Molecular Sorting in Early Endosomes

Tatsuya SAKAI

Department of Microbiology, Kawasaki Medical School, Kurashiki 701-0192, Japan

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ABSTRACT. The early endosome is an intracellular membrane organelle that specializes in the sorting and recycling of endocytosed macromolecules. To elucidate the molecular sorting reaction, early endosomes in living human epidermal carcinoma A431 cells were stained with fluorescently-labeled transferrin and individual endosomes were continuously observed under a real-time confocal microscope. Early endosomes were observed as organelles with various different shapes, such as vesicles, tubules and complexes of these. However, the accumulation of sorted molecules and fission of the endosomal membrane, which are essential for molecular sorting, were predominantly observed in tubular endosomes. These observations indicate that tubular endosomes are key elements for the molecular sorting reaction.

Key words : endosome — molecular sorting — transferrin

Receptor-mediated endocytosis is a type of cellular eating process and is involved in various biological reactions, such as cholesterol removal, immune responses and virus infection, etc.[1,2] Various kinds of macromolecules in the extracellular fluid become attached to their specific receptors on the cell surface membrane and are then internalized into the cell through clathrin-coated pits. Endocytosed molecules are sorted from each other and delivered to different destinations. For example, low density lipoprotein (LDL) and insulin are delivered to lysosomes, whereas LDL receptors, transferrin and transferrin receptors are returned to the plasma membrane and then reused. The early endosome is an organelle that specializes in the molecular sorting and recycling.[3,4] In the process of molecular sorting, molecules to be delivered to the same destination are accumulated with each other, and then segregated from other molecules to be delivered to different destinations by membrane fission. However, the mechanisms of the molecular accumulation and membrane fission remain unclear. In electron microscopic studies, early endosomes have been observed as organelles of various shapes and sizes such as vesicles (diameter : 0.1-1.0 μm), tubules (length: 2-10 μm) and tubulovesicular structures (a vesicle with tubular arms).[5-8] Regarding the molecular sorting in early endosomes, two hypotheses have been proposed. One is that the sorting occurs in tubulovesicular endosomes through division of a tubular structure from a vesicular body.[4,5] The other is that the sorting is mediated by small bud

堺 立也
e-mail : sakai@med.kawasaki-m.ac.jp

structures in the tubular endosomes.[9]

Here, in order to address the sorting process, individual early endosomes were continuously observed in living human carcinoma A431 cells under a real-time laser scanning confocal microscope (LSCM), and the endosome motion was categorized to identify the molecular accumulation and membrane fission events responsible for molecular sorting.

MATERIALS AND METHODS

Cell Culture

A431 cells were grown in DMEM containing 10% fetal calf serum at 37°C under 5% CO_2, and transferred every 3 days. Two days before use, the cells were plated onto polylysine-coated glass coverslips.

Observation of the Static Features of Endosomes

Cells were incubated with 5 μg/ml tetramethylrhodamine-labeled transferrin (TRITC-transferrin) in DMEM at 37°C for 10 min, and then washed three times with 10 mM HEPES-buffered Hanks' balanced salt solution (HEPES-HBSS), pH 7.3. The receptor-specificity of TRITC-transferrin internalization under these loading conditions was tested by competitive inhibition experiments in which excess (1 mg/ml) non-labeled transferrin was added simultaneously with the tracer during the loading periods.

A glass coverslip with living cells was mounted on a metal block observation chamber[10] filled with HEPES-HBSS. Fluorescence microscopic images of endosomes were obtained at room temperature using an LSCM (LSM410 ; Zeiss) with a x63 oil plan-apochromat lens (N.A. 1.4 ; Zeiss). Optical sections were obtained every 0.3 μm along the z-axis as digital images, from which projection images were reconstructed for presentation.

