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Two Distinct Kinds of Tubular Organelles Involved in the Rapid Recycling and Slow Processing of Endocytosed Transferrin

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The tubular structures of endosomes are thought to mediate the sorting and recycling of endocytosed macromolecules. These structures have been reported to show considerable morphological variety. However, it is not clear whether they are functionally identical. To address this question, we applied quantitative imaging analysis to characterize tubular organelles loaded with a recycling protein marker, fluorescent transferrin, in living human carcinoma HEp2 cells, using laser scanning confocal microscopy. High-resolution images of the cells demonstrated two types of tubular structures with a distinct morphology and showing a time dependency in their appearance: the fine tubular element and the extensive tubular element. Fine tubular elements 2 - 10 μm long were distributed throughout the cytoplasm after 10 min of loading with the tracer. Extensive tubular elements 5 - 20 μm long radiated from the cytocenter after 2 h of loading, but not after 10 min. Time-lapse imaging analysis demonstrated that the half-life of transferrin in the fine and extensive tubular elements was 12 min and ~50 min, respectively, at 33°C. Double labeling experiments using fluorescent transferrin and epidermal growth factor indicated that the extensive tubular element was neither a late endosome nor a lysosome. From these results, we conclude that the fine tubular and extensive tubular elements are distinct organelles: the former comprising the sorting endosome and recycling compartment which mediate the rapid recycling of transferrin, and the latter being part of a novel pathway of slower transferrin processing.

Endocytosis is a cellular membrane process by which various macromolecules are taken in from the cell surface and delivered to their intracellular destinations. The endosome has a central role in the sorting and delivery of endocytosed molecules (1–3). Among the various morphologies assumed by this organelle, the tubular structures are thought to mediate molecular sorting and recycling. Geuze et al. identified the compartment of uncoupling of receptors and ligands (CURL) which has a tubulovesicular shape and contains many recycling receptors in the endosomal tubular portions, allowing them to be segregated from lysosome-directed ligands in the globular portions (4).

The morphologies of the tubular structures of endosomes have been examined in several previous EM studies (4–9), which showed the tubules to be of various lengths ranging from the submicrometer to micrometer order. Using video-fluorescence microscopy, Hopkins et al. demonstrated a longer tubular endosome which formed an extensive network (10). These previous observations raise the question of whether all these tubular structures belong to the same kind of endosome.

The endosomes have been thought to comprise an early and a late endosome, only the former being considered to play a role in molecular sorting and recycling (11). The early endosome was further classified into two kinds of organelle, the sorting endosome and the recycling compartment (12–14), which were recently characterized in terms of their kinetic properties in molecular trafficking through quantitative analysis by laser confocal microscopy (LSCM) (15, 16). The sorting endosome and recycling compartment have been shown to have tubulovesicular and tubular morphologies, respectively (12–14). However, the question of whether all the tubular structures reported in earlier studies can be categorized into the sorting endosome and recycling compartment with a tubular appearance still remains to be solved.

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Abbreviations used: EGF, epidermal growth factor; EM, electron microscope; HBSS, Hanks' balanced salt solution; LDL, low-density lipoprotein; LSCM, laser scanning confocal microscope; OM, optical microscope; TGN, *trans*-Golgi network; TRITC, tetramethylrhodamine isothiocyanate.

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To address this question, we characterized endosome tubular structures containing a recycling molecule marker, fluorescent transferrin, in living HEp2 cells, by quantitative imaging analysis using LSCM. We detected two tubular elements with different morphologies and showing a time dependency in their appearance. Using time-lapse imaging analysis of transferrin recycling in living cells, we determined transferrin turnover in whole cells, intracellular regions and single organelles. In particular, it was anticipated that the turnover in each type of tubular organelle would help to identify its role in transferrin processing. Our results demonstrated two types of tubular organelle with distinct morphology and function; one is involved in the rapid transferrin recycling pathway, and the other in slower transferrin processing.

