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EGF stimulates annexin 1-dependent inward vesiculation in a multivesicular endosome subpopulation

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Here we show that EGF and EGF receptor (EGFR) are trafficked through a subpopulation of multivesicular endosomes/bodies (MVBs) that are distinct from morphologically identical vacuoles that label for the late endosomal marker lyso-bisphosphatidic acid (LBPA). EGF stimulation increases both MVB biogenesis and inward vesiculation within EGFR-containing MVBs. Deletion of annexin 1, a substrate of EGFR tyrosine kinase, abolishes the effect of EGF stimulation on inward vesiculation. This phenotype is reversible by transfection with wild-type but not Y21F phosphorylation mutant annexin 1. Deletion of annexin 1 has no effect on EGF-stimulated MVB biogenesis, suggesting that MVB biogenesis and inward vesiculation within MVB are mediated by separate mechanisms. Loss or depletion of annexin 1 has no effect on EGF degradation and causes only a small delay in EGFR degradation, indicating that annexin 1 operates downstream of Hrs- and ESCRT-mediated sorting and is required solely for EGF-stimulated inward vesiculation. Annexin 1 accumulates on internal vesicles of MVB after EGF-stimulated inward vesiculation, suggesting that it may be required for a late stage in inward vesiculation.

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Introduction

Following internalisation from the plasma membrane, activated EGF receptors (EGFR) are sorted onto the internal vesicles of multivesicular endosomes/bodies (MVBs), while proteins that are to be recycled remain on the perimeter membrane. When all recycling proteins have been removed, the MVB fuses with the lysosome and the EGFR are degraded. Components of the machinery responsible for sorting within the MVB have recently been identified in yeast, where three protein complexes (endosomal sorting complex required for transport (ESCRT) I, II and III) are thought to act sequentially in the selection of cargo proteins for inclusion on the internal vesicles of MVB (Katzmann *et al*, 2001; Babst *et al*, 2002a, b). Homologues for many of the ESCRT components have been identified in mammalian cells, suggesting conservation in the core MVB sorting machinery. Vps27 and its mammalian homologue, Hrs, recruit ESCRT complexes to the endosomal membrane (Bache *et al*, 2003; Katzmann *et al*, 2003) and interference with Hrs expression or function affects both cargo selection and the generation of internal vesicles within MVB (Bilodeau *et al*, 2002; Raiborg *et al*, 2002; Urbe *et al*, 2003), suggesting a link between Hrs/ESCRT-mediated cargo selection and internal vesicle formation.

Although the mammalian ESCRTS are believed to act in a manner similar to that proposed in yeast, this has not been fully investigated. Moreover, in mammalian cells, MVBs sort receptor tyrosine kinases and, hence, play an important role in regulating mitogenic signalling. EGF-stimulated tyrosine kinase activity has previously been shown to be required for sorting of EGFR onto internal vesicles of MVB (Felder *et al*, 1990), but the effect of EGF stimulation on MVB formation or internal vesicle formation within MVB has not been reported.

We have previously used the EGFR itself as a marker of MVBs (Hopkins *et al*, 1990; Futter *et al*, 1996, 2001), and have therefore examined the regulation of MVB biogenesis solely in EGF-stimulated cells. Another frequently used marker of MVBs or late endosomes/lysosomes is lyso-bisphosphatidic acid (LBPA) (Kobayashi *et al*, 1998). We have shown that within EGFR-containing MVB, phosphatidyl inositol (PI) 3-kinase activity is required for generation of internal vesicles (Futter *et al*, 2001), while Bright *et al* (2001) have shown that inhibition of PI3-kinase activity does not prevent the accumulation of LBPA-containing internal membranes within late endosomes. Taken together, these data suggest that more than one class of MVB or more than one type of internal membrane within MVB might exist. The latter possibility is supported by the demonstration of separate PI3-phosphate (PBP)-enriched and LBPA-enriched areas of internal membranes within the same vacuole (Gillooly *et al*, 2000).

Here, we show that MVBs containing activated EGFR are distinct from those containing LBPA and, as EGF stimulation causes an increase in the number of MVBs/unit cytoplasm, at least a proportion of EGFR-containing MVBs are formed *de novo* as a result of EGF stimulation. Furthermore, we show that EGF stimulation promotes formation of internal vesicles specifically within EGFR-containing MVBs, and that this is dependent on tyrosine phosphorylation of annexin 1.

Results

EGF and LBPA coexist only in vacuoles in the later stages of the EGF degradation pathway

To investigate the possibility that EGF is trafficked to lysosomes through a specific subpopulation of vacuoles, cells

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were stimulated with fluorescent EGF and the relative localisation of fluorescent EGF and LBPA was determined by immunofluorescence microscopy. Vacuoles that contained fluorescent EGF and those that stained for LBPA were mutually exclusive 10 and 30 min after EGF stimulation (Figure 1A and B), but limited colocalisation was seen at 60 and 90 min (Figure 1C and D). By 60–90 min, the majority of EGF should have reached the lysosomal compartment in these cells (Futter *et al*, 1996).

To clarify the nature of these vacuoles, lysosomal staining was excluded by use of a previously characterised technique to load the lysosomal compartment with insoluble DAB reaction product (Futter *et al*, 1996). This technique accumulates HRP in vacuoles that are multilamellar and contain the bulk of the cell lysosomal associated membrane protein (LAMP 1) and acid hydrolase activity. We therefore term them lysosomes (see Discussion). Performing the DAB reaction in the living cells fills the lysosomes with insoluble reaction product preventing MVB–lysosome fusion. After EGF stimulation, EGF accumulates in vacuoles that are exclusively multivesicular, do not stain for LAMP 1 and do not degrade EGF (Futter *et al*, 1996). Antibody staining of antigens within the lumen of crosslinked vacuoles is eliminated. In the present study, cells were stimulated with fluorescent EGF without crosslinking or following lysosomal crosslinking, and stained with antibodies to LBPA and LAMP 1. The crosslinking technique has no effect on LAMP 1 staining, as the epitope exists on the cytosolic domain of this limiting membrane protein.

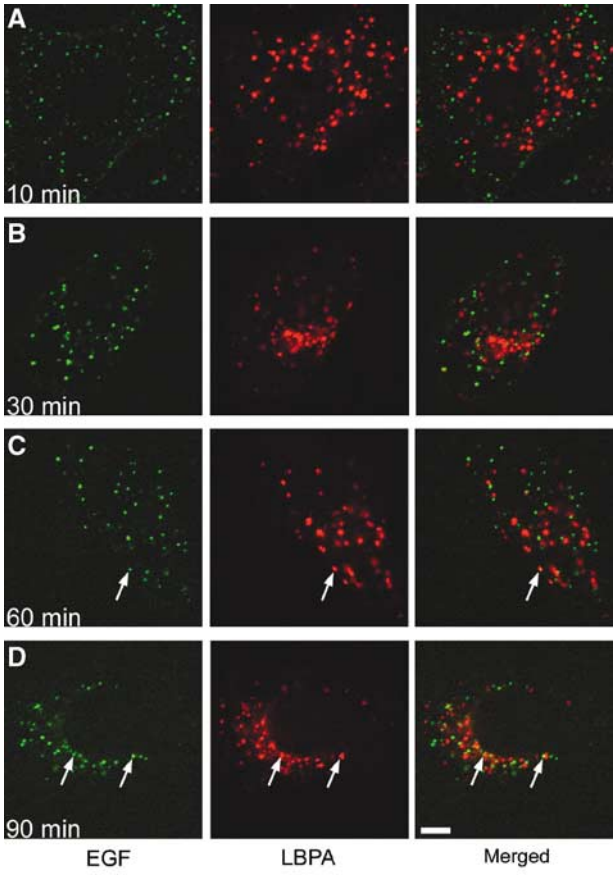

Figure 1 EGF does not enter LBPA-positive compartments till late in the endocytic pathway. HEp2 cells were stimulated with fluorescent EGF for the indicated times **A** = 10 min, **B** = 30 min, **C** = 60 min, and **D** = 90 min. Cells were fixed prior to permeabilisation and then stained with an antibody against LBPA. Colocalisation between EGF and LBPA (arrows) appears as yellow in the merged image. Colocalisation was limited and observed only after 60 and 90 min. Bar = 5 μm.

