ATPase-deficient hVPS4 impairs formation of internal endosomal vesicles and stabilizes bilayered clathrin coats on endosomal vacuoles

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Summary

Epidermal growth factor receptors (EGFRs) destined for lysosomal degradation are sorted in the early endosomal vacuole into small, lumenal vesicles that arise by inward budding of the limiting membrane. We have previously shown that, before their incorporation into internal vesicles, EGFRs are concentrated in flat bilayered-clathrin coats on the endosomal vacuole. Here, we show that an ATPase-deficient mutant of hVPS4 (hVPS4^{EQ}) increases the association of bilayered coats with endosomal vacuoles. In addition, hVPS4^{EQ} leads to a reduction in the number of internal vesicles in early and late endosomal vacuoles, and retention of EGFRs at the limiting membrane. Interestingly, hVPS4^{EQ} was predominantly found on non-coated regions of endosomal vacuoles, often at the rim of a coated area. In line with published data on Vps4p function in yeast, these results suggest that hVPS4 is involved in the release of components of the bilayered coat from the endosomal membrane. Moreover, our data suggest that disassembly of the coat is required for the formation of internal vesicles.

Key words: *VPS4*, Clathrin, Endosomes, Membrane domains, Immunoelectron microscopy

Introduction

Based on the time-dependent accessibility for endocytic tracers, the endocytic pathway can be subdivided into early endosomes (EEs), late endosomes (LEs) and lysosomes. From the EE vacuole – also known as the sorting endosome – proteins either recycle to the plasma membrane or are sorted to LEs/lysosomes. For many proteins, targeting to lysosomes requires incorporation into small, internal vesicles that form by inward budding of the limiting membrane of the endosomal vacuole by a process called microautophagy (reviewed by Geuze et al., 1998; Katzmann et al., 2002). For growth factor receptors, this step has an important regulatory role because it sequesters activated receptors away from the cytoplasm, which attenuates signaling. For an increasing number of proteins, ubiquitin is known to be a signal for sorting into these internal endosomal vesicles (Helliwel et al., 2001; Longva et al., 2001; Reggiori and Pelham, 2001; Urbanowski and Piper, 2001). In yeast, endosomal sorting is regulated by vacuolar protein sorting (VPS) genes, which encode for proteins that are required for the transport of biosynthetic as well as endocytic cargo to the vacuole, the yeast equivalent of the lysosome. Three VPS-protein-containing complexes have been identified (ESCRT-I, ESCRT-II and ESCRT-III), which are specifically involved in the ubiquitin-dependent intra-endosomal sorting of cargo. ESCRT-I, consisting of the class-E gene products Vps23p, Vps28p and Vps37p, is recruited to endosomes by Vps27p (Katzmann et al., 2003). Vps23p binds to ubiquitinated membrane proteins, after which interaction with ESCRT-II and ESCRT-III is required to direct cargo proteins into the vacuolar lumen (Katzman et al., 2001; Babst et al., 2002a; Babst et al., 2002b). Before inward budding, Vps4p removes the ESCRT components from the limiting membrane of the prevacuolar compartment (Babst et al., 1998; Babst et al., 2002a; Odorizzi et al., 2003).

In mammalian cells, an increasing number of orthologs of the class E VPS proteins is being identified and implicated in endosomal sorting (Babst et al., 2000; Bishop and Woodman, 2001; Bishop et al., 2002). As in yeast, the ortholog of Vps27p (Piper et al., 1995), hepatocyte-growth-factor-regulated tyrosine kinase substrate (Hrs), mediates the initial recruitment of ESCRT-I to endosomes (Bache et al., 2003). Hrs contains an ubiquitin-interacting motif (UIM) that binds ubiquitin and is required for mono-ubiquitination of Hrs itself and binding to ubiquitinated cargo (Polo et al., 2002; Bishop et al., 2002). In addition, Hrs contains a C-terminal clathrin box and, upon overexpression, increases clathrin recruitment onto endosomal vacuoles (Raiborg et al., 2001). The Hrs-positive clathrin coat on endosomes appears as flat lattices with a typical bilayered appearance – a dense inner laying lining the endosomal membrane and a more fuzzy, clathrin-containing outerlayer facing the cytoplasm (Louvard et al., 1983; Prekeris et al., 1999; Raposo et al., 2001; Raiborg et al., 2001; Sachse et al., 2002a). In yeast, no evidence has yet been obtained for an endosomal clathrin coat. In the bilayered coats, growth factor receptors destined for lysosomal degradation are concentrated, whereas recycling receptors like transferrin receptor (TfR) are not, suggesting that growth factor receptors routed to the lysosome are retained and concentrated in the coat before their incorporation in internal vesicles (Sachse et al., 2002a). It is tempting to speculate that

the bilayered coat will harbor the mammalian equivalents of the yeast ESCRT complexes.

The bilayered coat components clathrin and Hrs are not co-incorporated with growth factor receptors into the internal vesicles of the endosomes. Rather, inward budding profiles of the endosomal limiting membrane are only seen at the non-coated membrane areas, usually at the edge of a coated area (Sachse et al., 2002a). Recently, this notion was reinforced by three-dimensional electron tomography, showing that the coat exists as dispersed patches on the limiting membrane, with inward budding profiles always at the edge of the coat (Murk et al., 2003). Two models can explain these observations. First, the coat could exist as a more-or-less-stable unit, implying that concentrated cargo proteins have to move out of the coated area to enter a forming internal vesicle. Alternatively, the coat could disassemble locally to allow the formation of an inward budding vesicle. To distinguish between these two possibilities, we have studied the role of the mammalian class E VPS4 ortholog, hVPS4, a member of the AAA-ATPase family [ATPases associated with a variety of cellular activities, including organelle biogenesis and vesicle-mediated transport (Patel and Latterich, 1998)]. In yeast, Vps4p was shown to remove class E VPS gene products from the limiting membrane of the prevacuolar compartment before inward budding (Babst et al., 1998; Babst et al., 2002a; Odorizzi et al., 2003). A single point mutation in the ATPase domain leads to an ATPase-deficient form of Vps4p, which acts as a dominant-negative protein. Yeast cells expressing this mutant have an enlarged prevacuolar compartment (Babst et al., 1997; Finken-Eigen et al., 1997). Accordingly, the dominant-negative form of the mouse ortholog of Vps4p, SKD1, leads to enlarged endosomes in mammalian cells (Yoshimori et al., 2000). Overexpression of the ATPase-deficient form of the human ortholog of Vps4p, hVPS4, also results in swelling of endosomal vacuoles, on which the orthologs of the yeast ESCRT-I components Vps23p andVps28p, tsg-101 and hVPS28, respectively, accumulate (Bishop and Woodman, 2000; Bishop and Woodman, 2001).