MATERIALS AND METHODS

Cell culture. HEp2 cells were grown in Dulbecco's MEM with 10% fetal calf serum at 37°C under 5% CO_2, and then transferred every 3 days. Two days before use, the cells were plated onto polylysine-coated glass coverslips.

Fluorescent tracers. Human transferrin conjugated with tetramethylrhodamine isothiocyanate (TRITC) was purchased from Molecular Probes. Human epidermal growth factor (EGF) conjugated with Cy5 (Amersham) was prepared by a modification of the method recommended by the manufacturer. Briefly, human EGF (100 μg; Sigma) was fluorescently labeled by incubation with Cy5 monoreactive reagent (200 μg) in 0.5 ml of 0.1 M sodium carbonate (pH 9.3) for 30 min at room temperature. Unconjugated Cy5 was removed by Sephadex G-25 (Pharmacia) gel-filtration and dialysis in phosphate-buffered saline (pH 7.3).

Fluorescence labeling of endocytic compartments. The cells were incubated with 5 μg/ml TRITC-transferrin in Dulbecco's MEM at 37°C for 10 min or 2 h, then washed three times with 10 mM Hepes-buffered Hanks' balanced salt solution (Hepes-HBSS), pH 7.3. Under both loading conditions, the receptor-specificity of TRITC-transferrin internalization was tested by competitive inhibition experiments in which non-labeled transferrin was added simultaneously with the tracer during the loading periods.

Imaging analysis of endosomes in living HEp2 cells. Cells on a glass coverslip were mounted on a metal block observation chamber (17) filled with Hepes-HBSS. Fluorescence microscopic images of the cells were obtained at room temperature (about 20°C) using LSCM (LSM410, Zeiss) with a ×63 oil plan-apochromat lens (N.A. 1.4, Zeiss). Optical sections were obtained at 0.2 - 0.3 μm intervals, from which three-dimensional and projection images were reconstructed.

Time-lapse imaging was performed as follows. Specimens in an observation chamber were kept at 33°C by water circulating through a pair of chamber-holders (17) and air circulating through a plastic box enclosing the stage. Images of fluorescent transferrin-loaded cells were recorded in the same optical plane at various times after the addition of 1 mg/ml non-labeled transferrin.

The fluorescence intensity from several cells or from subcellular regions was estimated on serial digital images using the public domain NIH Image program (W. Rasband, available to Internet users from zippy.nimh.nih.gov).

Double labeling with fluorescent transferrin and EGF. To distinguish the extensive tubular element from the late endosome and lysosome, the morphology of TRITC-transferrin-loaded organelles was compared with that of Cy5-EGF-loaded organelles in double labeling experiments. For comparison with the late endosome, the cells were incubated with 5 μg/ml TRITC-transferrin for 1 h at 37°C, washed three times with Hepes-HBSS, and then incubated with 0.5 μg/ml Cy5-EGF and 100 μg/ml non-labeled transferrin for 15 min at 37°C. For comparison with the late endosome and lysosome, the cells were incubated simultaneously with 5 μg/ml TRITC-transferrin and 0.5 μg/ml Cy5-EGF for 1 h at 37°C, washed three times with Hepes-HBSS, and then incubated with 100 μg/ml non-labeled transferrin for 15 min at 37°C.

RESULTS

Loading of Fluorescent Transferrin for Ten Minutes

To investigate the structure and distribution of the endocytic compartments involved in transferrin recycling, HEp2 cells were incubated with fluorescent transferrin (TRITC-transferrin) for 10 min at 37°C. This incubation period is sufficient to load the tracer into all the elements involved in transferrin recycling, since previous kinetic studies have revealed that the half-life of transferrin recycling at 37°C is ~10 min (18–21).

