The exosome pathway in K562 cells is regulated by Rab11

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Summary

During maturation, reticulocytes lose some membrane proteins that are not required on the mature red cell surface. The proteins are released into the extracellular medium associated with vesicles that are formed by budding of the endosomal membrane into the lumen of the compartment; this process results in the formation of multivesicular bodies (MVBs). Fusion of MVBs with the plasma membrane results in secretion of the small internal vesicles, termed exosomes. K562 cells release exosomes with similar characteristics to reticulocyte exosomes, in particular the transferrin receptor (TfR) is found associated with the vesicles. Interestingly, this cell line has been shown to possess high amounts of Rab11 compared with other Rab proteins. To assess the regulation of transferrin receptor release via exosome secretion by Rab11 in this cell type, K562 cells were stably transfected with GFP-Rab11wt or the GTP- and GDP-locked mutants. The distribution of the proteins was assessed by fluorescence microscopy. Transferrin recycling and the number of TfRs present on the surface of the transfected cells were reduced by overexpression of either Rab11wt or the mutants. The amount of released exosomes was analyzed by measuring different molecular markers present on these vesicles either biochemically or by western blot. Overexpression of the dominant-negative mutant Rab11S25N inhibited exosome release, whereas the secretion of exosomes was slightly stimulated in cells transfected with Rab11wt. Taken together, the results demonstrate that in K562 cells Rab11 modulates the exosome pathway although the exact step involved is still not known.

Key words: Exosomes, Multivesicular body, Rab11, Transferrin receptor

Introduction

Exosomes are small membrane vesicles (60-80 nm) secreted by several cell types as a consequence of fusion of multivesicular bodies (MVBs) with the plasma membrane (Johnstone, 1992). The MVBs are formed by inward budding from the endosomal membrane and subsequent pinching off of small vesicles into the luminal space. The internal vesicles present in the MVBs are then released into the extracellular media as so-called exosomes. Upon maturation, reticulocytes externalize obsolete membrane proteins such as the transferrin receptor (TfR) by means of exosomes (Johnstone et al., 1991; Harding et al., 1983; Harding et al., 1984). Although other plasma membrane proteins (e.g. acetylcholinesterase) are lost during maturation of the red cell, reticulocyte exosomes are largely devoid of soluble proteins and proteins associated with lysosomes or other intracellular organelles. This indicates that only a selected group of proteins is lost via this pathway. The selective loss of membrane proteins appears to be preceded by the formation of MVBs. Interestingly, an ATP-binding protein structurally related to the clathrin-uncoating ATPase Hsc70 is highly enriched in exosomes (Davis et al., 1986; Thery et al., 1999). It had been proposed that this protein plays a role in targeting proteins for secretion (Johnstone, 1992), routing the selected proteins to the MVBs. However, as recently shown, Hsc70 is more probably sorted as a consequence of its binding to unfolded TfR (Géminard et al., 2001). TfR receptors have been found in all species where reticulocyte exosomes have been analyzed (Johnstone et al., 1989; Johnstone et al., 1991), suggesting that this is a major route for the maturation-associated loss of this receptor. Other hematopoietic cell types also secrete exosomes. Activated platelets release exosomes whose function is largely unknown. Platelet-derived exosomes are enriched in proteins implicated in signaling and adhesive functions (Heijnen et al., 1999; Denzer et al., 2000), and thus may have a role at sites of vascular injury. Cytotoxic T cells and B lymphocytes also secrete exosomes. Therefore, exosomes may be involved in diverse extracellular functions such as targeting molecules for cell killing (Peters et al., 1989) or antigen presentation (Raposo et al., 1996; Zitvogel et al., 1998), depending on the cell type from which they originate. For the same reason, it is also likely that the compartment involved in exosome biogenesis might be different.

Rab proteins are small GTPases that play an essential role in the regulation of membrane traffic (for a review, see Schimmoller et al., 1998; Brennwald, 2000). Different members of the Rab family are localized to distinct membrane-bound compartments where they are thought to have a central role in the proper targeting to and fusion of transport vesicles with the correct destination membrane (Brennwald, 2000). Rab11 has been shown to be associated with post-Golgi membranes, secretory vesicles (Urbe et al., 1993) and the pericentriolar recycling endosome (Ullrich et al., 1996; Ren et

al., 1998), as well as with the apical recycling system in polarized cells (Casanova et al., 1999). Transfection experiments with CHO or BHK cells indicate that Rab11 regulates Tf recycling through the pericentriolar endosomal compartment (Ullrich et al., 1996; Urbe et al., 1993). Rab11 function in exocytic trafficking has been deduced from the Rab11 requirement for transport from the trans-Golgi network to the plasma membrane (Chen et al., 1998). In addition, in a recent publication it was shown that Rab11 regulates transport from early endosomes to the trans-Golgi network (Wilcke et al., 2000), suggesting that Rab11 may control the interconnection between the endocytic and secretory pathways.

K562 cells release exosomes with similar characteristics to reticulocyte exosomes (Johnstone, 1996) and are therefore a useful model to study the secretion of these small vesicles from erythroid cells. One of the most significant similarities is the presence of the TfR, which is absent in other exosomes (e.g. from platelets, antigen presenting cells, cytotoxic T lymphocytes), suggesting a biogenesis from an early endosomal compartment. Moreover, we have shown by subfractionation analysis that reticulocyte MVBs contain early endosomal markers (e.g. rab4 and rab5) and not classical markers of late endosomes (e.g. rab7, CI-M6PR) (Dardalhon et al., 2002). Interestingly, K562 cells have been shown to possess high amounts of Rab11 compared with other Rab proteins (Green et al., 1997). Its relative abundance in K562 cells led us to hypothesize that Rab11 may have a specific function in this hematopoietic cell type, perhaps related to the formation and release of exosomes.

Since Rab proteins are key elements of the molecular machinery that controls membrane traffic, we examined the role of Rab11 in the exosome pathway in the K562 cells. The role of Rab11 in TfR trafficking was also analyzed. For this purpose, K562 cells were stably transfected with GFP-Rab11wt and mutants. As shown in other cell types, expression of the different forms of Rab11 inhibited TfR recycling. Furthermore, the number of TfR present on the plasma membrane of transfected K562 cells was markedly reduced. Expression of Rab11S25N, the dominant-negative mutant of Rab11, substantially inhibited the amount of released exosomes, whereas in cells transfected with the wild-type protein, exosome secretion was slightly enhanced. Therefore, our results indicate that Rab11, probably because of its function of regulating membrane recycling from early endosome and/or trans-Golgi network (TGN) compartments, is involved in the secretion of exosomes in K562 cells. This study provides new insights into the intracellular mechanisms that modulate not only the maturation of red cells but also the mechanism of exosome secretion in other cell types.

Materials and Methods

Materials

RPMI cell culture medium and fetal calf serum (FCS) were obtained from Gibco Laboratories (Grand Island, NY). Iron-loaded human transferrin (Sigma) was labeled with ^{125}I using the Iodo-Gen method (Fraker and Speck, 1978) to a specific activity of 2×10^6 cpm/μg protein. N-(lissamine rhodamine B sulfonyl)-phosphatidylethanolamine (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. Bodipy-TR ceramide was from Molecular Probes (Eugene, OR). All other chemicals were purchased from Sigma (St. Louis, MO).

