Regulation of exosome secretion by Rab35 and its GTPase-activating proteins TBC1D10A–C

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Oligodendrocytes secrete vesicles into the extracellular space, where they might play a role in neuron–glia communication. These exosomes are small vesicles with a diameter of 50–100 nm that are formed within multivesicular bodies and are released after fusion with the plasma membrane. The intracellular pathways that generate exosomes are poorly defined. Because Rab family guanosine triphosphatases (GTPases) together with their regulators are important membrane trafficking organizers, we investigated which Rab GTPase-activating proteins interfere with exosome release. We find that TBC1D10A–C regulate exosome secretion in a catalytic activity–dependent manner. We show that Rab35 is the target of TBC1D10A–C and that the inhibition of Rab35 function leads to intracellular accumulation of endosomal vesicles and impairs exosome secretion. Rab35 localizes to the surface of oligodendroglia in a GTP-dependent manner, where it increases the density of vesicles, suggesting a function in docking or tethering. These findings provide a basis for understanding the biogenesis and function of exosomes in the central nervous system.

Introduction

After endocytosis, proteins and lipids that are destined for lysosomal degradation are first incorporated into intraluminal vesicles of multivesicular bodies (MVBs) and are then delivered to lysosomes for degradation (Gruenberg and Stenmark, 2004; Piper and Katzmann, 2007). Alternatively, MVBs can directly fuse with the plasma membrane, leading to release of the intraluminal vesicles into the extracellular environment as exosomes, where they play an important role in processes such as protein turnover, intercellular signaling, transfer of mRNA, angiogenesis, and tumor spreading (Lakkaraju and Rodriguez-Boulan, 2008; Schorey and Bhatnagar, 2008; Buschow et al., 2009; Korkut et al., 2009; Simons and Raposo, 2009; Théry et al., 2009).

How proteins and lipids are sorted to these subsets of MVBs directed either for lysosomal degradation or for secretion as exosomes is presently unknown. Because the Rab family GTPase proteins show a characteristic subcellular distribution and represent an important determinant of organelle identity (Stenmark, 2009), we address herein the question of cargo separation by studying the role of Rab proteins in exosome release in oligodendroglial cells.

Although previous work has shown that the subsequent activity of Rab5 and -7 is required to transport cargo through the endosomal system to lysosomes to mediate its degradation (Stenmark, 2009), much less is known about Rabs required for delivery of exosomal cargo. To address this issue, we used Oli-neu cells, an oligodendroglial cell line that contains a large number of MVBs and secretes substantial amounts of exosomes as a model system. In these cells, the proteolipid protein (PLP), the major protein of myelin of the central nervous system, is localized to a large extent in MVBs, from where it can be transported back to the plasma membrane to be secreted in association with granules.
catalytic domain, the TBC (Tre/Bub2/Cdc16) domain, this approach leads to the selective inactivation of the different Rab proteins (Fuchs et al., 2007; Yoshimura et al., 2007). We coexpressed PLP-EGFP with EGFP fusion proteins of all 38 predicted Rab GAPs, confirmed their expression by Western blotting with anti-GFP antibodies in the cell lysates, and determined the amount of PLP-EGFP in the exosomal membrane fraction in three independent experiments. We did not focus on the GAPs that seemed to enhance exosome secretion because an increase of membrane in the extracellular medium could also be caused by more cell debris as a result of cell toxicity. The positive candidates that reproducibly reduced the release of PLP-GFP with exosomes were reevaluated by comparing the effects of the wild-type GAPs with that of the catalytically inactive point mutations. We found that TBC1D10B, RN-tre, TBC1D10A, TBC1D10C, and TBC1D15 reduced PLP-EGFP recovery from the exosomal membrane fraction in a catalytic activity–dependent manner (Fig. 1). RN-tre has been reported to act on Rab5 (Lanzetti et al., 2000) and Rab43 (Haas et al., 2005), TBC1D15 on Rab7 (Zhang et al., 2005), TBC1D10A on Rab27a (Itoh and Fukuda, 2006), and TBC1D10C on Rab35 (Patino-Lopez et al., 2008). We reexamined the effects of the TBC1D10 family toward specific Rab proteins by testing their ability to accelerate GTP hydrolysis in a biochemical assay with a wide set of Rabs (Fig. 2). These data clearly show that all three members of the TBC1D10 family have strong and Rab35-specific GAP activity. TBC1D10B, which reduced exosome secretion most potently, showed the strongest activity toward

with exosomes (Trajkovic et al., 2006, 2008; Krämer-Albers et al., 2007).

