Rab11 Promotes Docking and Fusion of Multivesicular Bodies in a Calcium-Dependent Manner

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Multivesicular bodies (MVBs) are membranous structures within 60–100 nm diameter vesicles accumulate. MVBs are generated after invagination and pinching off of the endosomal membrane in the lumen of the vacuole. In certain cell types, fusion of MVBs with the plasma membrane results in the release of the internal vesicles called exosomes. In this report we have examined how an increase in cytosolic calcium affects the development of MVBs and exosome release in K562 cells overexpressing GFP-Rab11 wt or its mutants. In cells overexpressing the Rab11Q70 L mutant or Rab11 wt, an increase in the cytosolic calcium concentration induced by monensin caused a marked enlargement of the MVBs. This effect was abrogated by the membrane permeant calcium chelator BAPTA-AM. We also examined the behavior of MVBs in living cells by time lapse confocal microscopy. Many MVBs, decorated by wt or Q70L mutant GFP-Rab11, were docked and ready to fuse in the presence of a calcium chelator. This observation suggests that Rab11 is acting in the tethering/docking of MVBs to promote homotypic fusion, but that the final fusion reaction requires the presence of calcium. Additionally, a rise in intracellular calcium concentration enhanced exosome secretion in Rab11 wt overexpressing cells and reversed the inhibition of the mutants. The results suggest that both Rab11 and calcium are involved in the homotypic fusion of MVBs.

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Multivesicular bodies (MVBs) are modified endosomal structures within which 60–100 nm diameter vesicles accumulate (for a review see (1,2)). MVBs are generated after invagination and pinching off of the endosomal membrane into the lumen (3,4). Membrane proteins can be sorted into these invaginating vesicles, which are delivered to the lumen of lysosomes by fusion of MVBs with the lysosomal compartment (5). MVBs are therefore believed to be a critical compartment for receptor down-regulation (6). Indeed, the formation of luminal vesicles ensures that both the luminal and cytosolic domains of transmembrane proteins, such as EGF receptors, are sequestered and eventually degraded in lysosomes. The molecular mechanisms underlying the formation of the internal vesicles have been, in part, elucidated. In endosomes, the protein Hrs recruits clathrin to retain ubiquitinated proteins in microdomains destined for degradation (7,8), for a review see (9). The sorting of ubiquitinated receptors into luminal vesicles of the MVB is mediated by three protein complexes, the ESCRT-I, II and III (Endosomal Sorting Complex Required for Transport) (10–12), for a review see (13)).

Many studies have shown that in cells of hematopoietic origin, MVBs fuse with the plasma membrane rather than degradative compartments. This process results in the release of the internal vesicles (exosomes) into the extracellular space (4,14–17). This is an alternative mechanism for down-regulation of some surface receptors. In most cell types, endocytosed transferrin receptor (TfR) is efficiently recycled back to the cell surface, but in differentiating and maturing red cells such as reticulocytes, TfR is targeted to MVBs and sorted into the internal vesicles. Subsequently, these MVBs fuse with the plasma membrane and the exosome-associated TfRs are released outside the cell (18). Antigen-presenting cells also secrete exosomes which carry peptide-loaded MHC molecules, functioning as intercellular vehicles for antigen material (15,16,19). Some of these molecules are released in the extracellular medium via their association with lipid microdomains present in the exosomal membrane (20).

Recent work indicates that exosome release is likely a regulated process. In most cell types, a rise in the intracellular calcium concentration is necessary to induce regulated secretion (reviewed in (21)). We have recently shown that exosome release in K562 cells is a calcium-dependent event (22). We have also found that binding of transferrin to its receptor enhances exosome secretion in a calcium-dependent manner, which constitutes a physiological stimulus for exosome release in K562 cells. The cytosolic calcium increase initiated by this stimulus involves the production of IP_3, which induces the release of calcium from intracellular stores. Subsequently, this event triggers capacitative calcium entry. Our results suggest that calcium is required for membrane fusion events at different stages in the exosome pathway.
The Rab family of small GTPases plays a key role in the regulation of vesicle trafficking between different compartments along endo-lysosomal and secretory pathways (23). Each member of this family associates with a specific membrane compartment, where they are believed to correctly target and fuse with vesicles with the proper acceptor membrane (24). Since more than 60 members of this family have been identified in mammals and implicated in the regulation of both exocytic and endocytic transport (25), these processes are considered to be highly complex. It has been shown that K562 cells possess large amounts of Rab11 compared with other members of this protein family (26). Numerous studies have shown that Rab11 is involved in the exocytic pathway as it is associated with post-Golgi membranes and secretory vesicles, and is required for TGN-to-plasma membrane transport (27,28). This protein has also been shown to regulate the recycling of the TFR through the pericentriolar endosomal compartment (29). We have previously shown that overexpression of the GDP-constitutive mutant, Rab11 S25N, inhibited exosome release, whereas this process was slightly stimulated in K562 cells overexpressing Rab11 wt (30). These results demonstrate that Rab11 participates in exosome secretion by regulating the interconnection between endocytic, recycling and secretory pathways.