Cell culture and transfection

K562, a human erythroleukemia cell line, 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 David Sabatini, New York University) were subcloned into the vector pEGFP as fusion proteins with the green fluorescent protein (GFP). K562 cells were transfected with Transfast (Promega) 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). Stably transfected cells were selected with geneticin (0.5 mg/ml) and separated by flow cytometry using a FACS Vantage SE-TSO (INSERM U475, Montpellier).

Antibodies

Rabbit anti-rab11 serum was a generous gift from Bruno Goud (Institut Curie, Paris, France). The rat monoclonal anti-Hsc 70 antibody was purchased from StressGen (Victoria, Canada), and the rabbit polyclonal anti-Lyn antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). The mouse anti-transferrin receptor was kindly provided by Sebastian Amigorena (Institut Curie, Paris). Anti-Man6P receptor (CI-MPR) was a generous gift from Stuart Kornfeld (Washington University, St. Louis, MO). The rabbit antibody against TGN46 was kindly provided by Franck Perez (Institut Curie, Paris). Peroxidase-conjugated antibodies were purchased from Jackson Immunochemicals (West Grove, PA).

Fluorescence microscopy

pEGFP-Rab11-transfected K562 cells were analyzed by fluorescence microscopy using an inverted microscope (Nikon Eclipse TE 300, Germany) equipped with a filter system (excitation filter 450-490, barrier filter 515). Images were obtained with a CCD camera (Orca I, Hamamatsu) and processed using the program MetaMorph 4.5 (Universal Images Corporation).

Labeling with Transferrin-rhodamine

For colocalization studies, cells were incubated in serum-free RPMI containing 20 μg/ml human Tf (tetramethyl rhodamine-conjugated, Molecular Probes) for 45 minutes at 37°C and washed twice with ice-cold PBS. Cells were mounted on coverslips and immediately analyzed by fluorescence microscopy using the following filters: excitation filter 510-560 nm, barrier filter 590 nm.

Visualization of MDC-labeled vacuoles

Autophagic vacuoles were labeled with monodansylcadaverine (MDC) by incubating cells with 0.05 mM MDC in PBS at 37°C for 10 minutes. After incubation, cells were washed four times with PBS, mounted on coverslips and immediately analyzed by fluorescence microscopy using the following filter system: excitation filter V-2A 380-420 nm, barrier filter 450 nm.

Labeling of acidic compartments with Lysotracker

Acidic compartments were labeled by incubating the cells with 1 μM LysoTracker (Molecular Probes) in serum-free RPMI medium for 10 seconds at room temperature. After incubation, cells were extensively washed with PBS, mounted on coverslips and immediately analyzed by fluorescence microscopy using the following filter system: excitation filter 510-560 nm, barrier filter 590.

Labeling with BODIPY-TR ceramide

Membranes of the Golgi apparatus were labeled with 2.5 μM

BODIPY-TR ceramide (Molecular Probes), essentially as described by Pagano and collaborators (Pagano et al., 1991).

Indirect immunofluorescence for Man-6-P receptor (CI-MPR) and TGN46

Cells were fixed in suspension with 1 ml of 2% paraformaldehyde solution in PBS for 30 minutes at room temperature. Cells were washed with PBS and blocked by incubating with 0.1 M glycine in PBS. Cells were permeabilized with 0.05% saponin in PBS containing 0.2% BSA and were then incubated with a rabbit antibody against CI-MPR (dilution 1:50) or with the antibody against TGN46 (dilution 1:100). Bound antibodies were subsequently detected by incubation with Texas-Red-conjugated goat anti-rabbit secondary antibody. The cells were mounted with 50% glycerol in PBS and analyzed by fluorescence microscopy.

Exosome isolation

Exosomes were collected from the media of 15 ml K562 cells cultured for 24 hours. The culture media was placed on ice and centrifuged at 800 ***g*** for 10 minutes to sediment the cells and subsequently was centrifuged at 12,000 ***g*** for 20 minutes to remove the cellular debris. Exosomes were separated from the supernatant by centrifugation at 100,000 ***g*** for 2 hours. The exosome pellet was washed once in a large volume of PBS and resuspended in 100 μl of PBS (exosome fraction).

Analysis of exosomes

Acetylcholinesterase assay

Acetylcholinesterase activity was assayed by standard procedures (Ho and Ellman, 1969). Briefly, 15 μl of the exosome fraction were suspended in 100 μl phosphate buffer and incubated with 1.25 mM acetylthiocholine and 0.1 mM 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.

Fluorescent N-Rh-PE measurement

The fluorescent phospholipid analog N-(lissamine rhodamine B sulfonyl) phosphatidyl ethanolamine (N-Rh-PE) was inserted into the plasma membrane as previously described (Willem et al., 1990). 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 minutes at 4°C. After this incubation period, the medium was removed and the cells were extensively washed with cold PBS to remove excess unbound lipids. Labeled cells were cultured in complete RPMI medium to collect exosomes. 50 μl of the exosomal fraction were solubilized with 1.5 ml PBS containing 0.1% Triton X-100 to measure N-Rh-PE using a SLM Aminco Bowman Series 2 luminescence spectrometer at 560 nm and 590 nm excitation and emission wavelengths, respectively.

Transferrin recycling assay

Cells were pre-incubated for 1 hour at 37°C in serum-free RPMI medium supplemented with 1% BSA to deplete endogenous transferrin (Tf). They were then incubated with ^{125}I-labeled human Tf (2 μg/ml, 2×10^6 cpm/μg protein) in serum-free RPMI medium supplemented with 1% BSA for 1 hour at 37°C. Cells were then washed four times with cold PBS and once with an acetate buffer (200 mM sodium acetate and 150 mM NaCl, pH 4.5) for 2 minutes on ice to remove surface-bound Tf. Cells were aliquoted (0.5×10^6 cells per tube) and incubated at 37°C in 0.5 ml of serum-free RPMI medium containing 100 μM deferoxamine mesylate and 20 μg/ml unlabeled human Tf. At different times, the incubations were stopped by adding 1 ml ice-cold PBS and immediately placing the samples on ice. The samples were centrifuged for 20 seconds at 14,000 ***g*** to sediment the cells, and the medium was collected. The cell pellet was then washed with the acetate buffer (see above) to remove surface-bound Tf. The radioactivity in both the medium and the cell pellet was determined using a γ-counter (Packard Cobra). Nonspecific radioactivity (not competed by incubation with a 100-fold excess of unlabeled Tf) did not account for more than 10% of the total cell-associated radioactivity.

Transferrin receptor measurements

Ligand-binding studies were performed as previously described (Sainte-Marie et al., 1991). Cells were suspended in ice-cold serum-free RPMI medium at 3×10^6 cells/ml. Binding reactions were prepared with 100 μl of this cell suspension containing 50-500 nM ^{125}I-Tf, with or without 25 μM unlabeled Tf. Samples were incubated at 4°C for 90 minutes, after which 1 ml of cold PBS-BSA was added and the cells pelleted at 14,000 ***g*** for 1 minute. The medium was removed and the cells resuspended in 50 μl PBS-BSA and centrifuged through a water-impermeable layer of dibutyl phtalate on 15% sucrose at 14,000 ***g*** for 3 minutes. The tubes were quickly frozen using liquid nitrogen, and the tips containing the cell pellets were cut off and counted in a γ-counter. The amount of nonspecifically bound ^{125}I-Tf associated in the presence of excess unlabeled Tf was subtracted from samples incubated in the absence of cold ligand to determine the amount of specific ^{125}I-Tf bound. Specific binding data were analyzed by the Scatchard method to determine the number of Tf binding sites. The total number of transferrin receptors (TfRs) was determined by western blot of whole cell fractions.