TRITC fluorescence images after this short loading time showed numerous fine tubular elements distributed throughout the cytoplasm (Fig. 1a). Higher-magnification images (Fig. 1b) demonstrated that the fine tubular elements were 2 - 10 μm long and less than 0.3 μm wide, with many bends and branches. In addition, each element had 2 - 6 bright globular portions with a diameter slightly larger than that of the tubular portions. The appearance of the tubules with globular portions was essentially consistent with those observed previously by EM (4, 7, 9). Some globular elements obviously free from the tubules were also observed.

To confirm that the fine tubular elements were involved in transferrin recycling, the 10-min loading with TRITC-transferrin was followed by incubation with 1 mg/ml non-labeled transferrin. After incubation for 30 min, most of the fluorescent elements had disappeared (Fig. 1c).

Hopkins et al. (10) previously pointed out that the finer tubules in the endosomal reticulum were fragmented by ultraviolet illumination, low temperature, increased pH or other suboptimal culture conditions. However, under our experimental conditions using LSCM, the length of the fine tubular elements was sustained at 2 - 10 μm at 20–37°C (Fig. 1a and b).

Loading of Fluorescent Transferrin for Two Hours

Although the fine tubular elements that we observed were 2 - 10 μm long (Fig. 1b), Hopkins et al. have previously described longer tubular structures in HEp2 cells as an "extensive network of tubular cisternae", sometimes extending from the cytocenter to the periplasm. This difference may have been due to the longer loading time used in the earlier study (≥ 1 h) than in