Since both Rab11 and calcium participate in the regulation of the exosome pathway we were interested in determining if there is a connection between these factors, or if they work at independent stages of the pathway. For this purpose we examined how the increase of cytosolic calcium affects the development of MVBS and exosome release in K562 cells overexpressing GFP-Rab11 wt and its mutants. In this report we show that treatment of transfected cells with monensin (MON), an agent that increases cytosolic calcium concentration (22,31), caused a marked enlargement of the MVBS and regulated exosome secretion in Rab11-overexpressing cells. Our results suggest that the concerted actions of Rab11 and calcium are required for generation of the giant MVBS.

Results

Monensin differentially affects the size of MVBS in Rab11 K562 transfected cells

K562 is a human erythroleukemic cell line that expresses a large amount of mRNA for Rab11. As a result, 0.05–0.1% of the total cell membrane protein is Rab11 (26). We have previously observed that Rab11 labels distinct compartments in K562 cells (30). One of these compartments is the pericentriolar recycling compartment. Rab11 was also observed to be associated with large vesicular ring-shaped structures distributed throughout the cytoplasm, which have been identified as MVBS (30). To further analyze the subcellular compartments labeled by Rab11, we used stably transfected K562 cells overexpressing GFP-Rab11 wt, the mutants GFP-Rab11 Q70L (active mutant) and GFP-Rab11 S25N (inactive mutant), and the vector pEGFP (control). Exogenously administered N-lissamine rhodamine B sulfonyl-phosphatidylethanolamine (N-Rh-PE) was used to visualize MVBS (32). K562 transfected cells were incubated with the fluorescent lipid N-Rh-PE for 3 h. As shown in Figure 1A (top panel), numerous MVBS appear decorated with GFP-Rab11 wt and the chimera GFP-Rab11 Q70L. As expected, in cells overexpressing the dominant-negative mutant GFP-Rab11 S25N, MVBS were not labeled with the fluorescent chimera since this mutant is mostly cytosolic.

MON is an ionophore that induces the enlargement of MVBS (22,33). We observed that MON caused an increase in the size of MVBS that became evident after 30 min in the presence of the drug and was maximal after 120 min (data not shown). The enlarged MVBS were visualized by confocal microscopy. We took advantage of this attribute and used MON to further characterize the biogenesis of MVBS in cells overexpressing Rab11 and its mutants. For this purpose, transfected cells were incubated for 3 h with N-Rh-PE in the presence of MON. In Figure 1A (bottom panel) the MON effect on MVBS size is transfected cells is shown. Addition of 7 μM MON noticeably increased the size of MVBS, especially in GFP-Rab11 wt and Q70L overexpressing cells. It is important to mention that at the MON concentrations there was no change in cell viability – as assessed by Trypan blue exclusion – nor was morphological evidence of apoptotic nuclei observed in K562 cells (22). Interestingly, in the case of GFP-Rab11 wt and Q70L overexpressing cells, almost all MVBS that accumulated N-Rh-PE were decorated by GFP-Rab11 (see also the 3D reconstruction, Figure S1 available at http://www.traffic.dk/video_gallery.asp), whereas no decorated MVBS were observed in cells overexpressing the dominant-negative mutant GFP-Rab11 S25N (Figure 1A, bottom panel). In order to confirm that the large structures formed in the presence of MON were indeed MVBS they were labeled with an antibody against lysobisphosphatidic acid (LBPA) which is particularly concentrated in the internal membranes (34,35). Figure 1B upper panel clearly shows the labeling of the internal vesicles with LBPA. This is consistent with the observation that LBPA is present in B-cell derived exosomes (36). Since exosomes are also rich in cholesterol (37,38), the MVBS were stained with filipin that was clearly localized to the internal vesicles labeled with N-Rh-PE (Figure 1B, lower panel).

Since we observed that MON induced a striking enlargement of MVBS we next determined the size of the MVBS in cells overexpressing GFP Rab11 wt or mutants. The area of individual MVBS was determined using the META-MORPH program. As shown in Figure 2, in cells overexpressing GFP-Rab11 wt and Q70L, MON markedly enhanced the size of MVBS compared with the control.
pEGFP vector overexpressing cells). Our results indicate that MON has a differential effect on MVBs morphology in cells overexpressing GFP-Rab11 wt and its mutants.