SDS-PAGE and western blotting

Samples of the total cell pellet (100 μg protein) or exosomal fraction (15 μl) were solubilized in reducing SDS loading buffer and incubated for 5 minutes at 95°C. Samples were run on 7.5% or 10% polyacrylamide gels and transferred to Immobilon (Millipore) or BioBlot-NC (Costar) membranes. The membranes were blocked for 1 hour in Blotto (5% non-fat milk, 0.1% Tween 20 and 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.

Electron microscopy

K562 cells were incubated with Tf-coated colloidal gold particles (10 nm) for 45 minutes at 37°C. Cells were washed twice with cold PBS to remove unbound particles and processed for electron microscopy as previously described (Harding et al., 1984). Briefly, the samples were fixed in 2% glutaraldehyde in 0.15 M sodium cacodylate buffer for 20 minutes, washed, post fixed in 2% OsO_4 in cacodylate, pH 7.4, rinsed, stained and embedded as previously described (Harding et al., 1983).

Results

Rab11 wildtype and mutants are differentially localized in K562 transfected cells

In the human erythroleukemic K562 cell line the small GTPase Rab11 is highly expressed relative to other Rab proteins (Green et al., 1997). In an effort to gain insights into the function of Rab11 in this cell type, stably transfected K562 cells were

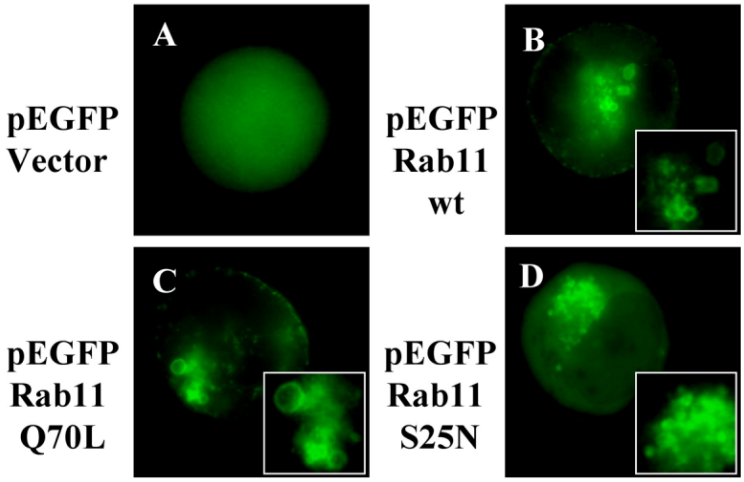

Fig. 1. Subcellular distribution of GFP-Rab11 in K562 cells. K562 cells were transfected with the plasmid pEGFP (control) or with the constructs pEGFP-Rab11wt, pEGFP-Rab11Q70L and pEGFP-Rab11S25N. The stably transfected cells were mounted on coverslips and immediately analyzed by fluorescence microscopy without fixation using an inverted microscope (Nikon Eclipse TE 300, Germany) equipped with a filter system (excitation filter 450-490, barrier filter 515). Images were obtained with a CCD camera (Orca I, Hamamatsu) and processed using MetaMorph 4.5 (Universal Images Corporation).

generated to overexpress GFP-tagged Rab11 wildtype (wt) and two mutants forms or the vector pEGFP (control). Expression of the transfected proteins was visualized by fluorescence microscopy. As expected, in cells transfected with the vector alone the GFP was distributed diffusely throughout the whole cell, including the nucleus (Fig. 1A). However, the chimeric protein GFP-Rab11wt was enriched in vesicular structures localized mainly to the perinuclear region, but was also observed in vesicles distributed throughout the cytoplasm and in punctate structures in close proximity to the plasma membrane (Fig. 1B). Interestingly, a few large vesicles with a typical ring shape were also observed. A similar distribution pattern was found with the mutant Rab11Q70L, with even larger vesicular ring-shaped structures in this case (Fig. 1C). In contrast, in cells overexpressing the dominant-negative mutant GFP-Rab11S25N, the protein showed a diffuse distribution throughout the cytoplasm, although it also localized to vesicular structures in the perinuclear region. No labeling was observed at the plasma membrane (Fig. 1D).

In order to determine if the transfected cells were expressing similar levels of the Rab11 GFP-tagged proteins, cells were analyzed by western blotting using an antibody against the c-terminal domain of Rab11. As shown in Fig. 2, equivalent levels of overexpressed Rab11, quantified by densitometry, were observed in the transfected cells with either wt or mutants. We were unable to detect the endogenous Rab11 by this technique using the amount of protein loaded in the gel. The higher molecular weight band that appears in the vector-transfected cells is a nonspecific band that is also present in the Rab11-transfected cells. To work with similar levels of overexpressed proteins, the transfected cells were frequently checked by FACS.

As it is known that Rab11 associates with transferrin-containing recycling compartments (Ullrich et al., 1996; Ren et al., 1998; Green et al., 1997), we studied the localization of the chimeric Rab11 proteins with rhodamine-transferrin internalized by endocytosis for 45 minutes at 37°C. Consistent with previous observations, a marked colocalization with GFP-Rab11wt was observed in the perinuclear and peripheral small vesicles (Fig. 3), however no colocalization was observed in the large ring-shaped structures in the case of cells overexpressing Rab11wt or the mutant Rab11Q70L (Fig. 3, bottom panels). In the case of the mutant Rab11S25N, colocalization was exclusively observed in the pericentriolar region.

The observation that the large vesicles in the transfected cells do not colocalize with internalized transferrin might be explained by the fact that only a very small amount of this protein, which is unable to be detected by fluorescence microscopy, is directed to this compartment.

The large ring-shaped vesicles are labeled by TGN markers

In order to determine the nature of the large vesicles observed in cells overexpressing the mutant Q70L, colocalization studies with markers of different vesicular compartments were carried out. As shown in Fig. 4, the large vesicles were neither labeled with Lysotracker, a marker of acidic compartments, nor with monodansylcadaverine (MDC), an autofluorescent compound

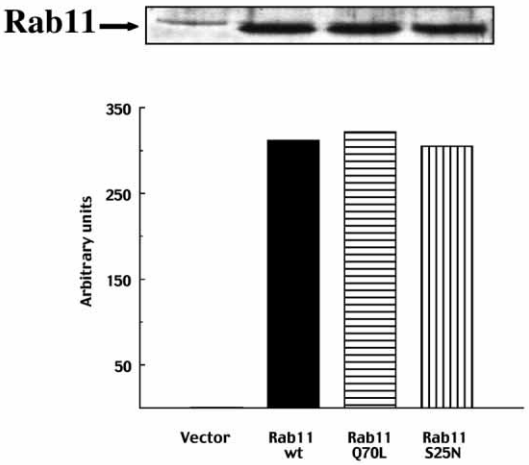

Fig. 2. Expression levels of GFP-Rab11 wt and mutants. Stably transfected K562 cells (1×10^5 cells) were lysed with PBS containing 1% Triton X100. Samples were subjected to SDS-PAGE and transferred onto a nitrocellulose membrane as described in the Materials and Methods. The membrane was incubated with a rabbit anti-Rab11 serum and a corresponding HRP-labeled secondary antibody and developed with a enhanced chemiluminescence detection kit (Pierce). The intensity of the bands was quantified by densitometry and was expressed as arbitrary units.

