab11a was distributed in vesicles and tubules throughout the cell, Rab11a was redistributed into a condensed network of Rab11-FIP1A(T197A)-containing membranes. This condensed phenotype of Rab11a with GFP-Rab11-FIP1A(T197A) was also seen with co-expression of Cherry-Rab11a (Figure 5). Co-expression of Cherry-Rab11a with GFP-Rab11a-FIP1A(T197A) did not alter the morphology of the collapsed membrane cisternum. With structured illumination microscopy, we observed the colocalization of GFP-Rab11-FIP1A and Cherry-Rab11a for both wild-type and mutant T197A Rab11-FIP1A. Finally, with live cell imaging, wild type GFP-Rab11-FIP1A and Cherry-Rab11a moved together in vesicles. While GFP-Rab11-FIP1A(T197A) and Cherry-Rab11a localized together, we saw little movement of the two proteins in the perinuclear collapsed membrane cisternum (Figure 5, Supplementary Video 1, Supplementary Video 2).

Given the dramatic alteration of Rab11a localization in the presence of GFP-Rab11-FIP1A(T197A), we investigated other aspects of endosomal trafficking. We next evaluated the effects of the T197A mutation in Rab11-FIP1A on the localization of other Rab proteins and Rab-interacting proteins that reside on endosomes. First, we over-expressed GFP-Rab11-FIP1A or GFP-Rab11-FIP1A(T197A) in HeLa cells and evaluated the endogenous localization of Rab11a, Rab5, Rab8a, Rab14, and Rab11-FIP5 (Figure 6). By structured illumination microscopy we saw that all of these proteins were localized on vesicles throughout the cell in the presence of wild type GFP-Rab11-FIP1A. However, these particular Rab and Rab-interacting proteins were condensed into the perinuclear membrane structure created by expression of GFP-Rab11-FIP1A(T197A).

However, not all endosomal Rab proteins involved in trafficking were affected by GFP-Rab11-FIP1A(T197A) expression. Rab4a localization was unchanged or even appeared more dispersed in the presence of GFP-Rab11-FIP1A(T197A) (Figure 7A). Endogenous Rab4a remained on vesicles throughout the cell, although GFP-Rab11-FIP1A(T197A), along with Rab11a, was condensed near the nucleus. Furthermore, DAKAP2, a previously described scaffolding protein that binds Rab11a and Rab4 but not Rab5 [28], was also unaffected by overexpression of GFP-Rab11-FIP1A(T197A) (Supplementary Figure 2). These observations reveal that the single point mutation at T197A in Rab11-FIP1A selectively affects the trafficking of specific Rab proteins and Rab11 interacting proteins and that these proteins including Rab5, Rab8a, Rab10, Rab14, and Rab11-FIP5 likely function in the same pathway as Rab11-FIP1A.

To quantify the localization changes of Rab and FIP proteins created by the GFP-Rab11-FIP1A(T197A) overexpression, we performed Pearson's Correlation analyses (Figure 7B). We compared colocalization values between GFP-Rab11-FIP1A(T197A) and several endogenous Rab and Rab11-interacting proteins labeled by antibodies. We found that the highest correlation occurred with GFP-Rab11-FIP1A(T197A) and Rab11a (using either a rabbit or mouse antibody), while there was little colocalization between GFP-Rab11-

FIP1A(T197A) and Rab4a. This result is expected for Rab11a because it is a known interactor of Rab11-FIP1A. However, we found that Rab5, Rab8a, Rab10, Rab14, and Rab11-FIP5 showed significantly increased correlations compared to Rab4a. These correlations agree with our fluorescence images, which depict a dramatic redistribution of Rab11a, Rab5, Rab8a, Rab14, and Rab11-FIP5 with no change in localization of Rab4a.

