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A role for Rab5 activity in the biogenesis of endosomal and lysosomal compartments

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Abstract

Rab5 is a small GTPase that plays roles in the homotypic fusion of early endosomes and regulation of intracellular vesicle transport. We show here that expression of GFP-tagged GTPase-deficient form of Rab5b (Rab5bQ79L) in NRK cells results in the sequential formation of three morphologically and functionally distinct types of endosomes. Expression of GFP-Rab5bQ79L initially caused a homotypic fusion of early endosomes accompanying a redistribution of the TGN-resident cargo molecules, and subsequent fusion with late endosomes/lysosomes, leading to the formation of giant hybrid organelles with features of early endosomes and late endosomes/lysosomes. Surprisingly, the giant endosomes gradually fragmented and shrunk, leading to the accumulation of early endosome clusters and concurrent reformation of late endosomes/lysosomes, a process accelerated by treatment with a phosphatidylinositol-3-kinase (PI(3)K) inhibitor, wortmannin. We postulate that such sequential processes reflect the biogenesis and maintenance of late endosomes/lysosomes, presumably via direct fusion with early endosomes and subsequent fission from hybrid organelles. Thus, our findings suggest a regulatory role for Rab5 in not only the early endocytic pathway, but also the late endocytic pathway, of membrane trafficking in coordination with PI(3)K activity.

Keywords: Rab5; Endosomes; Lysosomes; Ptdlns (3)-kinase; Wortmannin; Hybrid organelle; Lysosome biogenesis

Rab proteins constitute a subfamily of small GTPases that play important roles in the regulation of intracellular vesicle transport [1]. An intriguing early proposal was that different compartments in the exocytic and endocytic pathways contain distinct Rab GTPases on their surfaces [2]. Rab5 is the most thoroughly characterized member of the Rab GTPase family and is mainly localized to the early endosomal compartments [3]. It is now believed that Rab5 is essential for the homotypic fusion of early endosomes and is required for the endocytic pathways mediating the transport of clathrin-coated vesicles from the plasma membrane to the early endosomes [4,5].

There is less evidence to show that Rab5 affects the biogenesis of lysosomes. Lysosomes are dynamic organelles receiving materials from the biosynthetic, endocytic and autophagic pathways [6]. They are regarded as storage organelles for acid hydrolases and are capable for fusing with late endosomes to form hybrid organelles where the digestion of endocytosed macromolecules occurs. The reformation of lysosomes from the hybrid organelles involves a condensation of content and probably the removal of some membrane proteins by vesicular transport. Rosenfeld and colleagues [7] showed that lysosomal proteins were redistributed to endosomes during the expression of a GTPase-defective Rab5a (Rab5aQ79L) in macrophages. Little is known, however, about how the expression of Rab5aQ79L affects lysosome biogenesis. Therefore, in the present study we have investigated the involvement of Rab5 in the biogenesis and maintenance of late endosomes/lysosomes in more detail.

Materials and methods

Cell culture. Normal rat kidney (NRK), COS-1 and HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented

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with 10% (v/v) FBS (Gibco BRL, Grand Island, NY, USA), 2 mM glutamine and 1% penicillin–streptomycin in humidified 95% air and 5% CO_2 at 37 °C. The cells were plated onto 13 mm glass coverslips the day before infection.

DNA constructs. The cDNAs for human wild-type Rab5b and Rab5bQ79L inserted into the vector pCMV5-Flag [8] were subcloned in between the XhoI and HindIII sites of pEGFP-C1 (Clontech, Palo Alto, CA, USA).

Generation of adenoviruses. Adenoviruses encoding both Cre recombinase and GFP-Rab5bQ79L or GFP-Rab5b wild-type were generated as previously described [9]. We used Ψ5 as a donor to supply the viral backbone. The DNA sequence inserted between the Ψ site and the *loxP* site was incorporated into recombinant viruses in a Cre recombinase-catalyzed recombination between pAdlox and Ψ5. GFP-Rab5b was subcloned in between the KpnI and XbaI sites of pAdlox. GFP-Rab5bQ79L was subcloned in between the XbaI and EcoRI sites of pAdlox.

Adenoviral infection. NRK cells were infected with adenovirus for 6 h at 37 °C at a m.o.i. (multiplicity of infection) of 2×10^3 plaque-forming units/cell. After infection, the viral medium was removed, washed twice with medium, replaced with medium and chased for a given period.

Antibodies. Rabbit polyclonal antibodies to rat LGP85, rat MPR 300 and rat cathepsin D have been described [10–12]. Mouse monoclonal antibodies to EEA1, GM130, TGN38, syntaxin-6 and γ-adaptin were obtained from BD Transduction Laboratories (Lexington, KY, USA). Cy-3 or Cy-5-conjugated mouse monoclonal or rabbit polyclonal antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA, USA).