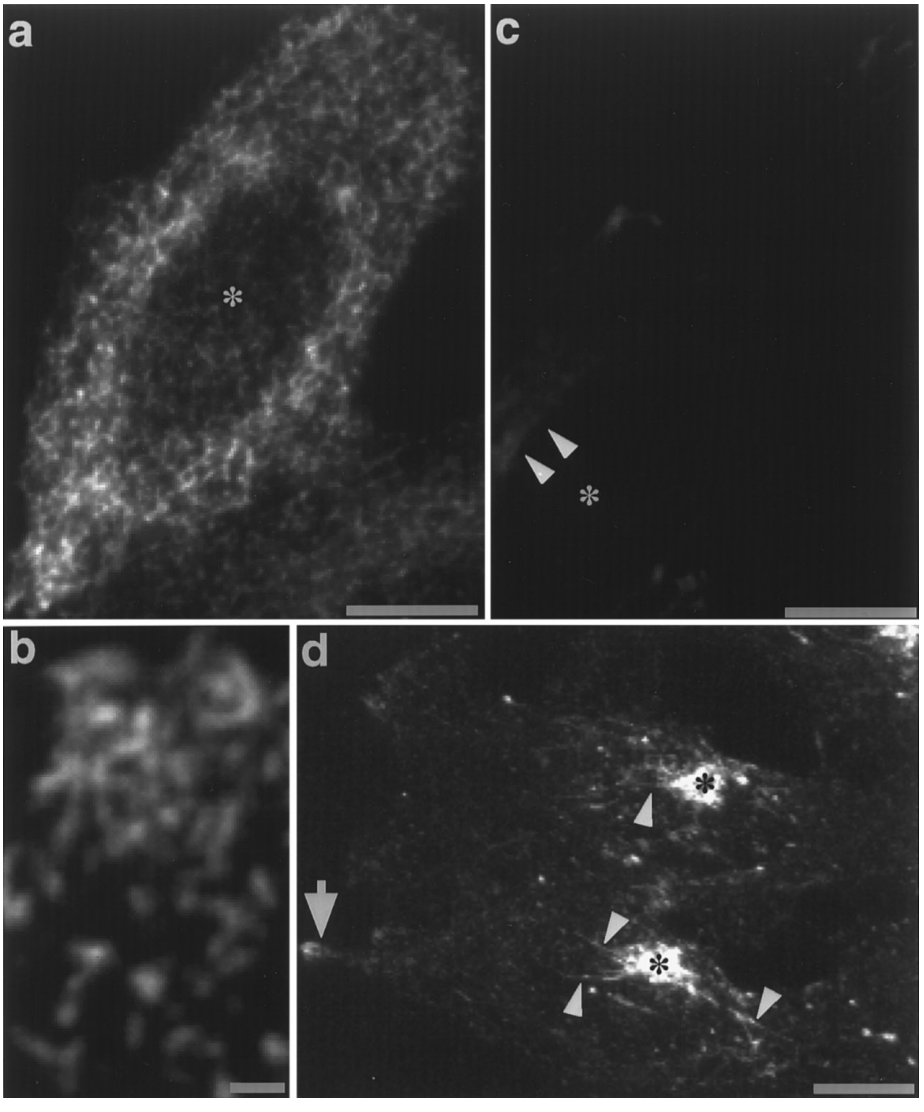

FIG. 1. (a) HEp2 cells loaded with TRITC-transferrin for 10 min at 37°C. Numerous fluorescent tubular elements are evident throughout the cytoplasm. The position of the cell nucleus is marked with an asterisk. bar = 10 μm. (b) High-magnification image of endosomes after a 10-min loading period. Fine tubular elements are 2 - 10 μm long and have many globular portions. bar = 1 μm. (c) HEp2 cells incubated with non-labeled transferrin after a 10-min loading period. Most of the fluorescence has disappeared within 30 min. Only dim fluorescence (arrowhead) remains close to the nucleus (*). bar = 10 μm. (d) HEp2 cells loaded with tracer for 2 h at 37°C. Several extensive tubular structures (arrowhead) are evident. These elements usually extend radially from the cytocenter. Fluorescent aggregates are also observed in the cytocenter (*) and protrusions (arrow). In this experiment, the sensitivity of the detector used was lower than that in a and b to avoid overexposure of the extensive tubular elements and cytocenter aggregation. Therefore, the fine tubular elements appear dimmer than those in a and b. bar = 10 μm.

ours (10 min). Therefore, to investigate the effect of prolonged loading time, we applied TRITC-transferrin to HEp2 cells for 2 h at 37°C.

After this incubation period, fluorescent elements were distributed throughout the cytoplasm, and were particularly dense in the cytocenter region, the cell margin and the protrusions (Fig. 1d). Fine tubular structures similar to those seen in the 10-min loading experiments were observed. However, the extensive tubular structure 5 - 20 μm long was most prominent (Fig. 1d). These extensive tubular elements showed a different time relationship from the fine tubular elements because they were not observed after the 10-min loading period (Fig. 1a). This indicated that the extensive tubular elements are involved in transferrin processing which is slower than transferrin recycling ($t_{1/2} \sim 10$ min).

Furthermore, the morphology of the extensive tubular elements differed from that of the fine tubular elements as follows: (a) These tubules were longer,

brighter, and therefore apparently thicker than the tubular elements seen after the 10-min loading period. (b) The number of extensive tubules in a single cell was usually 2 - 10. (c) Most of the extensive tubules radiated from the fluorescent aggregate in the cytocenter to the periplasm, sometimes reaching the edge of the cell. (d) The extensive tubules were straight, with fewer bends and branches than the fine tubular elements. (e) The extensive tubules were occasionally accompanied by large swollen structures.

According to these observations, we regarded the two tubular elements as distinct organelles. The extensive tubular elements observed here are similar to the longer tubular cisternae described by Hopkins et al. (10).

Time-lapse Imaging Analysis

The time-dependency of the appearance of the fine tubular elements (Figs. 1a and c) indicated that these are involved in the rapid transferrin recycling pathway. To confirm this result quantitatively, we examined TRITC-transferrin turnover by time-lapse imaging analysis.