We next asked whether Rab11-FIP2 was affected by GFP-Rab11-FIP1A(T197A). Rab11-FIP2 is a binding partner of Rab11a and is known to function in various trafficking pathways such as plasma membrane recycling and establishment of polarity in polarized epithelial cells. Two mutants of Rab11-FIP2, Rab11-FIP2(SARG) and Rab11-FIP2(129-512) inhibit plasma membrane recycling. Furthermore, several members of the Rab11-FIP proteins are known to heterodimerize. First, we overexpressed either GFP-Rab11-FIP1A or GFP-Rab11-FIP1A(T197A) in HeLa cells and used a Rab11-FIP2 antibody to evaluate endogenous Rab11-FIP2 localization. With wild-type GFP-Rab11-FIP1A, we found little overlap among vesicles displaying GFP-Rab11-FIP1A and endogenous Rab11-FIP2 (Figure 8A). When GFP-Rab11-FIP1A(T197A) was expressed, we also saw little change in the localization of Rab11-FIP2, suggesting it is not affected by expression of the mutant Rab11-FIP1A (Figure 8B). Furthermore, when we co-expressed GFP-Rab11-FIP1A(T197A) along with Rab11-FIP2-Cherry, we found that the condensed GFP-Rab11-FIP1A(T197A) did not alter Rab11-FIP2-Cherry localization (Figure 8C), with little appreciable overlap between GFP-Rab11-FIP1A(T197A) and Rab11-FIP2-Cherry in membranes.

Because Rab5 displayed the highest colocalization correlation with GFP-Rab11-FIP1A(T197A) other than the previously known interactor Rab11a, we sought to investigate this potentially novel association. When GFP-Rab11-FIP1A was compared with endogenous immunostaining Rab5 and another early endosome protein, EEA1, very little overlap was seen with these on vesicles. However, when GFP-Rab11-FIP1A(T197A) is overexpressed, Rab5 and EEA1 localization changed such that the both Rab5 and EEA1 staining clearly overlapped with GFP-Rab11-FIP1A(T197A) expressed in perinuclear collapsed membranes (Figure 9A). To examine further the relationship between Rab11-FIP1A and Rab5, we co-expressed GFP-Rab11-FIP1A and Cherry-Rab5a in HeLa cells for live cell imaging. By watching both proteins move on vesicles in the cell periphery, we detected an overlap between wild type GFP-Rab11-FIP1A and Cherry-Rab5a in dynamic vesicles that contained both proteins (Figure 9B, Supplementary Videos 9 and 10). Also, we found GFP-Rab11-FIP1A(T197A) and Cherry-Rab5a exhibited significant overlap in collapsed membrane cisternae containing both chimeric proteins. Furthermore, we observed dynamic movement within this condensed GFP-Rab11-FIP1A(T197A) and Cherry-Rab5a-containing membrane cisternum in the perinuclear region as well as in smaller Cherry-Rab5a-containing vesicles moving in and out of this structure. These dynamic interactions suggest a novel role for Rab5a with Rab11-FIP1A in the recycling pathway.

To examine the dynamics of vesicle entry into the collapsed recycling system cisternae, we analyzed the movement of GFP-Rab11-FIP1A compared to GFP-Rab11-FIP1A(T197A) by fluorescence recovery after photobleaching (FRAP). HeLa cells expressing either GFP-Rab11-FIP1A or GFP-Rab11-FIP1A(T197A) were exposed to 100% laser for 5 seconds to

bleach a region of interest (ROI) in the perinuclear area that encompassed vesicles or the condensed T197A spot (Figure 4A). After bleaching, fluorescence recovery to the ROI was measure over 5 minutes. We observed that wild type GFP-Rab11-FIP1A recovered to 85% of original fluorescence by 5 minutes, while GFP-Rab11-FIP1A(T197A) recovered to only 65% by 5 minutes (Figure 4B).

Additionally, we noted that vesicles moved in and out of the FRAP ROI in wild-type expressing cells, but vesicles were rarely seen in T197A expressing cells and almost never moved out of the T197A structure. While the dynamics and overall structure were very distinct, vesicle movement was observed in both GFP-Rab11-FIP1A and GFP-Rab11-FIP1A(T197A) expressing cells. These data suggest new protein is moving into the Rab11-FIP1A trafficking system to allow for recovery, while at the same time little protein is allowed to escape the GFP-Rab11-FIP1A(T197A)-induced structure, consistent with the concept that GFP-Rab11-FIP1A(T197A) blocks recycling system trafficking.