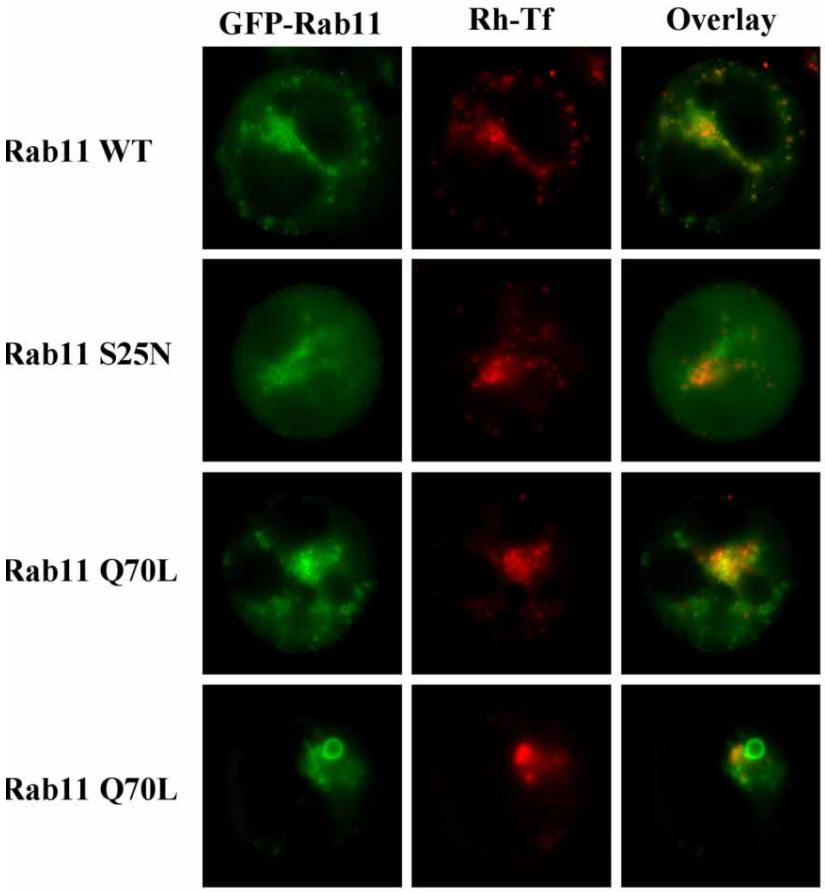

Fig. 3. Colocalization of GFP-Rab11 wt or mutants with rhodamine-transferrin. Cells were incubated in RPMI (serum free) containing 20 μg/ml human Tf (tetramethyl rhodamine-conjugated) for 45 minutes at 37°C and washed twice with ice-cold PBS. Cells were mounted on coverslips and immediately analyzed by fluorescence microscopy. Images were obtained with a CCD camera (Orca I, Hamamatsu) and processed using MetaMorph 4.5 (Universal Images Corporation). Left panels: GFP-Rab11wt and mutants. Middle panels: Rhodamine-transferrin (Rh-Tf). Right panels: overlay. In the first three panels, the focus was set on the small Rab11 structures that colocalize with the internalized Rh-Tf. In contrast, in the bottom panels the focus was set on the large ring-shaped structures formed in cells transfected with the mutant Q70L. Rh-Tf was not detected in these structures.

that specifically accumulates in autophagic vacuoles (Biederbick et al., 1995). Also, no colocalization with the mannose-6-phosphate receptor was observed, indicating that these structures are not late endocytic compartments. In contrast, the ring-shaped vesicles were labeled with the fluorescent compound Bodipy-TR ceramide, a lipid that preferentially accumulates in the Golgi, suggesting that these structures may originate from specialized regions of the Golgi apparatus, perhaps the TGN.

In order to address this possibility, we have used monensin, a ionophore that alters transport from the TGN to the plama membrane (Tartakoff and Vassalli, 1977; Tartakoff, 1983) and also blocks transferrin recycling in K562 cells (Stein et al., 1984). Monensin causes a marked distention of the Golgi membranes and also the formation of dilated MVBs in the perinuclear region of the cell (Stein et al., 1984).

Exosomes were labeled with the fluorescent lipid analog N-(lissamine rhodamine B sulfonyl) phosphatidyl ethanolamine (N-Rh-PE). This lipid is efficiently internalized via endocytosis but does not return to the cell surface. Sucrose gradient analysis and immunoisolation experiments have demonstrated that N-Rh-PE accumulates in exosomes that are eventually secreted into the extracellular medium (Vidal et al., 1997). Cells overexpressing GFP-Rab11Q70L were incubated with monensin and the lipid N-Rh-PE to label exosomes for 4 hours. As shown in Fig. 5A, the large multivesicular bodies were clearly decorated with GFP-Rab11 Q70L and contained numerous red dots that probably represent exosomes. Images of two typical cells are shown.

To confirm that the large MVBs originated, at least in part, from the Golgi apparatus, we have used an antibody generated against TGN46, a specific TGN marker for human cells. TGN46 was detected by indirect immunofluorescence and, as depicted in Fig. 5B, there was a marked colocalization of this protein with the ring-shaped structures decorated by GFP-Rab11Q70L. It is important to mention that for immunofluorescence studies, cells are fixed and fixation alters the size and shape of the GFP-labeled structures,

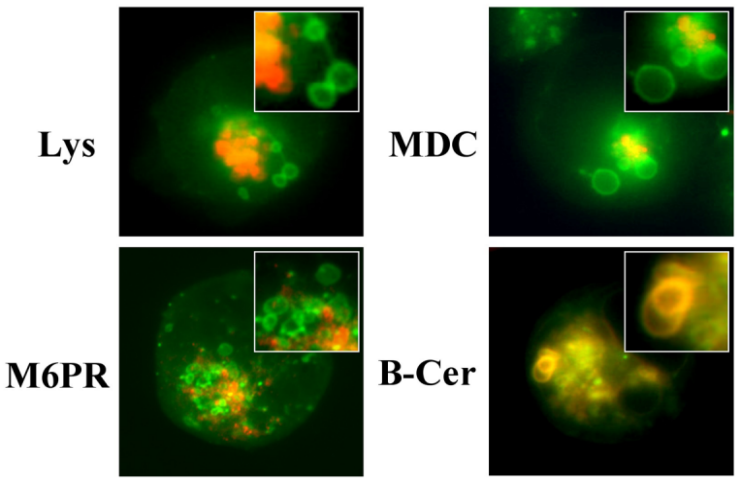

Fig. 4. The large ring-shaped vesicles observed in the GFP-Rab11Q70L-transfected cells are labeled with Bodipy-ceramide. Stably transfected cells overexpressing the mutant GFP-Rab11Q70L were labeled with Lysotracker (Lys) or monodansylcadaverine (MDC) as described in the Materials and Methods. Late endosomes were labeled using an antibody against the mannose-6-phosphate receptor (M6PR) and a Texas-Red-conjugated goat-anti rabbit secondary antibody. Labeling with Bodipy-TR ceramide (B-Cer) was performed as described in the Materials and Methods. Cells were mounted on coverslips and analyzed by fluorescence microscopy. Images were obtained as described in Fig. 3.