Cells that had not had their lysosomes crosslinked showed significant but not total colocalisation between LBPA and LAMP 1 (Figure 2A). In HEp2 cells, LAMP 1 is a good marker of the degradative compartment (Futter *et al*, 1996), and so these results suggest that the majority of LBPA is in the lysosome. As before, very limited colocalisation was seen between EGF and LBPA; the few vacuoles that did stain positive for both EGF and LBPA also stained strongly for LAMP 1, suggesting that these vacuoles are lysosomes (Figure 2A). It is likely that much of the EGF that had reached the lysosome had been degraded, explaining the relatively low levels of colocalisation between EGF and LAMP 1.

The total population of vacuoles staining for LBPA was markedly reduced by lysosomal crosslinking (compare Figure 2A and B), again suggesting that the majority of LBPA-containing vacuoles are lysosomal. The remaining vacuoles that stained for LBPA contained very little LAMP 1 (Figure 2B), and therefore are not merely lysosomes that have failed to crosslink fully. Of all vacuoles that did not stain for LAMP 1, those containing EGF and those that stained for LBPA remained mutually exclusive despite the extended incubation time. In cells incubated with leupeptin to prevent EGF degradation, EGF and LBPA still remained mutually exclusive in crosslinked cells, indicating that if the LBPA-containing compartments that remain after lysosomal crosslinking are degradative in nature, they are not involved in the trafficking and degradation of EGF (see Supplementary data). These results have been reproduced in HeLa, A431, HEK293 and NRK cells, indicating that this distribution of LBPA and EGF is a widespread phenomenon.

Taken together, these results suggest that while there is a population of LBPA-containing vacuoles that neither become crosslinked nor stain for LAMP 1 and therefore are not lysosomes, EGF is not trafficked through these vacuoles.

Nonlysosomal vacuoles containing EGFR or LBPA are distinct yet morphologically indistinguishable, and are multivesicular

In order to more closely investigate the nature and morphology of EGF- and LBPA-containing vacuoles, cryo-immuno-electron microscopy (cryo-immuno-EM) was performed on EGF-stimulated cells. In cells where lysosomes had not been crosslinked, double labelling with antibodies to EGFR and LBPA labelled two morphologically distinct compartments: those that were wholly multivesicular and those with a multilamellar component. While in wholly multivesicular vacuoles, EGFR and LBPA labelling was again mutually exclusive (Figure 3A and B), some colocalisation was evident in vacuoles that were multilamellar (Figure 3C). All labelling of multilamellar vacuoles was abolished in crosslinked samples (Figure 3F), and once again EGFR staining and LBPA staining were mutually exclusive. Significantly, these remaining structures labelling for either LBPA or EGFR were multivesicular (Figure 3D and E). Some multivesicular vacuoles showed labelling for neither EGFR nor LBPA, suggesting that further subpopulations of multivesicular vacuoles may exist.

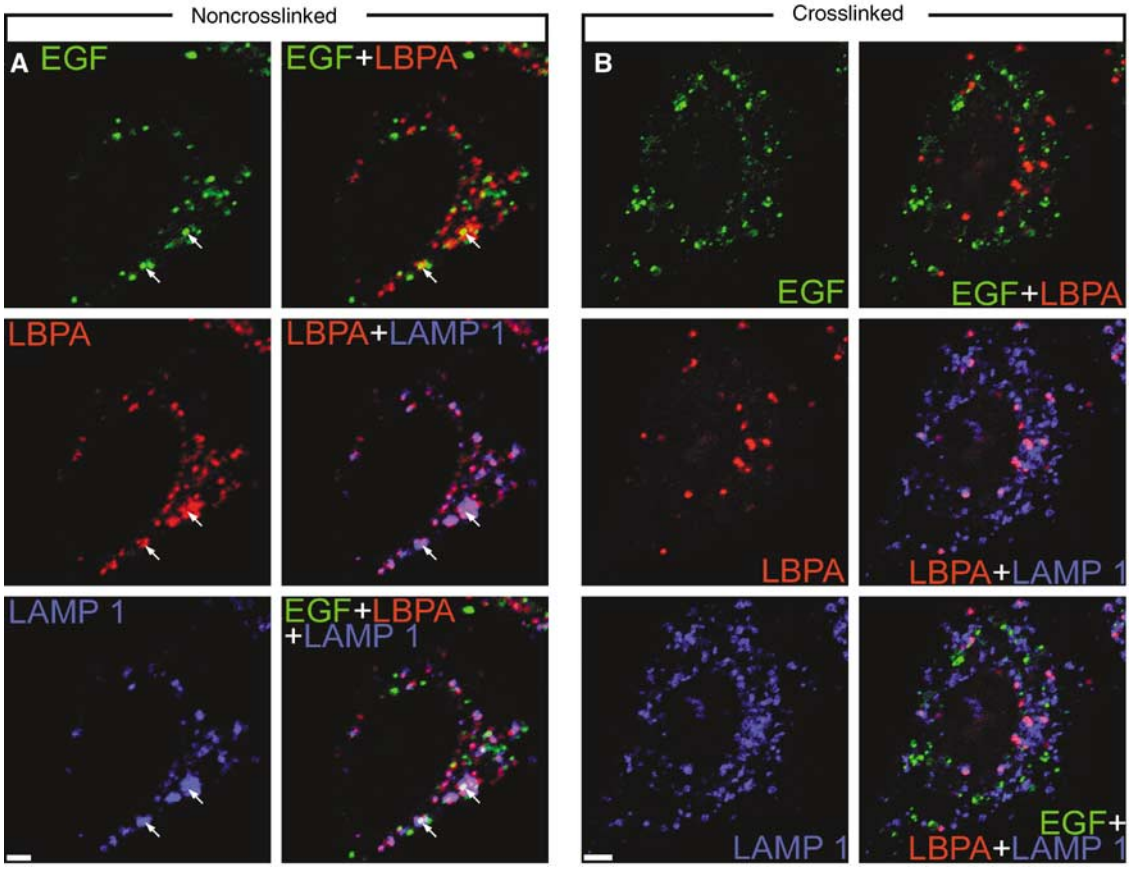

Figure 2 Inhibition of MVB–lysosome fusion inhibits delivery of EGF to LBPA-positive lysosomes. HEp2 cells were loaded with HRP in preparation for crosslinking. Noncrosslinked cells treated without DAB/H_2O_2 were stimulated with fluorescent EGF at 37°C for 60 min (**A**). Cells that were crosslinked with DAB/H_2O_2 were stimulated with fluorescent EGF for 180 min at 37°C (**B**). Cells were fixed prior to permeabilisation and then stained with antibodies against LBPA and LAMP 1. Colocalisation between EGF and LBPA appears as yellow in the merged images, LBPA and LAMP 1 as magenta, and colocalisation between all three (examples shown by arrows) as white. Vacuoles staining for both EGF and LBPA in noncrosslinked cells always also stain for LAMP 1. Crosslinking of lysosomes abolishes both the majority of LBPA staining and any colocalisation between EGF and LBPA. Bars = 5 μm.

Triple labelling experiments showed that both EGFR- and LBPA-containing populations also stained for the tetraspanin CD63 (Figure 3G and H), which has also previously been characterised as a late endosomal marker localised to internal vesicles (Fukuda, 1991; Escola *et al*, 1998; Kobayashi *et al*, 1999).

These findings confirm that EGF and its receptor exist in a subpopulation of MVBs that, while morphologically very similar to those containing LBPA, are distinct. Furthermore, the presence of CD63 in both of these populations indicates that while not exclusive to MVBs, CD63 may be a more accurate marker of MVBs than other molecules that appear to be present only in certain subpopulations.

EGF stimulates MVB biogenesis

That a subpopulation of MVBs that contains EGF and EGFR exists raises the question of whether EGF is trafficked into a pre-existing population following internalisation or whether that population is formed *de novo* as a result of stimulation. We therefore examined the effect of EGF stimulation on all populations of MVBs within cells. After lysosomal crosslinking, cells were incubated in the presence or absence of EGF for 60 min. Both samples were incubated in the presence of BSA gold as a fluid phase marker to aid in the identification of all MVBs accessible from the cell surface during the period of treatment (Figure 4A). MVBs were defined as vacuoles containing BSA gold that were $\geqslant$200 nm in diameter and contained $\geqslant$1 internal vesicles.

When combined, the results from three separate experiments showed a remarkably consistent basal level of MVB existence, as judged by the percentage of total cytoplasmic area that was MVBs (Figure 4B). This level was almost doubled in cells stimulated with EGF for an equal time period. The question of whether this increase in total MVB area, and therefore cellular MVB volume, is brought about by the formation of new MVBs or an increase in the size of pre-existing MVBs was also addressed. While there was some increase in the mean area of individual MVBs measured following EGF stimulation (Figure 4D), the number of MVBs per unit cytoplasm was also significantly increased (Figure 4C). Therefore, treatment with EGF does stimulate MVB biogenesis.