Formation of enlarged MVBs depends on cell type and the Rab11 level
The results showed in Figure 2 indicate that, even in cells overexpressing vector alone, MON caused a large increase in the size of MVBs. Interestingly, it has been shown that K562 cells have high amounts of endogenous Rab11 compared with other Rab proteins (26). Thus, we wondered whether the levels of Rab11 were critical for the development of the enlarged MVBs. We assessed the effect of MON in other cell types by the quantitation of the percentage of cells with enlarged MVBs after MON treatment. Strikingly, in CHO cells (not shown) and in RAW macrophages (Figure 3) practically no large MVBs were generated in untransfected cells incubated with MON. In contrast, in RAW cells overexpressing Rab11 wt, very large MVBs, labeled with the lipid N-Rh-PE, were formed by MON treatment (Figure 3A,B). However, only 25% of the Rab11 transfected RAW cells had large MVBs compared with 60% of the cells in the case of the K562 (Figure 3C). Furthermore, only a few of these large MVBs were decorated by GFP-Rab11 (not shown), indicating that the localization of Rab11 on MVBs is cell type specific. These results clearly indicate that formation of the large MVBs depends on the presence of Rab11 and that the levels of this protein are critical for MON action. We also measured the amount of exosomes released by RAW cells. As expected, these cells released a very limited number of exosomes compared with K562. Interestingly, transfection of RAW macrophages with Rab11 wt

Figure 1: Monensin generates giant MVBs that are decorated by EGFP-Rab11. K562 cells were transfected to overexpress EGFP-Rab11 wt and the mutants Q70L and S25N. A) Stably transfected cells were incubated with the fluorescent lipid analog N-(lissamine rhodamine B sulfonyl) phosphatidyl ethanolamine (N–Rh–PE) to label the MVBs. The lipid was internalized via endocytosis for 3 h. Cells were mounted on coverslips and immediately analyzed by confocal microscopy (top panel). A set of cells were incubated with 7 μM MON and analyzed by confocal microscopy (bottom panel). B) EGFP-Rab11Q70L transfected K562 cells were incubated with 7 μM MON for 3 h at 37°C. Cells were attached to polylysine-embedded coverslips, fixed with 3% paraformaldehyde and then incubated with a mouse monoclonal anti-lysobisphosphatidic acid (LBPA) antibody (upper panel). Images were taken by confocal microscopy. Cells in the lower panel were labeled with the lipid N-Rh-PE as indicated above, fixed and then incubated with 50 μg/ml filipin for 30 min at room temperature. Cells were analyzed by fluorescence microscopy.
increased exosome release by 50% (not shown), indicating that Rab11 overexpression favors the shedding of vesicles into the extracellular media via fusion of MVBs with the plasma membrane.

A calcium-dependent fusion of MVBs is involved in
the enlargement of MVBs decorated by GFP-Rab11

We have previously observed in K562 cells that treatment with MON provokes a marked and sustained rise in cytosolic calcium concentration. This originates from both intracellular calcium stores and capacitative entry (22). To analyze whether the formation of MVBs in Rab11 overexpressing cells was dependent on the level of cytosolic calcium, transfected cells labeled with the lipid N-Rh-PE were incubated with MON for 3 h. In each case, a set of cells was also incubated in the presence of 30 μM BAPTA-AM, a membrane permeable calcium chelator. As shown in Figure 4 (bottom panel), the presence of BAPTA-AM hampered the formation of the gigantic MVBs generated by MON-treatment (compare with Figure 4, top panel). Nevertheless, numerous small vesicles with a typical ring-shaped structure were observed when GFP-Rab11 wt or the positive mutant GFP-Rab11 Q70L were over-expressed in the cells. This suggests that a calcium-dependent fusion event among MVBs could contribute to their enlargement.