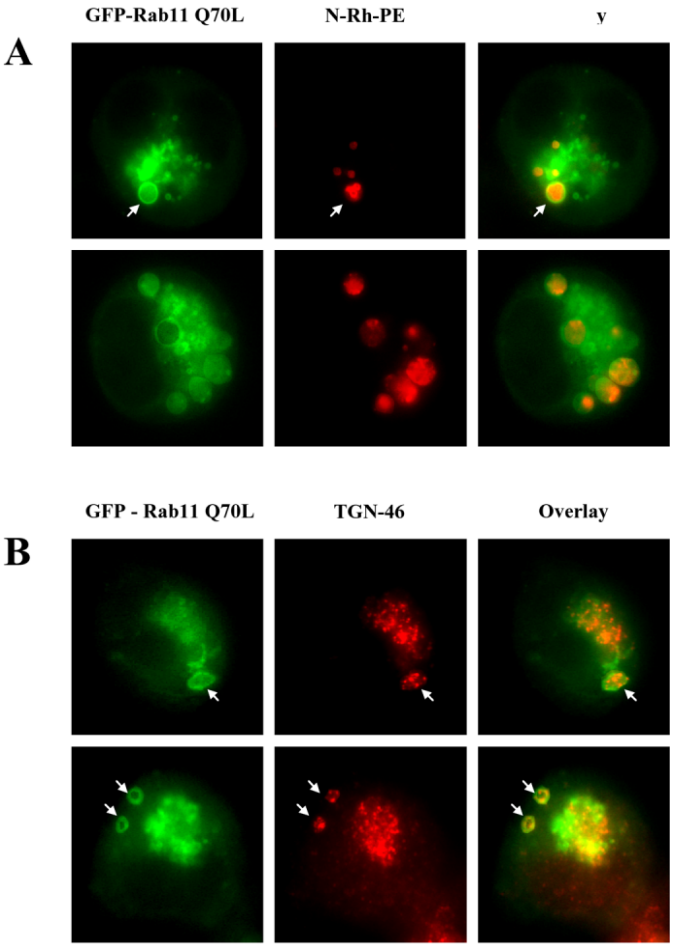

Fig. 5. Monensin causes the formation of large MVBs labeled with the trans-Golgi marker TGN46. (A) Stably transfected K562 cells overexpressing Rab11Q70L were incubated with monensin (1 μM) for 4 hours and with the fluorescent lipid analog N-(lissamine rhodamine B sulfonyl) phosphatidyl ethanolamine (N-Rh-PE) to label exosomes. The lipid was internalized via endocytosis. Cells were mounted on coverslips and immediately analyzed by fluorescence microscopy. Images were obtained as described in Fig. 3. Two sets of images show MVBs that are labeled with the lipid N-Rh-PE (depicted in red) and decorated with GFP-Rab11Q70L (green). (B) Cells overexpressing Rab11Q70L were incubated with monensin (1 μM) for 4 hours. Cells were mounted on coverslips, fixed and subjected to immunofluorescence with a rabbit antibody against TGN46 and a Texas-Red-conjugated goat-anti rabbit secondary antibody. Cells were analyzed by fluorescence microscopy. Two sets of images clearly show that TGN46 (red) colocalizes with the ring-shaped structures decorated by GFP-Rab11Q70L.

therefore they are not visualized as clearly as in nonfixed cells.

Taken together these results clearly indicate that the TGN contributes to the biogenesis of the MVBs involved in the formation and release of exosomes.

Transferrin recycling and cell surface delivery of Tf-receptors is impaired by overexpression of Rab11wt and mutants

The colocalization of Rab11 with rhodamine-transferrin in the recycling compartment suggests a role for Rab11 in directing the transport of Tf through this compartment in K562 cells, as described in other cell types (Ullrich et al., 1996; Ren et al., 1998). Therefore, we examined the function of Rab11 in the Tf pathway by studying the recycling of this protein and the delivery of TfRs to the cell surface. To study the kinetics of Tf recycling, K562 cells stably transfected with the vector alone or the chimeric Rab11wt and mutants were incubated with ^{125}I-labeled human Tf for 1 hour at 37°C and then transferred to fresh medium to measure the return of the labeled Tf to the medium. As shown in Fig. 6 in K562 cells transfected either with Rab11 wt (Fig. 6A) or with the mutants Rab11 Q70L and Rab11S25N (Fig. 6B,C) transferrin recycling was partly inhibited. In the case of the dominant-negative mutant although the effect was small it was consistently observed.

Transferrin (apotransferrin) recycles back to the cell surface bound to the TfR. Since recycling of transferrin was partly inhibited by overexpression of Rab11 constructs, it is possible that part of the TfRs remains trapped in internal compartments and is unable to reach the plasma membrane. In order to test this possibility we measured the number of TfRs on the cell surface. As shown in Fig. 7, the number of TfRs on the plasma membrane was markedly reduced in K562 cells overexpressing Rab11 wt or the mutants Rab11 S25N and Rab11 Q70L. In contrast, the total number of TfRs was not modified (data not shown). These results suggest that TfRs are probably trapped in internal compartments.

Overexpression of mutant Rab11S25N causes inhibition of the amount of exosomes released

Like reticulocytes, K562 cells release small vesicles termed exosomes into the extracellular medium after fusion of MVBs with the plasma membrane (Johnstone, 1996). Reticulocyte exosomes are enriched in proteins such as the TfR, Hsc70 and acetylcholinesterase (AChE) (Johnstone et al., 1989). They also contain the tyrosine kinase Lyn (C. Géminard and M.V., unpublished). Therefore, we have quantified the amount of exosome secretion by measuring the levels of TfR, Lyn and Hsc70 by western blot analysis. For this purpose, exosome fractions were harvested from the culture medium of Rab11-transfected K562 cells as described in the Materials and Methods. As shown in Fig. 8A, there was an increase in exosome release by K562 cells transfected with Rab11wt compared with cells transfected with the dominant-negative mutant of Rab11 (Rab11S25N), where the secretion of exosomes was inhibited. A slight inhibition was also observed with the mutant Q70L. The inhibitory effect caused by overexpression of Rab11S25N was observed with all the exosome markers used. The quantification by densitometry of several western blots is shown in Fig. 8B.

We also measured the activity of AChE in the harvested exosomes (see the Materials and Methods). Consistent with the results obtained by western blot, there was a decrease of exosome secretion in cells overexpressing the mutant Rab11S25N, whereas overexpression of Rab11wt stimulated the amount of exosomes released as determined by AChE activity (Fig. 8C). Similar results were obtained when the exosomes were labeled with the fluorescent lipid analog N-Rh-PE (see above). As shown in Fig. 8D, there was a marked decrease in the amount of exosomes collected from cells