Interestingly, HIV virus particles can assemble at LE/multivesicular bodies and/or at the plasma membrane by using the ESCRT/VPS4 machinery (Pornillos et al., 2002; Pelchen-Matthews et al., 2003; Raposo et al., 2002; von Schwedler et al., 2003). For example, in infected T cells, HIV-1 recruits ESCRT-I complex to the plasma membrane, whereas the budding of viral particles depends on VPS4 function (Garrus et al., 2001; Martin-Serrano et al., 2003). Expression of an ATPase-deficient mutant of mammalian VPS4 results in the accumulation of viral budding profiles at the plasma membrane (Garrus et al., 2001). Importantly, the topologies of viral budding at the plasma membrane and internal vesicle formation at the endosome are similar. In both cases, opposite to clathrin-coated-vesicle formation at the plasma membrane, the membrane bends away from the cytosol.

Although the involvement of hVPS4 in endosome function is established, its relation to the bilayered coat has remained unclear. In order to examine the molecular morphological effects of loss of hVPS4 function with sufficient resolution to look into subendosomal morphology, we have performed immunoelectron microscopy (immuno-EM) on HeLa cells overexpressing the ATPase-deficient mutant of hVPS4 ($hVPS4^{EQ}$). We found that $hVPS4^{EQ}$ expression resulted in an increase in Hrs-positive coated areas on endosomal vacuoles, whereas the formation of internal vesicles was reduced. In addition, the incorporation of the epidermal growth factor receptor (EGFR) into internal vesicles was decreased. Our results agree with a dynamic association of the bilayered coat to the endosomal vacuole and suggest a role for hVPS4 in local disassembly of the coat and the formation of internal vesicles.

Materials and Methods

Plasmids and antibodies

Plasmids containing fusions between green fluorescent protein (GFP) and wild-type hVPS4 ($hVPS4^{wt}$) or $hVPS4^{EQ}$ were kindly donated by P. Woodman (Bishop and Woodman, 2000). Epidermal growth factor (EGF) was purchased from Invitrogen (Leek, The Netherlands). Rabbit polyclonal antisera against GFP, clathrin and Hrs were kind gifts from D. Shima (Cancer Research, UK), S. Corvera (University of Massachusetts, MA) and S. Urbé (University of Liverpool, UK), respectively. Mouse monoclonal antibody against CD63 was purchased from Pelicluster (Amsterdam, The Netherlands) and rabbit polyclonal antiserum against mouse IgG was obtained from DAKO (Glostrup, Denmark). Sheep antiserum against EGFR was obtained from Invitrogen and rabbit polyclonal antiserum against sheep IgG was purchased from Nordic (Tilburg, The Netherlands).

Cell culture and transfections

HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) containing 10% fetal calf serum, 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, supplemented with 2 mM L-glutamine. For transient transfections with GFP-$hVPS4^{wt}$, GFP-$hVPS4^{EQ}$ or GFP alone, cells were grown in 60 mm dishes. Fugene (Roche Diagnostics) was used according to the manufacturer's protocol. Cells were fixed for immuno-EM 20 hours after transfection. For uptake of EGF, cells were depleted of growth factors by incubation in serum-free DMEM containing 0.5% bovine serum albumin 4 hours before fixation. EGF (100 nM) was added 20 minutes before fixation.

Immuno-EM and quantitative analysis

Cells were fixed 20 hours after transfection, using 2% paraformaldehyde and 0.2% glutaraldehyde in 0.1 M phosphate buffer pH 7.4 for 4 hours at room temperature. Fixed cells were washed with PBS after which free aldehyde groups were quenched with 50 mM glycin in PBS. Cells were scraped in PBS containing 1% gelatin and pelleted in 12% gelatin. The cell pellets were solidified on ice and cut into small blocks, which were incubated for cryoprotection overnight at 4°C in 2.3 M sucrose. Afterwards, the blocks were mounted on aluminum pins and frozen in liquid nitrogen. Ultrathin cryosectioning and immunogold labeling were done as described (Raposo et al., 1997).

To measure the effect of GFP-$hVPS4^{wt}$ and GFP-$hVPS4^{EQ}$ expression on the endosomal bilayered coats, cell profiles were randomly selected for GFP label, after which all Hrs-positive vacuoles were photographed (final magnification 81,000×). For each transfection, at least three sets of 15 endosomal vacuoles were analysed. The lengths of the limiting membranes of the vacuoles was measured by placing a transparent overlay displaying a squared lattice of lines 5 mm apart over pictures. The membrane lengths were expressed (in arbitrary units) by counting the number of intersections with the line-lattice overlay (Weibel, 1979). The limiting membrane was divided into three categories: coated, non-coated and undefined. For the last category, it was not possible to determine whether a coat occupied these membranes or not. Because of the variation in size of endosomal vacuoles, 15 vacuoles were grouped to calculate an

average for each transfection. During our studies, we noted an enlargement of LE rather than EE vacuoles. To prove this observation, we loaded cells for 5 minutes with BSA conjugated to 5 nm colloidal gold (final optical density at 520 nm of 5) to mark EE vacuoles specifically. Before use, BSA-gold was dialysed overnight against DMEM at 4°C. Cells were incubated with BSA-gold in DMEM containing 0.5% BSA for 5 minutes and then washed twice with cold medium before fixation for EM. BSA-gold-positive vacuoles in transfected cells were randomly selected. For each transfection, 40 pictures were taken in which the length of the limiting membrane was determined as described. In parallel, we counted the number of internal vesicles in the BSA-gold-positive vacuoles. To establish the number of inward budding profiles at the limiting membrane of endosomal vacuoles, three sets of 20 vacuoles with the complete limiting membrane visible within the plane of the section were analysed per condition. The distribution of the EGFR over the endosomal vacuoles was quantified by counting the number of gold particles that were localized at the limiting membrane as well as those present on internal vesicles. Three sets of 15 vacuoles were analysed and the numbers were expressed as the relative distribution of EGFR over the endosomal vacuole (in percentages±s.d.).

