Multivesicular Endosomes Containing Internalized EGF–EGF Receptor Complexes Mature and Then Fuse Directly with Lysosomes

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Abstract. We have followed the transfer of EGF–EGF receptor (EGFR) complexes from endosomal vacuoles that contain transferrin receptors (TfR) to lysosome vacuoles identified by their content of HRP loaded as a 15-min pulse 4 h previously. We show that the HRP-loaded lysosomes are lysosomal-associated membrane protein-1 (LAMP-1) positive, mannose-6-phosphate receptor (M6PR) negative, and contain active acid hydrolase. EGF-EGFR complexes are delivered to these lysosomes intact and are then rapidly degraded.

Preactivating the HRP contained within the preloaded lysosomes inhibits the delivery of EGFR and degradation of EGF, and results in the accumulation of EGFR-containing multivesicular bodies (MVB). Within time these accumulating MVB undergo a series of maturation changes that include the loss of TfR, the continued recruitment of EGFR, and the accumulation of internal vesicles, but they remain LAMP-1 and M6PR negative. The mature MVB are often seen to make direct contact with lysosomes containing preactivated HRP, but their perimeter membranes remain intact.

Together our observations suggest that the transfer of EGF–EGFFR complexes from the TfR-containing endosome compartment to the lysosomes that degrade them employs a single vacuolar intermediate, the maturing MVB, and can be achieved by a single heterotypic fusion step.

In recent years considerable progress has been made in elucidating the mechanisms involved in the budding and fusion of transport vesicles in the exocytic pathway (Rothman, 1994). The same family of molecules is likely to be involved in vesicular transport in the endocytic pathway (Diaz et al., 1989; D’Souza-Schorey et al., 1995; Peters et al., 1995), but for further progress to be made, it is necessary to obtain a clearer view of where in this pathway vesicle formation, docking, and fusion take place.

It is well established that after endocytosis via clathrin-coated pits, internalized ligand–receptor complexes enter the endosomal compartment, a system of interconnected tubulovesicular elements (for review see Trowbridge et al., 1994). From the endosome compartment many membrane proteins are rapidly recycled to the plasma membrane while much of the fluid-phase protein in the luminal content proceeds towards the lysosome. Recycling of membrane proteins to the plasma membrane is constitutive (Mayor et al., 1993; Trowbridge et al., 1994), and so membrane proteins destined for the lysosome probably display some form of trafficking signal. As yet, however, although certain sequence motifs, such as two adjacent leucines, frequently occur within lysosomally directed membrane proteins, a generic sequence that is sufficient to redirect nonlysosomal proteins to this location has not been identified. For the EGF receptor (EGFR), which we follow in the present study, sequences containing proposed lysosomal targeting information and independent of the tyrosine kinase have been identified in the cytoplasmic domain of the EGFR (Opresko et al., 1995), and we have shown that the tyrosine kinase itself also plays a role in generating a lysosome targeting signal (Felder et al., 1990). During their processing within the endosome, EGF–EGF complexes accumulate on the inner vesicles of multivesicular endosomes (Hopkins et al., 1990; Felder et al., 1990), usually called multivesicular bodies (MVB), one of the most distinctive vacuolar structures of the endocytic pathway.

The transfer step between the MVB and the next stage in the endocytic pathway remains to be defined and has been the subject of considerable debate. One model proposes that MVB are carrier vesicles that bud from the en...
pose a maturation process in which MVB gradually lose characteristic endosomal constituents such as recycling proteins and acquire the characteristics of lysosomes, such as active acid hydrolases (Murphy, 1991; van Deurs et al., 1993). An added complication in these discussions has been the difficulty of defining the point at which the lysosomal compartment begins. Thus, although there is good evidence that mature lysosomes have a distinctive protein composition (Beaumelle et al., 1990), many lysosomal constituents are thought to reach the lysosome via the endosome (Braun et al., 1989; Kornfeld and Mellman, 1989; Ludwig et al., 1991) and should therefore be found en route in prelysosomal structures, such as MVB.

In the present study we have followed the development of MVB and clarified events at the endosome–lysosome border by loading transferrin receptor (TTR)-containing endosome vacuoles with tracers for EGFR and then following them as they move to the lysosome where they become degraded. We have previously shown that EGF and transferrin (Tf) tracers are internalized into the same endocytic vacuole (Futter and Hopkins, 1989; Hopkins et al., 1990). Here we show that after emergence from these vacuoles, EGF–EGFR complexes are delivered exclusively to a preexisting subset of endocytic vacuoles, identifiable by their content of fluid-phase HRP internalized several hours previously. We have been able to inhibit the delivery of EGF to these preloaded vacuoles by preactivating the HRP they contain, and show, as a consequence, that EGF degradation is inhibited and MVB accumulate. Over a period of 60 min, the majority of the accumulating MVB mature, losing the TTR that they contain initially, recruiting additional EGFR, and becoming filled with EGFR-rich internal vesicles. Frequently these EGF–EGFR-containing MVB become directly attached to the vacuoles that contain preloaded HRP. Since these preloaded vacuoles are lysosomal-associated membrane protein-1 (LAMP-1) positive, and mannose-6-phosphate receptor (M6PR) negative, contain active acid hydrolase, and can be shown to degrade the intact EGF they receive from MVB, we believe them to be fully formed, preexisting lysosomes. The fusion of mature MVB with these lysosomes appears to be the only heterotypic vesicle fusion step in the route taken by EGF–EGFR complexes from the time they leave the TTR-containing endosome to the time they are degraded.