During incubation of the cells with excess non-labeled transferrin at 33°C following the 10-min TRITC-transferrin loading period, time-lapse images were recorded in the same optical plane of the cells. The fluorescence intensity of 20 cells decreased in a single exponential manner after the addition of non-labeled transferrin (Fig. 2a, solid circles). The half-life of the intracellular TRITC-transferrin was 12 min, which is similar to that obtained at this temperature in cultured human carcinoma FL cells with a biochemical bulk assay (20), and somewhat longer than that (~7 min at 37°C) recently obtained in HEp2 cells by Ghosh et al. using imaging analysis of cells fixed under various conditions by LSCM (15). Because the elements containing TRITC-transferrin after the 10-min loading period are the fine tubular and free globular elements (Figs. 1a and b), the results shown in Fig. 2a confirm that these elements are indeed involved in the process of transferrin recycling.

By contrast, TRITC-transferrin in 20 cells after 2 h of loading did not decrease in a single phase: the decrease was rapid during the first phase (within the first 10 min) but then slowed down (Fig. 2a, clear circles). More than 30% of the initial TRITC-transferrin on the optical plane remained in the cells after incubation for 2 h with non-labeled transferrin. This multi-phase decrease seems to be due to the presence of several types of fluorescent elements, each with a different turnover.

To explore this heterogeneity, we next analyzed transferrin turnover in particular elements and intracellular regions. The characteristic structures observed after a 2-h loading period were extensive tubular elements and aggregates of many fluorescent elements in the cytocenters and protrusions (Fig. 1d).

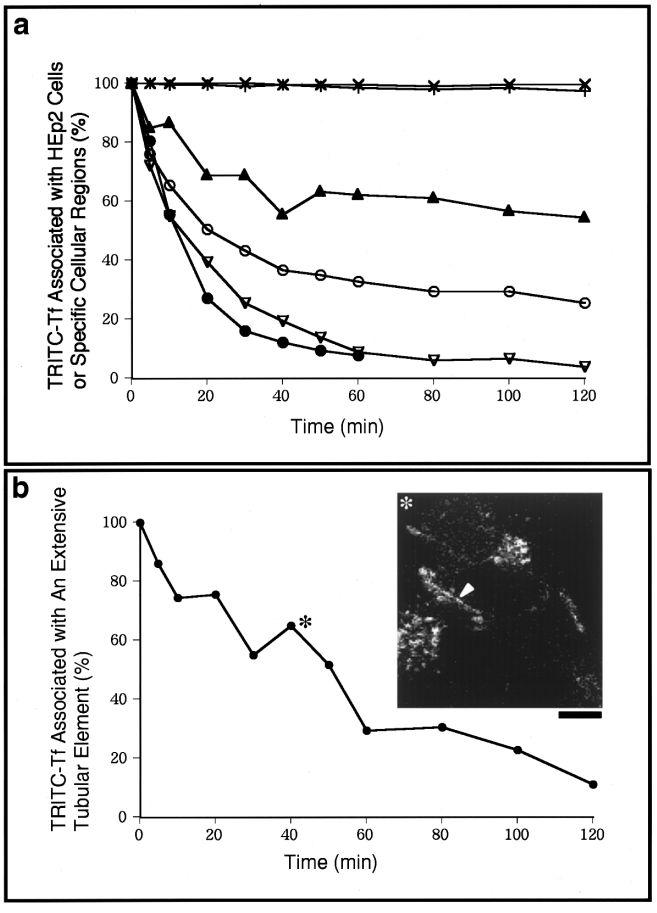

FIG. 2. (a) Time-lapse imaging analysis of TRITC-transferrin associated with HEp2 cells. The fluorescence intensity from 20 cells after a 10-min loading period (solid circles), 20 cells after a 2-h loading period (clear circles), six protrusions after a 2-h loading period (clear triangles), and six cytocenter aggregations after a 2-h loading period (solid triangles) were measured from images taken of a single optical plane in the cells at the various times indicated. The effect of photobleaching was estimated by scanning cells fixed after 10 min (×) or 2 h (+) loading. (b) Time-lapse imaging analysis of TRITC-transferrin associated with a single extensive tubular element. *inset:* a single optical section of a cell after 40 min of incubation (*). An extensive tubular element (arrowhead) is evident. bar = 10 μm.