Observation of the Dynamic Features of Endosomes

Cells were incubated with 5 μg/ml Texas Red-labeled transferrin (TxR-transferrin) in DMEM at 37°C for 2 h, and then washed three times with 10 mM HEPES-HBSS, pH 7.3. Prolonged incubation was required to completely occupy the transferrin receptors on the cells with the tracer. TxR-transferrin was chosen for the continuous observations, since TxR is more resistant to photo-bleaching than TRITC. The receptor-specificity of TxR-transferrin internalization under these loading conditions was tested by competitive inhibition experiments as described above.

Endosomes were observed using an inverted microscope (IMT2 ; Olympus) equipped with a real-time laser confocal scanning unit (InSight plus ; Meridian) and a x100 oil plan-neofluor lens (N.A. 1.3 ; Zeiss). Laser irradiation affected the endosome motility, and in particular, an intense laser beam ceased the saltatory movement of endosomes within a few minutes. Therefore, the laser power was reduced to avoid any photodamage of the saltatory movement for up to 10 min, and all data recordings were terminated within 10 min.

A glass coverslip with living cells was mounted on the observation chamber as mentioned above. The chamber was set onto the metal stage-

holder and its temperature was maintained at 37°C by water circulation.[10] The stage of the microscope was covered with a plastic box and maintained at 37°C by air circulation. Fluorescence real-time images of endosomes were captured for 10 min with a SIT camera (C-2400-08; Hamamatsu), enhanced with an image processor (Argus 10; Hamamatsu) and recorded using a video tape recorder (BR-S810; JVC). The video images were digitized using the PIAS3 image editing system (PIAS). Digital images were analyzed using the public domain NIH Image program (written by W. Rasband, and available to Internet users from zippy. nimh. nih. gov).

RESULTS

Static Features of Early Endosomes in Living Cells

To visualize early endosomes in living cells, fluorescently-labeled transferrin was applied to the cells. After endocytosis, transferrin molecules remain bound to transferrin-receptors in early endosomes until they return to the cell surface.[1] Consequently, fluorescently-labeled transferrin, such as TRITC-transferrin or TxR-transferrin, is a potent marker of early endosomes.

A431 cells were incubated with TRITC-transferrin at 37°C for 10 min. After the incubation, early endosomes were seen as abundant fluorescent structures distributed throughout the entire cytoplasm (Fig 1a). Early endosomes consisted of various structures such as tubules, vesicles and complexes of these (Fig 1b). The tubular structures were 2-10 μm long and the vesicular structures had a diameter of 0.2-1 μm (Fig 1b). In many

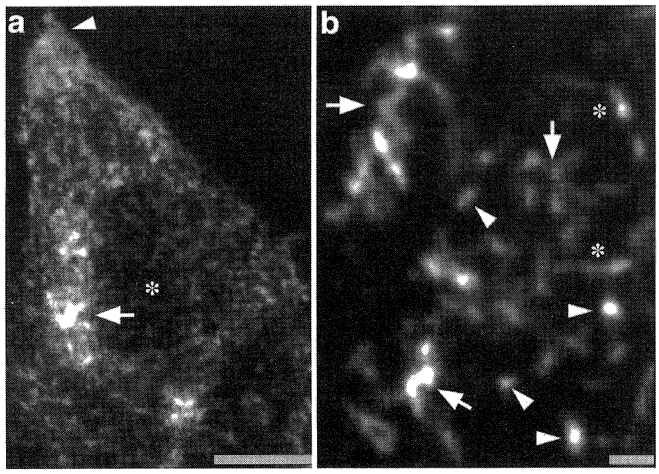

Fig 1. a) Early endosome distribution in a living cell. Early endosomes were stained by incubating A431 cells with TRITC-transferrin for 10 min at 37°C. Numerous fluorescent structures are evident throughout the cytoplasm, and are relatively abundant in the cytocenter (arrow) and at the cell edge (arrowhead). The position of the cell nucleus is marked with an asterisk. Bar=10 μm.
b) High-magnification image of early endosomes. Tubular endosomes (arrows) are 2-10 μm long. Vesicular endosomes (arrowhead) with a diameter of 0.2-1 μm are also present. Tubular endosomes usually have 2-6 globular domains. Some tubules that have one larger globule (*) appear to be tubulovesicular endosomes. Bar=1 μm.