**Materials and Methods**

**Reagents**

Human recombinant EGF, human iron-saturated Tf, type II HRP, and DAB were from Sigma Chemical Co. (Poole, UK). EGF and Tf were iodinated to specific activities of ~70 and 7 μCi/μg, respectively, using Na125I from Amersham Intl. (Little Chalfont, UK) and iodobeads from Pierce Chemical Co. (Chester, UK) according to the manufacturers instructions. HRP was conjugated to Tf using SPDP (Sigma Chemical Co.), free HRP was removed from the conjugate using the FreeZyme conjugate purification kit (Pierce Chemical Co.), and HRP was conjugated to EGF using a maleimide conjugation kit (Pierce Chemical Co.). Fluorescein-conjugated Tf (Tf-FITC) was prepared as described in Hopkins et al. (1994). Biotinylated EGF conjugated to Texas red streptavidin (Tx) (EGF-Tx) was from Molecular Probes, Inc. (Eugene, OR). Ferritin was from Miles Laboratories (Kankakee, IL). The mAb (108) to the extracellular domain of the EGFR (Bellot et al., 1990) was a generous gift from J. Schlessinger (New York University Medical Center, New York). The mAb to LAMP-1 (HA3) was a generous gift from Dr. T. August (Johns Hopkins University, Baltimore, MD). rabbit polyclonal antibodies to M6PR were generous gifts from Dr. G.E. Linehard (Dartmouth Medical School, Hanover, NH) and Dr. W.I. Brown (Cornell University, Ithaca, NY), and the rabbit polyclonal anti-HRP antibody was from Sigma Chemical Co. Gold particles (12 nm) were prepared using the method of Slot and Geuze (1985) and were stabilized with the mAb 108, according to standard procedures (DeMey, 1986).

**Cell Culture and Incubation Conditions**

HEp-2 cells were maintained in DME containing 10% FCS in a 5% CO2 atmosphere. To load lysosomal compartments, cells were incubated with either HRP (5 mg/ml) or ferritin (5 mg/ml) for 15 min at 37°C in serum-free DME containing 1% BSA. Cells were then chased for 4 h in DME containing FCS in a 5% CO2 atmosphere. For incubation at 20°C and subsequent transfer to 37°C, cells were incubated in Hank's balanced saline containing 20 mM Hepes and 0.2% BSA. 125I-EGF was used at a concentration of 10 ng/ml. Tf-HRP was used at that concentration that gave maximum cross-linking of 125I-EGF after loading of both tracers at 20°C. EGF-HRP was used at that concentration that gave maximum uptake of HRP activity, >75% of which could be prevented by coincubation with an excess of free EGF. EGF-Tx was used at a concentration of 1 μg/ml.

**DAB Cross-linking**

To determine the amount of radiolabeled ligand or enzyme activity in the same compartment as internalized HRP, cells were washed at 4°C with TBS and were then incubated in TBS containing 100 μg/ml DAB and 0.015% H2O2 at 4°C in the dark for 30 min. Cells were then washed with TBS and lysed in TBS containing 1% Triton X-100 and 0.2% azide. After centrifugation at 14,000 g for 10 min, the supernatant was either counted in a gamma counter or assayed for n-acetyl glucosaminidase activity (Beardmore et al., 1987). The percentage of cross-linking was determined by comparing the radioactivity or enzyme activity in supernatants from cells that had been incubated with HRP with those from cells that had not been incubated with HRP.

To inactivate lysosomes in the living cell, cells were incubated with DAB and H2O2 as above, except that the H2O2 concentration was reduced to 0.0025%, and cells were then washed thoroughly at 4°C with Hank's balanced salt solution before subsequent incubation.

**Measurement of EGF Degradation**

The intracellular degradation of 125I-EGF results in the generation of TCA-soluble products of degradation that diffuse out of the cell and can be collected in the extracellular medium. After incubation of cells with 125I-EGF, the extracellular medium was collected and precipitated with 10% TCA at 4°C for 2 h. TCA-precipitable proteins were pelleted by centrifugation at 14,000 g at 4°C, and pellet and supernatant were counted. By also counting the radioactivity remaining associated with the cells, the percentage of degradation of EGF could be determined.

**Immunofluorescence**

After incubation with fluorescent ligands, cells on coverslips were fixed in 3% paraformaldehyde, quenched in 15 mM glycine, and permeabilized in 0.2% saponin in PBS containing 1% BSA. All subsequent incubations in primary antibody and FITC-labeled secondary antibody were performed in PBS containing 1% BSA and 0.2% saponin. Cells were examined in a laser scanning confocal imaging system (MRC-600; BioRad Laboratories, Hemel Hempstead, UK) with an argon/krypton mixed gas laser. Final images were merged using Photoshop (Adobe Systems, Inc., Mountain View, CA) and photographed using a Sapphire Slide Recorder (Management Graphics, Minneapolis, MN).

**Electron Microscopy**

Cells were fixed in Karnovsky fixative, incubated with hydrogen peroxide and DAB as described by Graham and Karnovsky (1966), and then...
rinsed, osmicated, dehydrated, and embedded by standard procedures (Hopkins and Trowbridge, 1983). Where treated with tannic acid, cells that had been postfix in reduced osmium (1% osmium tetroxide containing 1.5% potassium ferricyanide) were incubated with 1% tannic acid for 45 min at room temperature. Thin sections were stained with lead citrate and viewed in a transmission electron microscope (CM12; Philips Electronic Instruments, Mahwah, NJ). For immunolabelling of cryosections, cells were fixed in 4% paraformaldehyde in 5% sucrose, scraped, pelleted, and processed for cryoimmuno-EM. Small blocks of gelatine embedded cells were infused for 4 h in 2.3 M sucrose, placed on specimen holders, and frozen in liquid nitrogen. Ultrathin cryosections were cut and immunolabeled as described (Slot et al., 1991), except that anti-LAMP-1 and anti-HRP antibodies were coincubated, followed by goat anti-mouse antibody conjugated to 15 nm gold and goat anti-rabbit antibody conjugated to 10 nm gold. Control incubations eliminated the possibility of cross-reactivity between antibodies.

Results

The standard procedure adopted in this study involved preloading lysosomes with fluid-phase tracer by a 15-min incubation at 37°C followed by a 4-h 37°C chase and then loading the endosome with EGFR and TfR tracers by incubating for 60 min at 20°C. During the 20°C incubation the endosome compartment is expected to become loaded to steady state with internalized TfR tracer (Futter and Hopkins, 1989), and the EGFR-EGFR complexes become concentrated within endosomal vacuoles. This protocol partially synchronizes the processing of the EGFR so that on transfer to 37°C, when they move out of the TfR-containing endosome compartment, subsequent events can be followed in detail.