Results and discussion

We started our screen for Rab GTPases in exosome secretion by performing a proteome analysis of purified exosomes using liquid chromatography (LC) coupled to tandem mass spectrometry (MS; LC-MS/MS). A total of 301 proteins were identified, of which approximately one third have been previously found in exosomes from other cell types (Barile et al., 2005; Segura et al., 2005; Aoki et al., 2007; Valadi et al., 2007; Conde-Vancells et al., 2008), confirming the purity of the preparation. Among the identified proteins was a relatively large number of Rab GTPases (Rab1a, -1b, -2a, -5b, -5c, -6a, -7, -8b, -10, -11b, and -35), many of which have previously been implicated in endosomal membrane trafficking (Stenmark, 2009). To analyze the relative abundance of the Rab GTPases in exosomes, we expressed all of the identified Rabs as EGFP fusion proteins in Oli-neu cells and compared the levels with PLP. As compared with PLP-EGFP, Rab proteins were found at relatively low levels in exosomes, but among the most abundant was EGFP-Rab35 (Fig. S1 and Table S1).

To define the requirement of Rab proteins in exosome biogenesis, a Rab GTPase-activating protein (GAP) library was screened for the ability of each Rab GAP to reduce the secretion of PLP-EGFP in association with exosomes. Because Rab GAPs promote GTP hydrolysis of Rabs requiring a conserved catalytic domain, the TBC (Tre/Bub2/Cdc16) domain, this approach leads to the selective inactivation of the different Rab proteins (Fuchs et al., 2007; Yoshimura et al., 2007). We coexpressed PLP-EGFP with EGFP fusion proteins of all 38 predicted Rab GAPs, confirmed their expression by Western blotting with anti-GFP antibodies in the cell lysates, and determined the amount of PLP-EGFP in the exosomal membrane fraction in three independent experiments. We did not focus on the GAPs that seemed to enhance exosome secretion because an increase of membrane in the extracellular medium could also be caused by more cell debris as a result of cell toxicity. The positive candidates that reproducibly reduced the release of PLP-GFP with exosomes were reevaluated by comparing the effects of the wild-type GAPs with that of the catalytically inactive point mutations. We found that TBC1D10B, RN-tre, TBC1D10A, TBC1D10C, and TBC1D15 reduced PLP-EGFP recovery from the exosomal membrane fraction in a catalytic activity–dependent manner (Fig. 1). RN-tre has been reported to act on Rab5 (Lanzetti et al., 2000) and Rab43 (Haas et al., 2005), TBC1D15 on Rab7 (Zhang et al., 2005), TBC1D10A on Rab27a (Itoh and Fukuda, 2006), and TBC1D10C on Rab35 (Patino-Lopez et al., 2008). We reexamined the effects of the TBC1D10 family toward specific Rab proteins by testing their ability to accelerate GTP hydrolysis in a biochemical assay with a wide set of Rabs (Fig. 2). These data clearly show that all three members of the TBC1D10 family have strong and Rab35-specific GAP activity. TBC1D10B, which reduced exosome secretion most potently, showed the strongest activity toward
Rab35 (>2,000 pmol/h of hydrolyzed GTP; Fig. 2). We coexpressed the GTP-locked Rab35Q67A mutant, which is resistant to GAP activity, with TBC1D10B and observed an approximate twofold increase in exosome release of PLP (Fig. 3, A and B).

Because our results from the Rab GAP screen pointed to a possible role of Rab35 in the exosome pathway, we used the dominant-negative mutant of Rab35 (Rab35N120I) to interfere with its function and found that it significantly decreased release of PLP with exosomes (Fig. 3, C and F). We continued the analysis of the function of Rab35 by performing RNAi knockdown experiments. Knockdown of Rab35 by two rounds of siRNA nucleofection efficiently depleted Rab35 from the cell lysate and resulted in a significant reduction of PLP recovery from the exosomal membrane fraction (Fig. 3, D–F), confirming the findings with the dominant-negative mutant. Relatively little is known about the function of Rab35 except of its role in receptor recycling, cytokinesis, and actin reorganization (Kouranti et al., 2006; Patino-Lopez et al., 2008; Sato et al., 2008; Walseng et al., 2008; Zhang et al., 2009).