Cytoskeletal alteration associated with GFP-Rab11-FIP1A(T197A) expression

We sought to examine the potential cytoskeletal changes caused by overexpression of GFP-Rab11-FIP1A(T197A). First, we treated HeLa cells expressing either GFP-Rab11-FIP1A or GFP-Rab11-FIP1A(T197A) with nocodazole for 1.5 hours and looked for disruption of GFP-Rab11-FIP1A structures and endogenous Rab11a localization (Figure 10). As previously shown, we found that GFP-Rab11-FIP1A vesicles were dispersed following nocodazole treatment. In the case of GFP-Rab11-FIP1A(T197A), we saw an intermediate phenotype. Rab11-FIP1A(T197A)-containing membranes were broken up, but not as dramatically as wild-type Rab11-FIP1A vesicles. Importantly, we found that Rab11a remained associated with both GFP-Rab11-FIP1A and GFP-Rab11-FIP1A(T197A), even in the presence of nocodazole. This result points to the stability of the interaction between GFP-Rab11-FIP1A and Rab11a both in the wild-type and mutant T197A form.

Second, we overexpressed GFP-Rab11-FIP1A or GFP-Rab11-FIP1A(T197A) and evaluated endogenous α -tubulin. The α -tubulin staining displayed a normal distribution of microtubules radiating from the perinuclear region in HeLa cells expressing GFP-Rab11-FIP1A. However, in cells expressing GFP-Rab11-FIP1A(T197A), α -tubulin-stained microtubules were dispersed to the edge of the cell, suggesting a redistribution of the microtubule cytoskeleton (Figure 11A).

Third, we evaluated the F-actin cytoskeleton by phalloidin staining in cells overexpressing either GFP-Rab11-FIP1A or GFP-Rab11-FIP1A(T197A) (Figure 11B). We saw little change in the F-actin cytoskeleton between wild type and mutant-expressing cells.

DISCUSSION

The Rab11 Family Interacting Proteins (Rab11-FIPs) are a diverse group of Rab11a-associated proteins that regulate aspects of plasma membrane recycling. Previous work from our lab showed that the Rab11-FIP1 isoforms are localized to distinct tubulovesicular populations within the cell such that Rab11-FIP1A and Rab11-FIP1C only partially overlap [16]. While expressed Rab11-FIP1A, Rab11-FIP2, and Rab11-FIP5 are found on dynamic

vesicular and tubulovesicular membranes, expressed Rab11-FIP1B, Rab11-FIP1C, and Rab11-FIP3 localize in perinuclear tubules [16]. In particular, the Rab11-FIP1 sub-family is composed of at least three splice variants that appear to regulate different aspects of plasma membrane recycling [16, 23]. Rab11-FIP1B and Rab11-FIP1C/RCP both contain amino-terminal C2-domains [10, 21, 23]. Interestingly, while Rab11-FIP1A does not have an amino-terminal C2-domain, it associates closely with internal membranes enriched in phosphatidylserine [19, 24]. Previous work has demonstrated that Transferrin enters the Rab11-FIP1A-containing compartment early in the process of recycling [16]. However, the exact role of Rab11-FIP1A in these pathways is less clear. Here we have identified a point mutant of Rab11-FIP1A that causes a strong blockade of the recycling system when expressed in HeLa cells. The accumulation of Rab5, but not Rab4, inside recycling system membranes containing Rab11-FIP1A(T197A) has revealed an unrecognized pathway that may indicate that cargoes can traffic directly from Rab5-containing early endosomes into Rab11a-containing recycling endosomes.

To investigate the function for Rab11-FIP1A, we performed a split-ubiquitin Yeast Two Hybrid assay to identify novel interactors. We were interested to find an interaction with 14-3-3 protein, because it is known to bind phosphorylated proteins. Several members of the Rab11-FIPs are phosphorylated [8, 29, 30]. Previous work has shown that phosphorylation of Rab11-FIP2 is essential for its role in the establishment of polarity [30, 31]. While we found that Rab11-FIP1A is capable of being phosphorylated *in vitro* (unpublished data), we did not find significant phosphorylation at the potential 14-3-3 interaction site, Threonine 197. However, when we mutated Threonine 197 to Alanine, we found a dramatic phenotype that altered the localization of the overexpressed GFP-Rab11-FIP1A. We also noted that T197D and T197E mutations had no effect on the localization of GFP-Rab11-FIP1A, corroborating the notion that phosphorylation at Threonine 197 is not necessary for Rab11-FIP1A function.