cases, endosomal elements were observed as complexes of the tubules and vesicles. These structures were usually observed as tubules with 2-6 small globular domains (Fig 1b). Some tubules had one large ($\geqq$0.5 μm) globular structure (Fig 1b) and appeared similar to the previously reported tubulovesicular endosomes.[5,6] Although early endosomes were seen throughout the cytoplasm, they were relatively abundant near the cell margins and nuclei, where the tubules appeared to form networks (Fig 1a). These morphologies are identical to those of the early endosomes previously observed in HEp2 cells.[11]

Dynamic Features of Early Endosomes in Living Cells

To explore the membrane dynamics of individual early endosomes, endosomes containing TxR-transferrin were observed continuously in living cells using a real-time LSCM. Cells were incubated with TxR-transferrin at 37°C for 2 h. After the prolonged incubation, the typical structures of early endosomes were more clearly stained than after the 10-min incubation described above. In addition to these early endosome structures, however, very long tubular organelles that extended radially from the cytocenter were also stained (please refer to the legend for Fig 4). These long tubules have been reported previously and were demonstrated to be functionally and morphologically different from early endosomes.[11,12] Hereafter, only the typically shaped early endosomes were focused on.

A. Accumulation of Transferrin in Tubular Endosomes

Accumulation of sorted molecules in early endosomes is crucial for molecular sorting. To detect the accumulation of TxR-transferrin molecules, the distribution of TxR fluorescence in individual endosomes was monitored continuously. As a result, transferrin accumulation was observed in tubular endosomes. As shown in Fig 2, tubular endosomes migrated, and repeatedly retracted and elongated. During these motions, the fluorescence distribution in the tubular endosomes altered temporally. Bright domains

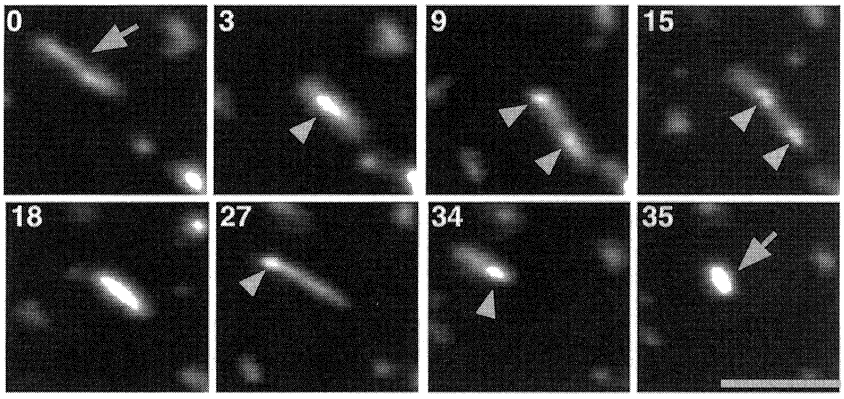

Fig 2. Transferrin accumulation in a moving endosome. Early endosomes were stained by incubating A431 cells with TxR-transferrin for 2 h at 37°C. The tubular endosome (arrow) repeatedly elongates and retracts. During the elongation and retraction, brighter domains (arrowhead) sometimes appear in this element. The numbers in the upper left corners show the time in seconds. Bar=5 μm.

transiently formed in the tubules, and some of these resembled the small globular domains of tubular endosomes seen in Fig 1b. Since the fluorescence intensity reflects the amount of TxR-transferrin, the formation of bright domains indicates transferrin accumulation. As shown in Fig 2, the accumulation seemed to occur independently of time or position in the endosomes. Moreover, many of the bright domains disappeared within several seconds.

If the random and transient accumulation of transferrin observed in tubular endosomes is involved in molecular sorting, the domains of accumulation should be segregated from tubular endosomes by membrane fission. Thus, the membrane fission sites in early endosomes were subsequently investigated.