To analyze where Rab35 may act in oligodendroglial cells, we analyzed the localization of EGFP-tagged Rab35 by confocal microscopy. Wild-type Rab35, the GTP-locked Rab35Q67A, and its GAP, TBC1D10B, were detected at the plasma membrane, whereas the nucleotide-free Rab35N120I and the GDP-locked Rab35S22N were mainly found within the cytosol and in vesicles that contained PLP and Lamp-1, identifying them as late endosomes/lysosomes (Fig. 4, A and B). Similar findings were obtained when Rab35 and its mutant were expressed in HeLa cells, with the exception of more intracellular wild-type EGFP-Rab35 in vesicles containing PLP-myc (Fig. S2).

We observed an expansion of the cell surface area after expression of either wild-type Rab35 or constitutively active GTP-locked Rab35Q67A (Fig. 4 A).

To obtain further evidence for the plasma membrane localization of Rab35, we analyzed the expression of Rab35 in myelin, a plasma membrane extension of oligodendrocytes which is formed during the development of the central nervous system by the spiral wrapping of glial membrane around the axons (Sherman and Brophy, 2005; Simons and Trotter, 2007; Barres, 2008). Indeed, we detected Rab35 at relatively high levels in the lysates of purified myelin by Western blotting (Fig. 4 D). Purified myelin did not contain glial fibrillary acidic protein (GFAP) or synaptophysin proteins specific to astrocytes and neurons, confirming the purity of isolated myelin (Fig. 4 E). Although the majority of myelin consists of tightly stacked membrane bilayers with no space for MVBs, a fraction of myelin is composed of more loosely organized membrane that contains larger cytoplasmic channels (e.g., paranodal...
loops and abaxonal space; Salzer, 2003). We performed electron microscopy to analyze whether these structures contained MVBs. MVBs were frequently found in noncompacted regions of myelin (Fig. 4 F; Trapp et al., 1989), and immunoelectron microscopy revealed that MVBs contained PLP (Fig. 4 F). Because the Rab35 antibody did not work reliably in immunohistochemistry, we purified noncompacted myelin from crude myelin by sucrose gradient centrifugations to determine the localization of Rab35 in myelin. Rab35 was not found in the light fraction, which is enriched in compact myelin, but was recovered from the heavier fraction, which also contained contactin, a protein which localizes to the paranodal junction (Fig. 4 E; Rios et al., 2000).

To define the steps at which Rab35 might act, we compared the effects of the dominant-negative Rab7 and Rab35 mutants. We found that only the expression of Rab7T22N but not Rab35N120I or Rab35S22N resulted in an enlargement of PLP-containing organelles and increased the colocalization of PLP and Lamp-1 (Fig. 4, A and C; and Fig. S3). In addition, we only observed a reduction of EGF degradation after Rab7T22N but not after Rab35N120I expression (Fig. S3), suggesting that lysosomal degradation is not regulated by Rab35.

To determine how Rab35 functions, we performed RNAi experiments to deplete Rab35 and analyzed the number and size of the PLP-containing intracellular vesicles. Although vesicle size was unaffected, the total number of vesicles increased by \(\sim 35\%\) after Rab35 knockdown (Fig. 5, A–C). These vesicles were identified as late endosomes/lysosomes based on their colocalization with Lamp-1. The intracellular accumulation of PLP in Lamp-1–positive vesicles and the reduction in exosome secretion after Rab35 depletion suggests that a pathway from the endosomal system to the plasma membrane is regulated by Rab35.