The dramatic mislocalization phenotype in cells expressing GFP-Rab11-FIP1A(T197A) is similar to several inhibitory mutants of trafficking. Two mutants in Rab11-FIP2, Rab11-FIP2(SARG) and Rab11-FIP2(129-512), inhibit trafficking through the apical recycling system in MDCK cells [12, 32]. Rab11-FIP2(SARG) appears to act at a more distal point in the apical recycling system. Similarly, the motorless MYO5B tail is a powerful inhibitor of recycling system trafficking [13, 14, 33]. Carboxyl terminal fragments of Rab11-FIP1 also inhibit plasma membrane recycling [19, 21, 22]. These previously-described inhibitory mutants potently accumulate Rab11a in collapsed tubulomembranous cisternae. In all of those cases, while Rab11a was pulled into the mutant, neither Rab5 nor Rab4 were observed in these inhibited recycling tubules. The association of Rab4 with the Rab11-FIP1 Rab11-binding domain has been controversial [21, 34]. We did not observe accumulation of Rab4 with the Rab11-FIP1A(T197A) mutant. We also did not observe any accumulation of DAKAP2, which can bind Rab11a and Rab4 [28]. More recent investigations have demonstrated that Rab14 also interacts with Rab11-FIP1C in its carboxyl-terminus [25, 35]. We observed accumulation of Rab14 with Rab11-FIP1A(T197A). Just as importantly, we also observed accumulation of both Rab5 and EEA1 in the tubular cisternae containing Rab11-FIP1A(T197A). These data have suggested that the Rab11-FIP1A mutant arrests

trafficking at a unique point at the transition between early and recycling endosomes. This transition does not involve Rab4 apparently, indicating that a pathway exists for direct trafficking of cargoes between early and late endosomes. We have never observed direct interactions between Rab5 and Rab11-FIP proteins [7]. Alternatively, the transition may involve an intermediate endosomal compartment containing Rab14, rather than Rab4.

Previous investigations have noted that the integrity of the microtubule cytoskeleton was necessary for proper morphology and function of the plasma membrane recycling system [36, 37]. Previously, we have reported the collapsed recycling system induced by expression of Rab11-FIP2(129-528) or Rab11-FIP2(SARG) was not affected by nocodazole. In contrast, the collapsed recycling system in the presence of expression of the motorless tail of MYO5B did show some fragmentation after nocodazole [13], albeit without the generalized dispersal of the recycling system seen in untransfected cells. Similarly, following treatment with nocodazole, GFP-Rab11-FIP1A(T197A)-containing membranes were partially disrupted. Just as importantly, we also saw a redistribution of microtubules in cells expressing GFP-Rab11-FIP1A(T197A), with loss of central radial microtubules generally associated with the centrosomes and a prominent presence of microtubule in the cell periphery. These peripheral microtubules were generally not associated with the GFP-Rab11-FIP1A(T197A)-containing membranes. A recent investigation has suggested that Rab11a-containing vesicles can stabilize microtubules [38]. Therefore, it is possible that the GFP-Rab11-FIP1A(T197A) disrupts the stabilizing effects of recycling system vesicles on the centrosomal-based microtubule cytoskeleton. These findings may be compatible with recent studies which have shown the association of Rab11a-containing recycling system elements with the appendages of the mother centrosome [39].

Taken together, all of these results support the importance of dynamic progression of trafficking through the plasma recycling system. Our studies indicate that there may be multiple entry and exit pathways associated with recycling endosomes. These decision points for trafficking are likely coordinated by microtubule based pathways and likely involve distinct trafficking decisions coordinated by Rab11-FIP proteins and other regulatory proteins. The net impact of these regulatory decisions leads to dynamic alterations in the recycling system morphology and function. Further investigations will be necessary to detail the complexity of the points for entry into the recycling system, processing through the tubular recycling system, and exit to recycling surfaces.

MATERIALS AND METHODS

Constructs

GFP-Rab11-FIP1A was described in Jin et al.[23] This construct was mutated to T197A by standard Quikchange (Stratagene) site directed mutagenesis protocols. Cherry-Rab11a was described in Baetz et al.[16] Cherry-Rab5a was described in Ducharme et al.[32] GFP-FIP2 was originally described in Hales et al.[19] From this construct, Rab11-FIP2 was subcloned into Cherry-N2 to create Rab11-FIP2-Cherry. Rab11-FIP1A and Rab11-FIP1A(T197A) were subcloned into pET30a for protein purification.

