The ubiquitin-dependent endocytosis motif is required for efficient incorporation of growth hormone receptor in clathrin-coated pits, but not clathrin-coated lattices

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SUMMARY

Endocytosis of the growth hormone receptor (GHR) requires an active ubiquitin-conjugation system. In addition, it depends on a 10 amino acid residues motif in the GHR-cytoplasmic tail, the ubiquitin dependent-endocytosis or UbE-motif. To gain insight into the role of ubiquitination in the early steps of endocytosis, we performed an ultrastructural analysis of GH-uptake in Chinese hamster cells expressing wild-type or mutant GHRs. In wild-type GHR cells, GH was found to be exclusively taken up via clathrin-coated pits. In early endosomes it was efficiently sorted from recycling transferrin and targeted to the degradative pathway. Mutation of all lysine residues of a truncated GHR (GHR-399K$^-$) precludes ubiquitination of the receptor, but internalization of GHR-399K$^-$ still depends on an active ubiquitin system. We found that GHR-399K$^-$ incorporates GH into clathrin-coated vesicles with the same efficiency as wild-type GHR. By contrast, a mutation in the UbE-motif (GHR-F327A) largely abolished incorporation of GH into clathrin-coated vesicles. Notably, access of GH to clathrin-coated lattices was not affected in GHR-F327A cells. These data corroborate and extend previous data that the UbE-motif but not ubiquitination of the receptor itself recruits GHR into clathrin-coated vesicles. Moreover, they suggest that incorporation of GHR into clathrin-coated lattices is differentially regulated from incorporation into clathrin-coated pits.

Key words: Growth hormone receptor, Endocytosis, Clathrin, Ubiquitination

INTRODUCTION

The ubiquitin-proteasome degradation pathway provides the major pathway for non-lysosomal degradation (Hershko and Ciechanover, 1998). It is involved in the degradation of cytoplasmic proteins and proteins, which do not pass the quality control of the ER, and plays a role in regulating a variety of cellular functions (Plemper and Wolf, 1999). Degradation is initiated by the ubiquitin-conjugation system, through which poly-ubiquitin moieties are attached to cytoplasmic proteins, after which the modified proteins are degraded by a multi-subunit protease, the 26S proteasome (Thrower et al., 2000). Recently, it became evident that for a restricted number of plasma membrane proteins, ubiquitination triggers internalization rather than proteasomal degradation (Strous and Govers, 1999). This pathway is best understood in yeast, where several plasma membrane proteins are endocytosed in an ubiquitin-dependent manner (Galan et al., 1996; Hicke and Riezman, 1996; Kölling and Hollenberg, 1994; Terrell et al., 1998; Hicke, 1999).

In mammalian cells, the best studied example of a protein undergoing ubiquitin-mediated endocytosis is the growth hormone receptor (GHR). When growth hormone (GH) is added to cells, it binds to two GHR molecules. After binding, phosphorylation of these receptors by JAK2 and ubiquitination occurs. Following uptake, GHR and its ligand remain bound until they are both degraded in lysosomes (Murphy and Lazarus, 1984). We have previously shown that ligand-dependent internalization of GHR is inhibited when the ubiquitin-activating enzyme E1 is inactivated (Strous et al., 1996). Ubiquitination and uptake of GHR depends on the integrity of a ^{322}DSWVEFIELD^{331} domain in the cytosolic tail of the receptor, named the UbE-motif (Govers et al., 1999). In this motif, the phenylalanine-residue at position 327 (F327) and the aspartic acid residue at position 331 (D331) are essential. Mutations at these positions affect ubiquitination as well as internalization of the receptor. Despite these findings, ubiquitination of GHR itself is not necessary for endocytosis. When all lysine residues in the cytoplasmic tail of a truncated GHR (GHR-399K$^-$) are mutated to arginines, which abolishes ubiquitination of the receptor, GH is still internalized and endocytosis remains ubiquitin system dependent (Govers et al., 1999; van Kerkhof et al., 2000).

Endocytosis of GHR is blocked when assembly of clathrin at the plasma membrane is disturbed (Govers et al., 1997; Strous et al., 1996), implying that GHR is taken up by clathrin-mediated endocytosis. In addition, a role for caveolae in GH endocytosis has been suggested (Lobie et al., 1999). The formation of a clathrin-coated vesicle starts with the assembly of clathrin at the plasma membrane (Kirchhausen 2000a; Schmid, 1997). By invagination of the plasma membrane, clathrin-coated pits are formed, which give rise to clathrin-

