

Human VPS34 is required for internal vesicle formation within multivesicular endosomes

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After internalization from the plasma membrane, activated EGF receptors (EGFRs) are delivered to multivesicular bodies (MVBs). Within MVBs, EGFRs are removed from the perimeter membrane to internal vesicles, thereby being sorted from transferrin receptors, which recycle back to the plasma membrane. The phosphatidylinositol (PI) 3'-kinase inhibitor, wortmannin, inhibits internal vesicle formation within MVBs and causes EGFRs to remain in clusters on the perimeter membrane. Microinjection of isotype-specific inhibitory antibodies demonstrates that the PI 3'-kinase required for internal vesicle formation is hVPS34. In the presence of wortmannin, EGFRs continue to be delivered to lysosomes, showing that

their removal from the recycling pathway and their delivery to lysosomes does not depend on inward vesiculation. We showed previously that tyrosine kinase-negative EGFRs fail to accumulate on internal vesicles of MVBs but are recycled rather than delivered to lysosomes. Therefore, we conclude that selection of EGFRs for inclusion on internal vesicles requires tyrosine kinase but not PI 3'-kinase activity, whereas vesicle formation requires PI 3'-kinase activity. Finally, in wortmannin-treated cells there is increased EGF-stimulated tyrosine phosphorylation when EGFRs are retained on the perimeter membrane of MVBs. Therefore, we suggest that inward vesiculation is involved directly with attenuating signal transduction.

Introduction

The binding of ligand to growth factor receptors on the cell surface initiates a series of events including receptor activation, the activation of target proteins, and endocytosis of the activated ligand–receptor complex. Both receptor activation and activation of target proteins begin on the plasma membrane. However, it has been known for some years that receptor tyrosine kinases remain phosphorylated and are potentially still active in the endosome (Wada et al., 1992; Futter et al., 1993; Burke et al., 2001), suggesting that signaling is not attenuated at the internalization step. Internalized growth factor receptors may be returned to the plasma membrane and can participate in further signaling, or they may be delivered to lysosomes and degraded, resulting in signal attenuation.

Recycling receptors, such as transferrin receptors (TRs)* and receptors destined for delivery to the lysosome, are

initially internalized into the same endocytic compartment where they are sorted (Trowbridge et al., 1993; Gruenberg and Maxfield, 1995; Mellman, 1996). EM shows that within multivesicular endosomes (multivesicular bodies [MVBs]) recycling TRs are confined to the perimeter membrane, and from this location they can return to the plasma membrane, whereas activated EGF receptors (EGFRs) accumulate on the internal vesicles of MVBs (Futter and Hopkins, 1989; Hopkins et al., 1990; Futter et al., 1996). When all the recycling receptors have been removed, the MVB fuses directly with the lysosome (van Deurs et al., 1995; Futter et al., 1996; Mullock et al., 1998), and EGF–EGFR complexes are rapidly degraded.

The vesiculation processes which give rise to internal vesicles within MVBs are distinctive because, unlike most vesiculation processes characterized to date, the vesiculating membrane buds away from the cytoplasmic matrix. Unlike coatomer- and clathrin-based mechanisms, the components regulating this kind of vesiculation are poorly understood, but in common with these mechanisms they are likely to require interactions which select and load cargo and rearrangements within the membrane that lead to the formation of free vesicles. Our previous data would suggest that tyrosine kinase activity of the EGFR is required

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*Abbreviations used in this paper: EGFR, epidermal growth factor receptor; MVB, multivesicular body; PDGFR, platelet-derived growth factor receptor; PI, phosphatidylinositol; SNX, sorting nexin; TR, transferrin receptor.

Key words: EGF receptor; VPS34; endosome; lysosome; phosphatidylinositol 3'-kinase

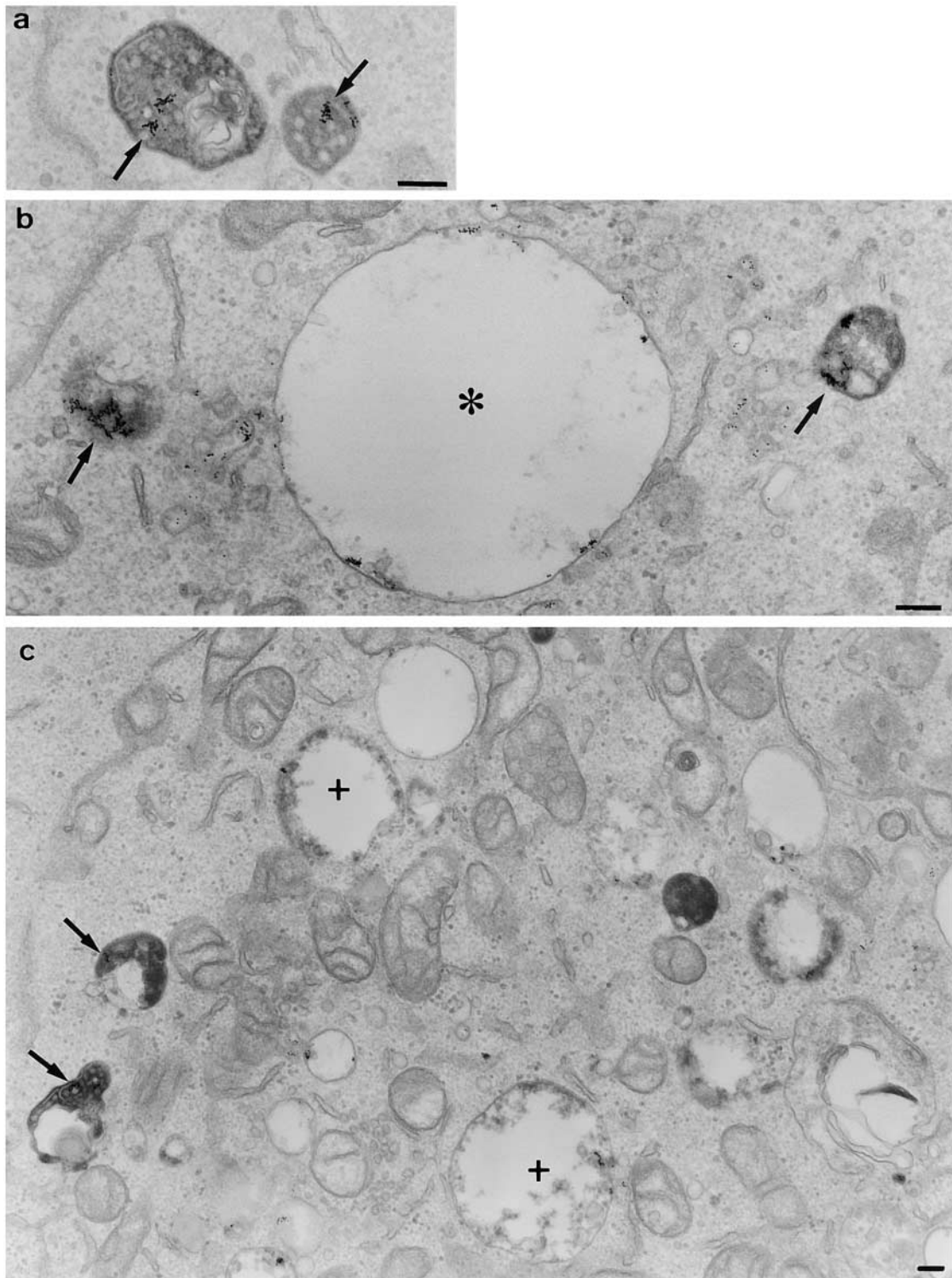


Figure 1. The effects of wortmannin on delivery of the EGFR to the lysosome. HEp-2 cells were incubated with HRP for 30 min at 37°C, chased for 3 h at 37°C, and then incubated with anti-EGFR gold and EGF for 1 h at 20°C, all in the absence of wortmannin. Cells were then chased for 1 h at 37°C in the absence (a) or presence of wortmannin (b and c). Note that in both the absence and the presence of wortmannin, the EGFR have, in many cases, reached the HRP-loaded lysosomes (arrows). In the presence of wortmannin, some MVBs (without HRP) are enlarged with comparatively few internal vesicles, and EGFRs on the perimeter membrane (asterisks) and some lysosomes (with HRP) are also enlarged (crosses). Bar, 0.2 μ m.