EGF stimulates internal vesicle formation

The effect of EGF stimulation on the number of internal vesicles within MVBs was also quantified. Internal vesicles were clearly visible in MVBs both in the presence or absence of EGF stimulation (Figure 4A). However, following

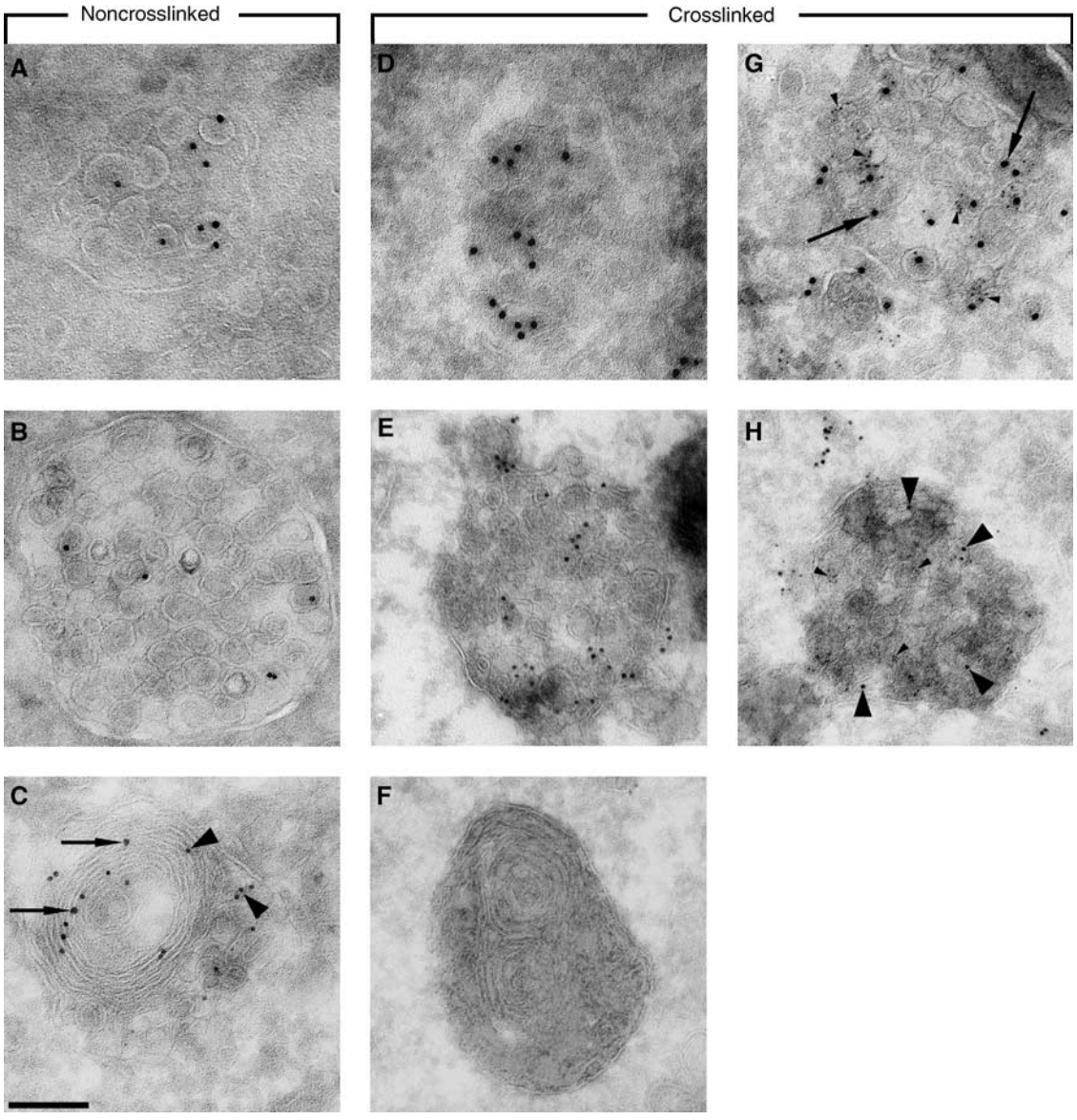

Figure 3 EGFR and LBPA appear in distinct MVBs that also contain CD63, and colocalise only in lysosomes. HEp2 cells were loaded with HRP in preparation for crosslinking. Noncrosslinked cells treated without DAB/H_2O_2 (**A–C**) were stimulated with EGF for 60 min at 37°C and those that were crosslinked with DAB/H_2O_2 (**D–H**) were stimulated with EGF for 180 min at 37°C. Cells were prepared for cryo-immuno-EM and ultrathin frozen sections either double labelled for EGFR with 15 nm gold (arrows), and LBPA with 10 nm gold (arrowheads) (A–F), or triple labelled for EGFR and LBPA as before, and also for CD63 with 5 nm gold (small arrowheads) (G, H). Panels A and D show MVBs labelling for EGFR, panels B and E MVBs labelling for LBPA and panels C and F lysosomes. Note that in the noncrosslinked cell, the lysosome labels for both EGFR and LBPA, whereas lysosomes in the crosslinked sample label for neither. Panels G and H show that MVBs containing EGFR (G) and those containing LBPA (H) also label for CD63. Bar = 200 nm.

stimulation with EGF, the average number of internal vesicles per MVB was greatly increased (Figure 5A). In untreated cells, more than half of MVBs contained 0–10 internal vesicles. In EGF-stimulated cells, over half of the MVB population contained 16–30 internal vesicles (Figure 5B). In conjunction with the effect of EGF on MVB numbers (Figure 4C), the increase in internal vesicle frequency leads to an almost three-fold increase in internal vesicle frequency as measured against cytoplasmic area.

EGF-stimulated inward vesiculation, but not biogenesis, requires annexin 1

We reasoned that substrates of the activated EGFR tyrosine kinase were good candidates for playing a role in EGF-stimulated MVB biogenesis and inward vesiculation. We previously identified annexin 1, a major substrate of the EGFR tyrosine kinase, in MVBs (Futter *et al*, 1993) but a role for annexin 1 in EGFR traffic has never been established. Given that membrane binding and vesicle fusion are characteristic properties of several annexins, we examined the effects of EGF stimulation on MVB biogenesis and inward vesiculation in cells lacking annexin 1. Mouse lung fibroblast cell lines have previously been derived from annexin 1 knockout and wild-type mice (Croxtall *et al*, 2003). Although these cells were transfected with human EGFR (to allow the use of a well-characterised human specific anti-EGFR antibody conjugated to colloidal gold), in the initial quantification all MVBs were examined, whether or not they