Results

Overexpression of hVPS4^{EQ} causes swelling of late endosomes

To study a possible regulatory role of hVPS4 in the disassembly of endosomal bilayered coats, we set out to establish the effects of hVPS4^{wt} and hVPS4^{EQ} overexpression on coat occurrence. HeLa cells were transfected with GFP or GFP-tagged hVPS4^{wt} or hVPS4^{EQ} constructs and cryosections were prepared and immunogold labeled for GFP. In accordance with previous studies (Bishop and Woodman, 2000), hVPS4^{wt} was only found in the cytosol with no significant membrane association (Fig. 1B). The endosomal morphology was essentially unchanged upon hVPS4 overexpression (Fig. 1A,B). Cells overexpressing hVPS4^{EQ} displayed cytosolic as well as membrane-associated label, mostly on the limiting membrane of endosomal vacuoles (Fig. 1C). Notably, like Hrs and clathrin (Sachse et al., 2002a), hVPS4^{EQ} was largely excluded from internal endosomal membranes. Occasionally, label was observed in seemingly internal, irregular membrane sheets, but these probably represent invaginations still connected to the limiting membrane (e.g. Fig. 1D, asterisk). Our studies were focused on the role of hVPS4 on sorting in the endosomal vacuole. It should be realized, however, that some hVPS4^{EQ} label was localized to vesicular/tubular membranes in close vicinity of endosomes as well as on vesicular/tubular membrane profiles in the Golgi area (not shown).

The hVPS4^{EQ}-positive endosomal vacuoles were often enlarged with two morphological distinguishable phenotypes. (1) Vacuoles with an irregularly shaped limiting membrane showing an unusually high number of inward budding profiles that were clearly connected to the limiting membrane (Fig. 1D, Fig. 4B). (2) Vacuoles with extended coated areas, covering a large part of the vacuolar limiting membrane (Fig. 3C). Some of the enlarged vacuoles had only a few internal vesicles. In addition, despite the fact that lipids are partially extracted during EM preparation procedures, we observed vacuoles containing many round, non-membranous lipid particles (Fig. 1C) (Möbius et al., 2002), which were also seen in non-transfected cells but with lower frequency. This finding is in agreement with previous studies showing that the expression of mutant hVPS4 leads to an accumulation of cholesterol in endosomes (Bishop and Woodman, 2000).

To establish the nature of the hVPS4^{EQ}-positive vacuoles, we performed double labelings for GFP and early or late endosomal marker proteins. As in control and hVPS4^{wt} cells (Fig. 2A), in hVPS4^{EQ} cells, most of the EE marker TfR was found in recycling tubules surrounding the EE vacuole (Fig. 2B), whereas small amounts were present at the EE vacuole. A portion of the hVPS4^{EQ}-positive endosomal vacuoles also contained TfR, defining them as EEs (Fig. 2B). Notably, despite the presence of mutant hVPS4 on these EE vacuoles, their morphology and size were very similar to those in cells expressing hVPS4^{wt} (Fig. 2A,B).

We next performed co-localization studies of hVPS4^{EQ} with a LE marker, the tetraspanin protein CD63. In non-transfected and hVPS4^{wt}-expressing HeLa cells, most CD63 is found on LEs and lysosomes, where it is enriched on the internal membranes (Fig. 2C-E). LEs are characterized by the presence of many internal vesicles (Fig. 2C,D), whereas lysosomes display internal membrane sheets (Fig. 2E) (Sachse et al., 2002b). Typically, CD63-labeled LEs and lysosomes were not reached by BSA-gold after a 5 minutes uptake (Fig. 2C), corroborating the late endocytic nature of the CD63-enriched compartments. Double-labeling experiments revealed that a subset of the hVPS4^{EQ}-positive vacuoles was indeed also labeled for CD63 (Fig. 2F). In contrast to the early hVPS4^{EQ} vacuoles, these late vacuoles were significantly enlarged compared with those in control cells. We conclude from these results that hVPS4^{EQ}-positive endosomal vacuoles compose a population of early and late endosomes, and that the hVPS4^{EQ}-induced enlargement of vacuoles occurs during endosomal maturation.

hVPS4^{EQ} increases association of bilayered clathrin coats on endosomal vacuoles

In control or GFP- or hVPS4^{wt}-expressing cells, the bilayered coats on EEs clearly show their characteristic flat, bilayered appearance, positive for Hrs (Fig. 3A,B) and clathrin (not shown). In cells transfected with hVPS4^{EQ}, the bilayered coats displayed the same organized morphology but often covered elongated areas of the limiting membrane of endosomal vacuoles; on some vacuoles, the coat even covered most of the limiting membrane (Fig. 3C,D). Interestingly, label for hVPS4^{EQ} was found not within the coated areas itself but at the non-coated edges (Fig. 3C,D). As shown in Table 1, upon overexpression of hVPS4^{EQ}, the average size of Hrs-positive vacuoles increases about 1.7 times compared with control cells expressing hVPS4^{wt} or GFP alone. This suggests that Hrs, which is normally predominantly localized to EEs (Komada et al., 1997; Sachse et al., 2002a), in hVPS4^{EQ} cells is also present on LEs, because EEs do not increase in size under these conditions (Fig. 2). Notably, both coated and non-coated areas of the limiting endosomal membrane increased 1.7-1.8 times in length. Thus, although endosomes are sometimes heavily coated (Fig. 3C,D), hVPS4^{EQ} does not on average change the ratio between coated and non-coated areas (1:5 in hVPS4^{wt} and hVPS4^{EQ} cells), indicating that these two endosomal subdomains are in a tightly regulated balance. Also, the labeling density (gold particles per arbitrary membrane unit)