for selection of EGFRs for inclusion in the internal vesicle, since mutant EGFRs lacking tyrosine kinase activity fail to accumulate on the internal vesicles and are recycled (Felder

et al., 1990). Sequences within the cytoplasmic domain of the EGFR, distinct from the kinase domain (Kornilova et al., 1996; Opresko et al., 1995; Kil et al., 1999), and c-Cbl-

mediated ubiquitination of the EGFR (Levkowitz et al., 1998) are also required for efficient lysosomal targeting of the receptor, although it is not yet known whether these are also requirements for sorting within MVBs. However, ubiquitination of carboxypeptidase S was shown recently to be required for sorting onto internal vesicles of MVBs in yeast (Katzmann et al., 2001; Reggiori and Pelham, 2001). Given that tyrosine phosphorylation of c-Cbl by the EGFR kinase is required for association with and ubiquitination of the EGFR, c-Cbl phosphorylation may explain at least one requirement for EGFR tyrosine kinase activity in sorting of EGFRs within the MVB. Additional proteins that may be involved in sorting within the MVB include annexin 1, which is phosphorylated by the EGFR kinase on the perimeter membrane of the MVB (Futter et al., 1993), Hrs, which is also a substrate of the EGFR kinase (Komada and Kitamura, 1995) and inhibits lysosomal targeting of the EGFR when overexpressed (Chin et al., 2001; Raiborg et al., 2001), and sorting nexin (SNX)1, which associates with the activated EGFR and enhances the efficiency of lysosomal targeting of the receptor (Kurten et al., 1996). Hrs contains a domain, which recognizes phosphatidylinositol (PI)(3)P, and several studies suggest a role for lipids in sorting within the MVB. Antibodies to lysobisphosphatidic acid (Kobayashi et al., 1998) or mutation of the Niemann-Pick type C gene, which encodes a protein with a sterol-sensing domain, cause the generation of aberrant MVBs that show defects in protein and cholesterol sorting (Kobayashi et al., 1999). Mutations in the yeast PI(3)P 5'-kinase, Fab1, cause defects in sorting within MVBs (Odorizzi et al., 1998), and in mammalian melanoma cells treatment with wortmannin causes the generation of enlarged vacuolar endosomes with a reduced number of internal vesicles (Fernandez-Borja et al., 1999), suggesting that the inward vesiculation process is likely to be regulated by a PI 3'-kinase.

In the current study, we have investigated the role of PI 3'-kinases in sorting within the MVB in HEP-2 cells and show that the lipid kinase hVPS34 is required for the formation of internal vesicles within the MVB but not for removing activated EGFR from the recycling pathway. Since we find enhanced tyrosine phosphorylation when EGFRs are retained on the perimeter membrane of MVBs in wortmannin-treated cells, we suggest that the incorporation of activated EGFRs onto the internal vesicles of MVBs is a regulatory step designed to terminate the EGFR signal more effectively and at a prelysosomal stage in the pathway.

Results

The effects of wortmannin on delivery of activated EGFRs to the lysosome

We identified lysosomes by incubating cells with a pulse of HRP followed by a 2–4 h chase. As previously described (Futter et al., 1996), in HEP-2 cells this protocol labels only lysosomes. After loading lysosomes with HRP, cells were incubated at 20°C with anti-EGFR antibody conjugated to colloidal gold, which in the presence of EGF follows the normal trafficking route of the EGFR. At 20°C, EGFRs are retained in the transferrin-containing endosome, but upon transfer to 37°C activated EGFRs move into the HRP-loaded lysosome, and after 1 h at 37°C the majority of anti-EGFR gold particles are in the lysosome (Fig. 1 a). When wortmannin is included in the 37°C incubation, most MVBs (defined by the presence of anti-EGFR gold but the absence of HRP) and some lysosomes (defined by the presence of HRP) become enlarged (Fig. 1, b and c). Both MVBs and lysosomes have fewer internal vesicles and internal membranes, but it is clear that many of the anti-EGFR gold particles reach HRP-loaded lysosomes in the presence of wortmannin. Counting the number of anti-EGFR gold particles that reached HRP-filled lysosomes after 1 h at 37°C showed that delivery of EGFRs to the lysosome was only slightly inhibited (6%) by wortmannin treatment and that the majority of EGFRs (81.9%) reached the lysosome in the presence of wortmannin (Table I). In these experiments, cells were allowed to take up EGFRs at 20°C in the absence of wortmannin (to eliminate possible effects of wortmannin on internalization of EGFRs), and wortmannin was only included in the 37°C incubation. PI(3)P is likely to be depleted rapidly from cells after wortmannin addition, since the PI(3)P binding protein, EEA1, was released from endosomal membranes within 2 min of wortmannin addition at 37°C (unpublished data). However, to eliminate the possibility that some EGFRs reached the HRP-filled lysosomes before depletion of PI(3)P cells were preincubated with wortmannin at 37°C for 30 min before loading with anti-EGFR–gold/EGF at 20°C and chase at 37°C, all in the continued presence of wortmannin. Under these conditions, there was a 12% reduction in the number of EGFRs reaching the lysosome, but the majority (76.7%) of EGFRs still reached the HRP-filled lysosome.

To further investigate the efficiency of delivery of EGF and EGFRs to the lysosome in the presence of wortmannin, the effects of wortmannin on ¹²⁵I-EGF degradation and

Table I. The effects of wortmannin treatment on delivery of EGFRs to HRP-loaded lysosomes

| Treatment | Number of anti-EGFR gold particles | | Percentage of anti-EGFR gold in HRP +ve vacuoles |
|--------------------|------------------------------------|------------------|--|
| | HRP –ve vacuoles | HRP +ve vacuoles | |
| –Wo | 157 | 1,084 | 87.3 |
| +Wo | 254 | 1,147 | 81.9 |
| +Wo + pretreatment | 327 | 1,078 | 76.7 |

HEP-2 cells were incubated with HRP for 30 min at 37°C, chased for 3 h at 37°C, and then incubated with anti-EGFR gold and EGF for 1 h at 20°C, all in the absence of wortmannin. Cells were then chased for 1 h at 37°C in the absence (–Wo) or presence of wortmannin (+Wo). A third group of cells was incubated as above except that wortmannin was added for 30 min at 37°C before addition of anti-EGFR gold, and EGF was included in the 20°C incubation and the 37°C chase (+Wo + pretreatment). The number of anti-EGFR gold particles in HRP –ve vacuoles (MVBs) and HRP +ve vacuoles (lysosomes) was counted in random thin sections, and the percentage of anti-EGFR gold particles in HRP +ve lysosomes was determined.

