Subfractionation of the endocytic pathway: isolation of compartments involved in the processing of internalised epidermal growth factor–receptor complexes

CLARE E. FUTTER and COLIN R. HOPKINS
Biochemistry Department, Imperial College of Science and Technology, London SW7 2AZ, UK

Summary

The aim of the present study was to isolate different parts of the endocytic pathway in order to examine the role of epidermal growth factor (EGF)–receptor internalisation in mediating the biological effects of EGF. We have used an antibody to the transferrin receptor complexed with colloidal gold to modify the density of the endocytic compartments so that they can be purified by sucrose density centrifugation. Using this technique, we have been able to isolate a highly purified preparation of endocytic vesicles from H.Ep.2 cells that contain internalised EGF. By employing pulse–chase protocols, it is possible to isolate the different parts of the endocytic pathway and show that they are temporally distinct with regard to the processing of EGF. It should now be possible to examine interactions between the EGF receptor and intracellular substrates in different parts of the endocytic pathway.

Key words: endosome subfractionation, epidermal growth factor–receptor complex, transferrin receptor.

Introduction

When epidermal growth factor (EGF) binds to its receptor, both receptor and ligand are rapidly internalised and pass through a series of endosomal compartments before being degraded in lysosomes (Carpenter and Cohen, 1976; Gorman and Poretz, 1987). The role of EGF-stimulated internalisation of the EGF receptor in mediating the mitogenic effect of EGF is not clear. Treatment of A431 cells with colchicine, which permits internalisation but retards degradation of the EGF–receptor complex, enhances the growth-promoting effects of EGF (Friedkin et al. 1979; Brown et al. 1980), suggesting that the endocytosed EGF–receptor complex makes a contribution to the transduction of the mitogenic signal. Binding of EGF activates the intrinsic tyrosine kinase activity of the receptor, resulting in phosphorylation of the receptor itself and various intracellular substrates (Ushiro and Cohen, 1980). Studies using in vitro mutagenesis of the human EGF receptor suggest that, while the tyrosine kinase activity of the receptor is necessary for the transmission of the biological effects of EGF (Chen et al. 1987; Honegger et al. 1987b), it is not required for the internalisation process itself (Honegger et al. 1987a). However, it is possible that internalisation of the ligand–receptor complex permits the phosphorylation of regulatory proteins in parts of the cell inaccessible from the plasma membrane. Indeed tyrosine kinase activity of the EGF receptor has been demonstrated in microsomal fractions of ligand-stimulated cells, consistent with the maintenance or initiation of hormone-dependent phosphorylation after receptor internalisation (Kay et al. 1986).

The aim of the present work was to devise a method for separating the 'early' and 'late' compartments of the endocytic pathway so that the biochemical properties of the internalised EGF–receptor complex could be examined in detail. Existing approaches for the isolation of different parts of the endocytic pathway rely on intrinsic differences in the density of endocytic vesicles, such as differences in density, permitting separation on density gradients (Gorman and Poretz, 1987) or differences in charge, permitting separation by free-flow electrophoresis (Schmitz et al. 1988). A density-shift protocol, using a colloidal gold-labelled antibody to the transferrin receptor that permits the isolation of a highly purified endosome preparation, has been described (Beardmore et al. 1987). The present work demonstrates that, by modifying this method, temporally distinct parts of the EGF–receptor processing pathway in H.Ep.2 cells can be isolated. Further, by employing pulse–chase protocols with the gold probe, we have been able to use this isolation procedure to demonstrate that the internalised EGF–receptor complex moves sequentially through the endocytic system. With this knowledge it should now be possible to study the transduction and trafficking signals...
of the EGF–receptor complex within these highly purified preparations.