coated vesicles. When clathrin assembly at the plasma membrane is blocked by K^+ depletion, no ubiquitination of GHR occurs (van Kerkhof et al., 2001). Yet, when clathrin assembly onto the plasma membrane is allowed but formation of clathrin coated vesicles is blocked, by cholesterol depletion or a mutant dynamin, ubiquitination of GHR still occurs. These data clearly indicate that ubiquitination of GHR can occur at the plasma membrane (van Kerkhof et al., 2001) and suggest a link between ubiquitination and recruitment of the receptor to clathrin-coated membranes. However, they do not establish the stage of internalization at which the ubiquitin machinery and the UbE-motif function. To gain more insight into the regulation of these very first steps of GHR endocytosis, we have used immunogold electron microscopy to study the uptake of GHR in situ. We addressed the role of ubiquitination herein, by using cell lines expressing wild-type or mutant GHRs. This approach allowed us to study the targeting of GHR to the different stages of clathrin-coated vesicle formation – lattices, pits and vesicles – without interfering with general uptake mechanisms. Our data show that an intact UbE-motif but not ubiquitination of the receptor itself is required to incorporate GH-GHR complexes in clathrin-coated pits and vesicles. Unexpectedly, our findings revealed that under conditions in which incorporation into clathrin-coated pits is significantly decreased, the occurrence of GHR in clathrin-coated lattices is relatively unaffected.

MATERIALS AND METHODS

Materials and antibodies

GH was a gift of Lilly Research Laboratories (Indianapolis). Sulfusuccinimidyl 6-(biotinamido) hexanoate (NHS-LC-biotin) for biotinylation of GH was obtained from Pierce (Rockford, IL). GH was biotinylated as described (Bentham et al., 1994). Guinea pig polyclonal antibody recognizing human GH was purchased from Biogenesis (Poole, UK), rabbit polyclonal antibody against biotin was obtained from Rockland (Gilbertsville, PA), rabbit antiserum against mouse Cathepsin D (Pohlmann et al., 1995) was a kind gift from Dr K. v. Figura (Göttingen). Polyclonal rabbit antibody against caveolin 1 and mouse monoclonal antibody against clathrin heavy chain were obtained from Transduction Laboratories (Lexington, KY) and polyclonal rabbit antibody against mouse IgG from DAKO (Denmark).

Cells

The Chinese hamster cell line ts20, containing a thermosensitive E1-enzyme (Kulka et al., 1988), was used in this study. It should be noted, however, that all experiments were carried out at the permissive temperature; the role of ubiquitination on endocytosis was studied only by mutations in the GHR itself and not by a general block. Construction of the mutant GHRs and transfection of ts20 cells with cDNA encoding either wild-type rabbit GHR, GHR 1-399 K271-362R (GHR-399K$^-$), or GHR 1-620 F327A (GHR-F327A), was as described previously (Govers et al., 1997; Govers et al., 1999). Cells were routinely grown at the permissive temperature of 30°C in Eagle's minimal essential medium (MEMα) supplemented with 4.5 g/l glucose, 10% fetal bovine serum, penicillin, streptomycin and 0.45 mg/ml geneticin. For experiments, cells were grown in 60 mm dishes in the absence of geneticin. To increase GHR expression, 10 mM sodium butyrate was added to the cells 18 hours before use.

Internalization of GH, BSA-gold and biotinylated transferrin

Cells were incubated for 1 hour in MEMα containing 0.1% bovine serum albumin (BSA) for serum depletion, after which 8 nM GH or biotinylated GH was added and cells were incubated for a further 30 minutes. Then cells were washed three times with MEMα containing 0.1% BSA, fixed and processed for immunoelectron microscopy as described below. BSA conjugated to 5 nm colloidal gold was used to mark the endocytic pathway (Slot et al., 1988). Before use, BSA-gold was dialyzed overnight against phosphate-buffered saline at 4°C. BSA-gold was added at a final optical density of 5 at 520 nm, after which the incubation was continued for 1 hour. Biotinylated transferrin was purchased from Sigma. Iron saturation was as described (Stoorvogel et al., 1987). Cells were incubated in MEMα containing 0.1% BSA for 1 hour at 30°C. Biotinylated Tf was added to a final concentration of 20 μg/ml, after which cells were incubated for a further 30 minutes.

Immunogold labeling of cryosections

Cells were fixed either in 4% paraformaldehyde or in 2% paraformaldehyde and 0.2% glutaraldehyde in 0.1 M phosphate buffer pH 7.4 for 30 minutes on ice, followed by 3 hours at room temperature. Processing of cells for ultrathin cryosectioning and immunolabeling according to the protein A-gold method was as described previously (Slot et al., 1991). In brief, fixed cells were washed with 0.02 M glycine in phosphate-buffered saline (PBS), scraped gently from the dish in 1% gelatine in PBS and pelleted in 12% gelatin in PBS. The gelatin cell pellet was solidified on ice and cut into small blocks. For cryoprotection, blocks were infiltrated overnight with 2.3 M sucrose at 4°C and afterwards mounted on aluminum pins and frozen in liquid nitrogen. To pick up ultrathin cryosections, a 1:1 mixture of 2.3 M sucrose and 1.8% methylcellulose was used (Liou et al., 1996).