To confirm that the calcium released by MON was required for MVB homotypic fusion and their subsequent enlargement, we used a permeable photolabile chelator, α-nitrophenyl EGTA (EGTA-NP-AM). This agent exhibits a high affinity for Ca2+, which is lost upon photolysis by UV illumination (the affinity decreases 12 500 fold). We incubated K562 cells overexpressing GFP-Rab11 Q70L with 30 μM EGTA-NP-AM for 15 min at 37 °C in the dark. Subsequently, 7 μM MON was added and the incubation was followed for 3 h at 37 °C. Before visualization by fluorescence microscopy, cells were illuminated with UV to destroy the chelator and release the calcium. Figure 5A shows a confocal image of a cell treated with MON that exhibits the typical gigantic MVBs (panel a). In panel b, a cell incubated with 30 μM EGTA-NP-AM and 7 μM MON, before the UV exposure is in which giant MVBs are absent. In panel c, an image taken just after the photolysis, some enlarging structures are visible. Many of these structures are docked, suggesting that a fusion process is in progress. Panel d shows a high magnification of a region of the cell in panel c showing fusion MVBs in more detail. Docking between two or more MVBs decorated by GFP-Rab11 Q70L was observed in the majority of the cells tested under these conditions. Progressive enlargement of the MVBs was observed after 15 and 30 min at 37 °C after UV exposure (panels e and f; see also video #1 at http://www.traffic.dk/suppmat/6_2.asp). These data clearly show that a calcium-dependent fusion event is involved, at least in part, in the enlargement of the MVBs.

In Figure 5B, two docked vesicles are clearly depicted showing an accumulation of GFP-Rab11 (brightly fluorescent mark) in the region between the fusing MVBs (panel a), suggesting that Rab11 is likely involved in the vesicle tethering/docking process (see also http://www.traffic.dk/suppmat/6_2.asp, video#2). Panel b shows the quantification of the fluorescence intensity along the dotted line in panel a. The fluorescence level in the region between the docked vesicles is almost four times the intensity of each individual vesicle membrane, indicating that the brightly fluorescent mark is not simply due to the superposition of membranes but represents an actual accumulation of the GFP protein at the docking site. Panels c and d show two images viewed by time lapse confocal fluorescent microscopy. The arrow points to a small vesicle that appears to be fusing with a larger one (see panel d).

We have frequently observed that the large MVBs emit tubular projections that are decorated by GFP-Rab11. A typical example is shown in Figure 5C (images were taken every 30 s). The arrows indicate the formation of a long tubule (see panels c–h) (see also the Image showing the 3D reconstruction and the video #3 at http://www.traffic.dk/suppmat/6_2.asp). It is likely that these Rab11 positive tubules are formed by extension of membrane protrusions along microtubules. The generation of

**Figure 2:** MVB size is differentially affected by MON in Rab11 transfected cells. K562 stably transfected cells overexpressing EGFP-Rab11 and its mutants were incubated with the fluorescent lipid analog N-Rh-PE. After 3 h of internalization, cells were mounted on coverslips and immediately analyzed by confocal microscopy. The size of N-Rh-PE-labeled MVBs was quantified in METAMORPH using the integrated morphometry analysis. Data represent the mean ± SEM (n=100 N-Rh-PE-labeled MVBs). *Differs from vector, p < 0.01.
these tubules required the normal functioning protein since in cells transfected with the dominant negative mutant of Rab11 no tubules were observed (not shown).

**A calcium increase stimulates exosome release in GFP-Rab11 transfected cells**

We have previously demonstrated that MON stimulates exosome secretion in K562 cells in a calcium-dependent manner.
manner (22). In an earlier work, we also reported that overexpression of GFP-Rab11 wt increases the release of exosomes in this cell type, whereas overexpression of the active and inactive chimeras, GFP-Rab11 Q70L and S25N, respectively, inhibited it (30). To gain insights into the role of calcium in the Rab11-dependent exosome release, we tested the effect of MON on this process using Rab11 transfected cells.

In hematopoietic cells, such as reticulocytes and K562 cells, some membrane proteins are known to diminish in the cell during the transition to erythrocytes and are released in exosomes while retaining their biological activities (39,40). Since acetylcholinesterase (AChE) is included amongst these proteins, its activity is used to measure the amount of exosome released (30). Exosomes from K562 cells as well as from reticulocytes are also enriched in proteins such as the TfR and the cytosolic chaperone Hsc70 (41). Released exosomes were quantified by measuring AChE activity and the levels of TfR and Hsc70 by Western blot analysis (see Materials and Methods). Exosomes were harvested from the culture medium of transfected cells after 7 h incubation with 7 μM MON and the activity of AChE was measured at 20 min in the exosomal fraction. As shown in Figure 6A, MON induced a marked increase in the amount of exosomes released (30). To confirm that the cytosolic calcium increase provoked by MON was the mechanism by which the exosome pathway was stimulated in Rab11 transfected cells, we increased intracellular calcium levels using the calcium ionophore A23187. Rab11 transfected cells were incubated with 1 μM A23187 and exosomes were collected and quantified by measuring AChE activity. A23187 stimulated exosome release in a manner similar to MON, although not to the same extent (Figure 7A). This suggests that MON might be activating an additional mechanism. The calcium requirement for Rab11-dependent exosome secretion in K562 cells was also evaluated by using the permeable calcium chelator BAPTA-AM (B-AM). As expected, incubation with 30 μM B-AM inhibited exosome secretion in all cases, reversing the stimulatory effect caused by overexpression of Rab11 wt (Figure 7B). Calcium is thus a key component in the regulation of Rab11-dependent exosome pathway.