Materials and methods

Antisera and reagents

The B3/25 monoclonal antibody, shown to be specific for the human transferrin receptor (Trowbridge and Omary, 1981), was a gift from Dr I. Trowbridge, Department of Cancer Biology, The Salk Institute, San Diego, CA 92128. Human transferrin (essentially iron-free) was from Sigma Chemical Co., Poole, Dorset. Na

Preparation of B3/25-gold complexes

Gold particles (10 nm) were prepared using the tannic acid method of Slot and Geuze, (1985) and were stabilised with B3/25 as described by Hopkins and Trowbridge (1983). The complexes were stored in 0.02% azide at 4°C and were washed by centrifugation in a Beckman AF-1M-3 airfuge (Beckman Instruments Inc., High Wycombe, Bucks.) at 30 lbf in \\

Electron microscopy

Cell monolayers were fixed in dilute Karnovsky fluid (Karnovsky, 1965), postfixed in 2% osmium tetroxide, and scraped off the dish in the minimum volume of lysis buffer using a rubber scraper. Finally, they were lysed on ice by 15 strokes with a 2 ml syringe and a 21 gauge needle. As judged by phase-contrast microscopy more than 95% of the cells were lysed by this procedure. Unbroken cells, nuclei and DNA aggregates were removed by centrifugation at 800 g for 5 min at 4°C. The resulting postnuclear supernatant was washed twice more times as above. The postnuclear supernatant (in a volume of 1 ml) was layered onto a 14 ml continuous sucrose gradient (26% to 52% sucrose in 10 mM-triethanolamine, 1 mM-EDTA, pH 7.4) in Beckman Ultra-Clear™ centrifuge tubes and was centrifuged in a Beckman SW40 swinging bucket rotor at 200 000 g for 15 h at 4°C in a Beckman L8-55 ultracentrifuge.

Results

Cosedimentation of internalised B3/25–gold complexes and 125I-labelled EGF

The first aim of this study was to determine whether an endocytic compartment involved in processing EGF could be isolated using an anti-transferrin receptor antibody. Cells were labelled with 125I-B3/25–gold for 60 min at 10°C, a temperature shown by preliminary morphological studies to permit binding of ligands to cell surface receptors but to inhibit their internalisation. Cells were then chased in the absence of ligand (5 min at 37°C) before removal of surface-bound ligand and lysis and fractionation as described above in Materials and methods. Of the total counts 80% were present in the postnuclear supernatant. Of these 65% were recovered in the pellet, compared with 2% when the B3/25-gold was omitted (Fig. 1B). The radiolabel that was not recovered in the pellet was

Isolation protocol

Cells were washed three times with lysis buffer (0.25 M-sucrose, 10 mM-triethanolamine, 1 mM-MgCl2, pH 7.4) and scraped off the dish in the minimum volume of lysis buffer using a rubber scraper. Finally, they were lysed on ice by 15 strokes with a 2 ml syringe and a 21 gauge needle. As judged by phase-contrast microscopy more than 95% of the cells were lysed by this procedure. Unbroken cells, nuclei and DNA aggregates were removed by centrifugation at 800 g for 5 min at 4°C. The resulting postnuclear supernatant was washed twice more times as above. The postnuclear supernatant (in a volume of 1 ml) was layered onto a 14 ml continuous sucrose gradient (26% to 52% sucrose in 10 mM-triethanolamine, 1 mM-EDTA, pH 7.4) in Beckman Ultra-Clear™ centrifuge tubes and was centrifuged in a Beckman SW40 swinging bucket rotor at 200 000 g for 15 h at 4°C in a Beckman L8-55 ultracentrifuge.

Gradients were fractionated from the bottom (0.5 cm above the pellet) into 0.8 ml fractions. The pellet was resuspended in 0.8 ml. The radioactive content of the fractions was counted immediately and, with the exception of galactosyl transferase activity (which was assayed in fresh fractions only), the fractions were stored at −20°C to await further analysis.

Enzyme assay

Marker enzymes are assayed according to published procedures: galactosyl transferase, using asialo and agalactosyl fetuin as acceptor (Howell et al. 1978); NADH-cytochrome c reductase (Omura and Takesue, 1979); 5′-nucleotidase; β-N-acetylglucosaminidase (Beardmore et al. 1987).