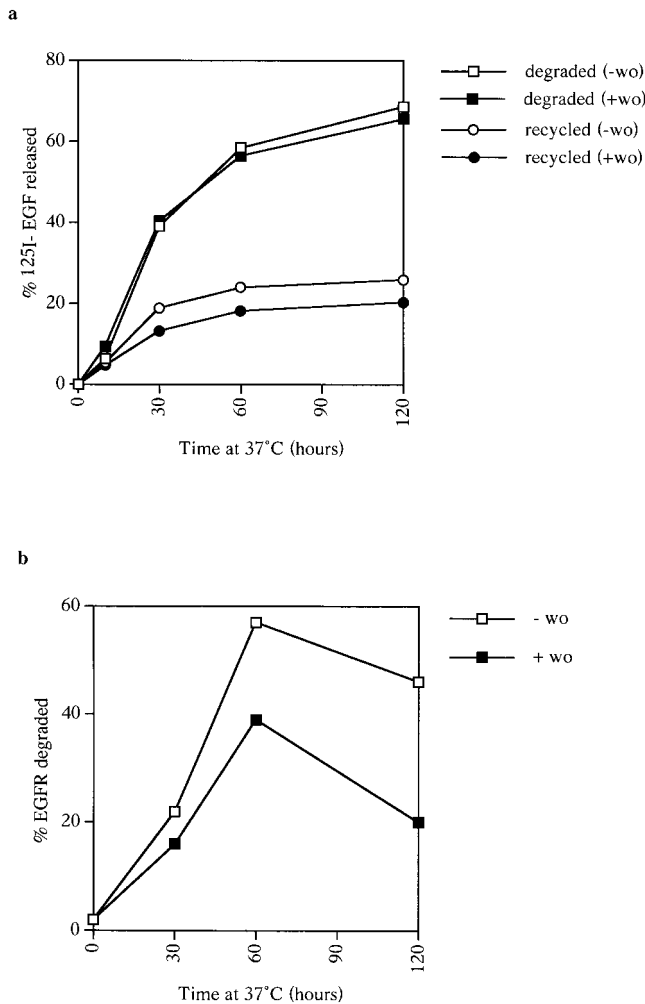


Figure 2. The effects of wortmannin on EGF and EGFR degradation. (a) Cells were incubated with ^{125}I -EGF for 1 h at 20°C, surface stripped, and chased at 37°C in the absence or presence of wortmannin (wo) for up to 2 h. Media samples were TCA precipitated to determine the percentage of degradation. (b) Cells were incubated with EGF for 1 h at 20°C and then chased at 37°C for up to 2 h in the absence or presence of wortmannin (wo). Cell lysates were analyzed by SDS-PAGE followed by Western blotting with an antibody against the cytoplasmic domain of the EGFR. Percentage degradation is calculated by comparison with the amount of EGFRs in cells not treated with EGF.

EGFR degradation were determined. In HEP-2 cells, $\sim 70\%$ of endocytosed ^{125}I -EGF is delivered to the lysosome and degraded to TCA soluble radioactivity, and 20% is released into the extracellular medium intact (Fig. 2 a). Wortmannin treatment had very little effect on the magnitude or kinetics of EGF degradation, although EGF recycling was inhibited. We have shown previously that MVB-lysosome fusion is required for EGF degradation to TCA-soluble products (Futter et al., 1996), and so these data together with the EM data described above indicate that wortmannin treatment does not inhibit MVB-lysosome fusion. EGFRs are normally delivered to the lysosome primarily on internal vesicles after MVB-lysosome fusion. In the presence of wortmannin, a considerable proportion of EGFRs are delivered to the lysosome on the perimeter membrane, and so EGF and the lu-

menal domain of the EGFR are exposed to a degradative environment, whereas the COOH terminus of the receptor remains exposed to the cytoplasm. Therefore, we determined the rate of degradation of the EGFR in the presence and absence of wortmannin by Western blotting with an antibody against the cytoplasmic domain of the EGFR. Although the rate of EGFR degradation was reduced in wortmannin-treated cells (Fig. 2 b), no fragments of the EGFR indicative of partial degradation were observed.

The effects of wortmannin on inward vesiculation in cells where lysosomes have been cross-linked

Incubation of living cells with DAB/ H_2O_2 crosslinks HRP-loaded lysosomes, preventing fusion between MVBs and lysosomes, and causes MVBs to accumulate (Futter et al., 1996). This technique allows the development of the MVB to be followed in the absence of endosome-lysosome fusion and is particularly useful for studying inward vesiculation. To quantify the effects of wortmannin on inward vesiculation, HRP-loaded lysosomes were cross-linked and cells were then incubated with anti-EGFR gold and EGF at 20°C followed by a 1 h chase at 37°C in the presence or absence of wortmannin. As shown in Fig. 3 a, in the absence of wortmannin MVBs with many internal vesicles accumulate, and the EGFR gold is primarily on the internal vesicles. In the presence of wortmannin enlarged anti-EGFR, gold-containing vacuoles accumulate (Fig. 3, b and c). These vacuoles contain very few internal vesicles, and the majority of the gold-labeled EGFRs is on the perimeter membrane. These EGFRs are not evenly distributed on the perimeter membrane but are clustered, and where an internal vesicle does form it is usually labeled with anti-EGFR gold (Fig. 3 b).

The major change in volume of MVBs induced by wortmannin treatment may alter the probability of sectioning through internal vesicles in any given thin section. To accurately quantitate the number of internal vesicles per MVB, we therefore analyzed serial sections to reconstruct individual MVBs in their entirety. As shown in Table II, MVBs formed in the presence of wortmannin have fivefold fewer internal vesicles than those formed in the absence of wortmannin and have approximately twice the diameter. As shown in Fig. 4, the number of internal vesicles per MVB is extremely variable, particularly in control cells, but the majority of MVBs have 10–40 internal vesicles per MVB. In contrast, in wortmannin-treated cells the majority of MVBs have less than five internal vesicles. Estimating the total membrane area of the MVB, assuming all internal vesicles to have a diameter of 50 nm, indicates that wortmannin-treated vacuoles have approximately twice as much membrane as control cells (Table II), suggesting that the vacuolar enlargement cannot be explained solely by a failure to inwardly vesiculate. Inhibition of exit from the MVB as has been suggested by others (Reaves et al., 1996; Kundra and Kornfeld, 1998) may, therefore, also contribute to the vacuolar enlargement. MVBs allowed to accumulate for 1 h in the presence of wortmannin have up to 50% fewer gold particles than those that accumulate in the absence of wortmannin. We can assume that the entry of EGFRs into the MVBs is not inhibited by wortmannin, since in cells where the lysosomes have not been cross-linked EGFRs are efficiently