### Discussion

In maturing red cells, TfR trafficking is an essential process to get the iron required for hemoglobin synthesis. TfRs are no longer needed when reticulocytes mature into erythrocytes and all of the hemoglobin has been synthesized. Therefore, after endocytosis, instead of recycling back to the cell surface, TfRs are specifically sorted to MVBs and subsequently targeted to the internal vesicles known as exosomes (30,42). This kind of MVB is able to fuse with the plasma membrane and shed the exosomes into the extracellular space (18,30), establishing a way to lose a number of membrane proteins and down-regulate the activity of some receptors. The present report shows that Rab11 and calcium regulate the exosome pathway in an interrelated fashion.
Figure 5: Homotypic fusion of MVBs labeled with GFP-Rab11 is calcium-dependent. A) EGFP-Rab11Q70L transfected K562 cells were loaded with a permeable photosensitive calcium chelator (EGTA-NP-AM) for 30 min at 37°C in the dark. Cells were washed and further incubated with 7 μM MON in the dark. Some cells were mounted on coverslips and immediately analyzed by confocal microscopy. An image showing the typical enlargement of MVBs after MON treatment is depicted in panel a. Resting cells were exposed for 1 min to UV illumination to destroy the chelator and release the calcium. Images were taken by confocal microscopy at different times after photolysis: time 0 (b), 30 s (c), 15 min (e) and 30 min (f). The inset in panel c is shown at a higher magnification to show in more detail the docking of MVBs labeled with EGFP-Rab11 Q70L (d). B) MVBs fusing after photolysis. Two docked MVBs are clearly depicted showing the accumulation of GFP-Rab11 at the contact zone between both vesicles (a). Panel b shows the quantitation of the fluorescence intensity following the dotted line in panel a. c) Several docked vesicles of different sizes. A small vesicle in panel c (arrow) disappears after 30 s by fusion with a bigger MVB (d). C) MVBs emit Rab11-decorated tubules. K562 stably transfected cells overexpressing EGFP-Rab11Q70L were loaded with EGTA-NP-AM and incubated with 7 μM MON in the dark as indicated above. The cells were then exposed to UV illumination for 1 min to destroy the chelator and release the calcium. Images were taken by confocal microscopy (a total of 30 slides every 11 s) starting at 30 min after photolysis. Arrows indicate the formation of long Rab11-decorated tubes that are generated from the enlarged MVBs.
We have previously shown that GFP-Rab11 wt overexpressed in K562 cells labels two different vesicle populations. One is the pericentriolar recycling compartment and the other the multivesicular compartment (30). In this report we present evidence that MVBs decorated by GFP-Rab11 undergo homotypic fusion. Rab11 stimulates this fusion event, since cells overexpressing GFP-Rab11 wt have enlarged of MVBs, which is more evident in cells overexpressing the positive mutant Q70L. Furthermore, treatment with the ionophore MON leads to the generation of gigantic MVBs labeled by Rab11, especially in the case of the Q70L mutant. In a recent report we have demonstrated that MON increases intracellular calcium level in K562 cells (22). Here we present evidence that the development of gigantic MVBs decorated by GFP-Rab11 induced by MON treatment depends on the calcium released by this ionophore, since incubation with the permeable calcium chelator (BAPTA-AM) prevented their formation. Interestingly, we have observed that many MVBs decorated by GFP-Rab11 wt and its positive mutant Q70L are docked and ready to fuse in the presence of a calcium chelator. This observation suggests that Rab11 is acting in the tethering/docking of MVBs to promote homotypic fusion, and calcium is required to complete this fusion event.

The requirement for Rab11 was further confirmed by the use of RAW macrophages. It is noteworthy that in Raw cells overexpressing the vector alone, MON treatment was unable to induce the formation of the giant MVB.
contrast, in RAW macrophages overexpressing Rab11 wt or the GTPase-deficient mutant, large MVBs were generated by MON treatment, although to a lesser extent than in K562 cells. This clearly indicates that Rab11 levels are critical for the formation of the enlarged MVBs and that the role of Rab11 in MVB formation is likely cell type specific.