Electron microscopy

Cell monolayers were fixed in dilute Karnovsky fluid (Karnovsky, 1965), postfixed in 2% osmium tetroxide, scraped from the dish, dehydrated and embedded in Epon as described previously (Hopkins and Trowbridge, 1983). Gold-loaded cell fractions were resuspended in dilute Karnovsky fluid, postfixed in 2% osmium tetroxide, and embedded and sectioned so that the full thickness of the preparation could be examined. Sections were stained in aqueous uranyl acetate and lead citrate and examined in a CM12 Philips electron microscope.
presumably in vesicles that contained insufficient gold to shift their density. We conclude from these data that at 37°C the B3/25-gold complexes and EGF internalise to a common compartment and that the enhanced density of the gold-loaded elements is sufficient to cause them to sediment.

When cells are incubated with B3/25-gold and 125I-EGF for 60 min at 10°C and lysed and fractionated, only 3% of 125I-EGF is sedimentable (Fig. 1C). Most of the radioactivity is found in a pink-coloured band near the top of the gradient (density ~1.01). We interpret these data to mean that under these conditions both ligands are bound to the plasma membrane but the amount of gold bound is insufficient to induce this boundary to sediment. The small amount of EGF recovered in the pellet may be the result of vesiculation of very deep heavily gold-loaded pits during the lysis and fractionation procedure.

Preparation of early and late endosomes using a pulse of B3/25-gold

The aim of this phase of the study was to isolate different parts of the endocytic pathway and show that they are temporally distinct. To prepare an early compartment cells were incubated for 60 min at 10°C with B3/25-gold and 125I-EGF followed by a ligand-free chase for 5 min at 37°C, a temperature shown by morphological studies to inhibit transfer of EGF from the endosome to the degradative compartments of the lysosome pathway (Hopkins and Trowbridge, 1983; Miller et al. 1986). Alternatively, to prepare a late compartment 90-min incubations with B3/25-gold and 125I-EGF at 22°C were followed by 30 min ligand-free chases at 22°C.

Under the incubation conditions used for the isolation of an early compartment 75% of the 125I-B3/25-gold and 13% of the 125I-EGF were found in the endosome pellet (Fig. 2A). Under the conditions used for the isolation of a late compartment 78% of the 125I-B3/25-gold and 41% of the 125I-EGF were found in the pellet (Fig. 2B). If the incubation with B3/25-gold was performed to prepare a late compartment but 125I-EGF was included for the last 10 min of the chase (so that only the early compartment was iodinated), only 2% of the radioactivity was recovered in the pellet (Fig. 2C). When the late compartment was labelled with 125I-EGF, and B3/25-gold was included for the final 10 min of the chase (so that the early compartment was isolated but the late compartment was radiolabelled), only 2% of the radioactivity was recovered in the pellet (Fig. 2C). These results demonstrate that, at 22°C, significant amounts of the internalised EGF and B3/25-gold are localised within the same compartments confirming earlier electron microscope studies (Beardmore and Hopkins, 1984). They also demonstrate that cross contamination between the isolated compartments is very low.

Efficiency of drainage of EGF from the plasma membrane to the early endosome

In order to demonstrate that the EGF contained within the early endosome fraction had been transferred directly from the plasma membrane, the rate at which the labelled peptide internalised from the plasma membrane in the intact cell was compared with the rate at which it appears in the isolated endosome fraction. Cells were incubated with 125I-EGF at 10°C for 60 min and transferred to 37°C for 0, 2.5, 5 or 10 min. After 60 min at 10°C 85% of the 125I-EGF was on the surface (as shown by acid rinsing) (Fig. 3A). Within 2.5 min at 37°C 60% of the surface-bound EGF was internalised. Thereafter, the rate of internalisation was slower.