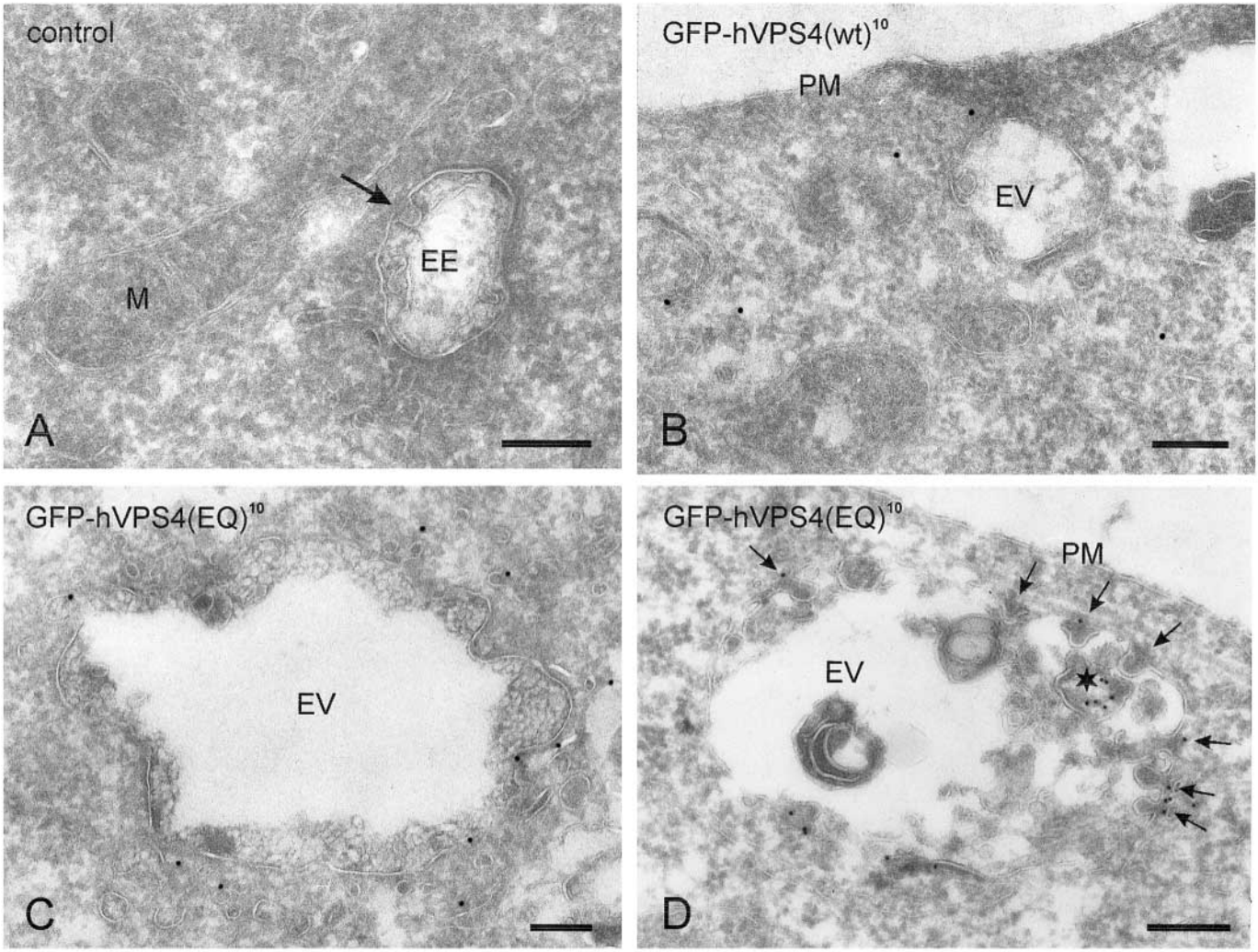

Fig. 1. Overexpression of hVPS4^{EQ} induces enlarged endosomal vacuoles. Cryosections of non-transfected HeLa cells or cells transfected with GFP-hVPS4^{wt} or GFP-hVPS^{EQ} were labeled for GFP (10 nm gold). (A) In non-transfected cells, EEs appear as electron-lucent vacuoles containing few internal vesicles. Only rarely can an inward budding profile (i.e. forming internal vesicle) be observed (arrow). (B) hVPS4^{wt} is mainly cytosolic and the morphology of endosomal vacuoles (EV) is unaffected. (C,D) Overexpression of the ATPase-deficient hVPS4^{EQ} leads to enlarged EVs. Some EVs display lipid particles without a limiting membrane (C). In others, unusually many inward budding profiles are seen (arrows in D). M, mitochondrion; PM, plasma membrane. Scale bars, 200 nm.

for Hrs at the limiting endosomal membranes was strikingly similar in control and hVPS4^{EQ} cells (Table 1). Because the length of the coats had increased, this indicates that the total amount of endosome-associated Hrs increases in hVPS4^{EQ} cells.

The findings that overexpression of a dominant-negative form of hVPS4 induces elongated stretches of Hrs-positive bilayered coat as well as an increased association of Hrs with LEs are both strong implications that hVPS4 plays a role in the dissociation of the bilayered coat.

hVPS4^{EQ} inhibits formation of internal vesicles and retains EGFR at the limiting membrane of endosomal vacuoles

In general, the process of invagination of the limiting endosomal membrane to form an internal vesicle is only occasionally visible in thin sections. In non-transfected HeLa cells or hVPS4^{wt}-expressing cells, these inward budding profiles were only rarely observed (e.g. Fig. 1A, Fig. 3B). However, as already mentioned above, in cells expressing hVPS4^{EQ}, we observed endosomal vacuoles that displayed a series of inward budding profiles (Fig. 1D). In contrast to control cells (Fig. 1A, Fig. 3B) (Sachse et al., 2002a; Murk et al., 2003), these inward budding profiles were not close to a bilayered-coated area. Sometimes, a marked electron-dense cytoplasmic staining was seen on parts of these endosomes including the inward budding profiles (Fig. 1D, Fig. 2F), which did not label for Hrs or clathrin (data not shown). hVPS4^{EQ} was sometimes present at the base of an inward budding profile (Fig. 1D). These dense areas are reminiscent of the inner layer of the bilayered coats in control cells, although more pronounced. However, the fuzzy outer layer of the bilayered coat (clearly seen in, for example, Fig. 3C,D) is absent from

