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Spatial segregation of degradation- and recycling-trafficking pathways in COS-1 cells

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Abstract

After endocytosis, most membrane proteins and lipids return to the plasma membrane (recycling pathway), but some membrane components are delivered to lysosomes (degradation pathway). These two pathways diverge in early endosomes. The recycling pathway involves recycling endosomes and the degradation pathway incorporates late endosomes and lysosomes. In many cell lines, these organelles often are located in the perinuclear region where they visually intermix. The present study, by tracking specific ligands (epidermal growth factor and transferrin) and expression of Rab proteins (Rab5, Rab7, and Rab11), demonstrated that, in COS-1 cells, the two pathways were spatially segregated. Recycling endosomes were mostly confined within the ring-shaped structure of the Golgi complex (''the Golgi ring''), whereas late endosomes and lysosomes were excluded from inside the Golgi ring. Thus, the unique organization of endocytic organelles in COS-1 cells can be utilized to visualize endocytic trafficking pathways in detail.

Keywords: Endocytosis; Early endosomes; Late endosomes; Lysosomes; Recycling endosomes; EGF; Transferrin; Rab5; Rab7; Rab11

Correct sorting of proteins and lipids is essential for many cellular activities. A primary function of endocytic membrane trafficking is to sort internalized ligands and receptors to different organelles [1–3]. For example, epidermal growth factor (EGF), low-density lipoprotein (LDL), and soluble components are delivered to late endosomes (LEs), and eventually to lysosomes for degradation [4–7], whereas transferrin (Tfn), the transferrin and LDL receptors are recycled to the plasma membrane [8–10]. The degradation and recycling pathways diverge in early endosomes (EEs) [1,3,11–13]. In EEs, cargo bound for recycling starts to separate from that destined for degradation and either is directly recycled to the plasma membrane or is transported to perinuclear recycling endosomes (REs) [14]. Because of their function, EEs also are referred to as sorting endosomes. EEs, REs, and LEs can be distinguished from each other at the molecular level, as different Rab GTPase proteins are primarily distributed on different endocytic organelles (Rab5: EEs; Rab7: LEs; and Rab11: REs) [15–19].

Although many studies have focused on molecular mechanisms underlying the sorting system or on the identity of endocytic organelles, a fundamental question remains unanswered: how are these organelles and the two corresponding endocytic pathways spatially arranged and associated with each other?

A recent, intriguing observation is that EEs are not uniform, but are comprised of two distinct populations: a dynamic population that matures quickly towards LEs and a relatively static population that matures much more

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slowly [20]. Cargo destined for degradation, such as LDL and EGF, is preferentially targeted to the dynamic EEs, whereas recycling cargo, such as Tfn, is non-selectively transported to both populations. Dynamic EEs apparently are associated with microtubules, suggesting that endocytic organelles of the degradation pathway may be physically linked by microtubule tracks. The spatial and physical connections between static EEs and dynamic EEs, and between static EEs and REs, remain unclear. Cell lines that have endocytic organelles with well-defined organization would greatly facilitate study of the connections between endocytic organelles. HeLa cells are the most widely used cell type in the study of endocytic pathways, but they have intermixed appearance in the endocytic organelles.

The present study demonstrated that COS-1 cells (green monkey kidney cells) have a unique endocytic organelle organization. Recycling endosomes were mostly confined within the ring-shaped structure of the Golgi complex (referred to as ''the Golgi ring''), whereas late endosomes and lysosomes were excluded from the Golgi ring. Based on the localization of transiently-expressed Rab5 and on the endocytic fate of EGF and Tfn, EEs mapped exclusively around the Golgi ring. Interestingly, a substantial amount of early-endosomal autoantigen-1 (EEA1), an authentic early-endosomal marker [21], was localized within the Golgi ring, suggesting a possible role of EEA1 in trafficking from EEs to REs, in addition to its proposed function in early-endosomal fusion [22,23]. The results of this study demonstrate the advantages of using COS-1 cells to study endocytic pathways.

Materials and methods

Plasmid and reagents. The expression vector, pAcGFP1-Golgi of green fluorescent protein fused human β1,4-galactosyltransferase (GalT-GFP), was purchased from Takara Clontech (Mountain View, CA). Mouse Rab5a, Rab7, and Rab11a were cloned as previously described [24] and introduced into the GFP-tagged protein expression vector, pEGFP-C1 (Takara Clontech). The following reagents were purchased from the manufactures as noted: sheep anti-TGN46 antibody (Serotec Ltd., Oxford, UK); monoclonal antibodies against GM130, EEA1, adaptin γ, and adaptin δ (BD Bioscience, San Jose, CA); monoclonal anti-LAMP-2 antibody (DSHB, Iowa University, IA, USA); EGF-Alexa488, dextran-Alexa568, donkey anti-sheep IgG antibody-Alexa555, goat anti-mouse IgG antibody-Alexa405, goat anti-mouse IgG antibody-Alexa488, Alexa488, and Alexa546 carboxylic acid succinimidyl ester (Invitrogen, Carlsbad, CA); human holo-Tfn (Sigma, St. Louse, MO). Fluorescent-labeled proteins (Tfn-Alexa488 and Tfn-Alexa546) were prepared according to the manufacturer's instructions (Invitrogen).