Protein Purification and CD Spectral Analysis

Rab11-FIP1A and Rab11-FIP1A(T197A) proteins were purified from *E. coli* using sonication to lyse bacteria and nickel beads to bind the protein. Beads were washed at least 3 times with 25 mM imidazole and eluted using 250 mM imidazole in a buffer containing 50 mM TRIS-HCl, pH 8, 500 mM NaCl, and 2 mM MgSO₄. Proteins were dialyzed to lower the NaCl concentration to allow for CD spectroscopy. This was achieved using a Slide-A-Lyzer® Dialysis Cassette (product # 66003, Thermo Scientific) and a multistep dialysis against decreasing NaCl concentrations (400 mM, 300 mM, 200 mM, 100 mM final concentration). Using a Jasco J-810 Spectropolarimeter (serial#B023160750) scans were made with the following parameters: 1.) Sensitivity: Standard (100 mdeg); 2.) Wavelength range: 260-195 nm for far-UV scan; 3.) Data Pitch: 1nm; 4.) Scanning Mode: Continuous; 5.) Scan speed: 50 nm/min; 6.) Response: 2 sec; 7.) Bandwidth: 1 nm; 8.) Accumulation: 6; 9.) Cell length: 0.1 cm. All scans were made at 4°C and the protein concentration for both wild type and mutant protein was 0.9 mol/L in 20 mM TRIS-HCl, pH 8, 100 mM NaCl.

Antibodies

The following primary antibodies were used: Rab4 at 1:100 (Abcam, ab13252), Rab5 at 1:1000 (Cell Signaling, 3547S), rabbit anti-Rab11a at 1:1000 [40], mouse monoclonal anti-Rab11a [41], Ranb11-FIP5 at 1:200 [42], α -tubulin at 1:2000 (Sigma, T5168), Alexa568-phalloidin at 1:100 (Invitrogen, A12380), Rab8a at 1:400 [33], Rab10 at 1:100 [33], Rab14 at 1:400 (Aviva, AVARP13107), Rab11-FIP2 polyclonal rabbit antibody at 1:1000 (Sigma-Aldrich, HPA037726), Rab11-FIP2 polyclonal rabbit antibody at 1:1000 (Proteintech Group, 18136-1-AP), Rab11-FIP2 polyclonal goat antibody at 1:1000 [43]. Secondary antibodies were obtained from Jackson ImmunoResearch.

HeLa Cell Transfections and Staining

HeLa cells were maintained and propagated by standard methods. Transient transfections were performed for approximately 18-24 hours with 200 ng total DNA using either Effectene transfection reagent (Qiagen) or PolyJet (SignaGen Laboratories). Cells were fixed for 20 minutes in either 4% paraformaldehyde in PBS or, for microtubule staining, with 200 mM Pipes, 2 mM EGTA, 2 mM MgSO₄, 0.2% TritonX-100, 60% glycerol pH 7.0, washed 3 times in PBS, and then blocked/extracted for 30 minutes at RT in 10% normal donkey serum (Jackson ImmunoResearch), 0.3% Triton X-100 in PBS. Coverslips were incubated for 2 hours at RT or overnight at 4° C with primary antibodies diluted in 1% normal donkey serum, 0.05% Tween-20 in PBS. Coverslips were washed 3 times for 15 minutes at RT with 0.05% Tween-20 in PBS (PBS-T), then incubated for 1 hour at RT with secondary antibodies from Jackson ImmunoResearch diluted at 1:200, washed 3 times in PBS-T, once in PBS, then rinsed in water and mounted with ProLong Gold (Invitrogen).

For quantitation of intracellular morphology induced by GFP-Rab11-FIP1A wild type or GFP-Rab11-FIP1A(T197A), HeLa cells were transfected as above and stained for DAPI and phalloidin. Fifty healthy, non-dividing cells were counted per construct. Cells were scored blindly as demonstrating multiple small vesicles, 1-2 large foci, or more than 2 large foci.

Structured Illumination Microscopy (SIM)

HeLa cells were fixed and stained as usual and used for SIM imaging on a DeltaVision OMX Blaze (GE) instrument equipped with cMOS cameras, a 60x objective lens (1.42 NA) and 405, 488, 514, 561, and 642 laser lines. Images were processed for SIM using Softworx software (Applied Precision). Images shown are maximum intensity projections. Colocalization done by Pearson's Correlation Coefficient were calculated on 3D volume images.