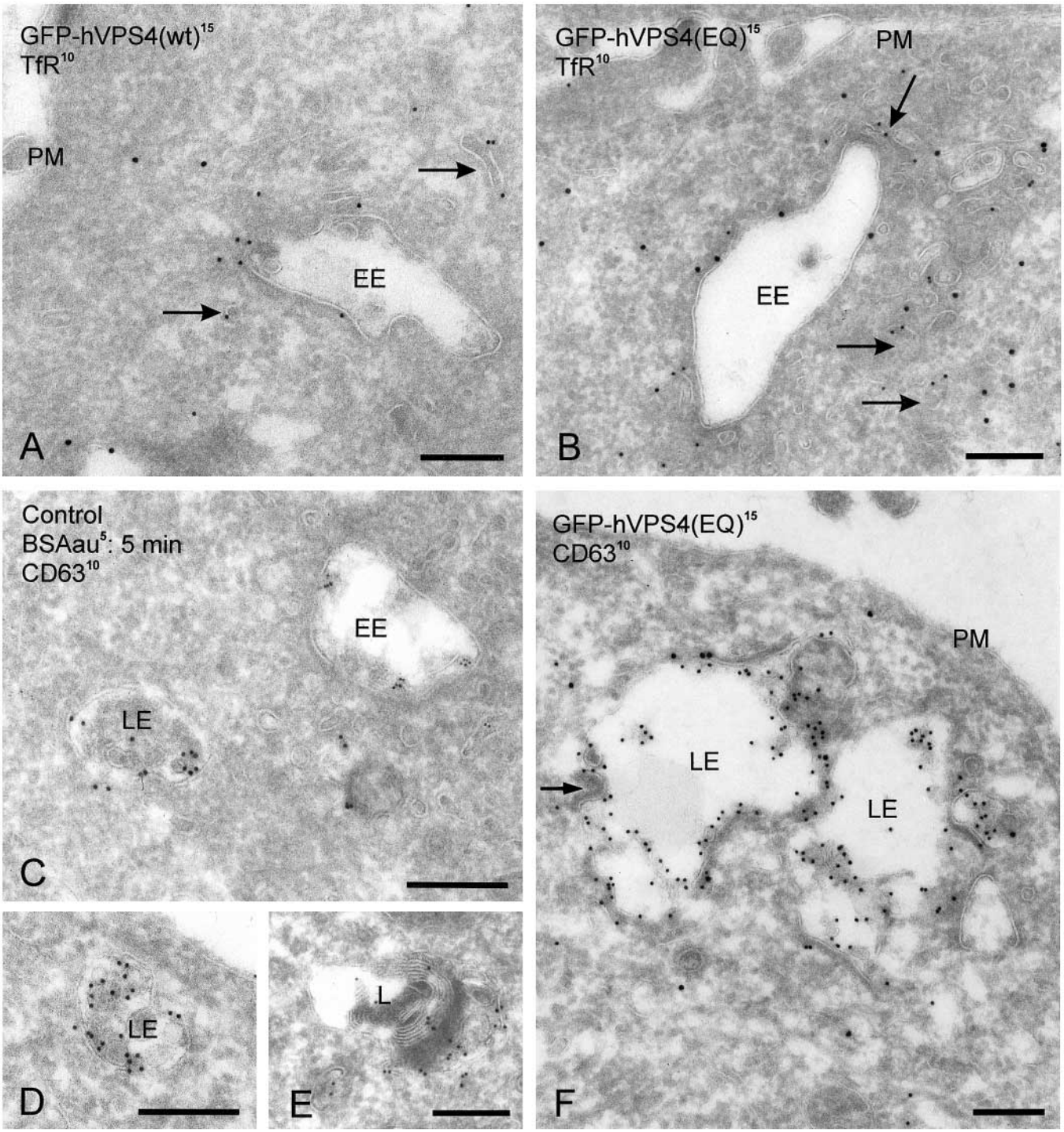

Fig. 2. hVPS4^{EQ} is present on early and late endosomes. (A) In hVPS4^{wt} (15 nm gold) cells, TfR (10 nm gold) is present on EE vacuoles and emerging recycling tubules (arrows). (B) In hVPS4^{EQ} cells, TfR labeling (10 nm gold) is present on normally shaped EEs that also contain hVPS4^{EQ} (15 nm gold). The arrows point to recycling tubules that contain most TfR. (C-E) HeLa cells incubated for 5 minutes with BSA conjugated to 5 nm gold and labeled for CD63 (10 nm gold). BSA-gold is found in EEs but not in LEs or lysosomes (L). CD63 is enriched on internal membranes of LEs and lysosomes, which differ by the presence of internal vesicles or membrane sheets, respectively. (F) hVPS4^{EQ} can also be found on swollen vacuoles that contain internal membranes positive for CD63 (10 nm gold), defining them as LEs. Label for CD63 is present on an inward budding profile (arrow) showing a distinct electron-dense cytoplasmic layer that is also seen at other parts of the limiting membrane. PM, plasma membrane. Scale bar, 200 nm.

these areas, concomitant with the lack of clathrin label. The nature of this dense coating is presently unclear, but the density of the staining indicates a high local protein concentration. Possibly, these areas include ESCRT components that are known to accumulate in endosomes upon hVPS4^{EQ} expression (Babst et al., 1998; Babst et al., 2002a; Bishop and Woodman,