B. Fission Events in Tubular Endosomes

Membrane fission is essential for completion of the molecular sorting. The real-time observation revealed two kinds of membrane fission that occurred in early endosomes: one was budding of small vesicles from tubular endosomes, and the other was division of tubular, but not tubulovesicular, endosomes.

As shown in Fig 3, small globular domains appeared and disappeared in tubular endosomes. Some of these were pinched off from the tubule. However, not all of the bright domains were pinched off and many of them disappeared before budding.

Molecular sorting in early endosomes has been thought to occur in tubulovesicular endosomes through membrane division between the tubular structure and the vesicular body.[4,5] Such binary division between a tubular

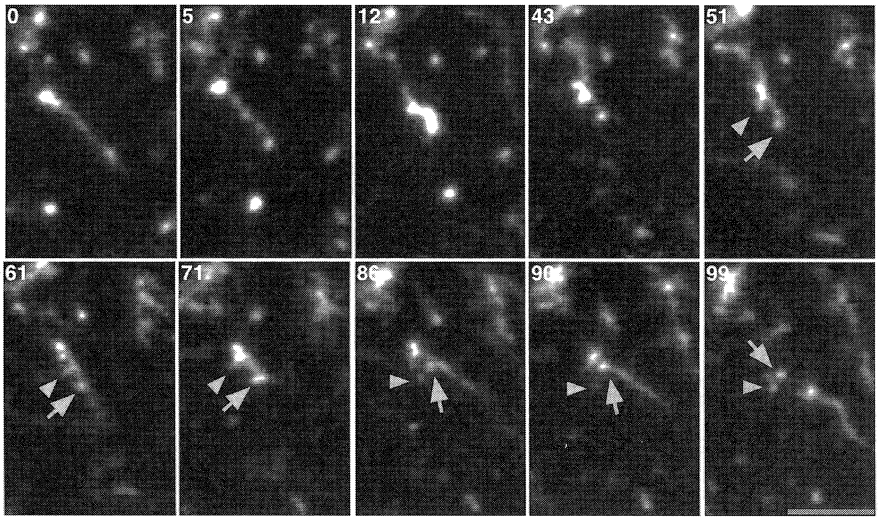

Fig 3. Formation and fission of globular domains in a tubular endosome. While the tubular endosome repeatedly elongates and retracts during migration, several globular structures are formed in the tubules (from 0 to 5 sec) and two of these (arrow and arrowhead) are released from the side of the tubule (from 61 to 99 sec). The tubular endosome then leaves these released vesicles behind. The numbers in the upper left corners represent the time in seconds. Bar=5 μm.

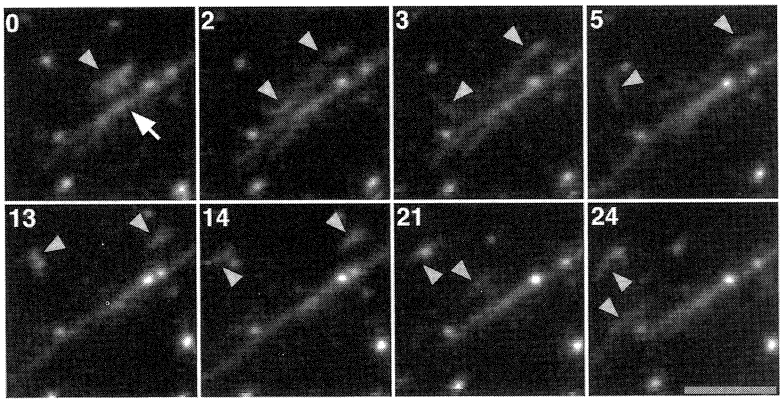

Fig 4. Binary fission of a tubular endosome. The tubular endosome (arrowhead) suddenly elongates and divides into two short tubules (0-3 sec). The divided tubules (arrowheads) then move independently of each other. Part of a long tubular organelle (arrow) can also be seen. This organelle is functionally and morphologically different from the early endosomes. In contrast with the early endosomes, the long tubule is immobile. The numbers in the upper left corners represent the time in seconds. Bar=5 μm.

structure and a large vesicular structure was not observed in this study. However, another kind of binary division was observed in tubular endosomes. As shown in Fig 4, both terminals of the tubule suddenly started to move in opposite directions and the tubule was elongated three-fold. This motion eventually caused the division of the endosome. However, this type of fission was rarely observed compared with the budding of small vesicles.