Immunofluorescence microscopy. Cells cultured on glass coverslips were fixed in 4% paraformaldehyde and processed for immunofluorescence microscopy as described previously [13]. Confocal images were acquired using a Zeiss confocal microscope (LSM 510 META) equipped with an argon/HeNe laser and a ZEISS 100×/1.4 Plan-Apochromat oil immersion lens. All images were created using Adobe Photoshop CS (Adobe Systems, San Jose, CA).

Texas red-dextran internalization. One milligram per milliliter of Texas red-dextran (M_r, 70,000 lysine fixable) (Molecular Probes, Eugen, OR, USA) was added directly to the medium for 1 h, then cells were washed and fixed or incubated in medium for an additional 6 h before washing and fixation.

Results and discussion

GFP-Rab5bQ79L causes the sequential formation of three types of endosome differing in size and morphology

Previous studies from our laboratory and others suggested that Rab5 participates in the biogenesis of late endosomes/lysosomes as well as early endosomes [7,14]. In order to clarify the involvement of Rab5 in more detail, we carried out a time-course analysis of the expression of GFP-Rab5bQ79L. We used here an adenoviral infection system to achieve efficient and reproducible expression of GFP-Rab5bQ79L in NRK cells. An infection time of 6 h was sufficient to detect significant amounts of GFP-Rab5bQ79L in endosomal compartments using confocal laser microscopy. With this system, GFP-Rab5bQ79L was expressed in more than 80% of cells. Immediately after the infection for 6 h, GFP-Rab5bQ79L was seen as numerous small punctate structures throughout the cytoplasm (Fig. 1A-e), which were apparently larger than those seen in cells infected wild-type GFP-Rab5b adenovirus (Fig. 1A-a). After 2 h of chase, the number of small dot-like structures observed after infection decreased and instead relatively large ring-like vacuoles ~2 μm in size began to appear (Fig. 1A-f). The GFP-Rab5bQ79L-positive vacuoles were of maximal size (an average diameter of ~5 μm) after 6 h of chase (Fig. 1A-g). Interestingly, after 12 h of chase some cells expressing GFP-Rab5bQ79L had an unique structure with 5–10 small ring-like vacuoles, resembling "a bunch of grapes" (Fig. 1A-h). Such structures were not seen in cells chased for up to 6 h. The suggestion that such morphological changes depend on the chase time rather than on the expression level of GFP-Rab5bQ79L was supported by Western blot analysis, which showed that there was little change in the expression levels of GFP-Rab5bQ79L during 12 h of chase (data not shown). Based on the size and morphology of vacuoles induced by the expression of GFP-Rab5bQ79L, we categorized these GFP-Rab5bQ79L-positive compartments into three types (Fig. 1B); type I: the small endosomes (<2 μm) observed primarily in cells chased for 0 or 2 h (Fig. 1A-e and f), type II: the giant endosomes (about 5 μm) seen in half of the cells expressing GFP-Rab5bQ79L chased for 6 h (Fig. 1A-g) or type III: the clustered endosomes (about 1–2 μm), the number of which increased significantly when cells were chased for 12 h (Fig. 1A-h). Thus morphological changes observed during a series of chase periods were quantitated and revealed that cells expressing the clustered endosomes increase in number time-dependently, whereas the number of cells expressing the giant endosomes declined as the chase time increased (Fig. 1C). These results imply that the Rab5bQ79L-positive endosomes have been gradually transformed from type I to type III endosomes via type II. The same results were obtained in COS-1 and HeLa cells (Fig. 1A-i–p, D and E). In addition to the results that the formation of giant and clustered vacuoles was never seen in cells expressing wild-type GFP-Rab5b (Supplementary Fig. 2), GFP alone nor GFP-tagged dominant negative Rab5b (Rab5bS34N) (data not shown), the formation of these morphologically distinct endosomes was also caused by a transient transfection of Rab5bQ79L tagged with FLAG instead of GFP in COS cells (Supplementary Fig. 1), indicating a specificity for the expression of Rab5bQ79L, but not secondary effects due to the adenoviral infection and GFP tagged to Rab5bQ79L.

Next, we examined the involvement of organelles within the central vacuolar system in the morphological changes to Rab5bQ79L-positive endosomal compartments. The steady-state distribution of several well-characterized organelle markers did not affect in cells infected wild-type GFP-Rab5b adenovirus (Supplementary Fig. 2). EEA1 contains Rab5- and FYVE finger-binding domains in the C-terminus, which are necessary and sufficient for the recruitment of EEA1 to early endosomes [15,16]. Thus, the binding of EEA1 to endosomal membranes is required for both PI(3)K activity and Rab5-GTP [15]. As expected, EEA1 colocalized exclusively with all three types of GFP-Rab5bQ79L-positive vesicles (Fig. 2A–D-a). MPR300, which is a receptor for soluble lysosomal enzymes, mainly localizes in the TGN and rapidly recycles between the TGN