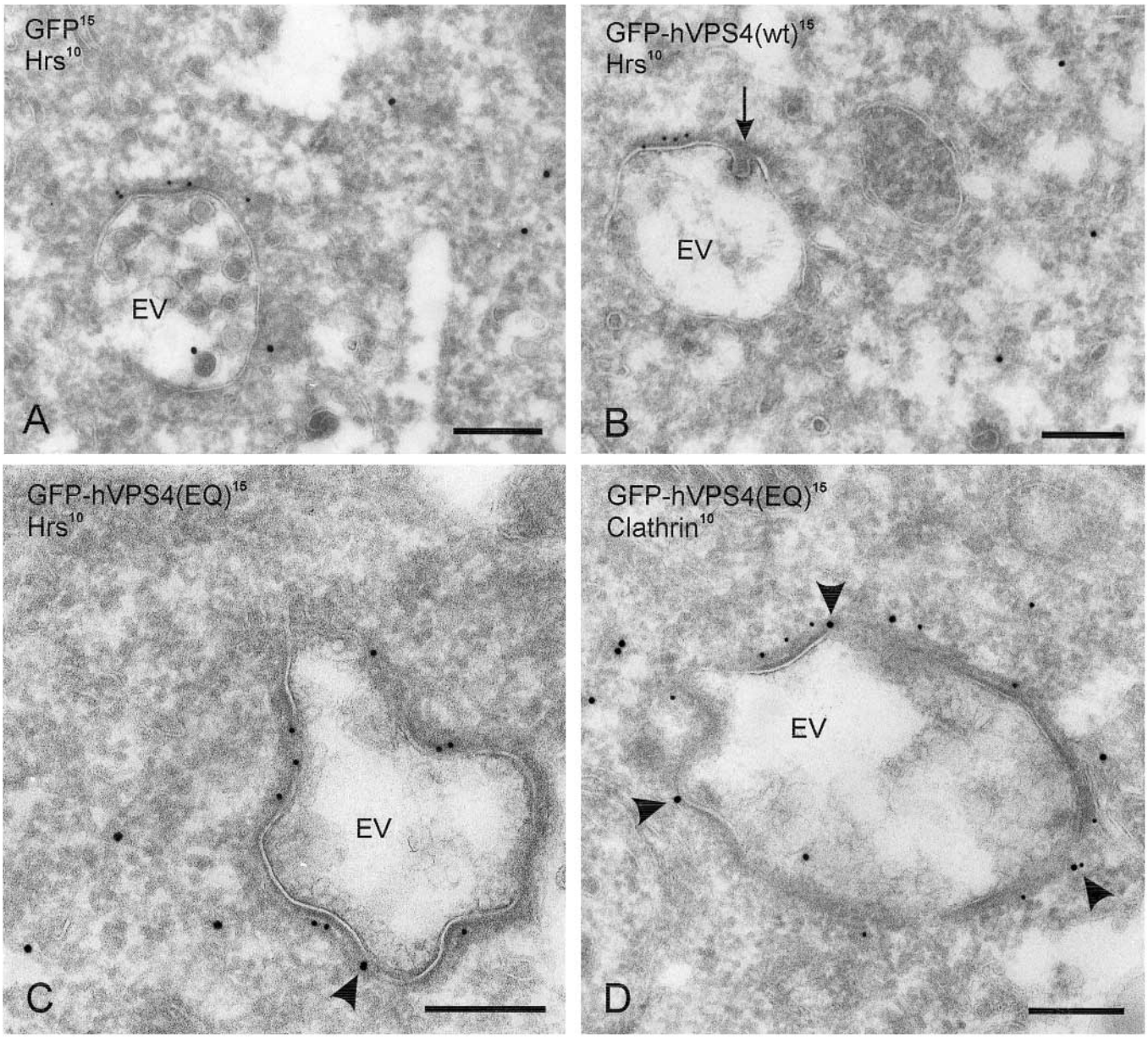

Fig. 3. hVPS4 function is required for the disassembly of bilayered clathrin coats on endosomal vacuoles. HeLa cells transfected with GFP, GFP-hVPS4^{wt} or GFP-hVPS4^{EQ} were double labeled for GFP (15 nm gold) and (A-C) Hrs (10 nm gold) or (D) clathrin (10 nm gold). (A) Cells expressing GFP (15 nm gold) show Hrs (10 nm gold) in the characteristic flat bilayered coats on endosomal vacuoles (EV). (B) Also, in hVPS4^{wt} cells, Hrs (10 nm gold) is present in flat bilayered coats. Inward budding profiles are formed next to the coat (arrow). (C,D) Cells overexpressing hVPS4^{EQ} show endosomal vacuoles positive for Hrs (10 nm gold in C) and clathrin (10 nm gold in D). The coat is assembled in an orderly fashion, displaying the characteristic two layers and label for hVPS4^{EQ} is present not within the coated areas but at the rims (arrowheads). Scale bars, 200 nm.

2000; Bishop and Woodman, 2001). Recently, a similar suggestion was provided for an electron-dense, clathrin-negative coat that appeared on the endosomal membrane upon HIV budding into LEs/multivesicular bodies (Pelchen-Matthews et al., 2003).

To validate the accumulation of inward budding profiles in hVPS4^{EQ} cells, we established the average number of inward budding profiles in hVPS4^{EQ} and hVPS4^{wt} cells. We found a tripling in the number of inward budding profiles in hVPS4^{EQ} cells (13±0.6 inward budding profiles per 20 vacuoles in hVPS4^{EQ} cells, compared with 4±1.2 in hVPS4^{wt} cells, $P<0.001$). The accumulation of inward budding profiles together with the apparent decrease in number of internal vesicles suggests that the formation of internal vesicles is inhibited in cells expressing hVPS4^{EQ}. To address this quantitatively, we labeled EEs by incubating cells for 5 minutes with BSA coupled to 5 nm gold (Sachse et al., 2002b) and counting the number of vesicles in BSA-gold-positive EEs. This analysis confirmed the observation that EEs do not change in size upon hVPS4^{EQ} expression and revealed a significant decrease in the number of internal vesicles compared with GFP or hVPS4^{wt} cells (Table 2). Because the average size and shape

Table 1. Overexpression of hVPS4^{EQ} increases the association of bilayered coats with endosomal vacuoles

	Length of endosomal subdomains			Total length of endosomal membrane
	Coated areas	Non-coated areas	n.d.*	
GFP	29±2.1	200±15.0	66±15.0	295±26.8
hVPS4^{wt}	33±3.8	178±4.9	74±10.3	285±17.2
hVPS4^{EQ}	59±11.3	296±70.2	129±42.6	483±116.8

Numbers represent the length of the individual subdomains of the endosomal limiting membrane in arbitrary units (mean±s.d.) and were obtained by analyzing at least three sets of 15 Hrs-positive endosomal vacuoles per transfection. The labeling density for Hrs (gold particles per arbitrary membrane unit) does not differ between GFP-, hVPS4^{wt}, and hVPS4^{EQ} cells (0.58, 0.55 and 0.6, respectively).

*Areas where the limiting membrane was not clearly visible in the plane of the section.

Table 2. hVPS4^{EQ} affects the number of internal vesicles but not the size of EEs

	Length of limiting membrane	Number of internal vesicles per vacuole
GFP	20.4	3.3±0.5
hVPS4^{wt}	23.5	3.4±0.4
hVPS4^{EQ}	20.8	2.1±0.4

HeLa cells were transfected with the indicated constructs and EEs were specifically labeled by a 5 minute incubation with BSA coupled to 5 nm gold. For each transfection, 40 BSA-gold positive vacuoles were analysed. Numbers represent the average length (arbitrary units) of the limiting membrane of a vacuole or the average number of internal vesicles per vacuole (±s.e.m.). In contrast to the total Hrs-positive endosome population (Table 1), EEs show no increase in size upon hVPS4^{EQ} expression, but internal vesicles are less frequent (P<0.05).