First, a single extensive tubule was examined (Fig. 2b). The half-life of TRITC-transferrin in this element was 50 min, obviously longer than that in the fine tubular elements. This result confirms that the extensive tubular elements are involved in slow transferrin processing, distinct from the rapid recycling pathway.

Second, the fluorescence intensity of six cytocenter aggregate regions was examined (Fig. 2a, solid triangles). Forty percent of TRITC-transferrin in these regions was depleted with a half-life of ~15 min, which was similar to that seen in the fine tubular and free

globular structures. However, 60% remained even after incubation for 2 h with non-labeled transferrin. These results indicate that the cytocenter aggregates contain organelles with a transferrin turnover different from those of the fine tubular and free globular elements (12 min), and those of the extensive tubular elements (~50 min).

Third, protrusion aggregates were examined (Fig. 2a, clear triangles). The transferrin turnover in six protrusions was similar to that observed in the 10-min loading experiment, suggesting that this region consists mainly of fine tubular and free globular elements, but not extensive tubular elements, as is the case for other peripheral regions (Fig. 1d).

Double Staining with Fluorescent Transferrin and EGF

Time-lapse imaging analysis demonstrated that the fine tubular element is involved in rapid transferrin recycling (Fig. 2). However, the function of the extensive tubular element remained unclear. To examine whether this element is the late endosome or lysosome, the cells were double-labeled with Cy5-EGF and TRITC-transferrin. According to previous kinetic studies, lysosome-directed molecules appear in the late endosome 8 min after endocytosis (21) and in the lysosome ~30 min after endocytosis (22).

First, to examine whether the extensive tubule is the late endosome, the cells were loaded with TRITC-transferrin for 60 min at 37°C, and then loaded with Cy5-EGF and excess non-labeled transferrin for 15 min at 37°C (Fig. 3a). Cy5-EGF fluorescence was observed in globular structures which were distinct from the extensive tubules. In addition, Cy5-EGF was not distributed in the extensive tubules containing TRITC-transferrin. Second, to compare the extensive tubule with the late endosome and lysosome, the cells were loaded simultaneously with Cy5-EGF and TRITC-transferrin for 60 min at 37°C, and then incubated with non-labeled transferrin for 15 min at 37°C (Fig. 3b). Cy5-EGF fluorescence was observed in globular elements accumulated in the cytocenter, most of which also contained TRITC-transferrin. However, Cy5-EGF was not found in the extensive tubules containing TRITC-transferrin. These results indicated that the extensive tubular element is an organelle different from the late endosome and lysosome.

DISCUSSION

In this study, we observed two types of tubular organelles with distinct transferrin-processing functions. The fine tubular elements are involved in rapid transferrin recycling (12 min) and can be regarded as components of early endosomes (11). The extensive tubular elements do not participate in rapid recycling, but appear to be involved in some slower mode of transferrin processing. These two tubular elements, which have previously been regarded as an identical organelle (3, 9, 10, 15), must be critically distinguished when discussing transferrin recycling.

The fine tubular elements were observed to be loaded with TRITC-transferrin within 10 min (Figs. 1a and b). Moreover, the turnover of TRITC-transferrin in the elements had a short half-life of 12 min (Fig. 2a), which was consistent with previous biochemical bulk assays of transferrin recycling (18–21). Our results indicated that the fine tubular elements have a function in the rapid transferrin recycling pathway, and that therefore they include both sorting endosomes and recycling compartments (12–16).

Previous studies of these two kinds of organelles in CHO cells demonstrated that the morphology of the sorting endosomes and recycling compartments were tubulovesicules and perinuclear condensed tubules, respectively (12–14). In the case of HEp2 cells, the latter were observed throughout the cytoplasm as dim structures, some of which were tubular (15, 16). The morphology of the fine tubular elements observed in HEp2 cells (Figs. 1a and b) is consistent with the previously reported morphologies of the sorting endosomes and recycling compartments.