The generation of giant MVBs by overexpression of GFP-Rab11 Q70L in conjunction with MON has provided an excellent opportunity to examine the behavior of MVBs in living cells. We have observed by time lapse confocal microscopy that after releasing the calcium sequestered by a photolabile calcium chelator, the docked vesicles begin fusing. Sometimes vesicles remain attached for a time; as fusion proceeds, one of the docked vesicles gradually decreases in diameter and is consumed by the larger acceptor vesicle. We have also occasionally observed fusion events where there was a rapid coalescence of the membranes, forming an enlarged vacuole. These two types of fusion are reminiscent of membrane fusion events previously described and termed ‘bridge fusion’ and ‘explosive fusion’, respectively (44), indicating that this is a common characteristic of membrane fusion events in the endocytic pathway. Since MVBs were decorated by GFP-Rab11, we have observed in both types of fusion reactions a highly fluorescent spot in the contact zone between fusing MVBs, suggesting that Rab11 is indeed engaged in vesicle tethering. Our observation is very similar to the phenomenon observed in endosome fusion events in cells overexpressing the active mutant GFP-Rab5 Q79L (44). These authors have clearly shown that the spot present in the contact zone was not simply due to the overlap of fusing membranes but to a specific accumulation of the Rab protein (44). We observed that, in the absence of calcium, MVBs remain docked, with the brightly fluorescent speck at the point where vesicles contact each other, but fusion was hampered until calcium was uncaged. It is noteworthy that in many cases more than two vesicles were docked (see Figure 5A), consistent with the idea that vesicles form aggregates and multiple fusion events take place (45).
We have also observed by confocal video microscopy the formation of long tubules decorated by Rab11 that emerged from the large MVBs. These tubules have also been observed in stimulated dendritic cells. It has been proposed that the tubules allow the return of MHC II from the internal vesicles to the plasma membrane (46).

Our observation that vesicles decorated with Rab11 remain docked until calcium is released is in agreement with numerous studies indicating the requirement for calcium in the last steps of membrane fusion (reviewed in (21)). It is well known that membrane fusion involves several events: docking, tethering, hemifusion and pore formation as the final stage. The involvement of Rab proteins in vesicle transport and membrane tethering is widely accepted but, in most membrane fusion events, SNARE action is also necessary for fusion to proceed (for a review see (47)). Different members of this protein family are located on both of the membranes to be fused. After membrane attachment, hemifusion (bilayer mixing) is initiated when both membranes are forced into close proximity by the formation of trans SNARE complexes. In many cases, this step has been shown to be calcium-dependent, since several calcium-binding proteins modulate SNARE complex formation by interacting with their constituents (48). The calcium control of membrane fusion has been depicted to be relevant not only for calcium triggered exocytosis, but also for intracellular 'constitutive' fusion events (49). Thus, the requirement for calcium in the exosome pathway is not unexpected. Indeed, in a recent study we have shown that exosome exocytosis in K562 cells is a stimulus-dependent phenomenon. When transferrin associates with its receptor at the plasma membrane it raises intracellular calcium levels through a signaling cascade that results in an increase in exosome release (22). On this basis, transferrin could be a physiological control for exosome secretion in maturing red cells via a calcium-dependent mechanism.

In the present report we have shown that calcium is required for homotypic fusion of MVBs. As mentioned above, a role for intravesicular Ca\(^{2+}\) in several secretory and intracellular fusion events has been proposed. Consistent with our observations, fusion of early endosomes seems to require release of luminal Ca\(^{2+}\) for fusion to occur (50,51). Our results indicate that calcium is involved in the earlier steps of MVB biogenesis, probably acting in a coordinated manner with Rab11. Treatment with BAPTA-AM inhibited exosome exocytosis, even in cells overexpressing GFP-Rab11 wt, where this process was stimulated. This indicates that the stimulatory effect of overexpressed Rab11 wt depends on the presence of calcium. Furthermore, calcium also seems to be important at the final step in the exosome pathway, when MVBs fuse with the plasma membrane, since exosome release was impeded in the presence of a calcium chelator. An increase in the cytosolic calcium concentration stimulated by MON or the calcium ionophore A23187 enhanced exosome secretion in cells overexpressing Rab11 wt or the mutants in which exosome secretion was impeded. It is believed that Rab mutants, such as the S25N, act as dominant-negative mutants because they sequester the Rab exchange factors. It is likely that calcium bypasses the restriction imposed by the S25N mutant because it may activate the Rab11 exchange factor, which will be now able to function on the endogenous Rab11, a protein that is expressed at high levels in K562 cells. This could also explain the effect of MON on the size of MVBs in dominant-negative mutant Rab11 overexpressing cells.