Endosomal Vacuoles Are Loaded with EGFR and TfR by Incubating 60 min at 20°C

At the end of the 20°C incubation, vacuoles containing EGF tracer were distributed throughout the cytoplasm. Light and electron microscopy showed that the majority of these structures contained TfR. This was confirmed quantitatively using a 60-min incubation at 20°C with Tf-HRP and 125I-EGF when 80% of the internalized radiolabel could be cross-linked by the activated enzyme (Fig. 1 a). Degradation of 125I-EGF during the 20°C incubation is negligible.

EM showed the vacuoles containing EGF tracers to be predominantly 0.2-0.5-μm diam MVB containing usually less than five internal vesicles. EM and biochemistry also showed that endosomal vacuoles loaded at 20°C are distinct from lysosomes loaded with fluid-phase tracer 4 h previously. In cells preloaded with the fluid-phase tracer, ferritin, 4 h previously, morphological quantitation showed that 90% of the EGF–HRP+ vacuoles formed by the end of the 60-min, 20°C incubation did not contain detectable ferritin (see Table 1). In cells containing preloaded HRP, at the end of a 60-min, 20°C incubation with 125I-EGF, only 3% of 125I-EGF was cross-linkable, although after subsequent incubations at 37°C, the HRP was able to cross-link up to 30% of the internalized 125I-EGF (Fig. 1 a and see below). By immuno-fluorescence neither M6PR nor LAMP-1 were detectable in these EGF+ vacuoles (Fig. 2, a and d).

Vacuoles Containing Fluid-phase Tracers Loaded 4 h Previously Are Lysosomes

By light and electron immunocytochemistry all vacuoles containing HRP loaded 4 h previously were strongly LAMP-1 positive but did not contain detectable M6PR (Fig. 3). The content of HRP within these vacuoles was used to inhibit their content of acid hydrolase activity. Thus, as shown in Fig. 4, in the presence of DAB, 55% of the total cell n-acetylglucosaminidase activity could be inhibited by the preloaded HRP. Together, these data show that these HRP-containing vacuoles are lysosomes.
Table I. Distribution of Tracers at 20°C and after Warming to 37°C

| HRP tracer | Incubation at 37°C | HRP only | Ferritin only | HRP and Ferritin |
|------------|-------------------|----------|---------------|-----------------|
|            | min | %    | %            | %              |
| Transferrin| 0   | 86.1 | 12.3         | 1.7            |
|            | 45  | 81.4 | 14.6         | 4.0            |
| EGF        | 0   | 60.5 | 32.5         | 7.0            |
|            | 45  | 23.0 | 14.4         | 62.6           |

All elements containing tracer were scored. Approximately 200 elements were counted for each treatment; e.g., for the distribution of vacuoles containing both Tf-HRP and ferritin after 45 min at 37°C, only 10 doubled vacuoles out of a total of 253 were found.

On Transfer to 37°C EGF-EGFR Separate from TfR, Enter the Preloaded Lysosome, and Become Degraded

After the 60-min, 20°C incubation and transfer to 37°C, TfR are lost from the EGF⁺ vacuoles with a half time of ~30 min (Fig. 1 a). Concomitantly, the amount of 125I-EGF in the lysosomes containing HRP increased and, within 30 min, the amount of radiolabeled ligand that could be cross-linked by the preloaded enzyme increased almost 10-fold (Fig. 1 a). After a 10-min delay, the degradation of EGF, as indicated by the appearance of acid-soluble 125I counts in the medium, became detectable, and by 60 min at 37°C, >50% had been degraded (Fig. 1 a). Upon transfer to 37°C, EGF-HRP moved into a compartment containing acid hydrolase activity with exactly the same kinetics as movement of 125I-EGF into the HRP-preloaded lysosome (Fig. 1 b). Comparison of the kinetics with which EGF emerges from the compartment containing Tf-HRP with those with which it is delivered to the lysosome show that the two processes occur almost simultaneously (Fig. 1 a).

The efficiency with which the EGF ligand entered lysosomes was assessed using ferritin rather than HRP-preloaded fluid-phase tracer, since this allowed the efficien-

Figure 2. Lysosomes were preloaded with HRP (15 min followed by 4-h chase at 37°C) and then incubated with EGF-Tx (red fluorescence) for 60 min at 20°C (a and d). Cells in b, c, e, and f were then transferred to 37°C for 45 min. Cells were then fixed and stained immunocytochemically for LAMP-1 (a–c) or M6PR (d–f) (green fluorescence). In c and f cells were incubated with DAB/H₂O₂ to prevent lysosome fusion, as described in Materials and Methods, before loading with EGF-Tx. At the end of the 20°C incubation, EGF-Tx-containing vacuoles do not stain for LAMP-1 (a) or M6PR (d), although there are many LAMP-1 and M6PR⁺ vacuoles elsewhere in the cytoplasm. After 45 min at 37°C, EGF-Tx vacuoles are strongly positive for LAMP-1 (b) but remain M6PR⁻ (e). Activation of the HRP contained within the preloaded lysosomes prevents endosomes from fusing with these lysosomes. Under these conditions, the EGF-Tx⁺ vacuoles become mature endosomes, but they remain LAMP-1⁻ (c) and M6PR⁻ (f). Bar, 10 μm.
cells with which EGF-HRP and Tf-HRP were incorporated to be directly compared (Table I). Within 60 min of being transferred to 37°C after the 60-min, 20°C incubation, >60% of the labeled vacuoles contained both EGF-HRP and ferritin, whereas after similar incubations with Tf-HRP, only 4% of labeled vacuoles contained both tracers (Fig. 5).

Taken together, these results show that on transfer to 37°C, the EGF-EGFR complexes internalized at 20°C move rapidly to lysosomal vacuoles preloaded with fluid-phase tracer, and within minutes their degradation begins. To be able to follow the sequence of events involved in this transfer process in more detail, a method was devised that inhibited the entry of the EGFR into the preloaded lysosomes.

**Inactivation of HRP-containing Lysosomes**

To prevent the lysosomal vacuoles preloaded with HRP from interacting with other endocytic elements, their content of HRP was “preactivated” by incubating living cells with DAB and H2O2, as described in Materials and Methods. After this preactivation, the internalization of radiolabeled transferrin and EGF proceeded with unaltered kinetics, and the recycling of the Tf was unaffected (Fig. 6 a). The degradation of EGF was, however, strongly inhibited (Fig. 6 b).