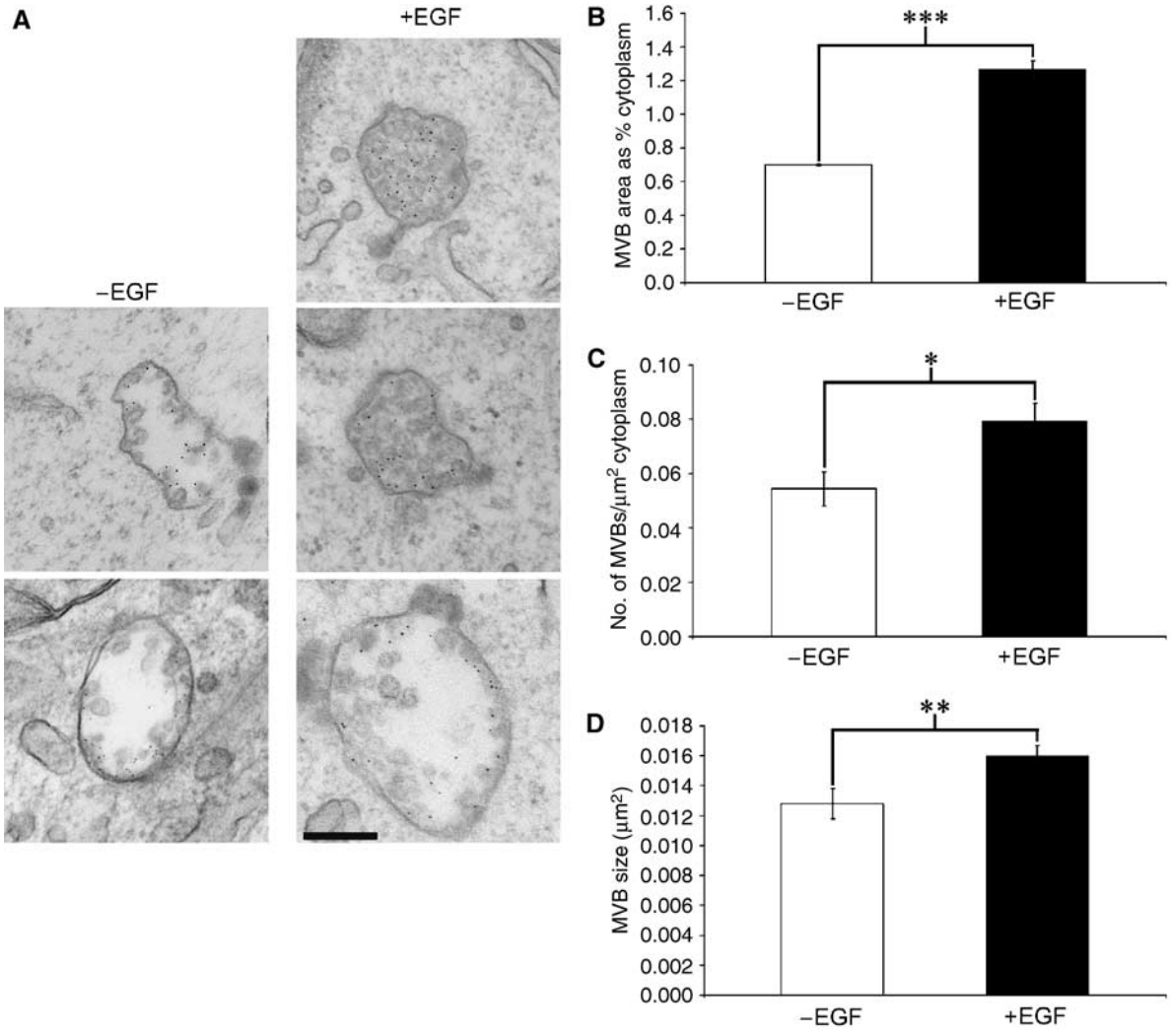

Figure 4 EGF stimulates the formation of MVBs. HEp2 cells with lysosomes crosslinked were incubated in the presence or absence of EGF for 60 min at 37°C in the presence of 10 nm BSA gold. (**A**) Electron micrographs of MVBs from both EGF-stimulated and nonstimulated samples. (**B**) Mean total MVB area as a percentage of total cytoplasmic area. (**C**) Mean numbers of MVBs/unit area cytoplasm. (**D**) Mean MVB sizes. There are significant increases in total area and frequency of MVBs, and a small but significant increase in MVB size upon EGF stimulation. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$. Data shown are the mean ± s.e.m. of three independent experiments. Bar = 200 nm.

contained EGFR. Wild-type cells showed a basal frequency of MVBs per unit cytoplasm comparable to that seen in HEp2 (Figures 4C and 6B). Upon stimulation with EGF, the frequency of MVBs showed a significant increase that was, again, comparable to that seen in HEp2. Annexin 1 knockout cells also showed a similar significant increase in MVB frequency following EGF stimulation and, while the results are not identical to those seen for wild-type cells, the small differences between annexin 1-positive and -negative cell lines are not statistically significant.

In the absence of EGF stimulation, there was no significant difference in MVB internal vesicle numbers between wild-type and annexin 1 knockout cells (Figure 6C). However, following EGF stimulation, wild-type cells revealed a marked increase in internal vesicle numbers, which was abolished in annexin 1 knockout cells, indicating that annexin 1 is required for EGF-stimulated inward vesiculation.

To determine whether EGF-stimulated annexin 1-dependent inward vesiculation is limited to the subpopulation of MVB that contain EGFR, the gold-conjugated anti-hEGFR antibody was used to distinguish between EGFR-containing and non-EGFR-containing subpopulations of MVB. A comparison of internal vesicle numbers in successfully transfected and untransfected cells showed that expression of human EGFR had no significant effect on inward vesiculation within MVB, indicating that the level of expression of the endogenous mouse receptor was sufficient to obtain maximum EGF-stimulated inward vesiculation (not shown). In successfully transfected cells, following EGF stimulation the anti-hEGFR gold was found in many, but not all, MVBs and those MVBs containing EGFR had significantly more internal vesicles than those MVBs lacking EGFR (Figure 6D). This difference was not apparent in EGF-stimulated annexin 1 knockout cells, indicating that EGF and annexin 1 only promote inward vesiculation in MVBs containing EGFR.

As further verification that annexin 1 mediates the effects of EGF on inward vesiculation, experiments were performed to rescue the wild-type phenotype by transfecting the annexin 1 knockout cells with annexin 1-GFP. Nucleofection of annexin 1 knockout cells with annexin 1-GFP gave a

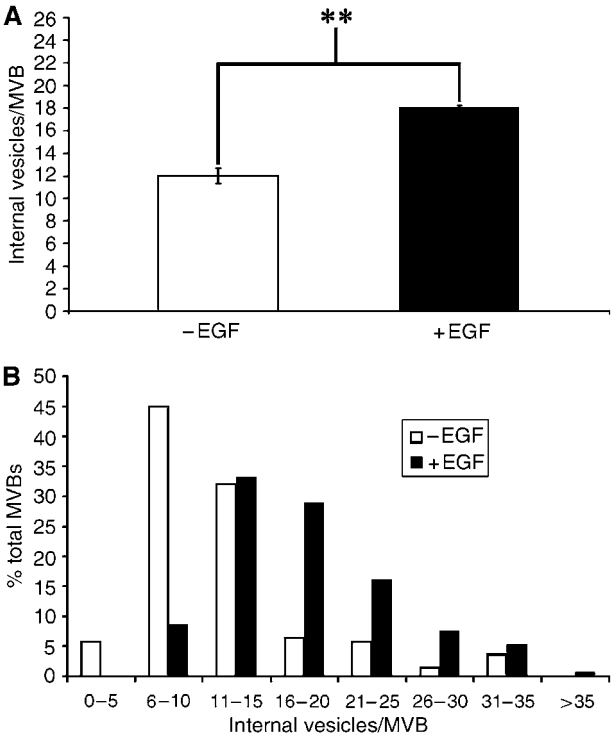

Figure 5 EGF stimulates the formation of internal vesicles within MVBs. HEp2 cells with lysosomes crosslinked were incubated in the presence or absence of EGF for 60 min at 37°C in the presence of 10 nm BSA gold. Sections were analysed by EM. Internal vesicle numbers were recorded from MVBs chosen randomly over three independent experiments. (**A**) Mean numbers of internal vesicles/MVB ±s.e.m. (**B**) Percentage of total MVBs containing different numbers of internal vesicles. $^{**}P<0.01$. Internal vesicle numbers are significantly increased upon stimulation with EGF.

>60% transfection efficiency and caused a significant increase in internal vesicles within MVBs in EGF-stimulated cells (Figure 7). That the rescue of the wild-type phenotype was only partial can be explained by the fact that not all the cells were successfully transfected. In contrast, expression of an annexin 1-GFP construct containing a point mutation at the site of EGF-stimulated tyrosine phosphorylation (Y21F) did not rescue the phenotype. Fluorescence and immunoblotting analysis indicated that the Y21F mutant and wild-type annexin 1-GFP were expressed at similar levels (results not shown) and so we conclude that Tyr21 within the N-terminus of annexin 1 is required for EGF-stimulated inward vesiculation.

These data confirm that the effect of EGF on MVB biogenesis and inward vesiculation is not unique to HEp2 cells. Furthermore, they show that while annexin 1 is not required for EGF-stimulated MVB formation, it is required for EGF-stimulated internal vesicle formation in the EGFR-containing subpopulation of MVBs and that tyrosine phosphorylation of annexin 1 is likely to be required for EGF-stimulated inward vesiculation.

Annexin 1 does not affect trafficking to the lysosome or degradation of EGF

To determine whether loss of annexin 1 affects lysosomal targeting of EGF/EGFR, the degradation of EGF and EGFR was studied in wild-type and annexin 1 knockout cells. Loss of annexin 1 had no effect on EGF degradation (Figure 8A) and caused a small, although not significant, delay in EGFR degradation (Figure 8B). To determine whether long-term loss of annexin 1 has led to compensatory mechanisms being utilised for the efficient degradation of EGF in annexin 1 knockout cells, annexin 1 was depleted in HeLa cells using siRNA. Although the depletion was incomplete, inward vesiculation was inhibited in EGF-stimulated annexin 1-depleted cells almost as much as in the annexin 1 knockout cells (Figure 8C). As with annexin 1 knockout, annexin 1 depletion had no effect on EGF degradation and caused only a small delay in EGFR degradation (Figure 8D and E). These data indicate that annexin 1 acts in the inward vesiculation process in MVBs, and does not play a further role in trafficking or MVB/lysosome fusion.