Cell culture and transfection. COS-1 cells were obtained from the American Type Culture Collection. COS-1 cells were cultured at 37 °C under 5% CO_2 in Dulbecco's modified Eagle's medium (Sigma) containing 10% heat-inactivated fetal calf serum. Transfection was performed using FuGENE 6 Transfection Reagent (Roche, Basel, Switzerland) according to the manufacturer's instructions. For transfection, COS-1 cells were grown on μ-dishes (Ibidi, Martinsried, Germany). COS-1 cells stably expressing GalT-GFP were constructed by transfection with the pAcGFP1-Golgi vector and were selected with medium containing 100 μg/ml hygromycin B.

Internalization of fluorescent probes. COS-1 cells grown on 35 mm glass-bottom dishes (Matsunami Glass Ind. Ltd., Osaka, Japan) were routinely starved in serum-free media at 37 °C for 30 min before internalization of fluorescent probes. Cells then were pulsed for an appropriate period of time in serum-free media containing either 2.5 μg/ml EGF-Alexa488, Tfn-Alexa546, or dextran-Alexa568 chased with serum-free media.

Immunofluorescence. Cells were fixed with 4% paraformaldehyde in PBS at room temperature for 20 min. Cells then were permeabilized with Triton X-100 in PBS for 5 min, washed twice with PBS, and treated with blocking buffer (5% BSA in PBS) at room temperature for 30 min. Fixed cells were incubated with primary antibodies diluted in blocking buffer (1:400 for anti-TGN46 antibody: 1:100 for the others) at 37 °C for 30 min. After washing with PBS three times, cells were incubated with Alexa-conjugated secondary antibodies diluted in blocking buffer (1:400) at 37 °C for 30 min. Cells then were washed with PBS three times and mounted with Aqueous Mounting Medium PERMAFLUOR™ (Beckman Coulter, Fullerton, CA).

Confocal microscopy. Confocal microscopy was performed using a laser scanning microscope (model LSM 5 PASCAL; Carl Zeiss Micoimaging, Inc., Germany) with a 63 × 1.4 Plan-Apochromat oil immersion lens. Excitation was performed with a 30 mV argon laser emitting at 488 nm and with a 1.0 mW helium/neon laser emitting at 543 nm. Emissions were collected using a 505–530 nm band-pass filter for Alexa488 and GFP, and a LP560 filter for Alexa546, 555, and 568. Triple color imaging was performed with a LSM510 META (Carl Zeiss Micoimaging, Inc., Germany) equipped with a UV laser (Enterprise II, Coherent Inc., Santa Clara, CA). Confocal images were taken at the optical section that showed maximum intensity of the Golgi ring. Images were processed using Adobe Photoshop CS2 software (Adobe Systems, Inc., CA).

Results and discussion

Degradation pathway in COS-1 cells

First, the trafficking pathway of EGF, an authentic degradative ligand, was investigated in COS-1 cells. EGF-Alexa488 was endocytosed at 37 °C for 5 min and then chased with EGF-free media. The Golgi complex was visualized by immunostaining for TGN46 (*trans*-Golgi network marker). As shown in Fig. 1A, some EGF remained on the plasma membrane at the end of the pulse (0 min). At this time, internalized EGF was at the cell periphery and near the Golgi ring as dispersed punctate structures. The amount of EGF on the plasma membrane drastically diminished during the chase, with a concomitant increase in internalized EGF. It is noteworthy that EGF-positive structures began to cluster near the Golgi ring after a 10-min chase, but never entered the interior regions of the Golgi ring throughout the chase. After a 55-min chase, EGF and lysosomal-associated membrane protein-2 (LAMP-2) co-localized consistently (data not shown), indicating that organelles belonging to the degradation pathway (EEs, LEs, and lysosomes) were excluded from inside the Golgi ring.

The fate of soluble components, which are delivered to lysosomes and not to REs, also was examined [3]. In this experiment, the Golgi complex was visualized by expression of galactosyltransferase-GFP (medial-*trans*-Golgi marker) [25]. As shown in Fig. 1B, internalized dextran-Alexa568 was mostly excluded from inside the Golgi ring throughout the uptake experiment, further supporting exclusion of degradative organelles from the Golgi ring.