It has been implied that MVBs can follow two distinct pathways: either to fuse with lysosomes (52,53) for the degradation of their contents, or to fuse with the cell surface to release the internal vesicles (39,54,55). Since Rab proteins and their effectors are believed to coordinate many transport events, such as driving vesicles to their appropriate target compartment, the final destination of MVBs (degradation or exocytosis) might be determined by specific Rab proteins. The results of this study hint that Rab11 is an essential molecular component in the regulation of MVB formation, interconnecting endocytic and exocytic pathways. Our results also suggest that Rab11 might function as the specific Rab driving MVBs to the plasma membrane, avoiding fusion with lysosomes. This mechanism is likely to be critical in those cell types that secrete exosomes. Furthermore, our results with the RAW cells also support this point, since these cells released very limited amounts of exosomes compared to K562 (not shown). Interestingly, transfection of RAW macrophages with Rab11wt increased exosome release by 50%, indicating that Rab11 overexpression favors the shedding of molecules in the extracellular media via fusion of MVBs with the plasma membrane. This interpretation is supported by a recent publication by Thomas Sudhof and collaborators that stated that Rab 11 has a specific role in neuronal cells, acting as a GTP-dependent switch between constitutive and regulated secretory pathways determining the secretory fate of a transport vesicle (56).

According to our previous and present results, the following working model for exosome release can be proposed. Rab11, which is found in abundance in K562 cells, inhibits membrane recycling from the recycling compartment and drives membrane proteins, such as the Tfr, to MVBs. To appropriately generate MVBs, this small GTPase facilitates homotypic MVB fusion and also a membrane flux from the TGN (30). Once formed, MVBs are driven to the plasma membrane to fuse with and secrete exosomes, an episode that probably is also regulated by Rab11. Both intracellular MVB fusion events as well as fusion of this compartment with the plasma membrane require calcium. The calcium level necessary to maintain the functioning of the exosome pathway is likely the result of a specific stimulus. Each time that transferrin binds to its receptor, a signaling cascade is triggered that results in a moderate rise in the

Savina et al.
intracellular calcium concentration, involving IP₃ channels and capacitative calcium entry (30). This discrete elevation of calcium levels would be sustained by continuous transferrin endocytosis and would ensure the fusion events regulated by Rab11. Therefore, transferrin would regulate its own active life, facilitating its elimination via exosomes. Since Rab11 is considered a key element in the control of TFR recycling, we propose a regulatory function for Rab11 in TFR intracellular traffic controlling its targeting to the exosome pathway. In a very recent publication, Vidal and collaborators (60) have shown that in maturing reticulocytes the protein Alix coimmunoprecipitated with the TFR. These results link TFR to the ESCRT sorting machinery, explaining the specific enrichment of TFR in reticulocyte exosomes compared to other cell types. Similarly, it is likely that in K562 the expression of high levels of Rab11 may represent an adaptation of this cell type to modulate the endocytic system facilitating the shedding of TFR via exosomes.

Materials and Methods

Materials

RPVM cell culture medium and fetal calf serum (FCS) were obtained from Gibco Laboratories (Grand Island, NY). N-Rh-PE was obtained from Avanti Polar lipids, Inc. (Birmingham, AL). Acetylthiocholine and 5,5'-dithio-bis(2-nitrobenzoic acid) were obtained from Sigma (St. Louis, MO). Bodipy-TR ceramide was from Molecular Probes (Eugene, OR). All other chemicals were purchased from Sigma. The rat monoclonal anti-Hsc 70 antibody was purchased from StressGen (Victoria, Canada). Peroxidase-conjugated antibodies were purchased from Jackson Immunoresearch (West Grove, PA). BAPTA-acetoxyethyl ester (BAPTA-AM), n-octophenyl EGTA-acetoxyethyl ester (EGTA-AM-NP) and A23187 were purchased from Molecular Probes. The anti-lysobisphosphatidic acid (LBPA) mouse monoclonal antibody was a generous gift from Jean Gruenberg (University of Geneva, Switzerland).

Cell culture and transfection

K562, an erythroleukemia cell line of human origin, was grown in RPMI supplemented with 10% FCS, streptomycin (50 μg/mL) and penicillin (50 U/mL). The CDNA of Rab11a and its mutants (a generous gift from Dr. David Sabatini, New York University) were subcloned into the vector pEGFP (kindly provided by Dr. Philip Stahl, Washington University, St. Louis, MO) as fusion proteins with the green fluorescent protein (GFP). K562 cells were transfected with Transfast (Promega, Madison, WI) according to the manufacturer’s instructions, with pEGFP (control vector), pEGFP-Rab11wt, pEGFP-Rab11Q70L (a GTPase-deficient mutant) and pEGFP-Rab11S25N (a GTP-binding deficient mutant). Stable transfected cells were selected with genetin (0.5 mg/mL) and subsequently cloned. For some experiments, transfected Raw macrophages overexpressing the vector alone (pEGFP), pEGFP-Rab11wt or pEGFP-Rab11Q70L were used.