Annexin 1 accumulates on the internal vesicles of MVB in EGF-stimulated cells

To determine whether EGF-stimulated annexin 1-dependent inward vesiculation is accompanied by movement of annexin 1 with the EGFR from the perimeter membrane to the internal vesicles of MVB, cryo-immuno-EM was performed on HEp2 cells stably transfected with annexin 1-GFP. In unstimulated cells, annexin 1 costained with transferrin receptor-positive endosomes and was only rarely found associated with MVBs where it was largely confined to the perimeter membrane (Figure 9C). In EGF-stimulated cells, annexin 1 was associated with EGFR-containing MVBs, and while some annexin 1 was on the perimeter membrane there was also a striking accumulation of annexin 1 on the internal vesicles of MVBs containing EGFR (Figure 9A). Annexin 1 was largely absent from non-EGFR-containing MVBs and from non-EGFR-containing internal vesicles within EGFR-containing MVBs (Figure 9A and B).

Discussion

EGFR-containing and LBPA-containing MVBs are distinct

LBPA has long been known to be enriched in lysosomes (Wherrett and Huterer, 1972; Poorthuis and Hostetler, 1976; Bleistein *et al*, 1980) and has also been identified on the internal membranes of late endosomes (Kobayashi *et al*, 1998) and has therefore sometimes been assumed to be a component of MVBs. When comparing previously published data with the results of the present study, it is necessary to consider the relationship between MVBs, late endosomes and lysosomes and whether these represent three distinct compartments. Protein markers of late endosomes/lysosomes tend to vary between cell types in how far they extend along the endocytic pathway. We have, therefore, preferred functional definitions and have termed the compartment in which EGF/EGFR are sorted onto internal vesicles but are not degraded as MVBs, and the compartment in which EGF/EGFR are degraded as lysosomes (Futter *et al*, 1996). These compartments can also be distinguished on the basis of morphology in that MVBs are wholly multivesicular while lysosomes are multilamellar (although can also contain internal vesicles).

Which of these compartments is the late endosome? A first step towards answering this question is to determine which contains LBPA. The present study shows that EGF only encounters LBPA at time points coincident with the onset of EGF degradation, suggesting that EGF and LBPA meet in the

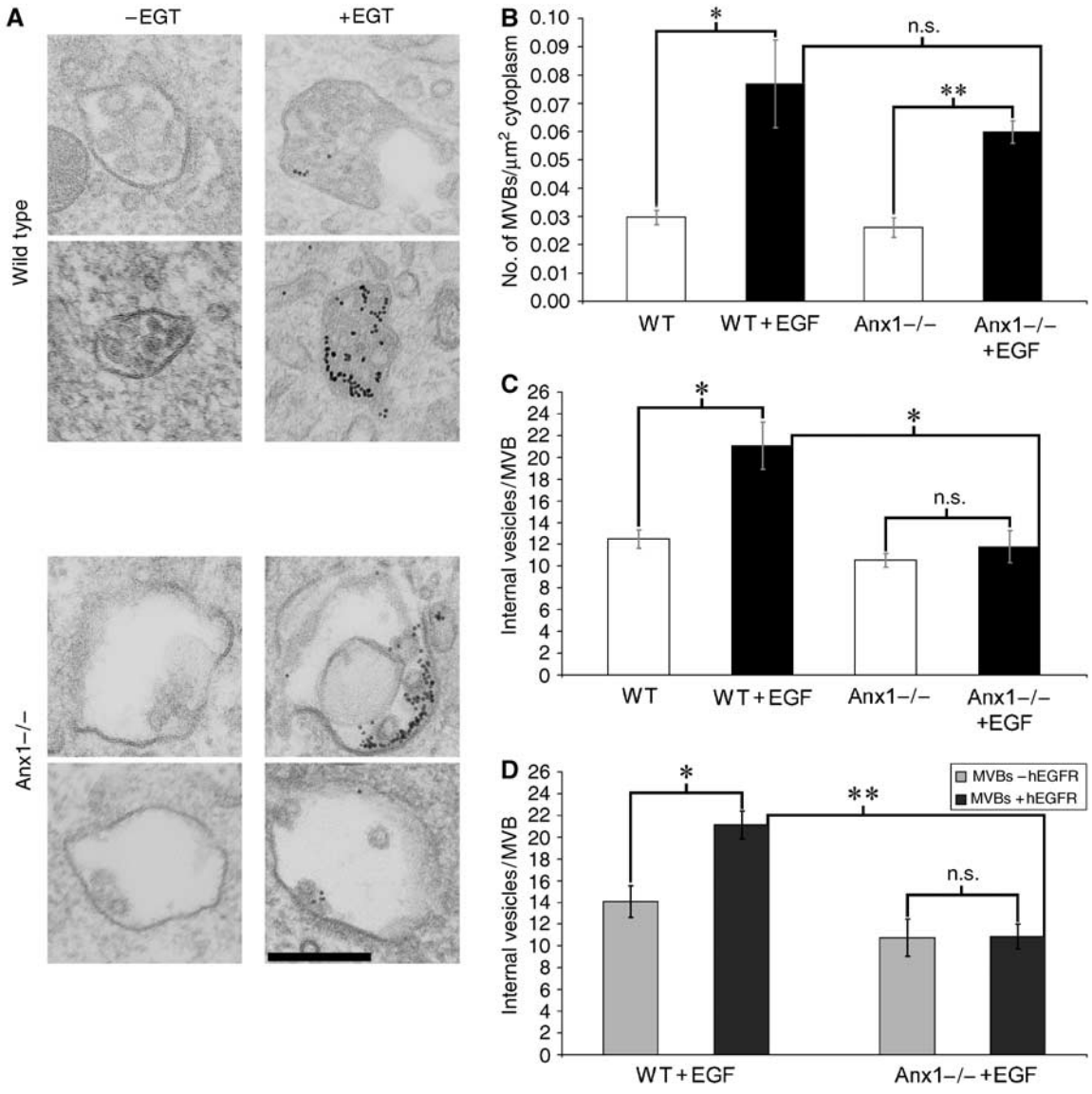

Figure 6 In annexin 1 knockout cells, EGF stimulates MVB formation, but not internal vesicle formation within MVBs. Wild-type (WT) and annexin 1 knockout (Anxl−/−) cells were transfected with hEGFR. Cells were incubated in the absence or presence of EGF, and hEGFR antibody conjugated to 10 nm gold for 60 min at 37°C. (**A**) Electron micrographs of MVBs from both EGF-stimulated and nonstimulated samples in wild-type and annexin 1 knockout cells. (**B**) Mean number of MVBs per unit area of cytoplasm. (**C**) Mean numbers of internal vesicles/MVB. (**D**) Mean numbers of internal vesicles/MVB from EGF–stimulated successfully hEGFR-transfected cells only. Mean numbers of internal vesicles are shown in MVBs either containing or not containing hEGFR. $^{*}P<0.05$, $^{**}P<0.01$, n.s., no significant difference. Data shown are the mean ± s.e.m. of three independent experiments. Annexin 1 knockout does not have a significant effect on the EGF-stimulated increase in MVB formation, but does prevent the EGF-stimulated increase in MVB internal vesicle numbers. The effect of EGF on internal vesicle formation in wild-type cells is limited to MVBs containing hEGFR. Bar = 200 nm.

lysosome rather than within MVBs. MVBs fuse directly with lysosomes (van Deurs *et al*, 1995; Futter *et al*, 1996; Mullock *et al*, 1998) and we have previously developed a technique to prevent this fusion that provides a simple way to distinguish between these two compartments (Futter *et al*, 1996). Cells are incubated with a short pulse of HRP followed by several hours chase. The compartment thus loaded is multilamellar and contains the bulk of the cells acid hydrolase activity and is therefore the lysosome. Performing the DAB reaction on the living cells fills the HRP-containing vacuoles with insoluble DAB reaction product preventing MVB–lysosome fusion. On EGF stimulation, EGFR-containing MVBs accumulate, which are exclusively multivesicular and do not degrade EGF. Lysosomal crosslinking prevents EGF from meeting LBPA, confirming that EGF and LBPA meet only in the lysosome. Comparing crosslinked with noncrosslinked cells shows that the bulk of the LBPA staining is in the lysosome, but a small population of LBPA-positive vacuoles, distinct from those containing EGF, remains. Prevention of EGF degradation by leupeptin treatment allows compartments in which EGF is normally rapidly degraded to be visualised. Under these conditions, in the absence of crosslinking, leupeptin treatment increases the amount of EGF visible in LAMP-positive vacuoles and upon crosslinking, the EGF-positive pre-lysosomal vacuoles that accumulate still fail to costain with LBPA indicating that LBPA-positive, LAMP-negative compartments have no place in the EGF trafficking pathway. EM shows that the bulk of the LBPA is in multilamellar lysosomes that are