To analyze the possibility that Rab35 controls vesicular movement, time-lapse experiments were performed using Lyso-Tracker as a probe to identify PLP/Lamp-1–containing endosomes (85.6 ± 2.7% of the LysoTracker-labeled vesicles contained PLP-EGFP; \(n = 1,522\) vesicles analyzed). The effects of the constitutive active and inactive mutants of Rab35 were compared in their ability to influence the mobility of endosomes. We used a recently developed algorithm to identify and track individual vesicles automatically in a video sequence (Westphal et al., 2008). Data from 47 videos from three independent experiments were pooled to provide a quantitative assessment of vesicle motility. Surprisingly, we found that active GTP-locked Rab35Q67A slightly reduced the motility of LysoTracker-labeled vesicles (by 14.6% ± 3.8% as compared with Rab35S22N; \(n = \sim 1,800\) vesicles; \(P < 0.001\), Welch’s
Figure 4. Localization of Rab35. (A) Oli-neu cells were transfected with PLP-myc together with EGFP-Rab35S22N, EGFP-Rab35N120I, EGFP-Rab35Q67A, or wild-type EGFP-Rab35 (WT) and analyzed by confocal microscopy. Wild-type and the GTP-locked Rab35Q67A were detected at the plasma membrane, whereas the GDP-locked Rab35S22N and the nucleotide-empty EGFP-Rab35N120I were mainly found within the cytosol and in vesicles that contained PLP and Lamp-1. (B) Oli-neu cells were cotransfected with EGFP-TBC1D10B and PLP-myc and analyzed by confocal microscopy. (C) Quantification of colocalization of PLP with Lamp-1 within 7 × 7-µm intracellular regions after coexpression with the different protein Rab35 mutants is shown (n = ~27–38). The values represent the mean ± SD. (D) Myelin (total) and myelin subfractions (light and heavy) were purified from the brain homogenates (brain) of adult mice, and the amounts of Rab35, PLP/DM20, and contactin were determined by Western blotting. Rab35 was detected in purified myelin (total) and in the subfraction of higher density (heavy). (E) Purified myelin did not contain relevant levels of GFAP or synaptophysin proteins, confirming the purity of isolated myelin. (F) Immunoelectron microscopy analysis of myelin in the spinal cord of adult mice with PLP antibodies. The left image shows an MVB within a cytoplasmic channel of compact myelin, and the right image shows an MVB in the abaxonal space. MVBs [boxed areas] are displayed at higher magnification in the insets. Bars: [A and B] 10 µm; [F] 200 nm.
Figure 5. Rab35 functions in recruitment of the endosomal vesicle to the plasma membrane. (A) Control (Ctl) or siRNA against Rab35 was delivered into cells by nucleofection, and the cells were imaged by immunofluorescence microscopy to detect PLP-myc. (B and C) Vesicle number and size are displayed in a histogram. Note that vesicle size was unaffected, whereas the number of vesicles increased after Rab35 knockdown (n = ~80 cells from three independent experiments). (D) To analyze vesicular movement, PLP-myc was cotransfected with wild-type or mutant EGFP-tagged Rab35, cells were stained with LysoTracker red DND-99, and time-lapse images were acquired at 1 frame every 2 s at 37°C. The motility of LysoTracker-labeled vesicles was slightly reduced when active GTP-locked Rab35Q67A was expressed (n = ~1,800 vesicles from three independent experiments). (E and F) The mean number of LysoTracker green DND-26–labeled vesicles in a field of 12.7 µm × 12.7 µm (unit area) was determined in the evanescent excitation field. Expression of the GTP-locked Rab35Q67A increased the number of vesicles in the TIRF evanesence field as compared with the GDP-locked Rab35S22N (n = 70 cells from three independent experiments; mean ± SD; ***, P < 0.001; Welch’s two-sample t test). (G) The mobility of LysoTracker green DND-26–labeled vesicles...
two-sample t test; Fig. 5 D). One possible explanation is that Rab35Q67A promotes vesicle docking/tethering. To test this possibility, cells were imaged with total internal reflection fluorescence (TIRF) microscopy to observe the vesicles just beneath the plasma membrane. Indeed, we found that that Rab35Q67A increased the number of LysoTracker-labeled vesicles within the evanescent excitation field (∼120 nm) by around 50% as compared with GDP-locked Rab35S22N (Fig. 5, E and F). To determine whether these vesicles were docked/tethered to the plasma membrane, we performed time-lapse TIRF experiments. To estimate vesicle motility, the temporal colocalization was determined by calculating the Pearson’s correlation coefficient of pairs of images separated by time (Huang et al., 2007). In such an analysis, the degree of temporal colocalization is inversely related to vesicle motility. Expression of Rab35Q67A increased vesicle immobilization as compared with the inactive form of Rab35 (Fig. 5 G).