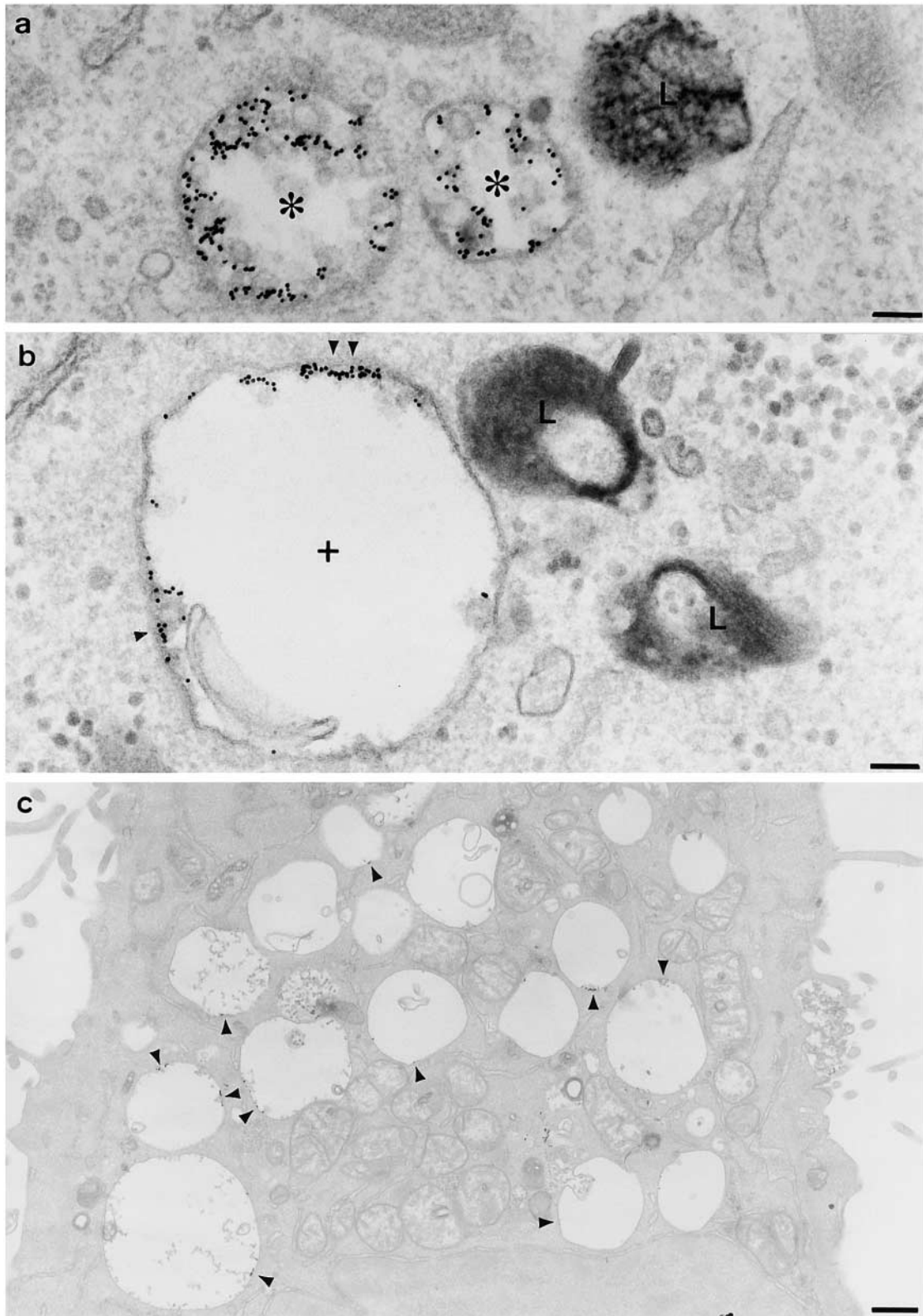


Figure 3. The effects of wortmannin on inward vesiculation in cells where the lysosomes have been cross-linked. HEP-2 cells were incubated with HRP for 30 min at 37°C, chased for 3 h at 37°C, and then incubated with DAB/H₂O₂ at 4°C to crosslink the lysosomes. Cells were then incubated with anti-EGFR gold and EGF at 20°C in the absence of wortmannin and then chased at 37°C in the absence (a) or the presence of wortmannin (b and c). Note that in the absence of wortmannin, MVBs with many internal vesicles and anti-EGFR gold predominantly on the internal vesicles accumulate (asterisks). In the presence of wortmannin, enlarged MVBs with very few internal vesicles accumulate (crosses), and the EGFR are clustered (arrowheads) on the perimeter membrane of the enlarged MVBs. L, lysosome. Bars: (a and b) 0.1 μm; (c) 0.5 μm.

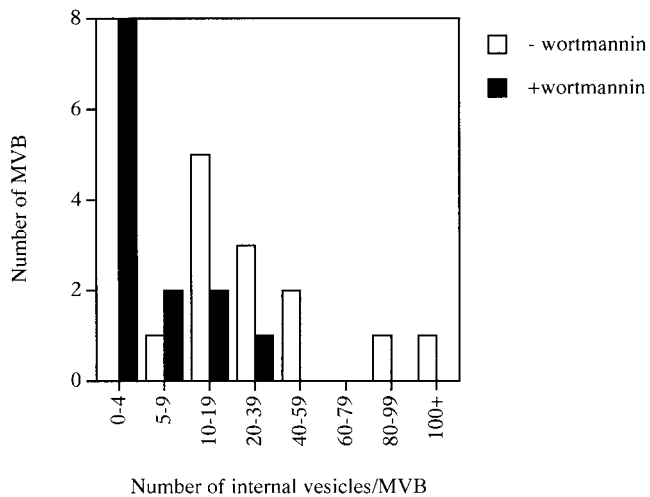


Figure 4. The effects of wortmannin on the number of internal vesicles per MVB. HEP-2 cells were incubated with HRP for 30 min at 37°C, chased for 3 h at 37°C, and then incubated with DAB/H₂O₂ at 4°C to crosslink the lysosomes. Cells were then incubated with anti-EGFR gold and EGF at 20°C in the absence of wortmannin and then chased at 37°C in the absence or the presence of wortmannin. The total number of internal vesicles in 13 MVBs per treatment was estimated by analysis of 70-nm serial sections.

delivered to the lysosome (see above). Therefore, it is likely that some loss of EGFRs from the perimeter membrane of the MVB does occur in wortmannin-treated cells when MVB–lysosome fusion is prevented.

To determine whether there is a general inhibition of exit from MVBs in wortmannin-treated cells or whether EGFRs are specifically retained on the perimeter membrane of MVBs, we determined the effect of wortmannin treatment on removal of TRs from MVBs. Lysosomes were cross-linked in the living cell as described above. Cells were then incubated with anti-EGFR gold and EGF at 20°C in the absence of wortmannin to allow EGFR internalization but retain EGFRs in the TR-containing endosome. Cells were then chased for 1 h at 37°C in the presence or absence of wortmannin. In the absence of wortmannin, TRs would be expected to be gradually removed from the maturing MVBs. Cells were then permeabilized and immunogold labeled using an antibody to the cytoplasmic domain of the TR as described in Materials and methods. As expected, mature MVBs accumulated in the absence of wortmannin for 1 h at 37°C have very few TRs compared with surrounding vesicles and tubules (Fig. 5, a and b). In wortmannin-treated cells,

although the MVBs are enlarged, there are also very few TRs on the perimeter membrane compared with surrounding vesicles and tubules (Fig. 5, b and c). Examining a single time point does not allow the effect of wortmannin on the rate of removal of TRs from the MVBs to be determined. However, counting the number of EGFR- and TR-gold particles in random thin sections shows that the ratio of EGFRs to TRs in MVBs accumulated in the presence of wortmannin compared with untreated cells is almost identical: 57:2 EGFRs to TRs in the absence of wortmannin and 51:2 in the presence of wortmannin ($n = 100$). Thus, although inward vesiculation is inhibited sorting of EGFRs from TRs continues.

The effects of microinjection of anti-PI 3'-kinase antibodies on inward vesiculation

To determine which PI 3'-kinase is involved in inward vesiculation, isotype-specific inhibitory antibodies to the p110 α and p110 β subunits of the type I kinases and to hVPS 34 (the type III kinase) were assessed for their effects on inward vesiculation. These antibodies have been shown to inhibit the respective PI 3'-kinase activities when microinjected into cells (Siddhanta et al., 1998). HRP-loaded lysosomes were cross-linked in the living cell, and then cells were microinjected with anti-PI 3'-kinase antibody and with 20 nm gold in order to locate the microinjected cells. Cells were then allowed to recover for a further 2 h at 37°C before incubation with anti-EGFR gold and EGF at 20°C. Cells were then chased at 37°C for 1 h before processing for EM. Control experiments were performed to confirm that the morphology of the cells, and the formation of MVBs was not affected by microinjection with 20 nm gold. The microinjected 20 nm gold was distributed frequently throughout the cytoplasm as single particles, although occasionally clusters of gold were observed in the cytoplasm or enclosed within a limiting membrane (Fig. 6 a). Microinjection of anti-p110 α antibody did not affect the morphology of the MVB at any dose of antibody (Fig. 6 c). Microinjection of anti-p110 β antibody did not appear to affect the morphology of the MVB at low doses. However, cells injected with larger doses of antibody had unusually small MVBs with very few internal vesicles, and EGFRs were often found in small vesicles and tubules rather than MVBs (Fig. 6 d). This suggests that p110 β is involved in early events in endocytic processing and may be involved in the delivery of membrane to the MVB. In cells microinjected with anti-hVPS34 MVBs had a reduced number of internal vesicles and the

Table II. The effects of wortmannin on inward vesiculation and membrane area per MVB

| Treatment | MVB diameter nm | Number of internal vesicles per MVB | Membrane area | | |
|-----------|--------------------|-------------------------------------|---------------|-------------------------------------|--------|
| | | | Perimeter | Internal vesicle μm^2 | Total |
| -Wo | 386 | 33.8 | 468.1 | 265.5 | 733.6 |
| +Wo | 698 | 6.3 | 1,530 | 49.5 | 1579.5 |

HEP-2 cells were incubated with HRP for 30 min at 37°C, chased for 3 h at 37°C, and then incubated with DAB/H₂O₂ at 4°C to crosslink the lysosomes. Cells were then incubated with anti-EGFR gold and EGF at 20°C in the absence of wortmannin and then chased at 37°C in the absence (-Wo) or the presence of wortmannin (+Wo). The total number of internal vesicles in individual MVBs was counted in serial 70-nm sections. The largest measured diameter of each MVB was assumed to be the diameter of the vacuole. Membrane areas were calculated assuming internal vesicles to have a diameter of 50 nm. 13 MVBs were measured for each treatment.