For fractionation cells were incubated with
Fraction 301

Fig. 2. A. The preparation of an early endocytic compartment using a pulse of B3/25-gold. Cells were incubated with $^{125}$I-B3/25-gold (○) or $^{125}$I-EGF and B3/25-gold (■) for 60 min at 10°C, followed by a 10 min chase at 22°C. B. The preparation of a late endocytic compartment using a long pulse of B3/25-gold. Cells were incubated for 90 min at 22°C with $^{125}$I-B3/25-gold (○) or $^{125}$I-EGF and B3/25-gold (■), followed by a ligand-free chase for 30 min at 22°C. C. Demonstration that the early and late compartments are distinct: cells were incubated with B3/25-gold for 90 min at 22°C, chased for 20 min at 22°C, incubated with $^{125}$I-EGF for 60 min at 10°C and chased for 10 min at 22°C (○). B3/25-gold and $^{125}$I-EGF at 10°C for 60 min and then transferred to 37°C for 0, 2.5, 5 or 10 min before lysis. In cells incubated with B3/25-gold and $^{125}$I-EGF at 10°C without subsequent chase only 3% of the radioactivity was recovered in the pellet (Fig. 3B). When cells were warmed to 37°C for 2.5 min the radioactivity in the pellet increased to 17.5%. However, maximum loading of the early endosome (33%) with $^{125}$I-EGF was achieved only after 5 min at 37°C.

Fig. 3. Efficiency of drainage of EGF and B3/25-gold from the plasma membrane to the early endosome. A. Cells were incubated with $^{125}$I-EGF for 60 min at 10°C and were then chased in ligand-free medium at 37°C for 0, 2.5, 5 or 10 min. The proportion of the cell-associated counts that were surface-bound and internalised was determined as described in Materials and methods. B. Cells were incubated with $^{125}$I-EGF and B3/25-gold for 60 min at 10°C and were then chased in ligand-free medium at 37°C for 0, 2.5, 5 or 10 min. The recovery of counts in the endosome pellet was determined.

The discrepancy between the rate at which EGF leaves the surface (maximum at 2.5 min) and appears in the isolated endosome (maximum at 5 min) may arise because there is more than one route of internalisation from the plasma membrane. This is suggested by morphological studies that have shown that in epidermoid cells (de Brabender et al. 1988; van’t Hof et al. 1989) EGF–receptor complexes enter the cell primarily via uncoated pits while transferrin or B3/25-gold–receptor complexes enter the cell exclusively through clathrin-coated pits (Hopkins et al. 1985). The vesicles containing newly internalised EGF and transferrin receptors may therefore arise as separate populations but they must nevertheless begin to fuse within 2.5 min of internalisation, since significant amounts of $^{125}$I-EGF are co-localised with B3/25-gold by this time.

Efficiency of drainage of EGF and B3/25-gold from the early endosome

In this phase of the study the rate of transfer of EGF and B3/25-gold through the early endosome compartment was assessed. When cells were incubated with $^{125}$I-EGF and B3/25-gold for 10 min at 37°C, in order to isolate and label the early endosome, 24% of the EGF was recovered in the endosome pellet (Fig. 4). When cells were incubated with $^{125}$I-EGF for 10 min, rapidly washed, and incubated with B3/25-gold for 10 min before endosome fractionation the $^{125}$I-EGF found in the
Fig. 4. Efficiency of drainage of EGF and B3/25-gold from the early endosome. Cells were incubated with $^{125}$I-EGF and B3/25-gold for 10 min at 37°C or were incubated with a 10 min pulse at 37°C of $^{125}$I-EGF, were rapidly washed, chased in ligand-free medium for 0–40 min and then incubated with a 10 min pulse of B3/25-gold (filled blocks). Cells were also incubated as above but with B3/25-gold first, followed by $^{125}$I-EGF (cross-hatched blocks). The recovery of counts in the endosome pellet was determined.

pellet was reduced to 10 % (indicating that about 60 % of the EGF in the early endosome is transferred along the endocytic pathway within 10 min). Thereafter, drainage of EGF from the early endosome is relatively slow, as shown by the very slow decline in the % EGF recovered in the pellet, when the pulses of EGF and B3/25-gold were separated by more than 10 min. This may be the result of some EGF recycling.

In a parallel experiment $^{125}$I-EGF was used to identify the early endosome (by using a 10 min incubation at 37°C immediately before lysis) and the rate of drainage of a 10 min pulse of B3/25-gold was followed (Fig. 4). Drainage of B3/25-gold from the early endosome is less efficient than that of EGF, especially during the first 30 min. This is probably because (as suggested in Fig. 3 and discussed below) a significant proportion of the B3/25-gold in the early endosome is recycled back to the plasma membrane and reinternalised.