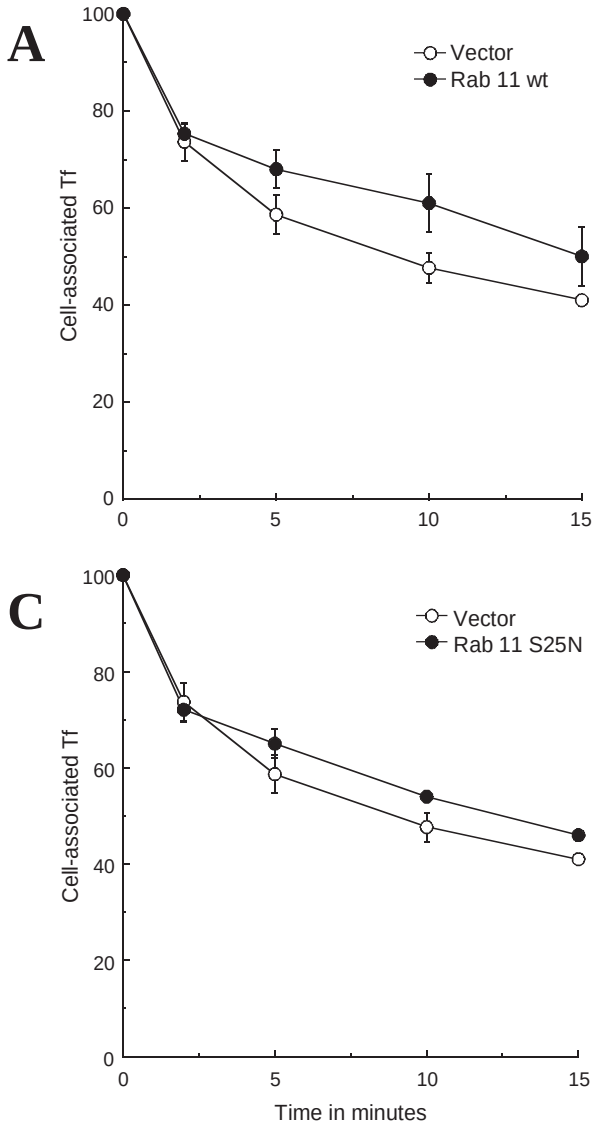

Fig. 6. Overexpression of GFP-Rab11wt and mutants inhibits transferrin recycling. Cells were first pre-incubated for 1 hour at 37°C in serum-free RPMI medium supplemented with 1% BSA to deplete endogenous transferrin (Tf) and then were allowed to internalize ^{125}I-labeled human Tf continuously for 1 hour at 37°C. Cells were washed at 4°C, and surface-bound transferrin was dissociated by acid treatment as described in the Materials and Methods. Cells were then further incubated at 37°C in serum-free RPMI medium containing 100 μM deferoxamine mesylate and 20 μg/ml unlabeled human Tf. At different times, the incubations were stopped by adding ice-cold PBS and placing the samples on ice. The samples were centrifuged to sediment the cells, and the medium was collected. The cell pellet was washed with the acetate buffer to remove surface-bound Tf. The radioactivity in both the medium and the cell pellet was determined by γ-counting. Data represent the mean±s.e.m. of three independent experiments.

transfected with Rab11 S25N, as determined by measuring the fluorescence of N-Rh-PE, whereas Rab 11wt increased exosome secretion.

The results indicate that overexpression of dominant-negative Rab11 mutant inhibits exosome release. As mentioned above, exosomes originate from inward budding of the endosomal compartment leading to the formation of MVBs, which by fusion with the plasma membrane induces the release of the included vesicles. Therefore, the inhibition in exosome secretion observed in the Rab11S25N transfected cells may have various causes, such as impairment of molecule sorting or inward membrane budding during MVBs formation, or it may involve a latter step such as fusion of the MVBs with the plasma membrane. In order to get insights into some of these possibilities, we have analyzed by electron microscopy cells transfected with either Rab11wt or the mutant Rab11S25N. The cells were incubated with transferrin-coated colloidal-gold particles (Tf-gold) to label the TfR present in the small inclusion vesicles. As shown in Fig. 9, MVBs labeled with Tf-gold are clearly observed in the K562 cells overexpressing Rab11wt (Fig. 9A,B). In cells overexpressing the mutant Rab11S25N, similar MVBs were observed (Fig. 9C). This suggests that overexpression of this mutant protein does not highly impede the formation of the MVBs. However, we can not discard the possibility that membrane inward budding or the biogenesis of certain membrane domains might be perturbed. It is likely that small changes in membrane-flow steady state, induced by transfection of the Rab11 constructs, are resulting in modification of exosome secretion.

Discussion

Concomitant with their maturation into erythrocytes, reticulocytes secrete membrane vesicles termed exosomes responsible for the clearance of obsolete proteins such as the TfR or integrin α4β1 (Rieu et al., 2000). More recently, other cells, mainly of hematopoietic origin, have been shown to secrete exosomes whose possible functions vary from adhesion and signaling to antigen presentation (for a review, see Denzer et al., 2000). The formation of MVBs leading to exosome secretion through their fusion with the plasma membrane is a common step in the process in all cell types. However the

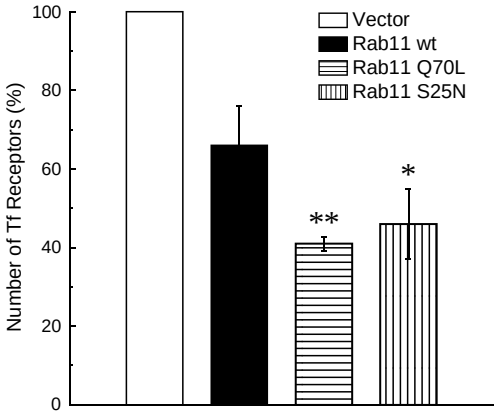

Fig. 7. Surface transferrin receptor measurements. Ligand binding studies were performed as described in the Materials and Methods. Briefly, control cells transfected with the vector alone (white bar) or with Rab11 wt (black bar) or mutants (hatched bars) were incubated with increasing concentrations of ^{125}I-transferrin for 90 minutes at 4°C. Cells were washed, and binding was determined by spinning the cells through dibutyl phthalate oil. Nonspecific binding was determined and subtracted for each point. Specific binding data were analyzed by the Scatchard method to determine the number of Tf-binding sites. The data represent the mean±s.e.m. of four independent experiments. Statistical analysis: *t*-test for single group mean. *Mean differs from control $P<0.05$; **mean differs from control $P<0.01$.

molecular mechanisms underlying this process are largely unknown. Of particular interest is the specific protein composition of exosomes released by different cell types that suggests budding/sorting of molecules from various intracellular compartments. For example, the TfR is a major protein component of reticulocyte exosomes and is present in vesicles secreted by erythroid cell lines such as K562 and HL-60 cells (Johnstone, 1996), although it is absent from the surface of vesicles secreted by antigen presenting cells (Raposo et al., 1996; Clayton et al., 2001). Another interesting point is the regulation of exosome secretion that is constitutive in some cells (reticulocytes, B lymphocytes), although it is triggered by specific stimuli in others (T cells, platelets, mast cells), suggesting a fine tuning in membrane traffic events. Essential components regulating the membrane transport such as Rab GTPases are thus likely to play a key role in these processes (Novick and Zerial, 1997; Schimmoller et al., 1998; Somsel and Wandinger-Ness, 2000). Tf recycling is regulated by Rab4, a Rab protein that colocalizes with Tf in early endocytic compartments (Daro et al., 1996; Sonnichsen et al., 2000; McCaffrey et al., 2001). In previous work, we have demonstrated an enrichment of Rab4 compared with Rab5 in reticulocyte exosomes (Vidal and Stahl, 1993). The presence of Rab4 in these vesicles supports the importance of Rab4 for TfR trafficking in reticulocytes. However, Rab4 was in the lumen of exosomes, indicating that this Rab protein was probably entrapped and shed from the reticulocyte via this pathway and, consequently, lost any regulatory function. Therefore, it raises the question of whether any other small GTP-binding protein of the Rab family is involved in the regulation of exosome release. This prompted us to study the possible relationship between the secretion of erythroid-cell-derived exosomes and Rab11, a Rab protein involved in Tf recycling from the pericentriolar recycling center to the plasma membrane (Ullrich et al., 1996; Ren et al., 1998; Sonnichsen et al., 2000).