The effect of preactivating the HRP in the lysosomes on the transfer of EGFR was quantitated morphologically using EGFR antibody–gold complexes to identify EGFR in the electron microscope. Thus, in control cells, after transfer to 37°C for 60 min >90% of EGFR gold-containing vacuoles contained HRP, indicating that they had been efficiently delivered to the preloaded lysosome (Fig. 7 a), but if the HRP in these lysosomes was preactivated, detectable HRP could be found in only 30% of the EGFR-containing vacuoles. Immunofluorescence also demonstrated (Fig. 2) that fluorescent EGF tracer does not gain access to LAMP-1+ lysosomes in cells in which the HRP within lysosomes has been preactivated. Concomitant with the inhibition of fusion, the number of endosomal vacu-
Ceils were incubated with ferritin for 15 rain at 37°C and chased for 4 h at 37°C. Cells were incubated with Tf-HRP at 20°C and then transferred to 37°C for 45 min in the continued presence of Tf-HRP. The pericentriolar area is shown. Ferritin is present in the lysosomes marked with an asterisk but absent from the dense bodies (db) that probably also belong to the lysosome compartment. Tf-HRP is present in the tubulovesicles of the recycling compartment (arrows) that surrounds the centriole. The inset shows a multivesicular endosome containing Tf-HRP surrounded by tubular vesicles with which, in other planes of section, it is probably connected. Bar, 0.2 µm.

In preactivated cells there were frequent profiles indicating that the EGFR-containing vacuoles were able to make direct contact with HRP⁺ lysosomes, but in most instances, it was clear that the perimeter membranes of the connected vacuoles remained intact (Fig. 7).

These experiments show that preactivation of the HRP contained within the preloaded lysosomes inhibits the transfer of EGF-EGFR complexes at 37°C. They strongly suggest that EGFR-containing vacuoles fuse directly with HRP-containing lysosomes and that EGF degradation proceeds only if fusion occurs. Because fusion with HRP-containing lysosomes could be inhibited, it became possible to analyze in detail the rather poorly synchronized changes that take place within the EGFR-containing vacuoles before fusion and to follow most of them until they plateaued, that is, to completion.

**Maturation of MVB**

When fusion with HRP-containing lysosomes is inhibited and cells are transferred to 37°C, a series of morphological

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**Figure 5.** Cells were incubated with ferritin for 15 min at 37°C and chased for 4 h at 37°C. Cells were incubated with Tf-HRP at 20°C and then transferred to 37°C for 45 min in the continued presence of Tf-HRP. The pericentriolar area is shown. Ferritin is present in the lysosomes marked with an asterisk but absent from the dense bodies (db) that probably also belong to the lysosome compartment. Tf-HRP is present in the tubulovesicles of the recycling compartment (arrows) that surrounds the centriole. The inset shows a multivesicular endosome containing Tf-HRP surrounded by tubular vesicles with which, in other planes of section, it is probably connected. Bar, 0.2 µm.
changes take place in the EGFR-containing vacuoles that then accumulate. These changes are complete after 60 min when the majority of the vacuoles have the same morphology and a similar content of EGFR. Thus, as shown in Fig. 8, when HRP-preloaded, DAB/H2O2-pretreated cells were loaded with anti-EGFR-gold at 20°C and then incubated at 37°C, the diameter of EGFR+ MVB increased ~1.5-fold over the first 30 min but thereafter remained relatively constant. EGFR+ MVB present at the beginning of the 37°C incubation had relatively few internal vesicles, but the number increased linearly over the next 60 min until the majority contained between 10 and 15 vesicles (Figs. 8 c and 9). Assuming that these vesicles are, on average, 50 nm in diameter, this increase in internal vesicles, together with the increase in size of the MVB overall, represents an increase of approximately threefold in the total membrane contained within these vacuoles.

With transfer to 37°C, there is also a rapid increase in the number of gold-labeled EGFR within the MVB. This reaches a 50% loading within 10 min (Fig. 8 b) and is complete by 30 min. To determine the stage at which Tfr are removed, incubations were carried out with Tfr-HRP in the 60-min, 20°C incubation and in the 37°C chase. At the end of the 20°C incubation, 90% of EGFR+ MVB contained Tfr tracer, but after 60 min at 37°C, only 20% of them contained detectable Tfr. The EGFR+ MVB that contained Tfr tracer at 60 min contained less than five inner vesicles, indicating that they had remained at an early stage in the maturation process.

Together these observations show that EGFR-containing MVB present at the end of the 20°C loading period undergo a process of maturation even though fusion with HRP-containing lysosomes is inhibited. Over the first 30 min at 37°C, this maturation involves the removal of Tfr, the continued recruitment of EGFR, and the accumulation of internal vesicles. The recruitment of EGFR is complete within the first 30 min, but the accumulation of internal vesicles continues at a steady rate for at least 60 min. As shown in Fig. 2 f, the mature EGFR+ MVB that accumulate when fusion with the HRP-containing lysosome is inhibited for 60 min remain M6PR-.

Contact and Fusion between EGFR+ MVB and Lysosomes

In cells in which fusion with HRP-containing lysosomes was inhibited, direct contacts between mature MVB and lysosomes were often seen. These contacts were also seen in cells that had not been incubated with DAB/H2O2, demonstrating that they were not caused by the preactivation treatment. Because they were seen so frequently, it is probable that they are relatively stable and exist for some time. To examine these contacts in detail, we used cells in which lysosomes had been preloaded with ferritin, incubated with EGF-HRP 60 min at 20°C, and then transferred to 37°C. In these cells groups of EGFR+ MVB and ferritin+ lysosomes were found distributed throughout the cytoplasm, and within them close contacts were evident (Fig. 10). In the contact areas an ordered array of filaments connecting the perimeter membranes of the two vacuoles could be detected (Fig. 10, b and c). These arrays of filaments were similar to those seen on the free surfaces of MVB (Fig. 10 a). On both free and attached vacuoles, the filamentous coat usually appeared as an ordered fringe of short, hairlike fibers (1-2-nm diam, 5 nm long) extending from a clearly defined, sometimes flattened plaque-like area of the perimeter membrane. Similar contacts were also seen between adjacent lysosomes. In these cells “hourglass” profiles of combined MVB-lysosome vacuoles (with as yet unmixed content of the two tracers) were also observed, showing that fusion of their perimeter membranes does eventually occur (Fig. 10 a). However, these hourglass profiles did not display residual attachment plaques, and the relationship between these plaques and the site at which membrane fusion occurs could not, therefore, be determined.