Efficiency of drainage of EGF from the late endosome

The experiments described in Fig. 2 demonstrate that transferrin/transferrin receptor complexes and EGF/EGF receptor complexes follow essentially the same endocytic pathway during the stages of intracellular processing that occur at 22°C. It is known that at 37°C EGF complexes are degraded while transferrin complexes recycle. This separation must occur at a stage beyond the compartment that is isolated with B3/25-gold at 22°C. To examine how long the EGF/EGF receptor complexes take to reach a degradative compartment beyond the 22°C late endosome, cells were incubated with ligand for 90 min at 22°C, followed by a 30 min chase at 22°C (as for the preparation of late endosomes described above) and then warmed to 37°C. The degradation products of the EGF label appearing in the medium were assessed by acid precipitation. No degradation of transferrin, B3/25 or B3/25-gold (prepared using $^{125}$I-B3/25) was detectable following this procedure. The kinetics of their release into the medium three times faster than the antibody (Fig. 5A). However, this is not a direct reflection of the kinetics of recycling of these different labelled probes because (unlike transferrin) the antibody is not expected to dissociate from the receptor when it reaches the cell surface (Hopkins and Trowbridge, 1983).

A total of 80 % of the label appearing in the medium from $^{125}$I-EGF was acid-soluble at all time points at 37°C, suggesting that some EGF complexes move rapidly and probably directly from the 22°C late endosome to the degradative compartment. The time taken for significant amounts of internalised EGF to be degraded was, however, relatively prolonged (50 % still remained after 20 min), suggesting that significant amounts of intact EGF may be retained for extended periods within the late endosome. This is confirmed by the experiment shown in Fig. 5B where cells were again incubated with B3/25-gold and $^{125}$I-EGF for 90 min at 22°C, followed by a 30 min chase at 22°C before being warmed to 37°C for increasing lengths of time. It is evident that, while there is a rapid initial decline in counts in the endosome pellet during the first 5 min of incubation, up to 80 % of
Morphological characterisation of early and late endosomes in the intact cell and in isolated fractions

Cells were incubated with B3/25-gold for 10 min at 37°C (to label early endosomes) or for 90 min at 22°C, followed by a 30 min chase at 22°C (to label late endosomes) and processed for electron microscopy as described in Materials and methods. After 10 min at 37°C the majority of the B3/25-gold was contained within vesicles in the periphery of the cell, the remainder being bound to the cell surface (Fig. 6). The peripheral elements include typical clathrin-coated vesicles of ~50 nm diameter located just below the plasma membrane and smooth-surfaced profiles, of similar size, deeper in the cytoplasm. Some of the smooth-surfaced elements are tubular rather than vesicular. In addition there were larger (300–500 nm diameter) vacuolar structures, which also contained gold. These structures frequently appear as U-shaped elements with a peninsula of cytoplasm projecting into their internum (see Fig. 6). Usually these U-shaped elements display a clathrin lattice-like coat on their convex surface. They appear to be free in the cytoplasm, separate from the plasma membrane, but no intermediate profiles suggesting how they might arise were observed. Their clathrin-like coats suggest that they may arise from the fusion of coated vesicles. If this is indeed the case then the smooth-surfaced elements that occur in their vicinity may be derived from them and may therefore represent rapidly recycling elements.

In endosome fractions isolated from cells incubated to load ‘early’ endosomes, a range of vesicular elements similar to those of intact cells were identified (Fig. 6). Clathrin-like lattices were not apparent, however, and we presume that this is because they readily dismantle above pH 6.5. Lysis and centrifugation were carried out at pH 7.4. U-shaped elements are uncommon in fractionated preparations but profiles of vacuoles of similar size and containing single, large membrane inclusions are frequent (so that the vacuole has a ‘double ring’ profile). Similar double-ringed profiles are also seen in intact cells at later steps in the endocytic pathway and we presume that during lysis the inflected membrane (see Fig. 6) that forms the peninsula of the U-shaped vacuole pinches off into the vacuolar lumen.