In this report we used K562 cells, a human erythroleukemic cell line, to study the exosome pathway in relation to the expression of Rab11. Interestingly, K562 cells have been shown to possess high amounts of Rab11 compared with other Rab proteins, and Rab11 is associated with compartments involved in TfR recycling (Green et al., 1997). K562 cells stably transfected with GFP-Rab11wt and mutants support the involvement of Rab11 in TfR recycling. Consistent with previous observations (Ren et al., 1998), GFP-Rab11wt and GFP-Rab11Q70L presented a typical vesicular distribution in the perinuclear region, whereas the dominant-negative form GFP-Rab11S25N was diffusely distributed in the cytoplasm, although some perinuclear structures were also labeled. The distribution of the dominant-negative mutant is in accordance with the observation that this chimeric protein is enriched in the TGN (Chen et al., 1998). Our results indicate that transfection of K562 cells with either Rab11wt or mutants partly inhibited Tf recycling. This inhibition is concordant with data obtained from CHO cells (Ren et al., 1998) and BHK cells (Ullrich et al., 1996), although less pronounced. Consistent with this inhibitory effect, Scatchard analysis revealed a marked decrease in the TfR number on the surface of cells overexpressing the different forms of Rab11. This decrease could be caused by retention of TfR in intracellular compartments and/or by a loss of this receptor via exosomes. Indeed, K562 cells have been demonstrated to release exosomes that contain TfR (Ahn and Johnstone, 1993; Baynes et al., 1994). We recently found that Hsc70, AChE and Lyn are also associated with K562 exosomes, as well as vesicles released by reticulocytes (C. Géminard and M.V., unpublished). These four endogenous proteins were used as markers to evaluate exosome secretion by K562 cells transfected with the Rab11 alleles together with the fluorescent phospholipid analog (N-Rh-PE), which is incorporated into the surface of cells and is efficiently sorted after endocytosis and directed towards lysosomes (Willem et al., 1990) and exosomes (Vidal et al., 1997). The five markers indicated an inhibition of exosome secretion by K562 cells transfected with Rab11S25N. Conversely, we have observed an increase in vesicle release in Rab11wt overexpressing cells. Therefore it is likely that in the Rab11wt cells the decrease in the number of TfR on the cell surface is a consequence of re-routing TfR from the recycling pathway toward the exosome pathway. The results reported here suggest that exosome secretion could be at least one of the mechanisms involved in TfR downregulation from the cell surface. In contrast, in cells transfected with the dominant-negative mutant, exosome secretion was inhibited. Since exosomes originate from fusion of MVBs with the plasma membrane and subsequent release of the inclusion vesicles, the inhibition of exosome secretion observed in Rab11S25N transfected cells may be a consequence of a reduction in MVB formation or in fusion with the plasma membrane. The electron microscopy studies show that Tf-gold labeled MVBs are present in Rab11S25N-transfected cells, thus indicating that sorting of TfR and inward endosomal budding during MVBs formation are not completely blocked. However, we can not discard the possibility that the biogenesis of MVBs, including the formation of the internal vesicles, is somehow affected by

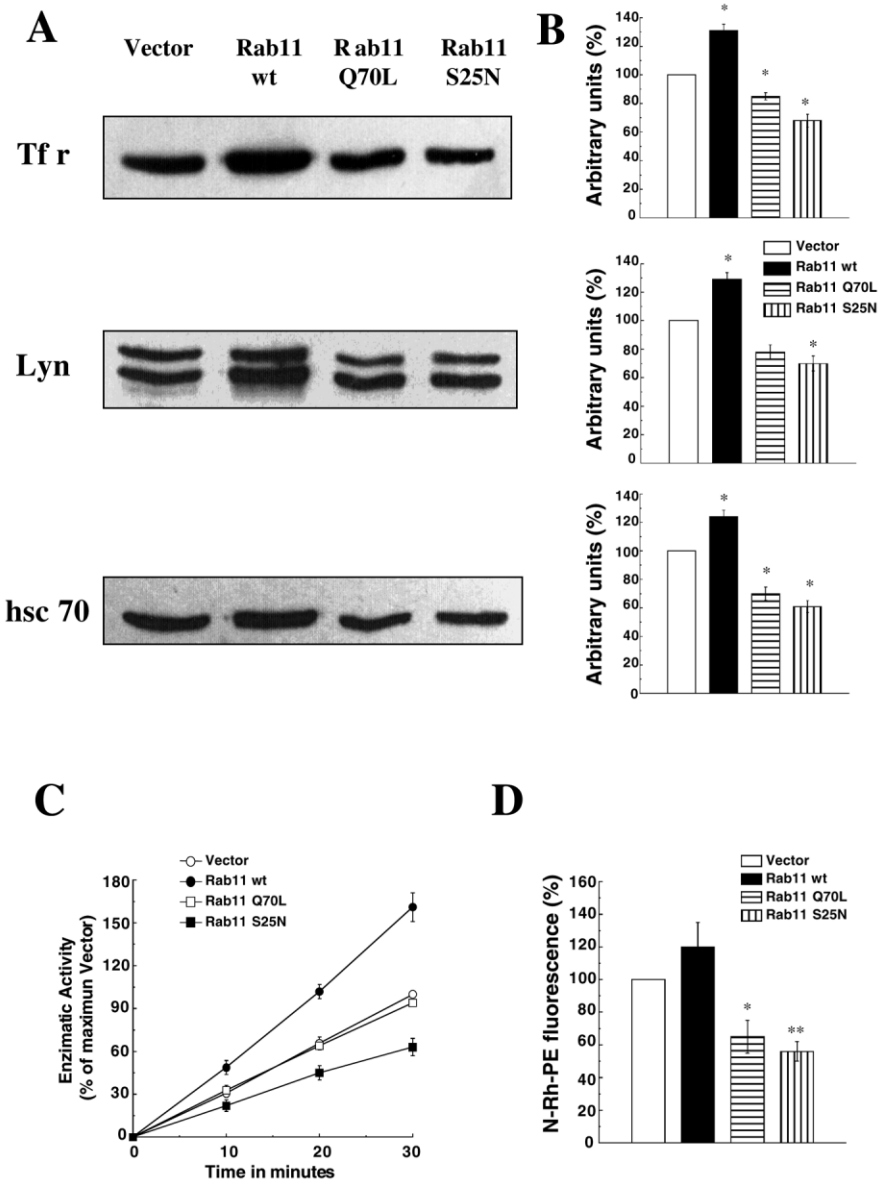

Fig. 8. Quantitation of exosome release by western blot and by measuring exosome-associated acetyl-cholinesterase and N-Rh-PE. (A) Samples of the exosomal fraction (see Materials and Methods) were solubilized in reducing SDS loading buffer, boiled and subjected to SDS-PAGE. The proteins were transferred to nitrocellulose membranes and detected with antibodies against the transferrin receptor (TfR), the protein Lyn and the heat shock protein Hsc70, respectively. The corresponding bands were detected using an enhanced chemiluminescence detection kit. A shows representative western blots of at least three independent experiments. Densitometric quantitation of the bands is depicted in B. Data are the mean±s.e.m. of three experiments expressed as a percentage of the control cells transfected with the vector alone. ∗P<0.05. (C) An aliquot of the exosome fraction (see Materials and Methods) was suspended in 100 μl phosphate buffer and incubated with 1.25 mM acetylthiocholine and 0.1 mM 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 for 30 minutes. Data were normalized as the percentage of the maximal activity measured at 30 minutes in the vector-transfected cells. Data represent the mean±s.e.m. of four independent experiments. (D) Cells were labeled for 60 minutes at 4°C with the fluorescent lipid analog N-(lissamine rhodamine B sulfonyl) phosphatidyl ethanolamine (N-Rh-PE) as described in the Materials and Methods. Cells were extensively washed with cold PBS to remove excess unbound lipid and were then cultured in complete RPMI medium to collect exosomes. A sample of the exosomal fraction was suspended in 1.5 ml PBS-TX100 (0.1%) and N-Rh-PE was measured by fluorometry. Data represent the mean±s.e.m. of three independent experiments expressed as a percentage of the control (vector transfected cells). Statistical analysis: t-test for single group mean. *Mean differs from control P<0.05; **mean differs from control P<0.01.