Deconvolution Microscopy

HeLa cells were fixed and imaged with a 100× oil immersion objective (1.4 numerical aperture) on a DeltaVision deconvolution microscope (Applied Precision, Issaquah, WA) and a CoolSNAP HQ² camera (Photometrics, Tucson, AZ). Single plane, widefield images were collected for DIC, FITC (GFP/Venus), and TRITC (mCherry). Images were collected at 512 × 512 pixels. Exposures were typically 0.2 to 0.5 seconds and were sufficient to achieve a minimum 5:1 signal-to-noise ratio. Individual raw files were deconvolved using Applied Precision Softworx deconvolution package. Colocalization was quantified as Pearson's Correlation Coefficient using the colocalization feature in Softworx, which measures a 3D volume. There was a statistically significant difference between groups (Rab4, Rab5, Rab8a, Rab14, and Rab11-FIP5) as determined by one-way ANOVA ($F(5,42) = 2.412, p = .05$).

Live Cell Imaging and Transferrin Trafficking

HeLa cells were grown on MateK dishes, transfected, and imaged on a DeltaVision deconvolution microscope (above) with a controlled box maintaining 37° C temperature and 5% CO₂. For transferrin trafficking, cells were incubated in serum free media for 1 hour. The cells were incubated on ice for 15 minutes with transferrin conjugated to Alexa 568 (Life Technologies), cells were washed quickly, dish was allowed to warm to 37° C for approximately 5 minutes, and cells were imaged for loss of transferrin from recycling compartments. Time lapse images were acquired every 0.5-1 minutes for a total of 1 hour.

Fluorescence Recovery After Photobleaching (FRAP)

HeLa cells were grown in MatTek dishes as for live cell imaging, transfected, and imaged by time lapse on a DeltaVision deconvolution microscope. Cells expressing GFP-Rab11-FIP1A or GFP-Rab11-FIP1A(T197A) were imaged pre bleach for 3 images every 5 seconds. Next, the same cells were subjected to a 488 nm laser at 100% for 5 seconds to bleach an ROI in the perinuclear area. Finally, cells were imaged every 5 seconds for 5 minutes to watch for fluorescence recovery. At least 4 cells were imaged for each transfected protein. Percent fluorescence recovery was calculated by averaging the fluorescence intensity of 3 pre bleach images and dividing the fluorescence intensity value for each post bleach image by this original pre bleach value to determine percent recovery. These percent recovery values are plotted along with SEM for each point.

Yeast two-hybrid assays

Full-length Rab11-FIP1A was cloned into the pCCW-SUC bait vector for use in a split ubiquitin yeast-two-hybrid library screen (DualSystems Biotech). The Rab11-FIP1A bait was used against an HCA-7 colon cancer cell line library (a gift of R. Coffey). Upon plating on -Trp-Leu-Ade-His plates and blue/white screening with X-gal, 17 colonies were repeatedly found to be positive for interaction with Rab11-FIP1A. Upon sequencing, 13 unique genes were identified including Rab11a, a known interactor for Rab11-FIP1A (Supplementary Table 1).

For binary yeast 2-hybrid assays, an overnight culture of Nym32 yeast was grown and diluted to $OD_{600}=0.2$, then allowed to grow to log phase. The yeast were pelleted at 2500Xg for 5 minutes. Cells were washed with 10ml of H₂O then pelleted again 2500Xg for 5 minutes after which the pellet was resuspended in 100ml of H₂O per reaction. A transformation solution of 750 ng of each plasmid, 36% yeast, 43% PEG, 0.1 M lithium acetate, and 2% salmon sperm DNA was vortexed for 20 seconds. The samples were then incubated for 30 minutes at 42° C then pelleted at 2500Xg for 5 minutes. The supernatant was discarded and the pellet was resuspended in 100ml of H₂O. Samples were then plated on Trp/Leu drop-out plates and grown for 3-4 days at 30° C. Once grown, 5-6 representative colonies were selected and diluted for spot testing. The same concentration of yeast was spotted on Trp/Leu/His drop-out plates with 10mM 3-AT and then grown for 3-4 days at 30° C.

Nocodazole treatment

HeLa cells were grown on coverslips and transfected after 24 hours. After 24 hours of transfection, cells were placed at 4° C for 1 hour. Next, nocodazole was added at a concentration of 33 μ M and incubated with the cells at 37° C for 1.5 hours. Cells were then fixed and used for immunofluorescence as described above.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. GFP-Rab11-FIP1A(T197A) localizes to a collapsed perinuclear membrane cisternum

A. HeLa cells were transfected with either GFP-Rab11-FIP1A or GFP-Rab11-FIP1A(T197A) and imaged live for deconvolution. Separately, cells were transfected, fixed, and imaged by Structured Illumination Microscopy (SIM). Wild-type GFP-Rab11-FIP1A shows vesicles concentrated in the perinuclear region as well as extended tubules throughout the cell, typical of vesicular proteins involved in endosomal trafficking. Mutant GFP-Rab11-FIP1A(T197A), however, was redistributed almost entirely to the perinuclear area and shows no tubule localization. See Supplementary Videos 1 and 2. B. The distribution of GFP-Rab11-FIP1A and GFP-Rab11-FIP1A(T197A) was evaluated in 50 cells and the distribution of transfected protein was scored as located in diffuse vesicles, in one to two condensed puncta, or in multiple large puncta.