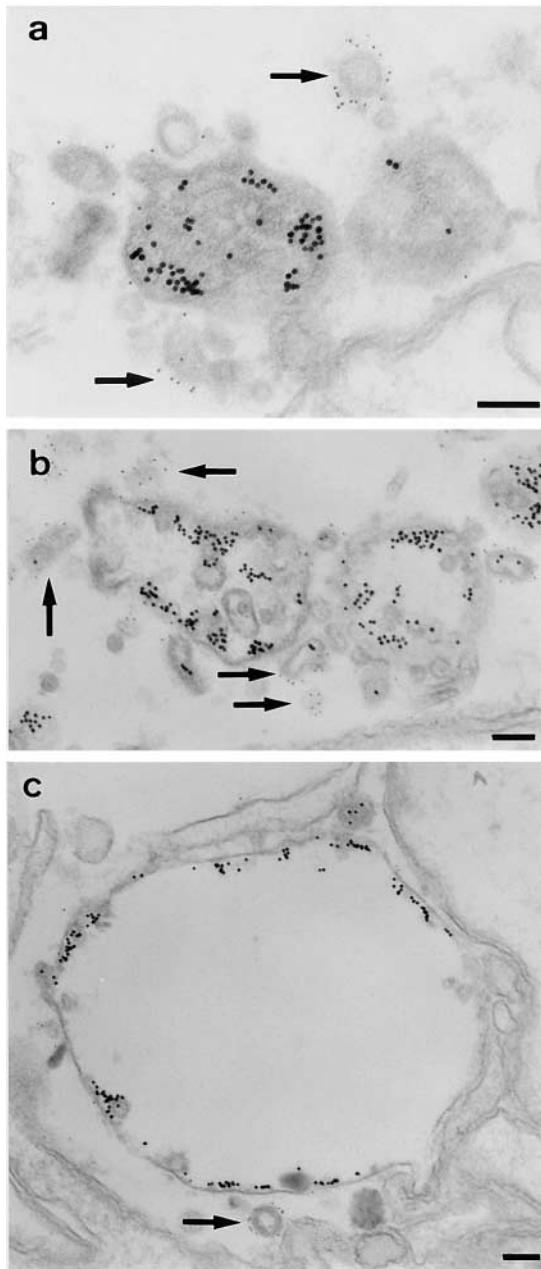


Figure 5. The effects of wortmannin on traffic of EGFRs and TRs through MVBs. HEp-2 cells were incubated with HRP for 30 min at 37°C, chased for 3 h at 37°C, and then incubated with DAB/H₂O₂ at 4°C to crosslink the lysosomes. Cells were then incubated with anti-EGFR gold (10 nm) and EGF for 1 h at 20°C in the absence of wortmannin and were then chased at 37°C for 1 h in the absence (a and b) or the presence of wortmannin (c). Cells were then permeabilized, fixed, and labeled with anti-TR antibody (5 nm gold). Note that in both the absence and the presence of wortmannin there are very few TRs on MVBs, but small vesicles labeling strongly for TRs (arrows) are often in close proximity. Bars, 0.1 μ m.

EGFRs were primarily on the perimeter membrane (Fig. 6 b). Although in some cases these MVBs were enlarged, they were not as large as those induced by treatment with wortmannin. It is possible that this difference in the results of anti-PI 3'-kinase antibody injection and wortmannin treatment could be explained by differences in the timing of PI 3'-kinase inhibition. Anti-PI 3'-kinase antibodies were in-

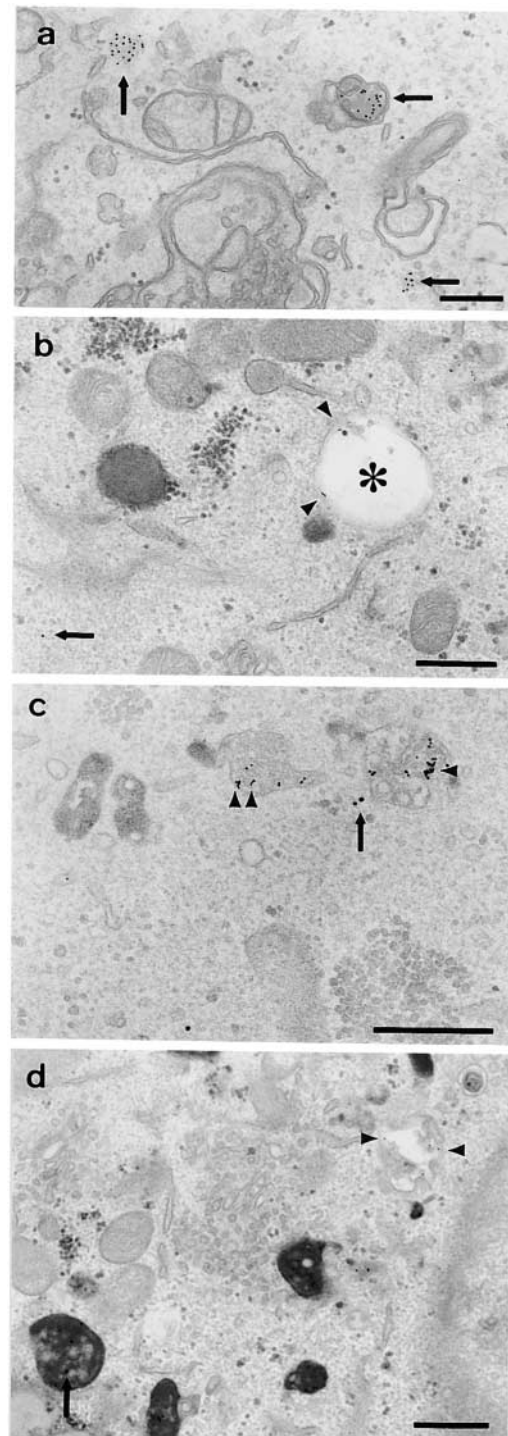


Figure 6. The effects of microinjection with anti-PI 3'-kinase antibodies on inward vesiculation in cells where the lysosomes have been cross-linked. HEp-2 cells were incubated with HRP for 30 min at 37°C, chased for 3 h at 37°C, and then incubated with DAB/H₂O₂ at 4°C to crosslink the lysosomes. Cells were then microinjected at 37°C with 20 nm BSA gold (arrows) alone (a) or coinjected with anti-hVPS34 antibody (b), anti-p110 α antibody (c), or anti-p110 β antibody (d). Cells were then incubated with 10 nm anti-EGFR gold (arrowheads) and EGF for 1 h at 20°C and were then chased at 37°C for 1 h. Microinjection with the anti-hVPS34 antibody caused the generation of enlarged MVBs containing few internal vesicles (asterisks). Microinjection with anti-p110 α had no effect on MVB formation, whereas microinjection with p110 β in some cases caused the generation of MVBs with comparatively few EGFRs, but they were not enlarged. Bars, 0.5 μ m.