On the other hand, the extensive tubular elements were observed after a 2-h loading, but not a 10-min loading period (Figs. 1a and d). The half-life of transferrin in the extensive tubular elements (~50 min) is much longer than that in the fine tubules (12 min), as shown in Fig. 2. This slower turnover clearly demonstrates that the extensive tubules are not components of the rapid pathway of transferrin recycling; i.e., they contain neither sorting endosomes nor recycling compartments.

Since it is possible that a small amount of endocytosed transferrin is localized in lysosomes (23), late endosomes, Golgi complexes and trans-Golgi networks (TGNs) (24), it was anticipated that the extensive tubular elements correspond to one of these organelles. However, such possibilities were excluded as follows. In the double-staining experiments with fluorescent transferrin and EGF, the extensive tubules were not loaded with EGF at an earlier ($\leq$ 15 min) and a later (~60 min) stage of EGF delivery to the lysosome (Fig. 3); therefore, they are neither late endosomes nor lysosomes. Furthermore, they are not Golgi complexes or TGNs, since the morphology of these elements is completely different from that of these other organelles stained with C6-NBD ceramide (data not shown). The delivery of TRITC-transferrin to the extensive tubules is not an artifact due to fluorescent tagging, because delivery was competitively inhibited by non-labeled transferrin (data not shown), and also because the