After a prolonged incubation at 22°C with B3/25-gold, labelled elements are concentrated in the juxtanuclear region of the cell, surrounding the centrioles (Fig. 7). These elements include vacuoles (with dense fibrillar contents and aggregates of gold particles) and multivesicular bodies (in which the gold particles are arranged along the limiting membrane). U-shaped vacuoles and double-ring profiles similar to these described above are present in addition to multivesicular bodies containing typical 30–50 nm vesicles. Smaller labelled profiles (~50 nm) often appearing as tubular extensions from the larger vacuoles are also common. In isolated fractions from similarly incubated cells representative profiles of all of these various elements can be identified (Fig. 7). There is no apparent preferential loss of any particular form of gold-loaded element but there appears to be some breakage of the larger, more heavily gold-loaded structures.

Discussion

Modifications of the endosome isolation technique of Beardmore et al. (1987) have permitted the production of a greater endosome yield from H.Ep.2 cells than that described from A431 cells, in terms of amount of endocytosed B3/25-gold recovered in the pellet. The major modification was the use of cells only 2 h after plating. Using cells at 2 h, rather than 24 or 48 h after plating, increased binding and uptake of ligands without affecting kinetics of internalisation, recycling or degradation (results not shown). Two hours after plating the cells are adherent but have not spread to the extent of cells 24 h after plating. An increased surface area and hence increased numbers of surface receptors may therefore be available for ligand binding. Marker enzyme analysis and electron-microscopic analysis of isolated fractions indicates that the increase in endosome yield is obtained without compromising the degree of purity achieved by Beardmore et al. In contrast to the work of Beardmore et al., high yields of B3/25-gold-loaded endosomes could be obtained in the absence of trypsinisation of the postnuclear supernatant prior to fractionation. The endosome preparation described here therefore represents the most effective method of isolating pure endosomes yet developed. This fractionation method also has the special advantage of being able to harvest from all parts of this highly pleiomorphic compartment.

By showing that significant amounts of 125I-EGF co-purified with B3/25-gold in endosome fractions, the feasibility of using this approach to isolate different parts of the EGF-processing pathway was directly demonstrated. The intracellular processing of transferrin and its receptor in A431 cells has been well characterised (Hopkins and Trowbridge, 1983) and co-localisation studies using a wide variety of probes for transferrin and EGF–receptor–ligand complexes have shown that while their ultimate destinations are different (plasma membrane versus lysosome) these two receptor populations are transported through the early and late endosome compartments together (Beardmore and Hopkins, 1984). However, the extent to which the transferrin receptor penetrates the endocytic pathway traversed by EGF varies with experimental conditions. Thus, while pulse-chase experiments demonstrate that transferrin can be recycled with a half-time of 5–7 min, longer incubations show that both transferrin and transferrin receptors can reach (and recycle from) the late endosome compartment in the juxtanuclear area. There are, therefore, at least two
Fig. 6. Morphological characterisation of early endosomes in the intact cell and in isolated fractions. Cells were incubated with B3/25-gold for 10 min at 37°C and were directly processed for electron microscopy (A and B) or endosomes were prepared and then processed for electron microscopy (C, D and E), all as described in Materials and methods. A shows coated invaginations at the plasma membrane (arrows); and B shows the variety of peripheral endosomal elements, including U-shaped vacuoles with coated convex surfaces and profiles of smooth-surfaced elements (arrows). D, E. Isolated elements derived from the smooth-surfaced elements and U-shaped vacuoles seen in the intact cell. Bar, 0.2 μm.
Fig. 7. Morphological characterisation of late endosomes in the intact cell and in the isolated fractions. Cells were incubated with B325-gold for 90 min at 22°C followed by a 30 min chase at 22°C. Cells were then either processed directly for electron microscopy (A) or endosomes were prepared and processed for microscopy (B, C, D and E), all as described in Materials and methods. A shows the concentration of gold-loaded endosomes in the pericentriolar area. Around the centriole (c) there are U-shaped vacuoles (u), multivesicular bodies (mvb) and smooth-surfaced vesicular-tubular elements (arrows). B–E illustrate the variety of components identified in the isolated fraction. Bar, 0.2 μm.
locations (the early and late endosomes) in which transferrin and EGF receptors co-localise before being separately sorted. Previous cell-fractionation studies of mouse fibroblasts (Gorman and Poretz, 1987) have identified internalised transferrin and EGF in vesicles of similar densities 2–5 min after internalisation but in vesicles of different densities 15 min after internalisation. This study, however, used conditions that should have loaded only the early endosome with transferrin. Our studies, which are designed to load either the early or the late endosome compartments with antibody–gold complexes, have allowed us to prepare two temporally distinct compartments of the endocytic pathway containing both the EGF and transferrin receptors.