overexpression of this Rab11 mutant. Further experiments are necessary to determine the exact step at which Rab11 regulates exosome secretion. It is important to mention that in our system we are measuring the final product of the exosome pathway, that is the released vesicles. Overexpression of Rab11 and its mutants may disturb either the formation of these small vesicles or the process of release of exosomes during the fusion with the cell surface.

Interestingly, Rab11 has also been reported to be involved in vesicle transport from the TGN to the plasma membrane (Urbe et al., 1993; Chen et al., 1998) and from the recycling endosome to the TGN (Wilcke et al., 2000). Consistently, overexpressing GFP-Rab11Q70L in K562 cells induced the formation of large ring-shape structures that were negative for Tf but Golgi related, as demonstrated by colocalization studies with a fluorescent ceramide. Furthermore, we have shown that these structures are also labeled with the antibody TGN46, a TGN marker. Lastly, in the presence of monensin the large ring-shape structures generated in cells overexpressing GFP-Rab11Q70L accumulated the fluorescent lipid N-Rh-PE internalized via endocytosis. Therefore, our results strongly suggest that these Rab11-labeled structures originate from the TGN, but they also receive material from the endocytic pathway. It is noteworthy that a close relationship between the endosomal compartment and the Golgi has previously been demonstrated in K562 cells (Stein and Sussman, 1986). Two distinct pathways involved in TfR recycling were described, one of which was found to be monensin sensitive. Treatment of K562 cells with 10^{-5} M monensin induced a marked reduction in cell surface TfR that accumulated in MVBs. Similarly, we have observed that overexpression of Rab11 markedly reduced the TfR present at the plasma membrane. This led us to the conclusion that, as previously stated by Sussman and collaborators (Stein et al., 1984), a coordinated interaction between MVBs and the Golgi

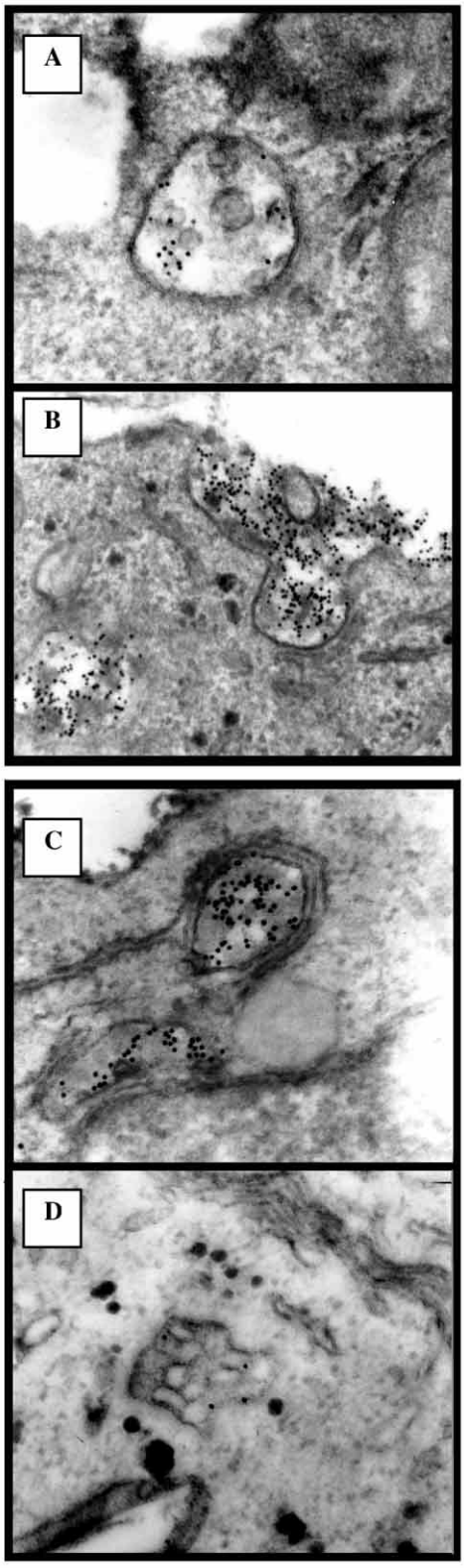

Fig. 9. Transferrin-coated colloidal gold is targeted to MVBs in K562 cells. Stably transfected K562 cells overexpressing GFP-Rab11 wt (A,B) or Rab11 S25N (C) were incubated with 10 nm Tf-coated colloidal gold particles (Tf-gold) for 45 minutes at 37°C and processed for transmission electron microscopy following standard techniques (see the Materials and Methods). (A) A typical multivesicular body (MVB) that contains vesicles labeled with Tf-gold is shown. A well defined membrane can often be seen surrounding these vesicles but since exosomes are small the large degree of curvature of this membrane might not be well cross-sectioned and consequently would not always be discernable. Magnification: 100,000×. (B) Two MVBs are shown, one of them has already fused with the plasma membrane, and Tf-receptor-containing exosomes are shed from the cell. Magnification: 100,000×. (C,D) Sections of a Rab11-S25N-transfected cell showing MVBs containing vesicles labeled with Tf-gold are depicted. Magnification: 110,000×.

apparatus is involved in the recycling of TfR in K562 cells. We have also observed by electron microscopy that in Rab11S25N the Golgi cisternae were markedly dilated (data not shown). Therefore, the evidence presented here suggest that this coordinated TfR traffic between MVBs and the trans-Golgi is probably modulated by Rab11.

It is thus tempting to speculate that the secretion of exosomes in K562 cells is caused by the fusion of these MVBs, described to be involved in TfR recycling, with the plasma membrane. This would be consistent with our results showing that the exosome pathway in these cells is regulated by Rab11 expression. However, the involvement of Rab11 in traffic between the recycling endosome and Golgi makes the picture more complex, and further experiments are necessary to unravel the precise mechanism by which Rab11 regulates exosome secretion. Thus, the hypothesis is that exosome release occurs in some cell types (e.g. erythroid cells) in which membrane outflow from the recycling endosome is used to secrete material. In circulating reticulocytes, this process would be a remnant process from the erythroid precursors. Moreover, it is also interesting to draw parallels with exosomes enriched with MHC class II molecules secreted by antigen presenting cells: (i) MHC class II molecules are major proteins in the function of antigen presenting cells as are TfR in the erythroid cell lineage, (ii) they are both ‘receptors’ transported from the endosomal compartment to the plasma membrane, (iii) for reasons that are as yet unknown, they both can be sorted in vesicles contained in MVBs, and finally (iv) these MVBs are connecting points between the endocytic and secretory pathways. Elucidation of variations around a common theme could be of great interest for the further development of exosomes as immunotherapeutic vehicles.

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