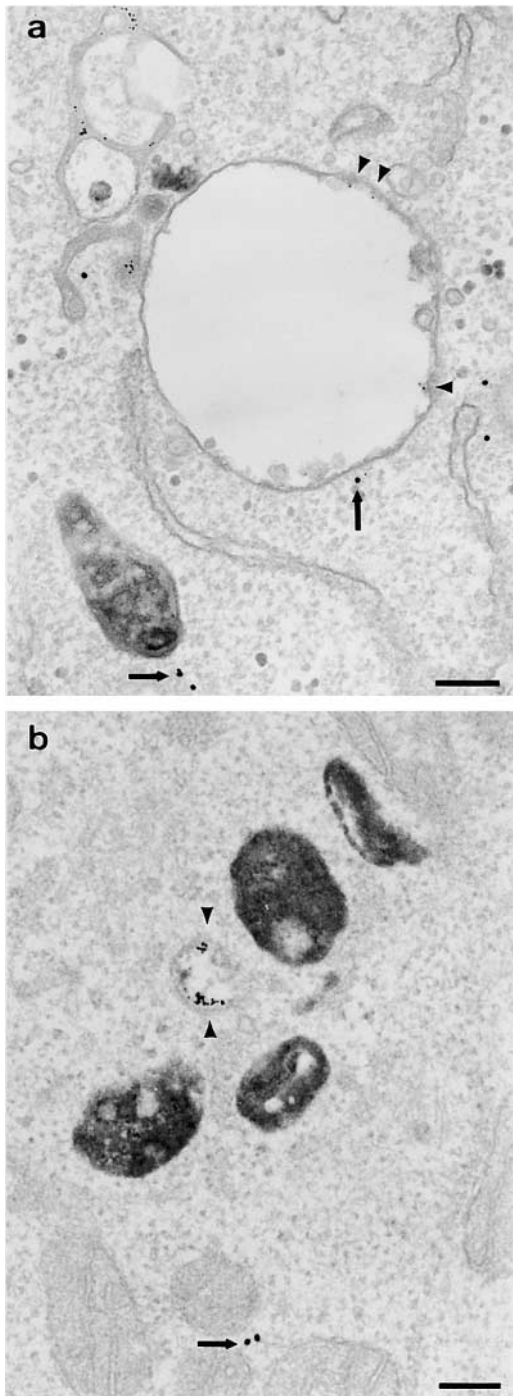


Figure 7. The effects of microinjection with anti-PI 3'-kinase antibodies after incubation with EGF at 20°C. HEp-2 cells were incubated with HRP for 30 min at 37°C, chased for 3 h at 37°C, and then incubated with DAB/H₂O₂ at 4°C to crosslink the lysosomes. Cells were then incubated with 10 nm anti-EGFR gold (arrowheads) and EGF for 1 h at 20°C and were then microinjected with 20 nm BSA gold (arrows) and anti-hVPS34 (a) or anti-p110β (b) at 20°C before incubation at 20°C for a further 30 min and then chase at 37°C for 1 h. Microinjection of anti-hVPS34 caused the generation of enlarged MVBs with very few internal vesicles and EGFRs on the perimeter membrane. Microinjection of anti-p110β antibody caused the formation of small MVBs with few internal vesicles and few EGFRs. Bars, 0.2 μm.

jected before the addition of anti-EGFR gold and EGF, whereas wortmannin was added to the cells after they had been incubated with anti-EGFR gold and EGF at 20°C.

Therefore, we performed further microinjection experiments to mimic the experiments using wortmannin as closely as possible. HRP-loaded lysosomes were cross-linked in the living cell, and then cells were loaded with anti-EGFR gold and EGF at 20°C. Cells were then microinjected with antibody and with 20 nm gold particles and were then incubated for a further 30 min at 20°C to allow time for the microinjected antibody to bind its antigen within the cell. Cells were then chased at 37°C for 1 h. Microinjection of the anti-hVPS34 antibody caused the formation of enlarged MVBs with very few internal vesicles and with EGFRs on the perimeter membrane, closely mimicking the effects seen with wortmannin treatment (Fig. 7 a). This effect appeared to be dose dependent. In cells with large amounts of injected 20 nm gold (indicating a larger volume of microinjected anti-PI 3'-kinase antibody), MVBs were large and had very few internal vesicles. Cells containing fewer injected gold particles seemed to have smaller MVBs with a few internal vesicles but still contained fewer internal vesicles than MVBs of control cells (unpublished data). Therefore, we conclude that the inhibition of inward vesiculation within the MVB by wortmannin is due to inhibition of the PI 3'-kinase hVPS34. Large doses of anti-p110β antibody sometimes led to a decrease in the size of MVBs and some accumulation of EGFRs in small vesicles and tubules (Fig. 7 b). This is an effect not observed with wortmannin treatment. p110β may be involved in an early step in the maturation of MVBs and is presumably more effectively inhibited by microinjection than by wortmannin treatment.

The effects of wortmannin on EGF-stimulated tyrosine phosphorylation

The above data suggests that EGFRs carry information that allows them to be retained on the perimeter membrane of MVB while TRs are being removed from this location to the recycling pathway, and that retention of EGFRs on the perimeter membrane is sufficient to target them for lysosomal delivery. This raises the question of the purpose of inward vesiculation. One possible purpose could be the removal of EGFRs from exposure to the cytoplasmic matrix and hence from substrates for their kinase activity. Therefore, we determined the effects of inhibition of inward vesiculation on the spectrum of proteins phosphorylated by the EGFR kinase. To enhance potential differences, lysosomes were cross-linked to prevent EGFR degradation, and then cells were incubated with EGF at 20°C followed by chase at 37°C in the presence or absence of wortmannin. MVBs with EGFRs on the perimeter membrane and exposed to the cytoplasm (in the presence of wortmannin) could be compared with MVBs with EGFRs on internal vesicles sequestered from the cytoplasm (in the absence of wortmannin). Tyrosine-phosphorylated proteins were detected by Western blotting cell lysates with antiphosphotyrosine antibody. As shown in Fig. 8, the pattern and strength of phosphorylation is increased significantly in wortmannin-treated cells. Since there is some loss of EGFRs from the perimeter membrane of MVBs in



Figure 8. The effects of wortmannin on EGF-stimulated tyrosine phosphorylation in cells were the lysosomes have been cross-linked. HEp-2 cells were incubated with HRP for 30 min at 37°C, chased for 3 h at 37°C, and then incubated with DAB/H₂O₂ at 4°C to crosslink the lysosomes. Cells were then incubated in the presence or absence of EGF at 20°C for 1 h and then chased at 37°C in the presence or absence of wortmannin (wo). Cell lysates were analyzed by SDS-PAGE followed by Western blotting with antiphosphotyrosine antibody.

wortmannin-treated cells in which the lysosomes have been cross-linked, we cannot be sure that all the tyrosine-phosphorylated proteins are on the perimeter membrane of MVBs in wortmannin-treated cells. However, these data are consistent with the possibility that inward vesiculation within MVBs removes EGFRs from potential cytoplasmic substrates.

Discussion

Wortmannin treatment has multiple effects on both endo- and exocytic pathways, including the stimulation of clathrin-dependent endocytosis (Martys et al., 1996; Spiro et al., 1996), the inhibition of endosome–endosome fusion *in vitro* (Jones and Clague, 1995; Li et al., 1995; Spiro et al., 1996), the inhibition of TR recycling (Martys et al., 1996; Shpetner et al., 1996; Spiro et al., 1996), the formation of enlarged late endosomes (Reaves et al., 1996; Bright et al., 1997; Kundra and Kornfeld, 1998; Fernandez-Borja et al., 1999), and inhibition of the perinuclear clustering and degradation of platelet-derived growth factor receptors (PDGFRs) (Joly et al., 1995; Shpetner et al., 1996). We have examined the effects of wortmannin on the endocytic pathway followed by activated EGFRs en route to the lysosome.

Wortmannin treatment inhibits inward vesiculation within MVBs but not EGFR delivery to the lysosome

In common with others, we find that wortmannin treatment causes the formation of enlarged endocytic vacuoles. After 1 h at 37°C, there is a minor enlargement of lysosomes, but the enlargement of vacuoles, recognizable as MVBs because they lack dense content and usually contain one or more small vesicles, is more marked. The enlarged MVBs frequently have comparatively few internal vesicles, and the EGFRs are found more frequently on the perimeter membrane compared with cells not treated with wortmannin. Wortmannin-induced enlargement of late endosomes and lysosomes has been proposed to be due to an inhibition of exit of proteins like mannose 6-phosphate receptor from these structures (Kundra and Kornfeld, 1998), an inhibition of reformation of lysosomes after endosome–lysosome fusion (Bright et al., 1997), or an inhibition of inward vesiculation (Fernandez-Borja et al., 1999). In the latter study on a human melanoma cell line, the authors found that wortmannin treatment generated enlarged vacuoles with few in-

ternal vesicles and proposed that wortmannin inhibited the invagination and/or pinching off of intraluminal vesicles of both MHC class II compartments and of other components of the endocytic pathway. A recent study from Bright et al. (2001) found that prolonged wortmannin treatment of NRK fibroblasts did not prevent the accumulation of internal vesicles within enlarged endocytic vacuoles and concluded that vacuolar enlargement was due to inhibition of exit of membrane from these structures.