Discussion

The Emergence of EGFR from the Tfr Recycling Pathway and the Maturation of MVB

The vacuoles that have formed after 60 min at 20°C proba-
Figure 8. Cells were incubated with a 15-min pulse of HRP, chased for 4 h, and then incubated with DAB/H2O2. Cells were then incubated with anti-EGFR-gold and EGF for 1 h at 20°C and then transferred to 37°C. Cells were processed for EM and the diameter (a), number of anti-EGFR-gold particles (b), and number of inner vesicles (c) per EGFR-containing MVB was determined. All vacuoles of >200-nm diam that had one or more inner vesicles and one or more anti-EGFR-gold particles were classed as EGFR-containing MVB. EGFR-containing MVB that contained DAB reaction product, indicating that they had fused with the lysosome, were not included. 58-60 MVB were measured at each time point.

Figure 9. Cells were treated as for Fig. 8. The variation in number of inner vesicles/MVB at 0 (cross-hatched columns) and 60 (filled columns) min is shown.

FIGURE 7. (a) Cells were incubated with a 15-min pulse of HRP, chased for 4 h, and then incubated with anti-EGFR-gold and EGF for 1 h at 20°C and then transferred to 37°C for 60 min. Single arrows indicate lysosomes containing HRP and gold, double arrows indicate HRP' lysosomes containing large aggregates of anti-EGFR-gold. (b) Cells were incubated as in a but were incubated with DAB/H2O2 to inhibit fusion with lysosomes before incubation for 60 min at 20°C with anti-EGFR-gold and chased for 60 min chase at 37°C. Double arrows indicate a vacuole that contains both HRP and anti-EGFR-gold, but it is clear that all of the other labeled vacuoles contain either HRP (single arrows) or gold (asterisks). The number of gold particles and internal vesicles within the MVB indicate their maturation is complete. (c and inset) As in b but treated with tannic acid to display membranes. EGF-gold-labeled MVB lie closely adjacent to HRP containing lysosomes (arrows). Arrowheads and small arrows (inset) indicate sites where contact occurs. Within the vacuoles the tracers remain unmixed and in favorable sections (inset), it is clear membranes remain intact. Bar, 0.2 μm.

bly correspond to “sorting endosomes” as described by Maxfield and his colleagues (Dunn et al., 1989), since they concentrate ligands destined for the lysosome and probably have a continuous flux of TfR passing through them. The changes that occur on transfer to 37°C reflect a maturation process similar to that documented in earlier work (Stoorvogel et al., 1991; Dunn and Maxfield, 1992) and they can be related to the previous studies on HEp-2 cells in which a fluid-phase tracer (HRP) and a nonspecific membrane marker (cationized gold) were employed (van Deurs et al., 1993). We have been able to extend these earlier studies by quantitating the changes that occur in a single cohort of MVB formed during the 20°C incubation and, by preventing the vacuoles fusing with lysosomes, we have been able to follow the maturation process to completion. We have shown that during this sequential process, the recruitment of EGFR occurs predominantly in the early stages of maturation. However, although these complexes are known to become concentrated on the internal vesicles (Hopkins et al., 1990), their recruitment to the MVB is not entirely responsible for generating internal vesicles in these MVB because these vesicles continue to increase in number in a linear fashion for at least 30 min after the full complement of EGFR is attained. The seven-fold increase in EGFR compared to the threefold increase in membrane that we show occurs during maturation cannot be achieved only by newly formed vacuoles fusing with each other. This suggests that maturing MVB continue to function as sorting endosomes for a considerable time after they emerge as discrete vacuolar structures. In vitro...
Figure 10. Cells were incubated with ferritin for 15 min at 37°C, chased for 4 h at 37°C, incubated with EGF-HRP for 1 h at 20°C, and then chased for 45 min at 37°C. "Hour-glass" fusion profile (a) with unmixed tracer, ferritin at one pole (F), and DAB reaction product (DAB) at the other. Some vacuoles contain only DAB reaction product (asterisks), others contain only ferritin (F), and still others contain both tracers (L). Small arrows indicate filamentous plaques present on the perimeter membranes of free MVB (a) and between many of the adjacent vacuoles (b and c). Bar, 0.2 μm.

studies support this suggestion since they show that newly formed endosomal vacuoles have little capacity for fusing with each other (Gruenberg et al., 1989).

The demonstration that newly synthesised acid hydrolases are delivered to an endosome compartment before reaching the lysosome (Braun et al., 1989; Kornfeld and Mellman, 1989; Ludwig et al., 1991), together with the demonstration that endosomes can acquire some of the characteristics of lysosomes in vitro (Roederer et al., 1990), has led to the suggestion that lysosomes might arise
directly from endosomes by an extended process of maturation (Murphy, 1991). Earlier published studies in HEp-2 cells also concluded that endosomal MVB mature into LAMP-1 positive lysosomes (van Deurs et al., 1993), and although later work showed that newly formed MVB can fuse with vacuoles that had been preloaded with fluid-phase tracer some hours previously, the identification of these vacuoles as lysosomes depended upon the observation that they were LAMP-1 positive (van Deurs et al., 1995). Our observations show that the EGFR+ vacuoles that arise as TfR+ sorting endosomes do not degrade EGF and remain LAMP-1 negative even when they are allowed to reach full maturity. These results, together with the electron microscopy that shows that docking but not fusion occurs between mature endosomal vacuoles and lysosomes containing preactivated HRP, clearly demonstrate that a discontinuity exists at the endosome–lysosome transfer stage in the endocytic pathway and that this step requires membrane–membrane fusion. Our results for EGFR-containing MVB are, therefore, more in keeping with the findings of Mullock et al. (1989, 1994) who have shown fusion between endosomes and lysosomes isolated from hepatocytes in vitro. They also agree with the recent study on macrophages stimulated with colony stimulating factor on macrophages stimulated with colony stimulating factor in which macroinosomes were shown to mature into vacuoles lacking TfR and then fuse with a stable, resident lysosome compartment (Racocisin and Swanson, 1993).