Because exosome release is known to be stimulated by intracellular Ca2+ levels (Savina et al., 2003; Krämer-Albers et al., 2007), we determined vesicular fusion after Ca2+ stimulation by whole-cell capacitance measurements (Cm; Voets, 2000). Cells were held in whole-cell patch-clamp configuration and dialyzed through the pipette with the Ca2+-caged nitrophenyl (NP)-EGTA and a mixture of two Ca2+-sensitive fluorescent dyes, Fura-4 and -2FF, which allows us to precisely measure [Ca2+]i, over a large concentration range (Voets, 2000). 2–3 min after the whole-cell configuration, a strong UV flash was applied to the cells, causing the photolysis of NP-EGTA and leading to a step-like increase of [Ca2+]i. After flash photolysis, the size of RRP of was reduced in Rab35S22N as compared with wild-type Rab35-expressing cells (Rab35: 1.12 ± 0.12 pF, n = 15; Rab35S22N: 0.48 ± 0.10 pF, n = 11; P < 0.001; Fig. 5 I). Interestingly, a recent study shows that Rab27 not only functions in melanosome biogenesis but also in the exosome secretion pathway (Stinchcombe and Griffiths, 2007; Idone et al., 2008; Delevoye et al., 2009). Interestingly, work in C. elegans showed that the combined knockdown of Rab35 and -11 leads to a dramatic enhancement of intracellular accumulation of endosomal cargo (Sato et al., 2008). Thus, it is plausible that Rab35 and -11 play a dual role in endocytic recycling rather than acting in sequential stages. Further work will be required to define the precise functional contributions of Rab35 and -11 in exosome biogenesis. It will also be important to find out whether the machinery involved in exosome secretion is related to the one required for the biogenesis of secretory lysosomes or melanosomes (Stinchcombe and Griffiths, 2007; Idone et al., 2008; Delevoye et al., 2009). Together with this study, these findings provide a molecular basis for understanding MVB trafficking and exosome biogenesis. This is important as these vesicles have been implicated in various different cellular functions (e.g., intercellular signaling and transfer of mRNA) and disease states (e.g., prion disease and tumor spreading). Thus, strategies to interfere with their biogenesis such as blocking Rab35 function will be important to get a better understanding of their physiological and pathological relevance. The function of glial-derived exosomes is also unclear. The finding that MVBs are not only found in the soma and the processes of oligodendrocytes but also within the periaxonal space of myelin points to the interesting possibility that they transfer cargo into the axon. The identification and characterization of Rab35 effectors is likely to yield new insights into how exosomes are generated and where the release occurs.

Materials and methods

Antibodies, plasmids, and other reagents

The following primary antibodies were used: rat anti–Lamp-1 (CD107a; BD), rabbit anti-myoc (Millipore), mouse anti-HA (16B12; Covance), mouse anti-PLP (3F4; a gift from K.-A. Nave, Max Planck Institute for Experimental...
Exosomes purified from Oli-neu cell line were separated by a 4–12% Bis-Tris gradient NuPAGE gel (Invitrogen) and stained with Colloidal blue (Invitrogen). Each gel lane was cut into 25 slices, and proteins were in-gel digested with trypsin. Tryptic peptides were analyzed on an LTQ XL Orbitrap (Thermo Fisher Scientific) coupled to an LC system (1100 series; Agilent Technologies). Peptides were separated at a flow rate of 200 nl/min on a re-versed phase column (C18; ReproSil). Elution of the peptides was performed with a 50-min gradient from 7–40% mobile phase B (80% acetic acid). Mass accuracy was 5 ppm for both parent ion and fragment ions. The proteins found in our exosome preparation were compared using Mascot version 2.2 (Matrix Science, Inc.) as a search engine. Mass accuracy was 5 ppm for both parent ion and fragment ions. The peptides were constrained to be trypsinous with a maximum of two missed cleavages. Carbamidomethylation of cysteines was considered a fixed modification, whereas oxidation of methionine residues was considered a variable modification. The proteins found in our exosome preparation were compared with proteins identified previously in exosomes from other cell types (Barile et al., 2005; Segura et al., 2005; Aoki et al., 2007; Valadi et al., 2007; Conde-Vancells et al., 2008).