We have developed a technique that allows the maturation of MVBs to be followed in the absence of MVB–lysosome fusion (Futter et al., 1996). By preincubation of HRP-loaded lysosomes with DAB/H₂O₂, which cross-links the lysosome in the living cell, MVB–lysosome fusion is prevented, but maturation of the MVB, in which inward vesiculation transfers EGFRs to the inner vesicle, proceeds. By including wortmannin in this protocol, we have demonstrated that there is a fivefold reduction in the accumulation of internal vesicles within the MVB and that EGFRs remain primarily on the perimeter membrane. Estimating the amount of membrane per MVB suggests that vacuolar enlargement cannot be explained solely by an inhibition of inward vesiculation. Therefore, it is likely that both inhibition of inward vesiculation and inhibition of exit of proteins, such as MPR, from the MVB contributes to the vacuolar enlargement. However, wortmannin-induced inhibition of exit from MVBs is selective, since TRs are efficiently removed from the maturing MVBs. This observation needs to be reconciled with several published studies which have reported that wortmannin treatment inhibits TR recycling (Martys et al., 1996; Shpetner et al., 1996; Spiro et al., 1996) and our finding that wortmannin inhibits the recycling of the small proportion of internalized EGF that is normally recycled. EM demonstrates that the majority of TRs in wortmannin-treated cells is in endosomal tubules, a compartment which is expanded in these cells (unpublished data; Shpetner et al., 1996) rather than in MVBs. Therefore, it is probable that it is within tubules rather than in MVBs that the trafficking of these recycling receptors is being inhibited.

Despite the inhibition of inward vesiculation, EGFRs are still delivered to the lysosome in the presence of wortmannin, and EGF degradation is largely unaffected. Although some proteolytic processing of EGF has been reported to occur in prelysosomal compartments (Schaudies et al., 1987; Renfrew and Hubbard, 1991), we have shown previously that MVB–lysosome fusion is required for the degradation of EGF to TCA-soluble products (Futter et al., 1996). Therefore, we conclude that MVB–lysosome fusion is not affected by wortmannin treatment. The EGFR is degraded with slower kinetics in wortmannin-treated cells. Similarly, the rate but not extent of degradation of Semliki Forest virus was found to be reduced by wortmannin treatment (Martys et al., 1996). We have been unable to detect degradation products of the EGFR indicative of partial degradation of the receptor, suggesting that the EGFR is unstable in the perimeter membrane of the lysosome. There is no indication in our studies of the means whereby the cytoplasmic domain of the EGFR is degraded in wortmannin-treated cells. Previous studies would support the notion that MVB–lysosome fusion can occur in the presence of wortmannin, since

Bright et al. (1997) identified lysosomes with a pulse of BSA gold and showed that a second pulse of BSA gold could reach the previously internalized gold even in the presence of wortmannin. These authors have shown that endosome–lysosome fusion results in the generation of a hybrid compartment from which dense core lysosomes must be reformed (Mullock et al., 1998). Wortmannin treatment reduced the number of dense core lysosomes within the cell, suggesting an inhibition of the reformation of dense lysosomes (Bright et al., 1997).

The delivery of PDGFRs to the lysosome has been reported to be inhibited by wortmannin (Joly et al., 1995; Shpetner et al., 1996). In the latter study, fluid phase markers could reach the lysosome in the presence of wortmannin, again indicating that wortmannin did not prevent endosome–lysosome fusion but rather that wortmannin inhibited the targeting of PDGFRs to that pathway. In contrast, we found that wortmannin treatment did not affect the targeting of EGFRs to the lysosomal pathway. We have proposed previously that movement of EGFRs onto the internal vesicles of MVBs is critical for removing EGFRs from the recycling pathway and targeting them to the lysosome. Inactivation of the tyrosine kinase activity of the EGFR inhibited movement of the EGFR onto the internal vesicles of MVB and, in this case, the EGFR recycled (Felder et al., 1990). In wortmannin-treated cells, the movement of the EGFR onto the internal vesicles is also inhibited, but in this case the EGFR is not recycled; it is delivered to the lysosome and degraded. Taken together, these results suggest that we have resolved two stages in the inward vesiculation process: (a) selection of EGFRs for inclusion in the internal vesicle, which requires tyrosine kinase activity but not PI 3'-kinase activity and is represented by the clusters of EGFRs on the perimeter membrane of MVBs in wortmannin-treated cells and (b) vesicle formation, which requires PI 3'-kinase activity. EGFR tyrosine kinase activity but not PI 3'-kinase activity is, therefore, necessary to retain the EGFR in the MVB and prevent recycling.

The fact that wortmannin treatment inhibits lysosomal targeting of PDGFRs (Shpetner et al., 1996) but not EGFRs (this study) could be because lysosomal targeting of PDGFRs and EGFRs are regulated differently. Alternatively, in our experiments wortmannin was only added after EGFRs had been internalized at 20°C, and so it is possible that a PI 3'-kinase is involved in an early step in the processing of EGFRs proximal to the 20°C block. Consistent with this possibility is our finding that when cells were treated with wortmannin before addition of EGF, there was a small reduction in the number of EGFRs reaching the lysosome, and also when anti-PI 3'-kinase antibodies were microinjected before addition of EGF, delivery of EGFRs to MVBs was, in some cases, partially inhibited (see below).

hVPS34 is the PI 3-kinase required for inward vesiculation

There are three classes of mammalian PI 3'-kinases, which differ both in their regulation and their substrate specificities (Rameh and Cantley, 1999). Class I and class III PI 3'-kinases have distinct roles in endocytic traffic. Microinjection of inhibitory anti-p110 α antibody inhibits TR recycling,

whereas microinjection of anti-hVPS34 but not anti-p110 α inhibits transit of internalized PDGFRs to a perinuclear compartment (Siddhanta et al., 1998). Here, we show that microinjection with inhibitory anti-hVPS34 but not anti-p110 α or anti-p110 β causes the generation of enlarged MVBs. These MVBs contain reduced numbers of internal vesicles, and EGFRs remain on the perimeter membrane. Therefore, we conclude that hVPS34 is the PI 3'-kinase required for inward vesiculation within MVBs. Some reduction in size of MVBs was observed in cells microinjected with anti-p110 β (and hVPS34 when microinjected before the addition of EGF), suggesting a possible role of these PI 3'-kinases in an early step in the sorting of EGFRs, which would be consistent with their recruitment by rab5 to early endosomes (Christoforidis et al., 1999). PDGFR mutants that fail to bind PI 3'-kinases are unable to enter the lysosomal pathway, suggesting a role of the class I PI 3'-kinases in this effect (Joly et al., 1995). On the other hand, microinjection of anti-hVPS34 inhibited accumulation of PDGFRs in the perinuclear area, indicating a role for this PI 3'-kinase in sorting of PDGFRs (Siddhanta et al., 1998). It is not possible to determine the roles of PI 3'-kinases in early events in the sorting of EGFRs from the results of our experiments, but both p110 β and hVPS34 may have roles in addition to that demonstrated here for hVPS34 in inward vesiculation.

The product of hVPS34, PI(3)P, is specifically recognized by proteins containing a cysteine-rich zinc finger domain, termed the FYVE domain. FYVE domains are found in several proteins implicated in membrane trafficking, including EEA1, Fab1, Vac1, Hrs (Stenmark and Aasland, 1999), and rabenosyn 5 (Nielsen et al., 2000). Association of EEA1 and rabenosyn 5 with endosomes involves binding to both PI(3)P and rab5-GTP (Patki et al., 1998; Simonsen et al., 1998; Nielsen et al., 2000). EEA1 is required for endosome–endosome fusion *in vitro* (Mills et al., 1998; Simonsen et al., 1998) and may act as a tethering factor (Pfeffer, 1999), mediating endosome–endosome docking (Christoforidis et al., 1999). Fab1 is a PI(3)P 5-kinase and therefore phosphorylates the product of VPS34. Mutations in yeast Fab1 cause expansion of the vacuole and retention of carboxypeptidase S, which is normally degraded within the lumen of the vacuole, on the perimeter membrane (Odorizzi et al., 1998). This shows a striking similarity with the phenotype observed for EGFRs in wortmannin-treated cells. Hrs has been localized to endosomes (Komada et al., 1997) where it can recruit clathrin (Raiborg et al., 2001), becomes tyrosine phosphorylated in response to several growth factors, including EGF, (Komada and Kitamura, 1995; Komada et al., 1997), and tyrosine phosphorylation causes the release of Hrs from endocytic vacuoles (Urbe et al., 2000). In addition to the FYVE domain, the PX domain contained in SNX3 has been shown recently to bind PI(3)P, and this domain is found in a family of proteins termed SNXs (Xu et al., 2001). SNX1 binds to the EGFR and can also interact with Hrs (Chin et al., 2001). Overexpression of SNX1 enhances EGFR degradation (Kurten et al., 1996), whereas overexpression of Hrs or the SNX1 binding domain of Hrs inhibits EGFR degradation, suggesting that Hrs may regulate the traffic of EGFRs through SNX1 (Komada and Soriano, 1999; Chin et al., 2001). Thus, there are multiple proteins containing