The kinetics with which the leading edge of the \(^{125}\)I-EFG pulse moves from the plasma membrane fraction to the early endosome (Fig. 4) and from the early endosome to the late endosome (Fig. 5) is entirely consistent with the morphological data obtained from intact epidermoid cells. The efficiency with which the label drains through the endocytic system from these membrane boundaries is relatively poor, but it is known that even at maximum receptor occupancy a significant proportion of EGF receptors do not internalise. If, in addition, a proportion of internalised EGF receptors recycle back to the plasma membrane or between endosome compartments, this too would tend to retard the rate at which EGF receptors drain through the early endosome system.

Our results suggest that there is some intracellular mixing of sequentially internalised probes. Ajoka and Kaplan (1986), using cell fractionation of HeLa cells, found no mixing of successively endocytosed transferrin, suggesting that, at least in this cell type, the endocytosed ligand is carried along the pathway as on a conveyor belt, rather than fusing with a pre-formed endocytic compartment. However, Salzman and Maxwell (1988), using CHO cells in a whole cell assay, found that, when a 2-min interval was allowed between pulses of transferrin and a fluid phase marker of endocytosis, 67% mixing occurred, while, after a 5-min interval, only 41% mixing occurred. Our results, which suggest that fusion takes place between sequentially internalised vesicles but not between compartments formed several minutes apart, support those of Salzman and Maxwell. The results of cell-free fusion assays, which have shown that endocytosed vesicles lose their ability to fuse beyond 5 min after internalisation (Braell, 1981; Gruenberg and Howell, 1986), also support the idea that early parts of the endocytic pathway involve fusion with previously endocytosed vesicles. The consensus view is, therefore, consistent with a scheme whereby endocytosed vesicles fuse with pre-formed endocytic vesicles in the periphery of the cell. From this early compartment endocytosed ligands and receptors are either routed back to the cell surface or are passed through a series of temporally distinct pre-lyosomal compartments. These 'late endosomes' are located in the juxtanuclear region of the cell and from here ligands are again either recycled to the cell surface or routed to the lysosomes. The rate at which EGF degradation begins in cells that are transferred to 37°C after having been preloaded at 22°C suggests that there is little opportunity for further processing after the EGF receptor leaves the late endosome. This is in keeping with the original studies on the hepatocyte by Dunn and Hubbard (1984) who also showed a rapid onset (2 min) of degradation of internalised ligand once the temperature was shifted from 20 to 37°C. At the present time the relationship between 'late' endosomes and lysosomes and their respective roles in degradative activity is unclear. Better methods of purifying elements in this area of the cell need to be designed before we can distinguish sites at which intact EGF may be temporarily sequestered and the site of EGF degradation.

The high degree of purity of the endosome preparations described here should now enable the biochemical interactions of trafficking proteins in the different parts of the endocytic pathway to be studied in detail. In our current studies we are examining the substrates of the receptor kinase as it moves through the different compartments, since there is evidence that the major substrates vary with time following EGF binding (Cohen and Fava, 1985). Also, since new experimental systems expressing mutated receptor proteins (lacking kinase activity and/or phosphorylation sites) are now available, it has become possible to use the fractionation procedures described above to analyse quantitatively the role of these regulatory features in the selective trafficking of the EGF receptor.

The authors thank Adele Gibson for excellent technical assistance. This work was supported by an MRC programme grant awarded to C.R.H.

References


(Received 20 July 1989 – Accepted 18 August 1989)