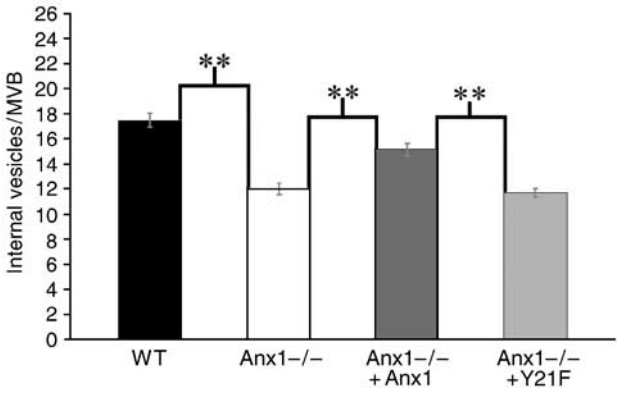

Figure 7 In annexin 1 knockout cells, ectopic expression of annexin 1, but not an annexin 1 tyrosine phosphorylation mutant, partially rescues the EGF-stimulated internal vesicle increase phenotype. Wild-type (WT), annexin 1 knockout cells (Anxl−/−), annexin 1 knockout cells transfected with annexin 1-GFP (Anxl−/− + Anxl) or a Y21F-GFP tyrosine phosphorylation mutant of annexin 1 (Anxl−/− +Y21F) were incubated for 60 min at 37°C in the presence of EGF and 10 nm BSA gold. Sections were analysed by EM. The figure shows mean numbers of internal vesicles/MVB ±s.e.m. of three independent experiments, $**P<0.01$. Expression of annexin 1-GFP in annexin 1 knockout cells caused a significant increase in MVB internal vesicle count partially rescuing the phenotype seen in wild-type cells. Expression of Y21F-GFP had no significant effect on the number of internal vesicles found when compared to untransfected knockout cells.

crosslinked by the above protocol, while the small population that is not crosslinkable is multivesicular and morphologically indistinguishable from MVBs containing EGF.

That the bulk of the LBPA is in multilamellar lysosomes is in agreement with a recent study comparing the distribution of LBPA with that of cholesterol in the EBV-transformed human B-cell line RN. That study found that the bulk of LBPA was in multilamellar vacuoles while the bulk of cholesterol was found in multivesicular vacuoles (Mobius *et al*, 2003). That not all MVBs contain LBPA is consistent with the findings of Wubbolts *et al* (2003). They found that purified exosomes derived from the internal vesicles of MVBs that have fused with the plasma membrane are enriched in CD63 but not LBPA. We found in all the cell types we tested that the majority of LBPA-positive vacuoles were ablated by lysosomal crosslinking, but a small population remained, which were distinct from those containing EGF/EGFR. Our results, together with those of Wubbolts *et al* and Mobius *et al*, suggest that the distribution of LBPA we have observed is a widespread phenomenon.

In a recent review, Gruenberg and Stenmark (2004) proposed a scheme whereby MVBs/endosomal carrier vesicles are defined as vacuoles that transport material from early to late endosomes, are multivesicular and LBPA negative. According to this scheme, late endosomes have both multivesicular and multilamellar regions and are positive for LBPA. We would suggest that the compartment that we crosslink and refer to as lysosomes corresponds to the late endosome according to Gruenberg and Stenmark (2004), as it is multivesicular, multilamellar and LBPA positive. The EGFR-containing MVBs that accumulate after crosslinking correspond to MVBs/endosomal carrier vesicles of Gruenberg and Stenmark (2004), as they are multivesicular and LBPA negative. Importantly however, while EGFR-containing MVBs do indeed act as carrier vesicles transporting proteins like EGFR from early endosomes to the lysosome, they also perform an important sorting function as shown by the gradual accumulation of lysosomally directed proteins, the gradual accumulation of internal vesicles and the gradual removal of recycling proteins (Hopkins *et al*, 1990; van Deurs *et al*, 1993; Futter *et al*, 1996). Thus, MVBs can be both early and late; early MVBs have few internal vesicles and still contain recycling proteins such as transferrin receptor, while late MVBs have many internal vesicles and lack recycling proteins.

We have previously shown that the PI3-kinase Vps34 is required for the generation of internal vesicles within EGFR-containing MVB (Futter *et al*, 2001), while Bright *et al* (2001) found that inhibiting PI3-kinase activity did not prevent the accumulation of internal membranes within LBPA-positive vacuoles. As described above, MVBs containing EGFR and MVBs/multilamellar lysosomes containing LBPA are distinct compartments and the internal membranes are presumably generated in different ways. Those within LBPA-containing vacuoles may utilise a mechanism involving LBPA itself and ALIX, as described in liposomes *in vitro* (Matsuo *et al*, 2004). The presence of a population of LBPA-containing MVBs in addition to the LBPA-containing lysosomes raises the intriguing question of what is the role of this population of MVBs.

EGF stimulates MVB biogenesis and the generation of internal vesicles within MVB

EGF stimulates both the number of MVBs/unit cytoplasm and the number of internal vesicles/MVB. To our knowledge, this is the first report of MVB formation being modulated by growth factor stimulation. Our experiments do not allow us to determine whether all EGFR-containing MVBs are formed as a result of EGF stimulation, but at least a proportion of them are formed *de novo* as a result of EGF stimulation. The increase in both MVB numbers and numbers of internal vesicles/MVB indicates that there is a substantial increase in the amount of cell membrane being delivered to the lysosome for degradation and could have important consequences for cell homeostasis, and possibly the degradation of components of downstream signalling cascades.

The mechanisms whereby EGF stimulation increases MVB formation and internal vesicle formation are likely to involve substrates of the EGFR tyrosine kinase or proteins that interact with the phosphorylated EGFR. Some years ago, we identified a major substrate of the EGFR tyrosine kinase within MVB as annexin 1 and proposed a role for this protein in sorting within MVB (Futter *et al*, 1993). Annexins are not expressed in yeast and so annexin 1 is unlikely to be part of the core MVB sorting machinery, but as a major substrate of the EGFR tyrosine kinase it could play a role in the regulation of the core machinery by EGF.

EGF stimulation of inward vesiculation requires annexin 1, and is specific to the EGFR-containing subpopulation of MVBs

Lung cell fibroblasts derived from an annexin 1 knockout mouse have altered morphology and attenuated responses to glucocorticoids (Croxtall *et al*, 2003). We have shown that in wild-type lung cell fibroblasts, as in HEp2 cells, EGF stimulation results in increased numbers of MVBs and increased numbers of internal vesicles/MVB. This demonstrates that