Table 3. hVPS4^{EQ} retains EGFR at the limiting membrane of endosomal vacuoles (EVs)

	EGFR in EVs (% of total endosomal label)		Internal vesicles per vacuole
	Limiting membrane	Internal vesicles	
hVPS4^{wt}	63±9.2	37±9.2	4.0
hVPS4^{EQ}	92±3.0	8±3.0	2.7

HeLa cells were transfected with the indicated constructs and incubated for 20 minutes with 100 nM EGF before fixation. For each construct, three sets of 15 EGFR-positive vacuoles were analysed. Numbers represent the relative distribution of EGFR at the endosomal vacuole (percentage ± s.d.). In total, 148 gold particles were counted for cells expressing hVPS4^{wt} and 119 for cells expressing hVPS4^{EQ}. The number of internal vesicles was also determined.

of the EEs were alike in all conditions, this decrease is not caused by dilution of the vesicles within the swollen vacuoles, implying that hVPS4 function is required for the formation of endosomal vesicles.

Finally, we studied the effect of hVPS4^{EQ} expression on the sorting of the EGFR. In hVPS4^{wt} cells after 20 minutes incubation with EGF, 37% of endosome-associated EGFR labeling was present on internal vesicles (Table 3, Fig. 4A). Under similar conditions in hVPS4^{EQ} cells, internalized EGFR was found almost exclusively at the limiting membrane of endosomal vacuoles (Fig. 4B): only 8% was incorporated into the internal vesicles (P<0.01, Table 3). Notably, the total amount of endosome-associated EGFR in hVPS4^{EQ} cells was only slightly lower than in hVPS4^{wt} cells (119 and 148 gold particles per 45 vacuoles in total, respectively). Finally, in hVPS4^{wt} cells, we found on average 4.0 internal vesicles per EGFR-positive vacuole, whereas in hVPS4^{EQ} cells we found 2.7. The decrease in sorting of EGFR into internal vesicles is therefore at least partially explained by the decrease in number of internal vesicles in hVPS4^{EQ} cells.

Discussion

We have recently characterized a bilayered clathrin coat on endosomal vacuoles and showed that ubiquitinated proteins destined for lysosomes are retained and concentrated in this coat before their incorporation into internal vesicles (Sachse et al., 2002a). The absence of coat material in the internal vesicles suggests that the coat disassembles before inward budding occurs. Alternatively, the coat could be stable while concentrated proteins move out to non-coated areas to be incorporated into an internal vesicle. Here, we show that overexpression of an ATPase-deficient mutant of hVPS4 (hVPS4^{EQ}) increases the association of bilayered clathrin coats with endosomal vacuoles, presumably by inhibiting coat disassembly. Moreover, we show for the first time that functional hVPS4 is required for the formation of internal vesicles. Together, these data imply that the coat undergoes dynamic cycles of assembly and disassembly, and that removal of the coat is required for internal vesicle formation. Finally, we found that the EGFR, which under control conditions is sorted into internal vesicles, is retained at the limiting membrane of endosomes upon hVPS4^{EQ} expression.

In yeast, Vps4p was shown to play a role in the disassembly of endosomal sorting ESCRT complexes as well as other class-E gene products from membranes of the prevacuolar compartment (Babst et al., 1998; Babst et al., 2002a; Bilodeau et al., 2002; Odorizzi et al., 2003). In yeast cells lacking *VPS4* function, binding of ESCRT components to endosomal membranes was increased, demonstrating that Vps4p is involved in the release of these proteins into the cytosol for further rounds of sorting (Babst et al., 1998; Babst et al., 2002a). In addition, expression of dominant-negative mutants of Vps4p results in the formation of malformed LEs, known as the class E compartment (Odorizzi et al., 1998). In agreement with its function in yeast, the hVPS4^{EQ}-induced increased coating of endosomes observed in this study is probably explained by a decreased uncoating. Moreover, our immuno-EM studies show that hVPS4^{EQ} is not or only occasionally present within the Hrs-positive bilayered coats; instead, it is localized at the rims of the coat and in the Hrs-negative areas. If hVPS4 does function in the assembly of coats, it is expected to reside within the coats, as is the case for Hrs (Sachse et al., 2002a). It is not likely that the lack of hVPS4 label in the coat is caused by epitope masking, because the other known bilayered-coat components are readily visualized by the cryosectioning technique, as are the components of other coats [e.g. AP-1, AP-2, GGAs (Golgi-localizing, γ-adaptin ear homology domain, ARF-binding proteins)]. Moreover, antibodies against the cytosolic tail of growth hormone