DISCUSSION

Based on these observations, the dynamics of early endosomes are summarized in Fig 5. Transferrin molecules are accumulated in small domains of tubular endosomes and segregated by budding of these domains. As reported in previous electron microscopic studies, recycling molecules such as transferrin were supposed to be accumulated in the tubular structures of tubulovesicular endosomes and segregated by division of the tubular structures from the vesicular body.[4,5,13] However, small bud structures (diameter, ~60 nm) were recently found on the tubular elements of early endosomes.[9] Since the small buds were coated with clathrin, they were proposed to mediate molecular accumulation and membrane fission. Due to the resolution limit of optical microscopes, the exact size and shape of the small globular domains observed in this study (Fig 1b and 3) remain undetermined. However, the small globular domains resemble the small buds reported previously in other morphological characters, such as the number and distribution in tubules. From the viewpoint of membrane dynamics, I can confirm that the small bud structures mediate molecular sorting in early endosomes.

Early endosomes are flexible and elastic organelles, as shown in Fig 2, 3 and 4. This nature should cause the polymorphic features of early

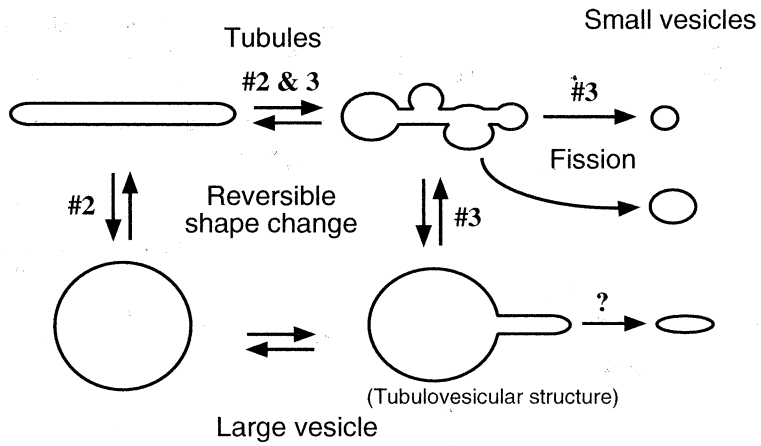

Fig 5. Schema of early endosome dynamics. Early endosomes consist of vesicular and tubular elements. Relatively large vesicular elements contain tubulovesicular endosomes. Tubular endosomes retract and elongate, and are sometimes converted into large vesicular structures by extreme retraction. In tubular endosomes, recycling molecules such as transferrin are transiently accumulated in small domains resembling small bud structures. The small domains are sometimes released from the tubules by membrane fission and form small vesicles. #X (X=2-4) indicates that identical or similar processes can be seen in Fig X.

endosomes. In earlier morphological studies, early endosomes were observed as vesicular, tubular and tubulovesicular structures,[5-8] and endosomes with different morphologies were assumed to have different functions. For example, tubulovesicular endosomes were thought to specialize in the sorting of recycled and lysosome-directed molecules, and large vesicular (diameter, 0.5-1.0 μm) and tubular endosomes were thought to transport molecules to lysosomes and the cell surface, respectively.[5,14-16] However, as shown in Fig 2 and 3, early endosomes in living cells frequently change their shape between tubular, vesicular and tubulovesicular elements. Thus, I conclude that flexibility and elasticity also contribute to the morphological variety of early endosomes.

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