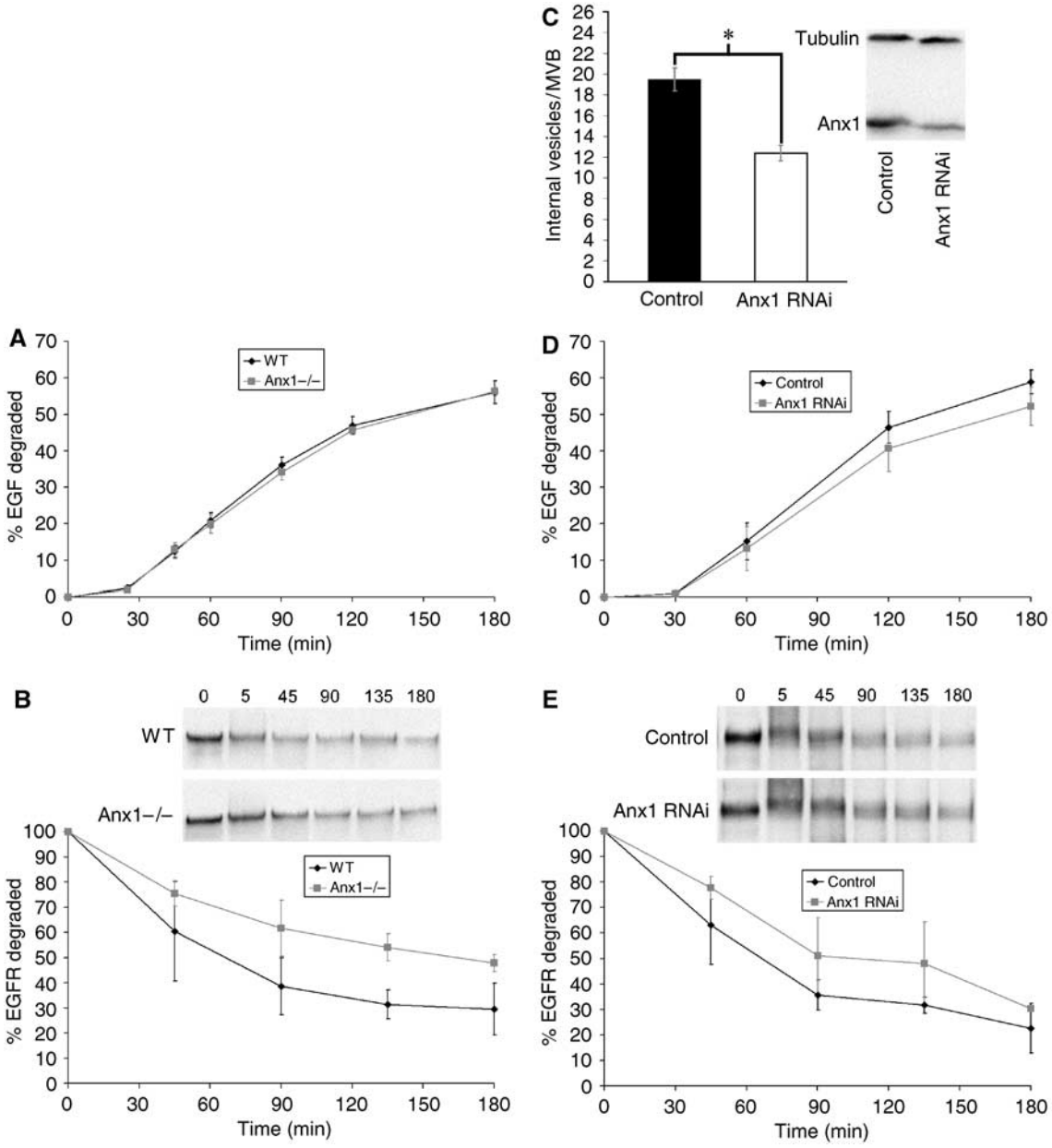

Figure 8 Annexin 1 depletion has no effect on EGF degradation, and causes only minor delays in EGFR degradation. Wild-type and annexin 1 knockout mouse lung fibroblast cells (**A, B**) and HeLa cells treated with control siRNA or annexin 1 siRNA (**C–E**) were incubated with ^{125}I-labelled EGF (1 ng/ml) for 10 min and chased for the times shown with serum-free media, and the % EGF degradation determined (A, D). The degradation of EGFR was also measured by immunoblotting for the presence of EGFR at the time points illustrated (B, E). All data points are mean$\pm$s.e.m. of three independent experiments. HeLa cells treated with annexin 1 siRNA showed reduced annexin 1 levels and display a similar phenotype to annexin 1 knockout cells in that EGF-stimulated inward vesiculation is blocked (C). Annexin 1 knockout or depletion has no effect on the levels or kinetics of EGF degradation, and induces only a small delay in the degradation of EGFR.

the growth factor modulation of MVB morphogenesis is not a phenomenon of only one cell type. The EGF-stimulated increase in internal vesicle numbers in lung cell fibroblasts was also shown to be specific to those MVBs that contain EGFR. Furthermore, we demonstrate that basal internal vesicle formation is not affected in cells lacking annexin 1, but EGF stimulation of internal vesicle formation is severely attenuated. This reduction is recoverable to a certain extent by transfection of annexin 1 knockout cells with annexin 1, confirming the role of annexin 1 in this process. These results demonstrate that annexin 1 is not an absolute requirement for internal vesicle formation, but that the EGF stimulation of internal vesicle formation is mediated through annexin 1. EGF stimulation of MVB formation as demonstrated by the number of MVBs/unit cytoplasm is reduced in annexin 1 knockout cells, but this reduction is not significant, suggesting that annexin 1-independent mechanisms may operate in MVB formation. Annexin 2 has recently been proposed to play a role in the biogenesis of MVBs (Mayran *et al*, 2003). However, annexin 2, unlike annexin 1, is not a good substrate for the EGFR tyrosine kinase, making it an unlikely candidate for playing a role in EGF-stimulated MVB formation. The lack of a pronounced phenotype in most annexin knockout mice has led to the suggestion that there is functional redundancy

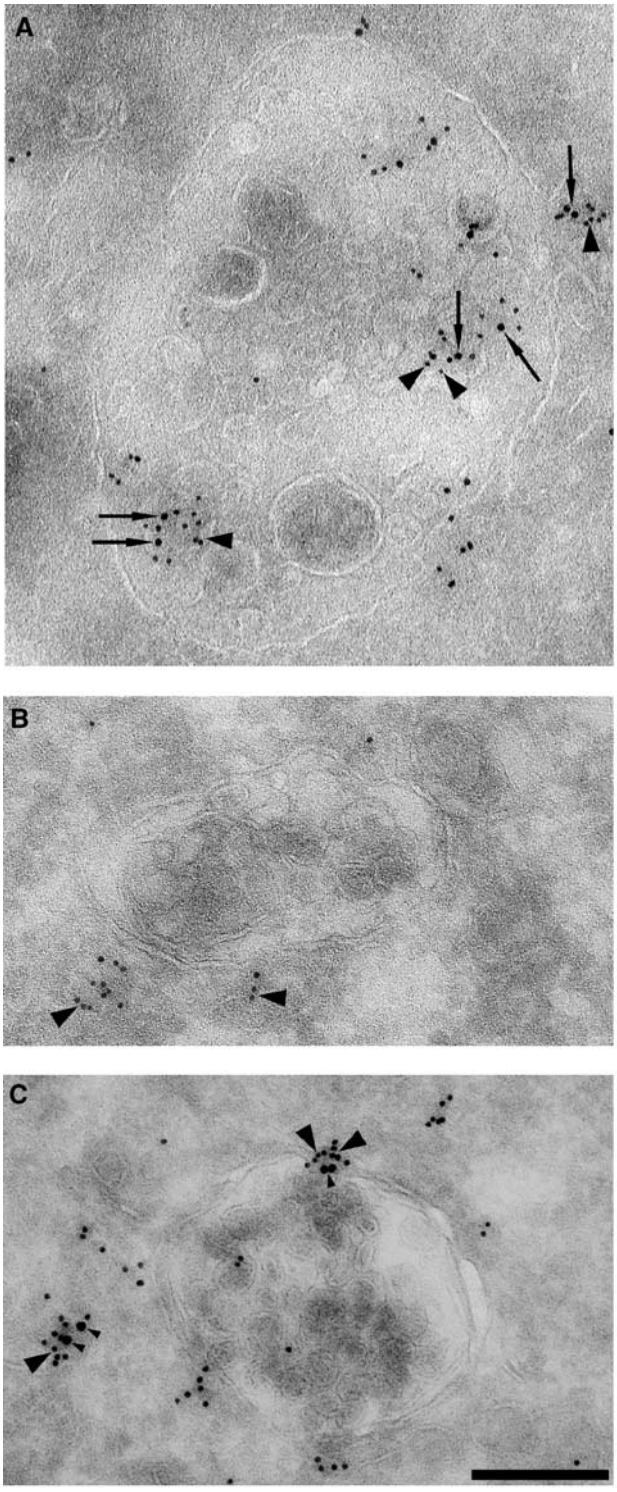

Figure 9 EGF stimulation causes annexin 1 to relocalise to MVBs containing EGFR. HEp2 cells stably expressing annexin l-GFP were incubated in the absence of EGF (C) or for 30 min at 37°C in the presence of EGF (**A, B**). Cells were prepared for cryo-immuno-EM and ultrathin frozen sections either double labelled for EGFR with 15 nm gold (arrows), and GFP with 10 nm gold (large arrowheads) (A and B), or double labelled for transferrin receptor with 15 nm gold (small arrowheads), and GFP with 10 nm gold (large arrowheads) (**C**). In the absence of EGF stimulation, annexin 1-GFP is found at the plasma membrane, or associated with transferrin receptor in small vesicles, or on the perimeter membrane of MVBs (C). Following EGF stimulation, annexin 1 localises to those MVBs that contain EGFR, where it is found on EGFR-containing internal vesicles (A), but not MVBs that do not contain EGFR (B).

among members of the annexin family (Tomas *et al*, 2003). Annexins 1 and 2 are closely related but our results demonstrate that annexin 2 cannot substitute for annexin 1 in the EGF stimulation of inward vesiculation, even though annexin 2 is upregulated in the cells derived from the annexin 1 knockout mouse (Croxtall *et al*, 2003).