Vesicular Intermediates in the Endocytic Pathway

Current models for the transfer of trafficking membrane proteins from one intracellular compartment to another on the exocytic pathway require the budding of transport vesicles from the donor compartment, followed by specific targeting to and fusion with an acceptor compartment. This process operates at the cell surface to produce vesicles from coated pits (and is probably required for the return of recycling proteins to the cell surface), but a role for small, 60–80-nm diam vesicular intermediates in transfers between the intracellular compartments that comprise the endocytic pathway remains to be clearly defined. In particular, the role of transport vesicles in removing or adding trafficking receptors to maturing MVB continues to be debated. Direct membrane connections between MVB and tubular elements have been well documented in HEp-2 and other epidermoid carcinoma cells (Miller et al., 1986; Hopkins et al., 1990; Tooze and Hollinshead, 1991), and it is possible that much of the traffic between these vacuoles and the rest of the endosome compartment is achieved by integral membrane proteins like TfR moving laterally within the lipid bilayers of their attached tubules. This possibility was originally proposed and discussed for receptor migrations in the armlike extensions of the compartment of uncoupling receptor and ligand (CURL) endosomes described in hepatocytes (Geuze et al., 1987). Results from more recent kinetic studies are conflicting because although fluorescence studies suggest that free, shuttling vesicles can transfer TfR between the sorting and the recycling subcompartments of the endosome in HEp-2 cells (Ghosh et al., 1994; Ghosh and Maxfield, 1995), studies using calmodulin antagonists suggest that if tubulation within the endosome compartment is reduced (Apodaca et al., 1994; de Figueiredo and Brown, 1995), Tf recycling is strongly inhibited. Morphological observations in our previous studies, in which the processing of EGFR tracers were followed in A431 cells (Miller et al., 1986), clearly showed that the MVB of EGF-stimulated cells continued to bear tubular extensions and/or budding vesicular profiles until the stage when they were full of internal vesicles, that is, as indicated in the present study, until their maturation was almost complete.

Docking and Fusion of EGFR+ MVB with Lysosomes

Amongst the events leading to the fusion of EGFR+ MVB with the lysosome, we have observed an attachment process that probably ensures that the MVB identifies the correct target membrane. The elaboration of the MVB surface that seems to be involved in this docking procedure has often been described in the literature (Friend and Farquhar, 1967; Hopkins et al., 1990), but there have been no previous indications of its function. This fusion process brings together two highly differentiated membrane boundaries and is likely to be mechanistically different from the fusion and docking processes being characterized at other intracellular transfer sites where short-lived 60–80-nm diam transport intermediates are generated (Malhotra et al., 1989; Barlowe et al., 1994). For EGFR, it provides the key for entry into the degradative environment of the lysosome and can, therefore, be expected to be tightly regulated.

The compartments to which EGF–EGFR complexes are delivered at 37°C contain preloaded HRP. These complexes are delivered intact to this compartment since their degradation can be inhibited >80% by DAB cross-linking. In other cell types, most notably the hepatocyte, it has been suggested that partial proteolytic processing of internalized EGF may occur at an early stage in the endocytic pathway (Renfrew and Hubbard, 1991). We cannot rule out the possibility that partial processing events occur before fusion of the MVB with the lysosome, but it is clear that the full degradation of EGF to TCA-soluble products, as in the hepatocyte (Renfrew and Hubbard, 1991), requires delivery to the lysosome. That the HRP only labels a subset of lysosomes is shown by the inhibition of only 55% of cell n-acetylglucosaminidase activity and by the light and electron microscopical observations that show that there are many LAMP-1 positive lysosomes that contain neither EGF nor HRP. EM suggests that the lysosomes preloaded fluid-phase tracers fail to reach are typical 0.5-μm diam dense bodies. Together these observations identify two distinct populations of lysosomes. Both contain active acid hydrolase and are LAMP-1 positive, but only one seems to be accessible to newly internalized tracers.

The Relationship between “Early” and “Late” Endosomes and the Endocytic Pathway Followed by EGFR

The system we have characterized in HEp-2 cells has many of the features of the endocytic pathways described in other cell types. The main difference is that we have shown that the TfR+, EGFR+ MVB that emerge from the
endosome compartment and fuse with M6PR−, LAMP+ lysosomes are vacuoles that undergo a maturation process. In some other systems MVB are considered to be carrier vesicles (Gruenberg et al., 1989) moving from a TIR-containing early endosome compartment to an M6PR-containing late endosome or prelysosome (Griffiths et al., 1988, 1990). In HeP-2 cells strongly staining M6PR+ vacuoles are present in the cytoplasm in all of the conditions we have examined, but we have been unable to obtain any evidence to suggest that they lie on the route between the TIR+EGFR+ MVB and the HRP+ lysosome. Even in cells in which fusion with preloaded lysosomes was prevented and the majority of the EGFR+ MVB became mature, we were unable to show that these vesicles contain M6PR. Similar difficulties in identifying an M6PR-rich compartment have been encountered in recent studies on other endocytic pathways (Racoosin and Swanson, 1993; Garcia-del Portillo and Finlay, 1994) and it is clear, therefore, that the relationship between M6PR processing pathways and the route from the cell surface via the endosome to the lysosome is complex and probably varies with cell type.

One explanation may be that there are multiple routes to the lysosome that use endosomal MVB, and that the route followed by EGFR is distinct from the routes that have the major responsibility for delivering acid hydrolases via M6PR to the lysosome. Our unpublished studies suggest, however, that EGF−EGFR complexes are carried to the lysosome in the same MVB that carry fluid-phase tracer and we believe, therefore, that it is more likely that the endosomal MVB in HeP-2 cells do carry M6PR but at levels too low to be detected. Like TIR and EGFR, they may be able to flux into and out of these vacuoles during the maturation stage. Either possibility would be compatible with the work of van Deurs (van Deurs et al., 1993) that showed that some MVB in HeP-2 cells are M6PR positive.