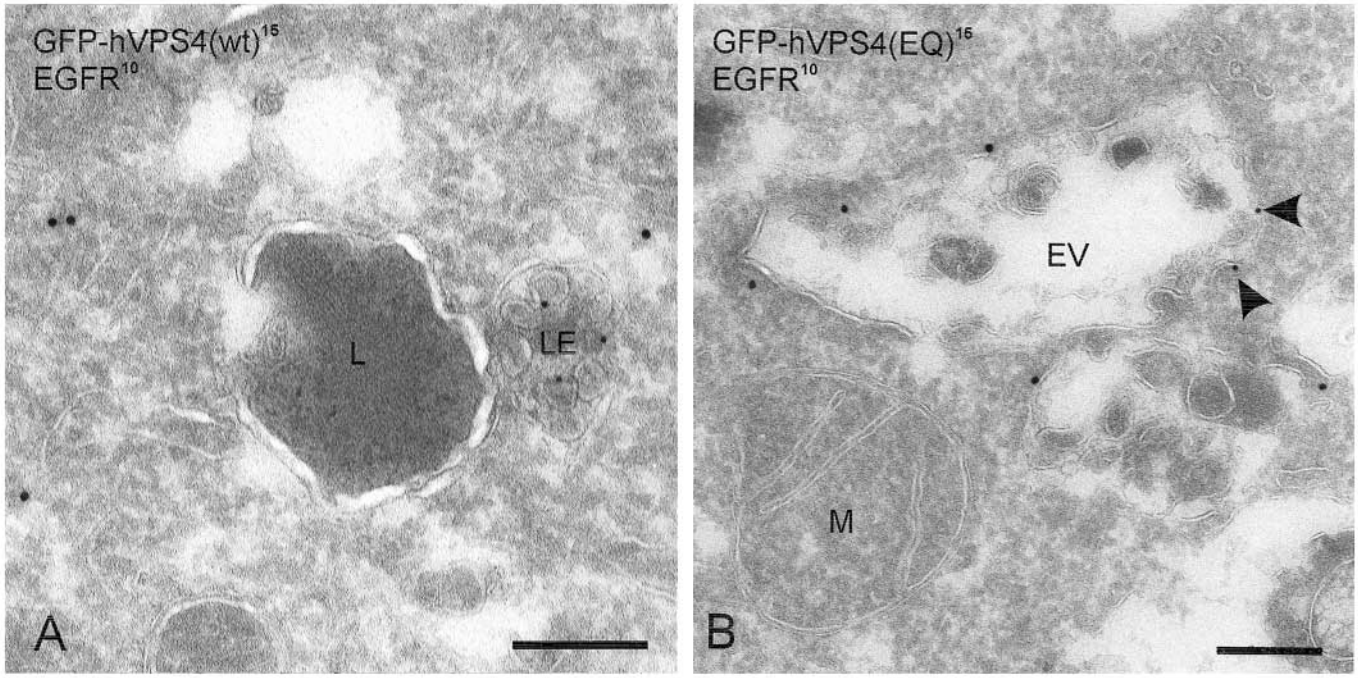

Fig. 4. Incorporation of EGFR into internal endosomal vesicles is inhibited by hVPS4^{EQ}. HeLa cells transfected with GFP-hVPS4^{wt} or GFP-hVPS4^{EQ} and incubated for 20 minutes with EGF were double labeled for GFP (15 nm gold) and EGFR (10 nm gold). (A) EGFR is found on internal vesicles of LEs in cells expressing hVPS4^{wt}. (B) In cells expressing hVPS4^{EQ}, EGFR (arrowheads) is retained at the limiting membrane of endosomal vacuoles (EV). L, lysosome; M, mitochondrion. Scale bars, 200 nm.

receptor detect receptors in the coat with the same efficiency as do antibodies against lumenal growth hormone (Sachse et al., 2002a). A decreased uncoating would also explain the presence of Hrs on LEs in hVPS4^{EQ} cells. Our data are therefore in favor of a role for hVPS4 in disassembly of the coat. How hVPS4 acts on coat removal, direct or indirect, remains to be established. For example, by inhibition of removal of one mammalian Class E orthologs (e.g. ESCRT-III), the entire endosomal sorting machinery might be retained on the limiting membrane. Such a scenario would be in agreement with our finding that, in hVPS4^{EQ} cells, inward budding profiles accumulate but are not in close vicinity to bilayered coated areas as in control cells (Murk et al., 2003; Sachse et al., 2002a).

Whether, under control conditions, disassembly is required for all ESCRT components remains to be seen. Tsg101, which is found on the secreted internal vesicles of LEs; the so-called exosomes (Thery et al., 2001), was recently by immunofluorescence localized to EE as well as LEs (Bache et al., 2003). Immuno-EM studies are required to provide the subendosomal distribution of Tsg101 in these different types of endosomes. Clathrin has also been identified on isolated exosomes (Wubbolts et al., 2003), but we are not able to detect by immuno-EM Hrs or clathrin on internal vesicles of control or hVPS4^{EQ} cells (Sachse et al., 2002a) (this study). Notably, in cells expressing mutant forms of Hrs (HrsΔUIM), we do find coat components on the internal vesicles (Urbé et al., 2003), indicating that our method allows the visualization of proteins when present in these vesicles. The most likely explanation for absence of clathrin labeling control or hVPS4^{EQ} cells is therefore that the concentration is under the detection limit for immuno-EM. A decrease of coat components from the limiting membrane of endosomal vacuoles to internal vesicles is in agreement with coat disassembly before inward budding.

The formation of inward budding profiles specifically at the rims of the coats in control cells (Sachse at al., 2002a) (this study) together with the finding that hVPS4^{EQ} expression increases coat formation and a reduction in the number of internal vesicles (this study) suggest that coat removal is a necessary step for internal vesicle formation. The seemingly contradictory observation that the number of inward budding profiles still connected to the limiting membrane increases in hVPS4^{EQ} cells is probably explained by the fact that hVPS4^{EQ} overexpression delays rather than inhibits the formation of internal vesicles. With a delayed budding process the chance to see these profiles in a ‘snapshot’ will increase. Furthermore, in hVPS4^{EQ} cells, the size and electron density of the internal vesicles was more irregular than in control cells, indicating an impaired, deregulated fission event rather than residual activity of endogenous hVPS4. Additional evidence that the presence of the coat inhibits internal vesicle formation was recently obtained in cells overexpressing Hrs, in which massive clathrin recruitment on EEs occurs (Raiborg et al., 2001; Urbé et al., 2003). Our recent studies (Urbé et al., 2003) showed that, as with hVPS4^{EQ}, this increased coating leads to a reduced formation of internal vesicles, reinforcing the link between coat assembly and internal vesicle formation.

In agreement with others (Bishop and Woodman, 2000; Yoshimori et al., 2000), we found an enlargement of LEs in particular upon hVPS4^{EQ} overexpression, whereas EEs were unaffected in size and morphology. The formation of internal vesicles starts at the EE and continues during endosomal maturation. Thus, when the budding of internal vesicles from

the limiting membranes is inhibited, this might well result in an increase in size of the vacuole that builds up during maturation. A similar enlargement of LEs was described when the formation of internal vesicles is blocked by inhibition of the phosphatidylinositol-(3)-phosphate kinase hVPS34 (Futter et al., 2001). In addition to the formation of internal vesicles, the transport of lipids also seems to be disturbed in hVPS4^{EQ} cells, because we observed an accumulation of lipid particles in the enlarged vacuoles. This provides an additional explanation for the increased size of endosomal vacuoles (e.g. that endocytosed lipids fail to exit the endosomal vacuoles). Finally, we found that EGFR is retained at the limiting membrane of endosomal vacuoles in cells expressing hVPS4^{EQ}. This retention can at least partially be explained by the reduced formation of internal vesicles, but we cannot exclude the possibility that hVPS4^{EQ} also interferes with an additional sorting step for the EGFR.

The ratio of coated versus uncoated membrane areas on endosomal vacuoles did not change in hVPS4^{EQ} cells compared with hVPS4^{wt} cells, and the non-coated areas enlarge in hVPS4^{EQ} cells. Overexpression of hVPS4^{EQ} does not inhibit endocytosis but the mouse ortholog of hVPS4^{EQ} inhibits recycling of endocytosed TfR back to the plasma membrane (Yoshimori et al., 2000). Recycling proteins enter the recycling pathway via endosomal tubules, which emerge from the non-coated areas of the endosomal vacuole (Sachse et al., 2002a). An inhibited membrane efflux from the endosomal vacuoles to recycling tubules could therefore explain the increase in non-coated areas upon hVPS4^{EQ} expression. The constant ratio between coated versus non-coated areas suggests that the balance between degradation versus recycling is tightly regulated.

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