Figure 2. T197A mutation in Rab11-FIP1A causes increased β -sheet structure

Diagram of Rab11-FIP1A protein with putative 14-3-3 binding site (A). CD spectra (B) of recombinant human Rab11-FIP1A (GREEN) and Rab11-FIP1A(T197A) (BLUE). The difference between the wild type protein and mutant protein is quite dramatic. The mutant protein shows a characteristic peak at 196 nm and a low at 218 nm, which is characteristic of a beta-sheet rich protein. Overall, the mutant protein displays much more secondary structure than the wild type protein.

Figure 3. GFP-Rab11-FIP1A(T197A) inhibits transferrin recycling

HeLa cells transfected with either GFP-Rab11-FIP1A or GFP-Rab11-FIP1A(T197A) were serum starved for one hour and then loaded with Transferrin-Alexa568 for 15 minutes on ice. Cells were chased with unlabeled Transferrin for 40 minutes and imaged in real time. Seven time points over 40 minutes are shown in the Transferrin-Alexa568 channel. Most of the Transferrin-Alexa568 was trafficked out of cells expressing wild type Rab11-FIP1A by 40 minutes, while GFP-FIP1A(T197A) cells still showed a concentration of Transferrin at 40 minutes. See Supplementary Videos 3 and 4.

Figure 4. GFP-Rab11-FIP1A(T197A) expressing membranes show slow recovery after photobleaching

Fluorescence Recovery After Photobleaching (FRAP) was performed on HeLa cells transfected with GFP-Rab11-FIP1A or GFP-Rab11-FIP1A(T197A). Three images were taken before bleach, cells were bleached for 5 seconds, and then cells were imaged every 5 seconds for a total of 5 minutes. A. Representative images pre-bleach, post bleach and after 5 minutes of recovery. B. Fluorescence recovery along with SEM is plotted for cells expressing GFP-Rab11-FIP1A or GFP-Rab11-FIP1A(T197A). Fluorescence in GFP-FIP1A cells recovered more rapidly than in GFP-FIP1A(T197A) cells. See Supplementary Videos 5 and 6.

Figure 5. Rab11a accumulates with GFP-Rab11-FIP1A(T197A) in a collapsed membranous cisternum

HeLa cells were transfected with either GFP-Rab11-FIP1A or GFP-Rab11-FIP1A(T197A), stained for endogenous Rab11a, and imaged by SIM. Cells imaged by Structured Illumination Microscopy showed a redistribution of Rab11a to the GFP-Rab11-FIP1A(T197A) spot compared to wild type (A). HeLa cells were transfected with Cherry-Rab11a along with either GFP-Rab11-FIP1A or GFP-Rab11-FIP1A(T197A) and were imaged in time lapse to observe the movement of vesicles (B). Cherry-Rab11a was condensed in the perinuclear area in the presence of the mutant protein, while it remains vesicular in the presence of the wild type protein. Cherry-Rab11a and GFP-Rab11-FIP1A vesicles were seen throughout the cell for wild type, but few vesicles were only found in the perinuclear region with the mutant. See Supplementary Videos 7 and 8.

Figure 6. Alteration of endosomal protein localization with GFP-Rab11-FIP1A(T197A) expression

HeLa cells were transfected with either GFP-Rab11-FIP1A or GFP-Rab11-FIP1A(T197A), stained for Rab5, Rab8a, Rab14, or Rab11-FIP5, and imaged by SIM. Rab5 was seen in vesicles in the presence of GFP-Rab11-FIP1A, but Rab5 localization was altered to the condensed regions in the presence of GFP-Rab11-FIP1A(T197A). Rab8a was seen in vesicles in the presence of GFP-Rab11-FIP1A, but Rab8a localization was altered to the condensed regions in the presence of GFP-Rab11-FIP1A(T197A). Rab14 was seen in vesicles in the presence of GFP-Rab11-FIP1A, but Rab14 localization was altered to the condensed regions in the presence of GFP-Rab11-FIP1A(T197A). Rab11-FIP5 was seen in vesicles in the presence of GFP-Rab11-FIP1A, but Rab11-FIP5 localization was altered to the condensed regions in the presence of GFP-Rab11-FIP1A(T197A). Quantitation is shown in Figure 7B.