The endocytic pathway we have followed consisted of only two structurally and functionally distinct compartments: the endosome and the lysosome. The endosome includes early components (which contain receptors, like TIR, which continuously recycle to the plasma membrane) and late compartments (which include EGFR+, TIR−, M6PR− MVB) that no longer contain these recycling receptors but have yet to fuse with lysosomes. The rate at which EGF is degraded during the 37°C incubation shows that the EGFR+ MVB in this pathway have half-lives of ~30 min and probably fuse with (or at least become attached to) lysosomes as soon as their TIR are removed. Perhaps in other cell types this later stage in the maturation process is more prolonged, and M6PR transiently accumulate before the vacuole fuses with the lysosome.

The major discrepancy between our findings and models that propose that MVB are carrier vesicles that fuse with a late M6PR-rich endosome is that we find MVB fuse with vacuoles that, by all the generally accepted criteria (Kornfeld and Mellman, 1989) are lysosomes (i.e., they are LAMP−1, M6PR−, contain active acid hydrolase, and degrade EGF). Our results do show that these vacuoles comprise a special subset of lysosomes because only they are involved in fusing with the MVB that carry labeled EGFR and thus are distinct from the rest of the LAMP-1 positive population. It is possible that there are additional, heterotypic fusion steps within the LAMP-1, acid hydrolase-containing lysosome compartment, but this does not mean that the lysosomal vacuoles that fuse with mature EGFR+ MVB should be regarded as late endosomes or prelysosomes. In terms of their position in the pathway to the lysosome and the time they take to appear, the earlier vacuolar stage of the EGFR+ TIR− MVB could be accurately described as late endosomes, but because this term has been so widely used to describe a stable compartment separated by a carrier vesicle-dependent step from the early endosomes, we would prefer to call these vacuoles mature endosomes. This term clearly indicates the way in which these MVB are formed but does not carry the implication that they acquire any new, stage-specific functions. Their defining characteristic is that they have matured to a point where the proteins of the early endosome that continually recycle to the cell surface (like TIR) can no longer be detected.

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References

Apodaca, G., C. Enrich, and K.E. Mostov. 1994. The calmodulin antagonist, W-13, alters transcytosis, recycling, and the morphology of the endocytic pathway in Madin-Darby canine kidney cells. J. Biol. Chem. 269:19005–19013.

Barkem, C., L. Orci, T. Yeung, M. Hoshohuchi, M.S. Hamamoto, N. Salama, M.F. Rexach, M. Ravazzola, M. Ambernt, and R. Schekman. 1994. COPII: a membrane coat formed by Sec proteins that drive vesicle budding from the endoplasmic reticulum. Cell. 77:985–997.

Beardmore, J., K.E. Howell, K. Miller, and C.R. Hopkins. 1987. Isolation of an endocytic compartment from A431 cells using a density modification procedure employing a receptor-specific monoclonal antibody complexed with colloidal gold. J. Cell Sci. 87:495–506.

Beamulle, B.D., A. Gibbon, and C.R. Hopkins. 1990. Isolation and preliminary characterization of the major membrane boundaries of the endocytic pathway in lymphocytes. J. Cell Biol. 111:1811–1823.

Bellot, F.B., W. Moolenaar, R. Kris, B. Mirakkrur, A. Ulrich, J. Schlessinger, and S. Felder. 1990. High-affinity epidermal growth factor binding is specifically reduced by a monoclonal antibody, and appears necessary for early responses. J. Cell Biol. 110:491–502.

Braun, M., A. Waheed, and K. von Figura. 1989. Lysosomal acid phosphatase is transported to lysosomes via the cell surface. EMBO (Eur. Mol. Biol. Organ.) J.8:3633–3640.

DeMey, J. 1986. The preparation and use of gold probes. In Practical Applications in Pathology and Biology. J.M. Polak and S. Van Noorden, editors. Wright, Bristol, UK. 115–145.

Diaz, R., L.S. Mayorga, P.J. Weidman, J.E. Rothman, and P.D. Stahl. 1989. Vesicle fusion following receptor-mediated endocytosis requires a protein active in Golgi transport. Nature (Lond.). 339:396–400.

D’Souza-Schorey, C., G. Li, M.I. Colombo, and P.D. Stahl. 1995. A regulatory role of ARF6 in receptor-mediated endocytosis. Science (Wash. DC). 267:1175–1177.

Dunn, K.W., and F.R. Maxfield. 1992. Delivery of ligands from sorting endosomes to late endosomes occurs by maturation of sorting endosomes. J. Cell Biol. 117:301–310.

Dunn, K.W., T.E. McGraw, and F.R. Maxfield. 1989. Iterative fractionation of recycling receptors from lysosomally destined ligands in an early sorting endosome. J. Cell Biol. 109:3003–3014.

Felder, S., K. Miller, G. Moehren, A. Ulrich, J. Schlessinger, and C.R. Hopkins. 1990. Kinase activity controls the sorting of the epidermal growth factor receptor within the multivesicular body. Cell. 61:623–634.

de Figueiredo, P., and W.J. Brown. 1995. A role for calmodulin in organelle membrane tubulation. Mol. Biol. Cell. 6:871–887.

Friend, D.S., and M.G. Farquhar. 1967. Functions of coated vesicles during protein absorption in the rat vas deferens. J. Cell Biol. 35:357–376.

Futcher, C.R., and C.R. Hopkins. 1989. Subfractionation of the endocytic pathway: isolation of compartments involved in the processing of internalized...
epidermal growth factor-receptor complexes. J. Cell Sci. 94:685-694.

García-del Portillo, F., and B.B. Finlay. 1995. Targeting of Salmonella typhimurium to vesicles containing lysosomal membrane glycoproteins bypasses compartments with mannose-6-phosphate receptors. J. Cell Biol. 129:81-97.

Geuze, H.J., J.W. Slot, and A.L. Schwartz. 1987. Membranes of sorting organelles display lateral heterogeneity in receptor distribution. J. Cell Biol. 104:1715-1723.

Ghosh, R.N., and F.R. Maxfield. 1995. Evidence for nonvectorial, retrograde transferrin trafficking in the early endosomes of HEP2 cells. J. Cell Biol. 128: 549-561.

Ghosh, R.N., D.L. Gelman, and F.R. Maxfield. 1994. Quantification of low density lipoprotein and transferrin endocytic sorting in H.Ep2 cells using confocal microscopy. J. Cell Sci. 107:2177-2189.