domains which specifically recognize PI(3)P. Some of these proteins have been implicated in the regulation of the traffic of the EGFRs, but as yet no role of any of these proteins in either selection of cargo for inclusion in internal vesicles or in vesicle formation has been demonstrated directly. Our data suggest that the generation of PI(3)P-rich domains on the perimeter membrane of the MVB is required for vesicle formation but not for lysosomal targeting of EGFRs. Given that Hrs and SNX1 have been implicated in lysosomal targeting of the EGFR, it is possible that these proteins play a role at an early step in the sorting of endocytosed EGFRs before the generation of internal vesicles within MVBs and that other PI(3)P binding proteins, such as Fab1, are required for inward vesiculation.

The generation of PI(3)P-rich domains on the perimeter membrane of the MVB requires the localization of hVPS34 to that domain. hVPS34 localization within the endosomal membrane may be regulated by rab5, since rab5 is localized to the endosomal membrane and hVPS34 interacts indirectly with activated rab5 (Christoforidis et al., 1999). Unlike the class I PI 3'-kinases, hVPS34 has not been shown to be regulated directly by growth factors. However, EGFR activation has been shown recently to induce activation of rab5a (Barbieri et al., 2000), and so EGF stimulation of rab5 may play a role in the localization of hVPS34. A recent study localizing PI(3)P at the light and EM level found this lipid to be highly enriched on early endosomes and on the internal vesicles of MVBs (Gillooly et al., 2000). This would be in keeping with the generation of patches of PI(3)P on the perimeter membrane, leading to inward vesiculation. Yeast studies suggest that PI(3)P must be transported to the vacuole/lysosome for turnover (Wurmser and Emr, 1998), and so PI(3)P would be expected to remain on the internal vesicles of MVBs until MVB-lysosome fusion.

Taken together, a model emerges whereby the rab5-dependent recruitment of hVPS34 results in the generation of microdomains rich in PI(3)P that recruit effector proteins, leading to inward vesiculation.

Sequestration of activated EGFRs within the lumen of MVBs removes the receptor from potential phosphotyrosine substrates

Retention on the perimeter membrane of MVBs is sufficient for the lysosomal targeting and degradation of activated EGFRs, raising the question of the purpose of the inward vesiculation process. Several studies suggest that EGFRs can continue to signal while they remain on the perimeter membranes of the endocytic pathway. Thus, it has been shown that EGFRs remain phosphorylated in the endosome (Wada et al., 1992) but become inactivated before degradation (Burke et al., 2001). Although some substrates of the EGFR kinase are phosphorylated on the plasma membrane, others, such as annexin 1 (Futter et al., 1993) and Eps8 (Burke et al., 2001), appear to be phosphorylated by the endosomal but not the plasma membrane EGFR kinase. Inhibition of endocytosis of EGFRs by expression of mutant dynamin inhibits Erk1/2 MAP kinase signaling (Vieira et al., 1996) and Erk2 activation can be initiated from the endosomal EGFR kinase in a cell-free system (Xue and Lucocq, 1998). Activated Raf-1 and Mek have been localized to endosomes after EGF stimulation

(Pol et al., 1998). Therefore, it is likely that endocytosed EGFRs, which continue to be exposed to the cytoplasm, continue to signal. When they become sequestered on internal vesicles of MVBs, their access to cytosolic substrates is presumably lost. We have addressed this question directly by comparing the activity of the EGFR kinase in cells in which EGFRs are retained on, with those in which they are removed from, the perimeter membrane. When EGFRs were retained on the perimeter membrane, tyrosine phosphorylation of a large number of proteins was enhanced, demonstrating that there are a large number of potential substrates of the EGFR kinase available to the cytoplasmically exposed EGFR. Therefore, a likely purpose of inward vesiculation is to sequester the activated EGFR away from cytoplasmic substrates and thereby attenuate its signal transduction.

Materials and methods

Reagents

Monoclonal antibody to the extracellular domain of the EGFR (108) and the cytoplasmic domain of the TR were gifts from J. Schlessinger (New York University Medical Center, New York, NY) and I. Trowbridge (The Salk Research Institute, San Diego, CA), respectively. Rabbit polyclonal antibodies against hVPS34, p110 α , and p110 β were as previously described (Siddhanta et al., 1998; Christoforidis et al., 1999). Rabbit polyclonal antibody against the cytoplasmic domain of the EGFR was raised by immunization with the peptide Cys-DVVDVADEYLIPQ coupled to KLH. Monoclonal antiphosphotyrosine antibody (PY20) was from ICN Biomedicals. HRP (type II) and wortmannin were from Sigma-Aldrich. Radiolabeled EGF and colloidal gold sols were made as described previously (Slot and Geuze, 1985; Futter et al., 1996).

Cell culture and incubation conditions

HEp-2 cells were maintained in DME containing 10% FCS in a 5% CO₂ atmosphere. Lysosomes were loaded with HRP and cross-linked in living cells as described in Futter et al. (1996). EGF was used at a concentration of 200 ng/ml, and wortmannin was used at a concentration of 200 nM.

EM

For conventional EM, cells grown on glass coverslips were incubated with HRP and gold probes under different conditions as described in the text and were then fixed, processed, and treated with tannic acid as described previously (Stinchcombe et al., 1995). Coverslips were embedded on Epon stubs, and the coverslips were then removed by immersion in liquid nitrogen. Cells were sectioned en face, stained with lead citrate, and viewed in Philips 400 or CM12 electron microscopes.

For morphometry, serial 70-nm sections were cut, the diameter of the MVB was measured, and the number of internal vesicles per MVB were counted. An MVB was defined as a vacuole that contained anti-EGFR gold particles and had a diameter of greater than 200 nm. The total membrane area per MVB was estimated by assuming the diameter of internal vesicles to be 50 nm.

To quantitate the numbers of TRs and EGFRs/MVBs, lysosomes were cross-linked and cells were incubated with anti-EGFR gold, EGF, and wortmannin as described above. Cells were then permeabilized and labeled with an antibody to the cytoplasmic domain of the TR as described in Futter et al. (1998). The number of anti-EGFR gold particles and anti-TR gold particles per MVB in random thin sections were counted.

Microinjection of anti-PI 3'-kinase antibodies

HEp-2 cells were grown on Celloclate glass-gridded coverslips (Eppendorf). Microinjection was performed using an Eppendorf semiautomated transfection system, and the needles were pulled using a Sutter p-97 micropipette puller. 1.5- μ l aliquots of anti-hVPS34, anti-p110 α , and anti-p110 β antibodies (3–4 mg/ml in PBS) were mixed with equal volumes of BSA-stabilized 20 nm gold and microinjected into the cytoplasm of cells. After embedding in Epon, ultrathin sections were cut from the appropriate grid area containing the microinjected cells and examined by EM.

Measurement of EGF degradation

The intracellular degradation of ¹²⁵I-EGF results in the generation of TCA-soluble products of degradation that diffuse rapidly 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 degradation of EGF could be determined.

Measurement of EGFR degradation

After incubation of cells with EGF, cell lysates were subjected to SDS-PAGE and Western blotted using a rabbit polyclonal antibody against the cytoplasmic domain of the EGFR. Western blots were visualized using ECL-Plus (Amersham Pharmacia Biotech) and quantitated using a Fuji PhosphorImager.

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