Cell culture, transfection, and RNAi

The oligodendroglial precursor cell line Oli-neu (provided by J. Trotter, National Institute of Molecular Cell Biology and Genetics, Dresden, Germany), was cultured as previously described (Trotter et al., 2006). The cells were seeded on poly-lysine–precoated plastic Petri dishes or glass coverslips and cultured at 37°C with 5% CO2 in SATO medium supplied with 5% horse serum. Transient transfections were performed using TransitLT1 transfection reagent (Mirus) according to the manufacturer’s instructions. siRNA was delivered into the cells by electrophoresis with a Basic Neuron Nucleofector kit (Lonza) in a two-step fashion as previously described (Trajkovic et al., 2006). For RNAi, we used the pooled oligonucleotides targeting four sequences of mouse Rab35 (D042604-01) and the pooled nontargeting oligonucleotides (D001810-10) as control from Thermo Fisher Scientific.

Exosome purification

Exosomes were prepared as described previously (Trajkovic et al., 2008). Before the preparation, the culture medium was replaced by serum-free medium, and media were then collected and centrifuged for 10 min at 3,000 g and 5°C for 10 min at 3,000 g before subjecting the supernatant to a centrifugation at 10,000 g for 30 min and at 100,000 g for 1 h. The pellets were resuspended in sample buffer (20% glycerol, 4 mM EDTA, 4% SDS, 4% 2-mercaptoethanol, and 100 mM Tris-HCl, pH 6.8). Cell lysates were incubated for 10 min on ice with lysis buffer (2% Nonidet P-40, 0.2% SDS, and 1 mM EDTA in PBS supplied with Complete protease inhibitor cocktail from Roche), scraped, and centrifuged at 100,000 g for 10 min. A fraction of the supernatant was mixed with sample buffer before subjecting it to 10 or 12% SDS-PAGE and transfer to nitrocellulose membranes. Western blots were revealed by enhanced chemiluminescence (Thermo Fisher Scientific), and bands were quantified using ImageJ software (National Institutes of Health).

Rab GAP assays

Rab GAP assays were performed on recombinant hexahistidine-FLAG-tagged human Rab GTases and hexahistidine-tagged TBC1D10A family proteins.
TILL Photonics), and a two-port epifluorescence condenser was used for coupling 80% of the flash light and 20% of the monochromator light into the microscope. Cells were bathed in extracellular solution (140 mM NaCl, 2.8 mM KCl, 4 mM CaCl2, 41 mM MgCl2, 10 mM Na-Hepes, and 2 mg/ml glucose, pH 7.2; osmolality ~310 mosM) and patched using a pipette solution containing the photocytosolic Ca2+ chelator Fluo-4 (100 mM Cr-glutamate, 20 mM Ca-Hepes, 2 mM MgATP, 8 mM NaCl, 0.3 mM Na-GTP, 10 mM NP-EGTA, 9 mM CoCl2, 400 µM Fura-4F [Invitrogen], pH 7.2; osmolality, ~300 mosM). Ca2+ measurements were performed by dual-wavelength ratiometric fluorometry as described previously (Voets, 2000) using a 1:1 mixture of the Ca2+-sensitive dyes Fura-4F (Kd = 1 µM) and Fluo-4 (Kd = 40 µM). The signal (ratio of fluorescence with excitation at 350 and 380 nm) was calibrated intracellularly by dialyzing the cells in whole-cell configuration with eight different pipette solutions with known [Ca2+]. Ca2+ ramps were elicited by the fluorescence excitation light, alternating between 350 and 380 nm, so that photolysis of NP-EGTA could be combined with simultaneous measurement of [Ca2+]. All measurements were performed at room temperature. Data analysis was performed using Axograph software (Axograph Scientific), and results were presented as mean ± SEM with the indicated number of experiments. Statistical significance was evaluated using the Mann-Whitney test. P < 0.05 was considered to be statistically significant.