Figure 7. Expression of GFP-Rab11-FIP1A(T197A) does not alter the distribution of Rab4
 HeLa cells were transfected with either GFP-Rab11-FIP1A or GFP-Rab11-FIP1A(T197A), immunostained for Rab4a and Rab11a, and imaged by SIM (A). In the presence of wild type GFP-Rab11-FIP1A, Rab4a was found on vesicles and rarely overlapped with GFP-Rab11-FIP1A and Rab11a. In the presence of GFP-Rab11-FIP1A(T197A), Rab4a distribution was unchanged and remained in dispersed vesicles despite the redistribution of GFP-Rab11-FIP1A(T197A) and Rab11a. The Rab4a immunostaining showed a non-specific variable nuclear background. Pearson's Correlation Coefficients (PCC) were calculated for GFP-Rab11-FIP1A(T197A) and endogenous Rabs and Rab11-FIPs shown in Figures 6-7 (B). PCC were plotted along with SEM. Rab11a showed the highest PCC and Rab4 showed the lowest. Rab4 was decreased compared to other Rabs and Rab11-FIP5. We found a statistically significant difference between groups (Rab4, Rab5, Rab8a, Rab14, and Rab11-FIP5) as determined by one-way ANOVA ($F(5,42) = 2.412, p = .05$).

Figure 8. Rab11-FIP2 does not associate with membranes containing GFP-Rab11-FIP1A(T197A)
 HeLa cells were transfected with GFP-Rab11-FIP1A, stained for Rab11-FIP2, and imaged by widefield deconvolution (A). GFP-Rab11-FIP1A and Rab11-FIP2 were not found on the same vesicles.

HeLa cells were transfected with GFP-Rab11-FIP1A, stained for Rab11-FIP2, and imaged by widefield deconvolution (B). GFP-Rab11-FIP1A and Rab11-FIP2 were not found on the same vesicles.

HeLa cells were transfected with GFP-Rab11-FIP1A(T197A) along with Rab11-FIP2-Cherry and imaged by widefield deconvolution (C). GFP-Rab11-FIP1A(T197A) and Rab11-FIP2-Cherry were not found on the same vesicles.

Figure 9. Rab5 accumulated on GFP-Rab11-FIP1A(T197A)-containing membranes

HeLa cells were transfected with either GFP-Rab11-FIP1A or GFP-Rab11-FIP1A(T197A), stained for Rab5 and EEA1, and imaged by widefield deconvolution (A). HeLa cells were transfected with either GFP-Rab11-FIP1A or GFP-Rab11-FIP1A(T197A) along with Cherry-Rab5a, and imaged live by widefield deconvolution. Still images from movies are shown (B). Both endogenous Rab5 and Cherry-Rab5a as well as EEA1 localization were altered in the presence of GFP-Rab11-FIP1A(T197A). See Supplementary Videos 9 and 10.

Figure 10. Disruption of microtubules only partially affects GFP-Rab11-FIP1A(T197A)
 HeLa cells were transfected with either GFP-Rab11-FIP1A or GFP-Rab11-FIP1A(T197A) and treated with nocodazole or DMSO (control). Cells were then stained for Rab11a and imaged on a widefield microscope. Disruption of microtubules caused a dispersion of GFP-Rab11-FIP1A and Rab11a vesicles (A). In the presence of GFP-Rab11-FIP1A(T197A), this dispersion was incomplete and the GFP-Rab11-FIP1A(T197A) cisternum was partially retained (B).

Figure 11. Expression of GFP-Rab11-FIP1A(T197A) alters the distribution of microtubules, but not F-actin

HeLa cells were transfected with either GFP-Rab11-FIP1A or GFP-Rab11-FIP1A(T197A). One set of cells was stained for α -tubulin (A) to view microtubules and a second set was stained for phalloidin to view F-actin (B). Note the peripheral distribution of microtubules in cells expressing GFP-Rab11-FIP1A(T197A) (A).