Exosome isolation

Exosomes were collected from the media of 10 mL K562 cells (1–1.5 × 10⁶ cells/mL) cultured for 7–15 h. The culture media was placed on ice and centrifuged at 800 x g for 10 min to sediment the cells and subsequently at 12,000 x g for 30 min to remove the cellular debris. Exosomes were separated from the supernatant by centrifugation at 100,000 x g for 2 h. The exosome pellet was washed once in a large volume of PBS and resuspended in 100 μL of PBS (exosome fraction).

Quantitation of exosomes

The amount of exosomes secreted was quantified by measuring proteins that are specifically targeted to these vesicles: acetylcholinesterase, TFR and the chaperone Hsc70 (57). Acetylcholinesterase activity was assayed by standard procedures (58). Briefly, 15 μL of the exosome fraction was suspended in 100 μL phosphate buffer and incubated with 1.25 μm acetylthiocholine and 0.1 μm 5,5'-dithio-bis(2-nitrobenzoic acid) in a final volume of 1 mL. The incubation was carried out in cuvettes at 37 °C, and the change in absorbance at 412 nm was followed continuously. TFR and Hsc70 were detected by Western blotting and subsequently analyzed by densitometry. Samples of the exosomal fraction (15 μL) were solubilized in reducing SDS loading buffer and incubated for 5 min at 95 °C. Samples were run on 7.5% or 10% polyacrylamide gels and transferred to Immobilon (Millipore Sao Palo SP, Brazil or BioBlot-NIC (Costar, Cambridge, MA, USA) membranes. The membranes were blocked for 1 h in Blotto (5% nonfat milk, 0.1% Tween 20, PBS) and subsequently washed twice with PBS. Membranes were incubated with primary antibodies and peroxidase-conjugated secondary antibodies. The corresponding bands were detected using an enhanced chemiluminescence detection kit from Pierce (Rockford, IL USA).

Labeling with N-Rh-PE

The fluorescent phospholipid analog N-Rh-PE was inserted into the plasma membrane as previously described (59). Briefly, an appropriate amount of the lipid, stored in chloroform/methanol (2:1), was dried under nitrogen and subsequently solubilized in absolute ethanol. This ethanolic solution was injected with a Hamilton syringe into serum-free RPMI (< 1% v/v) while vigorously vortexing. The mixture was then added to the cells and they were incubated for 60 min at 4 °C. After this incubation period, the medium was removed and the cells were extensively washed with cold PBS to remove excess unbound lipid. Labeled cells were cultivated in complete RPMI medium under several conditions for 3 h to allow internalized lipid to reach the MVBs. After this incubation, cells were washed in PBS and immediately mounted on coverslips and analyzed by confocal microscopy.

Immunofluorescence

Cells were attached to polystyrene embedded coverslips and fixed with 1 mL of 3% paraformaldehyde solution in PBS for 30 min at room temperature. Samples were washed several times with PBS and blocked by incubating with 50 μL NH₄Cl for 15 min in PBS. Cells were permeabilized with 0.05% saponin in PBS and incubated with a mouse antibody against LBPA (dilution 1: 250) in PBS containing 1% BSA. Bound antibodies were subsequently detected by incubation with Texas Red-conjugated goat-anti-mouse secondary antibody (dilution 1: 1000). Samples were mounted with 50% glycerol in PBS and analyzed by fluorescence microscopy.

Fluorescence microscopy

pEGFP-Rab11 transfected K562 cells were analyzed by fluorescence microscopy using a Nikon Confocal C1 and processed with the program EZ-C1. To quantitate the size of MVBs the METAMORPH 4.5 program (Universal Imaging Corp. West Chester, PA, USA) was used.

Videomicroscopy

In some experiments, confocal microscopy was used. However, to be able to visualize the formation of the enlarged MVBs continuously during a 30-min period after photolysis of the calcium chelator and to avoid photobleaching, we used a Leica DM IRBE epifluorescence microscope controlled by METAMORPH software (Universal Imaging), a cooled CCD camera (MicroMax, Princeton Instruments Inc., Princeton, NJ) and a halogen lamp. Coverslips with the cells were placed into a chamber on the microscope at 37 °C in a 5% CO₂ atmosphere. At time 1-min UV exposition was done to destroy the chelator. Every 25 s a stack of five z-planes (step 1 μm) were acquired with the green filter set. Images were taken using a 100 x 1.4 NA oil immersion objective.

Traffic 2005; 6: 131–143

141

Ca²⁺-Dependent Multivesicular Body Fusion
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