Myelin preparation

The myelin preparation was performed essentially as described previously (Larocca and Norton, 2007). In brief, the brains from 3–6-mo-old mice were homogenized in PBS containing protease inhibitors by sonication. The brain homogenate was put on top of two-step sucrose gradients (0.32 and 0.62 M sucrose interfaces, respectively). The fractions were washed with H2O and myelin fractions were collected at the 0.32/0.62 M and 0.62/0.8 M interface. The myelin pellet was resuspended with preparation buffer, put on top of a discontinuous sucrose gradient, and the protocol was repeated to obtain purified myelin.

EGF degradation

The EGF degradation assay was performed as described previously (Trajkovic et al., 2008). Cells transfected with EGFP-tagged EGF receptor (EGFP-EGF) were incubated with 50 ng/ml Alexa Fluor 555-labeled EGF (Invitrogen) for 15 min, washed, and incubated for 4 h in conditioned culture medium to allow EGF degradation. For quantification, images of randomly selected transfected cells were recorded at fixed settings below pixel-value saturation, and fluorescence intensities were quantified using Metamorph Imaging Series 6.1 software (MDS Analytical Technologies).

Immunoelectron microscopy

Immunoelectron microscopy was performed as described previously (Werner et al., 2007). Mice were fixed by transcardial perfusion with 4% formaldehyde and 0.2% glutaraldehyde in 0.1 M phosphate buffer containing 0.5% NaCl. Dissected optic nerves were infiltrated in 2.3 M sucrose in 0.1 M phosphate buffer overnight, mounted onto aluminum pins for ultramicrotomy, and frozen in liquid nitrogen. Ultrathin cryosections were picked up according to Liu et al. (1996) in a 1:1 mixture of 2% methylcellulose and 2.3 M sucrose. For immunolabelling, sections were incubated with antibodies specific for PIP, which was detected with protein A–gold (10 nm). Sections were analyzed with a LEO EM912 Omega (Carl Zeiss, Germany), and digital micrographs were obtained with an on-axis 2,048 x 2,048-pixel charge-coupled device camera (Proscan).

Statistical analysis

The significance of data was evaluated with the statistical program R using Welch’s two-sample t test or one-sample t test.

Online supplemental material

Fig. S1 shows the analysis of Rab GTPases (identified in the MS analysis) in the exosomal membrane fraction. Fig. S2 shows the subcellular localization of Rab35 in HeLa cells. Fig. S3 shows the inhibition of EGF degradation by dominant-negative Rab7 but not Rab35. Table S1, included as an Excel file, shows the MS analysis of Oli-neu–derived exosomes. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200911018/DC1.

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Figure S1. **Analysis of Rabs in exosomes.** The Rabs identified in Oli-neu–derived exosomes by MS were expressed as EGFP fusion proteins in Oli-neu cells. 16 h after transfection, the cells were switched to serum-free medium, and the medium was collected after ~4 h of further incubation before submitting it to sequential centrifugation steps. The amount of the EGFP-Rab proteins was determined in the cell lysates (CL) and 100,000 g exosome pellets (P100) by Western blotting. PLP was used as reference. One representative experiment is shown.

Figure S2. **Localization of Rab35 in HeLa cells.** HeLa cells were transfected with PLP-myc together with EGFP-Rab35S22N, EGFP-Rab35N120I, or wild-type EGFP-Rab35 (WT) and analyzed by confocal microscopy. Bars, 10 µm.
Table S1, included as an Excel file, shows the MS analysis of Oli-neu–derived exosomes.

Figure S3. Inhibition of Rab7 but not Rab35 inhibits EGF degradation. (A) Oli-neu cells were cotransfected with EGFP-Rab7T22N or wild-type GFP-Rab7 (WT) and PLP-myc and analyzed by confocal microscopy. (B) Quantification of colocalization of PLP and Lamp-1 is shown (n = 25 and 22). The values represent the mean ± SD (**, 0.001 < P < 0.05; Welch’s two-sample t test). (C) Cells were transfected with EGFP-Rab7T22N or EGFP-Rab35N120I or the respective wild-type constructs together with EGF receptor–EGFP, incubated with rhodamine-labeled EGF for 15 min, washed, and incubated for 4 h in conditioned culture medium to allow EGF degradation. For quantification, images of randomly selected transfected cells were recorded, and fluorescence intensities were quantified. Values represent the mean ± SD (n = 54–63 cells; ***, P < 0.001; Welch’s two-sample t test). Bars, 10 µm.