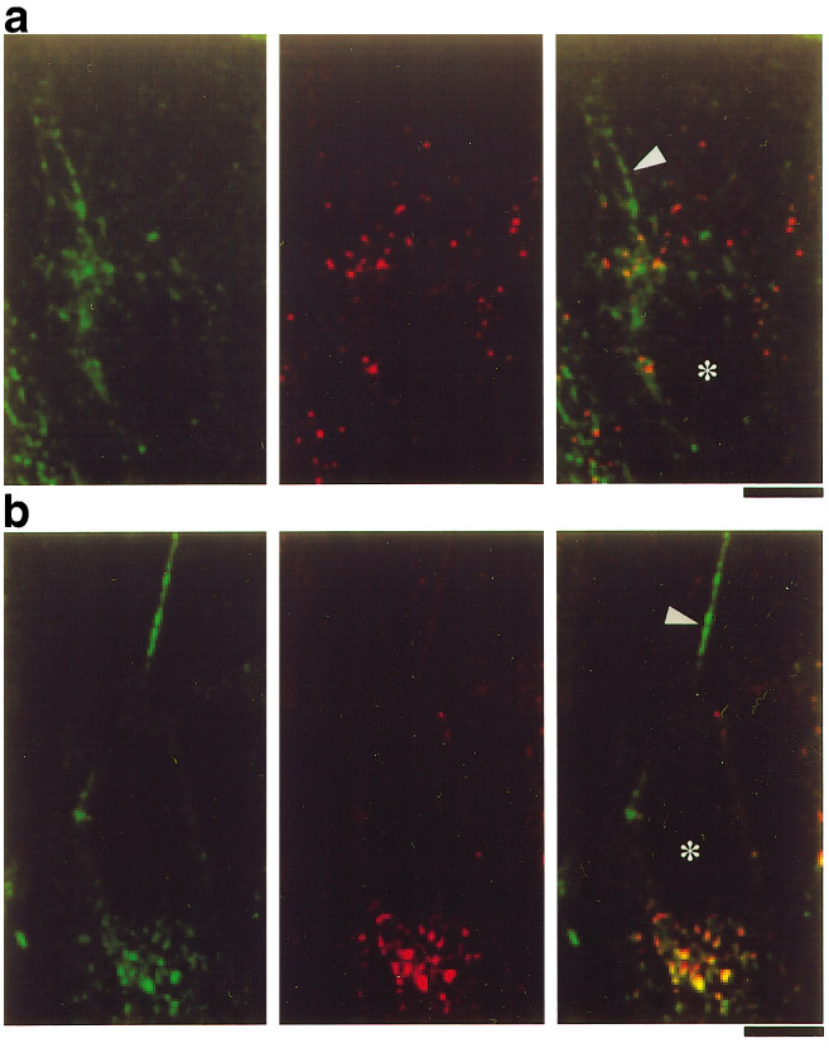

FIG. 3. (a) HEp2 cells were incubated with TRITC-transferrin for 60 min at 37°C and then with Cy5-EGF and non-labeled transferrin for 15 min at 37°C. TRITC-transferrin image (left), Cy5-EGF image (middle), and overlay image (right) are shown. Cy5-EGF is localized in globular structures which are distributed throughout the cytoplasm. Cy5-EGF is not localized in extensive tubular elements stained with TRITC-transferrin. Cell nucleus (*). bar = 10 μm. (b) HEp2 cells were incubated with TRITC-transferrin and Cy5-EGF for 60 min at 37°C and then with non-labeled transferrin for 15 min at 37°C. TRITC-transferrin image (left), Cy5-EGF image (middle), and overlay image (right) are shown. Cy5-EGF is localized in globular elements close to the nucleus (*). Cy5-EGF is not localized in extensive tubular elements stained with TRITC-transferrin (arrowhead). bar = 10 μm.

tracer in the elements was depleted when non-labeled transferrin was added afterward (Fig. 2b).

Transferrin is continuously internalized into cells under physiological conditions, which are more similar to the 2-h loading than to the 10-min loading experiment. Therefore, transferrin must be transported to the extensive tubular elements, in addition to the fine tubular elements. Hopkins et al. suggested that transport along extensive tubular structures is important for the trafficking of endocytosed molecules (10). The length (5–20 μm) of this structure appears to be sufficient for molecule trafficking over the long distance between the cytocenter and the periplasm. So far, we have not elucidated the function of the extensive tubular element, and further studies are needed in order to do this.

Cytocenter aggregates contained two kinds of elements differing in their transferrin turnover (Fig. 2a). Forty percent of TRITC-transferrin in this region was depleted with a half-life of ~15 min, indicating that the transferrin was localized in the sorting endosome and recycling compartment.

Sixty percent of transferrin in the cytocenter aggregates was not exported within 2 h (Fig. 2a), indicating that the aggregates include another organelle devoid of transferrin turnover. As shown by the double-stain-

ing of the cells for 60 min with transferrin and EGF, followed by chasing for 15 min (Fig. 3b), the cytocenter globular structures loaded with transferrin also contained EGF. This implies that the cytocenter aggregates include late endosomes or lysosomes, or both.

From the whole cell images after the 2-h loading experiment, such as Fig. 1d, the amount of transferrin localized in the cytocenter aggregates was quantified to be 16% of cell-associated transferrin. Because 60% of this amount did not undergo turnover, 9.6% of transferrin in the whole cells was considered to be such "dead-end" transferrin in the cytocenter aggregate. On the other hand, previous biochemical assays have demonstrated that degradation of transferrin and its receptor is very slow; e.g. the half-life of transferrin receptors is 60 h (22–24). If this is the case, it is estimated that only 2.3% of cell-associated transferrin is transferred to lysosomes after a 2-h loading period. Thus, not all of the dead-end transferrin in the cytocenter aggregate can be considered to exist in lysosomes. Alternatively, a substantial quantity of transferrin in the cytocenter aggregates may be localized in late endosomes.

A previous quantitative immunocytochemical EM study (24) revealed that under steady-state conditions a small amount of transferrin is localized in the late endosome, less in the Golgi complex and TGN, and is completely lacking in the lysosome. These observations imply that the organelle containing transferrin without turnover is the late endosome rather than the lysosome.

In conclusion, in addition to the early endosome involved in rapid recycling, two other organelles are loaded with endocytosed transferrin under continuous uptake. One is the extensive tubular element which is involved in the slow processing of transferrin and morphologically distinct from the fine tubules of the early endosome. The other, located in cytocenter aggregates, which is presumably the late endosome, contains transferrin without turnover.

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