Annexin 1 operates downstream of Hrs and the ESCRT proteins

That loss of annexin I had no effect on EGF degradation and caused only a small delay in EGFR degradation indicates that the role of annexin 1 in MVBs is limited to internal vesicle formation, rather than cargo selection and sorting. In contrast, interference with the function of Hrs or components of ESCRT-I has been shown to inhibit EGF or EGFR degradation (Babst *et al*, 2000; Chin *et al*, 2001; Bishop *et al*, 2002; Urbe *et al*, 2003; Bache *et al*, 2004), indicating that annexin 1 acts downstream of Hrs-and ESCRT-mediated sorting. The delay in EGFR degradation could be because EGFR delivered to the lysosome on the perimeter membrane might be less readily degraded than EGFR on internal vesicles. A similar delay in EGFR degradation was observed in wortmannin-treated cells where internal vesicles also fail to form (Futter *et al*, 2001).

In agreement with previous studies (Seemann *et al*, 1996; Rescher *et al*, 2000) in unstimulated cells, annexin 1 was localised to early transferrin-positive endosomes and a small amount was associated with MVBs. For annexin 1 to mediate EGF-stimulated inward vesiculation, the amount of annexin 1 associated with MVBs in EGF-stimulated cells would be expected to increase. We have found that this is indeed the case. Annexin 1 has recently been shown to co-immunoprecipitate with the EGFR in a reaction that is independent of the N-terminus and can occur in the absence of EGF stimulation (Radke *et al*, 2004). Constitutive association with the EGFR would explain the enhanced association of annexin 1 with MVBs in EGF-stimulated cells because both the number of MVBs and accumulation of EGFR within MVBs are increased following EGF stimulation. However, it is not clear how much of the cellular pool of annexin 1 co-immunoprecipitates with EGFR or whether this interaction is affected by EGF stimulation, neither is it clear whether the early endosome pool of annexin 1 shifts onto MVBs or whether a non-endosomal pool is recruited following EGF stimulation.

Annexin 1 is the first component of the MVB sorting machinery found to accumulate on the internal vesicles of MVBs but it does so only in EGF-stimulated cells and is only found on internal vesicles that contain EGFR. This localisation is in marked contrast to that of Hrs and the ESCRT complexes, which are believed to dissociate from the perimeter membrane of the endosome before inward vesiculation is complete. If ESCRT complexes play a role in inward invagination within MVB, it is likely to precede the role played by annexin 1.

Annexin 1 can mediate vesicle fusion *in vitro* in the presence of Ca^{2+} (Raynal and Pollard, 1994), which, together with our demonstration of the movement of annexin 1 onto internal vesicles following vesicle scission, suggests that annexin 1 might bring opposing membranes of the invaginating inward bud together to close the neck and allow scission to take place. Annexin 1 could then either mediate fusion/scission itself or bring the membranes close enough to allow other components to mediate fusion. Annexin 1 contains a

single tyrosine phosphorylation site in the N-terminus and our demonstration that this tyrosine is required for mediating inward vesiculation, together with our previous demonstration that annexin 1 is phosphorylated within EGFR-containing MVBs (Futter *et al*, 1993), strongly suggests that annexin 1 must be phosphorylated by the EGFR kinase to mediate inward vesiculation. Phosphorylation of annexin 1 renders the protein more susceptible to N-terminal proteolysis (Haigler *et al*, 1987). An N-terminal peptide produced by proteolysis of annexin 1 has been shown to mediate anti-inflammatory actions of extracellular annexin 1 (Parente and Solito, 2004) but an intracellular activity of this peptide has not yet been demonstrated. N-terminal proteolysis disrupts interaction of annexin 1 with its ligand, S100A11 (see Gerke and Moss, 2002 for model), and could also prevent interaction of annexin 1 with other lipid or protein components. Disruption of such interactions could be coupled to scission of the internal vesicle.

The revelation that separate populations of MVB exist raises the interesting question of what purpose each might serve. It is interesting to speculate that an MVB containing activated EGFR or other catalytically active proteins could be used as a 'signalling MVB' operating in a mobile, yet strictly controlled environment that is spatially separated from the plasma membrane. Annexin 1-mediated stimulation of internal vesicle formation by activated EGFR represents a method of directly attenuating signalling by removing the catalytic domain of the EGFR and potentially other signalling proteins from the cytoplasm.

Materials and methods

Cell culture and nucleofection

HEp2 cells and mouse lung fibroblasts were cultured as previously described (Futter *et al*, 1996; Croxtall *et al*, 2003). Stable cell lines of HEp2 cells were generated by transfection using the calcium phosphate method and transient transfections were performed by nucleofection (Amaxa Biosystems) according to the manufacturer's instructions (see Supplementary data). To deplete HeLa cells of annexin 1, cells were nucleofected on days 0 and 1 with siRNA directed against annexin 1 or control oligonucleotides (see Supplementary data) and assays performed on day 3. The efficiency of knockdown was measured by Western blotting of cell lysates with an anti-annexin 1 antibody.

Lysosomal crosslinking

Lysosomes were loaded with HRP and crosslinked in living cells as described by Futter *et al* (1996).

EGF stimulation

Fluorescent AlexaFluor 488-conjugated EGF and nonfluorescent EGF were both used at a concentration of 100 ng/ml in serum-free cell culture medium.

Antibodies

EGFR antibody used in cryo-immuno-EM was from Fitzgerald Industries International. 108 hEGFR antibody used in standard EM was a gift from J Schlessinger (Yale University School of Medicine, USA), LBPA antibody from Jean Gruenberg (University of Geneva, Switzerland), LAMP 1 antibody from Colin Hopkins (Imperial College, London, UK) and CD63 antibody from Mark Marsh (University College, London, UK). Annexin 1 antibody was a gift from J Croxtall.

Plasmids

hEGFR construct was a generous gift from Alexander Sorkin (University of Colorado, USA). Annexin 1-GFP construct was created by PCR of the annexin 1 gene from I.M.A.G.E. Consortium [LLNL] cDNA Clone lD 3459615 (Lennon *et al*, 1996). Site-directed mutagenesis was performed using the QuikChange^{TM} kit from Stratagene. For details of primers, see Supplementary data.

Immunofluoresence microscopy

Cells grown on sterile glass coverslips were fixed and quenched as described (Futter *et al*, 1996) and permeabilised in 0.1% saponin. Subsequent antibody incubations were performed in PBS containing 1% BSA and 0.01% saponin. Coverslips were mounted on 90% glycerol and 3% *N*-propyl galate in PBS.

Electron microscopy

Conventional EM was performed as previously described (Tomas *et al*, 2004). Cryo-immuno-EM was performed on cells fixed with 4% paraformaldehyde/0.1% glutaraldehyde in 0.1 M phosphate buffer pH 7.4, infused with 2.3 M sucrose and supported in 10% gelatin. Sections (70 nm) were cut at −120°C and picked up in 1:1 sucrose:methylcellulose. For labelling with mouse or sheep antibodies, primary antibody was followed by rabbit anti-mouse or rabbit anti-goat intermediate antibody, respectively. Sections were then labelled using protein A gold as described (Slot *et al*, 1991).

Quantification

Photographs were taken at random within cells. Where cytoplasmic area and MVB areas were measured, three separate experiments were performed for each treatment, and over 170 μm^2 of cytoplasm was examined in each case. For calculations of mean internal vesicle numbers, over 50 MVBs were examined across three experiments to obtain data for each sample.

EGF/EGFR degradation

Cells were incubated with ^{125}I-labelled EGF (1 ng/ml) for 10 min at 37°C, surface stripped at 4°C and then chased with serum-free media at 37°C. The % degradation of ^{125}I-labelled EGF in the medium was determined by TCA precipitation (see Supplementary data for more detail). To measure EGFR degradation, cells were incubated with unlabelled EGF, lysed, the EGFR immunoprecipitated and immunoprecipitates blotted with an anti-EGFR antibody.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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