Graham, R.C., and M.J. Karnovsky. 1966. The early stages of absorption of injected horseradish peroxidase in the proximal tubules of the mouse kidney: ultrastructural cytochemistry by a new technique. J. Histochem. Cytochem. 14:291-302.

Griffiths, G., B. Hoflack, K. Simons, I. Mellman, and S. Kornfeld. 1988. The mannose 6-phosphate receptor and the biogenesis of lysosomes. Cell. 52: 329-341.

Griffiths, G.R. Matteoni, R. Back, and B. Hoflack. 1990. Characterization of the cation-independent mannose 6-phosphate receptor-enriched prelysosomal compartment in NRK cells. J. Cell Sci. 95:441-461.

Gruenberg, J., G. Griffiths, and K.E. Howell. 1989. Characterization of the early endosome and putative endocytic carrier vesicles in vivo and with an assay of vesicle fusion in vitro. J. Cell Biol. 108:1301-1316.

Hopkins, C.R., and I.S. Trowbridge. 1983. Internalization of transferrin and the transferrin receptor on epidermoid carcinoma A431 cells. J. Cell Biol. 97: 508-521.

Hopkins, C.R., A. Gibson, M. Shipman, and K. Miller. 1990. Movement of internalized ligand-receptor complexes along a continuous endosomal reticulum. Nature (Lond.). 346:335-339.

Hopkins, C.R., A. Gibson, M. Shipman, D.K. Strickland, and I.S. Trowbridge. 1994. In migrating fibroblasts, recycling receptors are concentrated in narrow tubules in the pericentriolar area, and then routed to the plasma membrane of the leading lamella. J. Cell Biol. 125:1265-1274.

Kornfeld, S., and I. Mellman. 1989. The biogenesis of lysosomes. Annu. Rev. Cell Biol. 5:483-525.

Ludwig, T., G. Griffiths, and B. Hoflack. 1991. Distribution of newly synthesized lysosomal enzymes in the endocytic pathway of normal rat kidney cells. J. Cell Biol. 115:1561-1572.

Malhotra, V., T. Serafini, L. Orci, J.E. Shepherd, and J.E. Rothman. 1989. Puriﬁcation of a novel class of coated vesicles mediating biosynthetic protein transport through the Golgi stack. Cell. 58:329-336.

Mayor, S., J.F. Presley, and F.R. Maxfield. 1993. Sorting of membrane components from endosomes and subsequent recycling to the cell surface occurs by a bulk ﬂow process. J. Cell Biol. 121:1257-1269.

Miller, K., J. Beardmore, H. Kanoty, J. Schlessinger, and C.R. Hopkins. 1986. Localization of the epidermal growth factor (EGF) receptor within the endosome of EGF-stimulated epidermoid carcinoma (A431) cells. J. Cell Biol. 102:500-509.

Mullock, B.M., W.J. Branch, M. van Schaik, L.K. Gilbert, and J.P. Luzio. 1989. Reconstitution of an endosome-lysosome interaction in a cell-free system. J. Cell Biol. 108:2093-2099.

Mullock, B.M., J.H. Perez, T. Kuwana, S.R. Gray, and J.P. Luzio. 1994. Lysosomes can fuse with a late endosomal compartment in a cell-free system from rat liver. J. Cell Biol. 126:1173-1182.

Murphy, R.F. 1991. Maturation models for endosome and lysosome biogenesis. Trends Cell Biol. 1:77-82.

Opresko, L.K., C.-P. Chang, B.H. Will, P.M. Burke, G.N. Gill, and H.S. Wiley. 1995. Endocytosis and lysosomal targeting of the epidermal growth factor receptors are mediated by distinct sequences independent of the tyrosine kinase domain. J. Biol. Chem. 270:4325-4333.

Peters, P.J., V.W. Hsu, C.E. Ooi, D. Finazzi, S.B. Teal, V. Oorschot, J.G. Donaldson, and R.D. Klausner. 1995. Overexpression of wild-type and mutant ARF1 and ARF6: distinct perturbations of nonoverlapping membrane compartments. J. Cell Biol. 128:1003-1017.

Racoon, E.L., and J.A. Swanson. 1993. Macropinosome maturation and fusion with tubular lysosomes in macrophages. J. Cell Biol. 121:1011-1020.

Reid, C.A., and A.L. Hubbard. 1991. Sequential processing of epidermal growth factor in early and late endosomes of rat liver. J. Biol. Chem. 266: 4348-4356.

Roederer, M., J.R. Barry, R.B. Wilson, and R.F. Murphy. 1990. Endosomes can undergo an ATP-dependent density increase in the absence of dense lysosomes. Eur. J. Cell Biol. 51:229-234.

Rothman, J.E. 1994. Mechanisms of intracellular protein transport. Nature (Lond.). 372:55-63.

Slot, J.W., and H.J. Geuze. 1985. A new method for preparing gold probes for multiple labeling cytochemistry. Eur. J. Cell Biol. 38:87-93.

Slot, J.W., H.J. Geuze, S. Gigengack, G.E. Lienhard, and D.E. James. 1991. Immuno-localization of the insulin regulatable glucose transporter in brown adipose tissue of the rat. J. Cell Biol. 113:123-135.

Stoorvogel, W., G.J. Strous, H.J. Geuze, V. Oorschot, and A.L. Schwartz. 1991. Late endosomes derive from early endosomes by maturation. Cell. 65:417-427.

Tooke, J., and M. Hollishead. 1991. Tubular early endosomal networks in AtT20 and other cells. J. Cell Biol. 115:635-653.

Trowbridge, I.S., J.F. Collawn, and C.R. Hopkins. 1994. Signal-dependent membrane protein trafficking in the endocytic pathway. Annu. Rev. Cell Biol. 9:129-162.

van Deurs, B., P.K. Holm, L. Kayser, K. Sandvig, and S.H. Hansen. 1993. Multivesicular bodies in HEP-2 cells are maturing endosomes. Eur. J. Cell Biol. 61:208-224.

van Deurs, B., P.K. Holm, L. Kayser, and K. Sandvig. 1995. Delivery to lysosomes in the human carcinoma cell line HEP-2 involves an actin filament-facilitated fusion between mature endosome and preexisting lysosomes. Eur. J. Cell Biol. 66:309-323.