Semi-quantitative analysis of GH distribution

No differences in the overall labeling pattern of GH was observed when cells were fixed with or without glutaraldehyde. However, omission of glutaraldehyde resulted in a higher labeling efficiency, which is why we used this fixation condition for quantitative analyses. Addition of GH and detection with anti-GH antibody yielded identical labeling patterns as incubation with biotinylated-GH and detection with anti-biotin, and will therefore be discussed collectively. To establish the overall distribution of endocytosed GH (Table 1), areas of a grid were selected at low magnification for good overall ultrastructure and then scanned at a magnification of 12,000× along a linear track. All gold particles within a distance of 20 nm from a membrane were considered as membrane-associated and assigned to the compartment on which they were localized. In total, three independent counting sessions were performed for each cell line and the occurrence of GH over a compartment was expressed as percentage of total label. Clathrin was identified by immunolabeling and/or by its electron-dense appearance in the electron microscope. Clathrin-coated pits were defined as invaginations of the plasma membrane positive for clathrin. Clathrin-coated vesicles near the plasma membrane were counted as a separate category, but it should be noted that a subset of these vesicles, out of the plane of sectioning, might still be connected to the plasma membrane. Clathrin-coats at the plasma membrane were defined as 'lattice' when they lacked curvature. Caveolae appeared as non-coated, flask-like, sometimes branched invaginations. Immunolabeling of caveolin 1 corroborated this definition (not shown). To compare the number of clathrin-coated lattices and pits in wild-type GHR and GHR-F327A cells (Table 2A), we examined for each cell line 45 cell profiles and counted the number of lattices and pits at the plasma membrane. Plasma membrane length measurements (Table 2B) were carried out on randomly photographed cells with a visible nucleus as the only criterion. The length of the plasma membrane of wild-type GHR and GHR-F327A cells was compared by placing a transparent overlay displaying a squared line-lattice of 1 cm apart over the pictures (final magnification, 8100×) and counting the number of intersections (Weibel, 1979). To study the

relative distribution of GH over the three categories of clathrin-coated structures – lattices, pits and vesicles – 50 GH-positive clathrin-coated structures were analyzed and the number of structures of either category that was positive for GH, was expressed as percentage of the total of GH-positive structures (Table 3). For labeling density measurements (Table 4), pictures of 15 cell profiles with a visible nucleus and a minimum of 10 gold particles at the plasma membrane were randomly taken for wild-type GHR and GHR-F327A cells. A transparent overlay with a squared line-lattice of 5 mm apart was placed over the pictures (final magnification, 32,400×) and the length of the plasma membrane was measured by counting the number of intersections with the line lattice overlay. The length of the membrane was calculated as described by Griffiths (Griffiths, 1993). Labeling densities were then expressed as number of gold particles per μm membrane.

RESULTS

GH uptake by GHR

Because a detailed ultrastructural analysis of the pathway of GHR endocytosis was lacking, we initiated our studies by characterizing the endocytic compartments involved in GH uptake. To this end, ts-20 cells stably expressing wild-type rabbit GHR (wild-type GHR cells), were incubated with GH, after which GH was visualized by immunogold labeling of ultrathin cryosections. Incubation with GH leads to the formation of a ligand-receptor complex, which remains stable at endosomal pH values and only is destroyed by lysosomal breakdown (Roupas and Herington, 1986). Thus, visualization of the endocytic pathway of GH also delineates the pathway of GHR endocytosis. An important advantage of studying GH instead of GHR uptake, is that GHR in the biosynthetic pathway remains unlabeled, which largely facilitates interpretation of the labeling pattern.

After 30 minutes uptake by wild-type GHR cells, GH could be detected at the plasma membrane, in clathrin-coated pits and vesicles (Fig. 1A, Fig. 2B), primary endocytic vesicles (not shown) and in early and late endosomes (Fig. 1C,D). In endosomes, GH was seen on the limiting membrane as well as on internal vesicles. Caveolae were constantly devoid of label (Fig. 1B). Non-transfected cells were lacking label under these conditions (not shown), indicating that GH endocytosis was specifically mediated by exogenously expressed GHR. We next co-incubated and/or double-labeled GH with proteins specifically marking a subset of endosomal membranes. Exogenously added transferrin (Tf) binds to the transferrin-receptor (TfR) after which the complex quickly passes through the vacuolar part of early endosomes, also known as sorting endosomes, to enter tubular recycling endosomes for transport back to the plasma membrane (Prekeris et al., 1998; Sönnichsen et al., 2000; Stoorvogel et al., 1996). Double-labeling of GH and Tf showed that the two ligands co-localize in the vacuolar part of early endosomes (Fig. 1E), which appeared as electron-lucent vacuoles with few internal vesicles. As expected, the majority of Tf was detected in recycling tubules and vesicles near endosomes or close to the plasma membrane (Fig. 1E). In contrast to a previous report (Ilondo et al., 1992) but consistent with its targeting to lysosomes, GH was not found in these Tf-rich recycling endosomes (Fig. 1E).

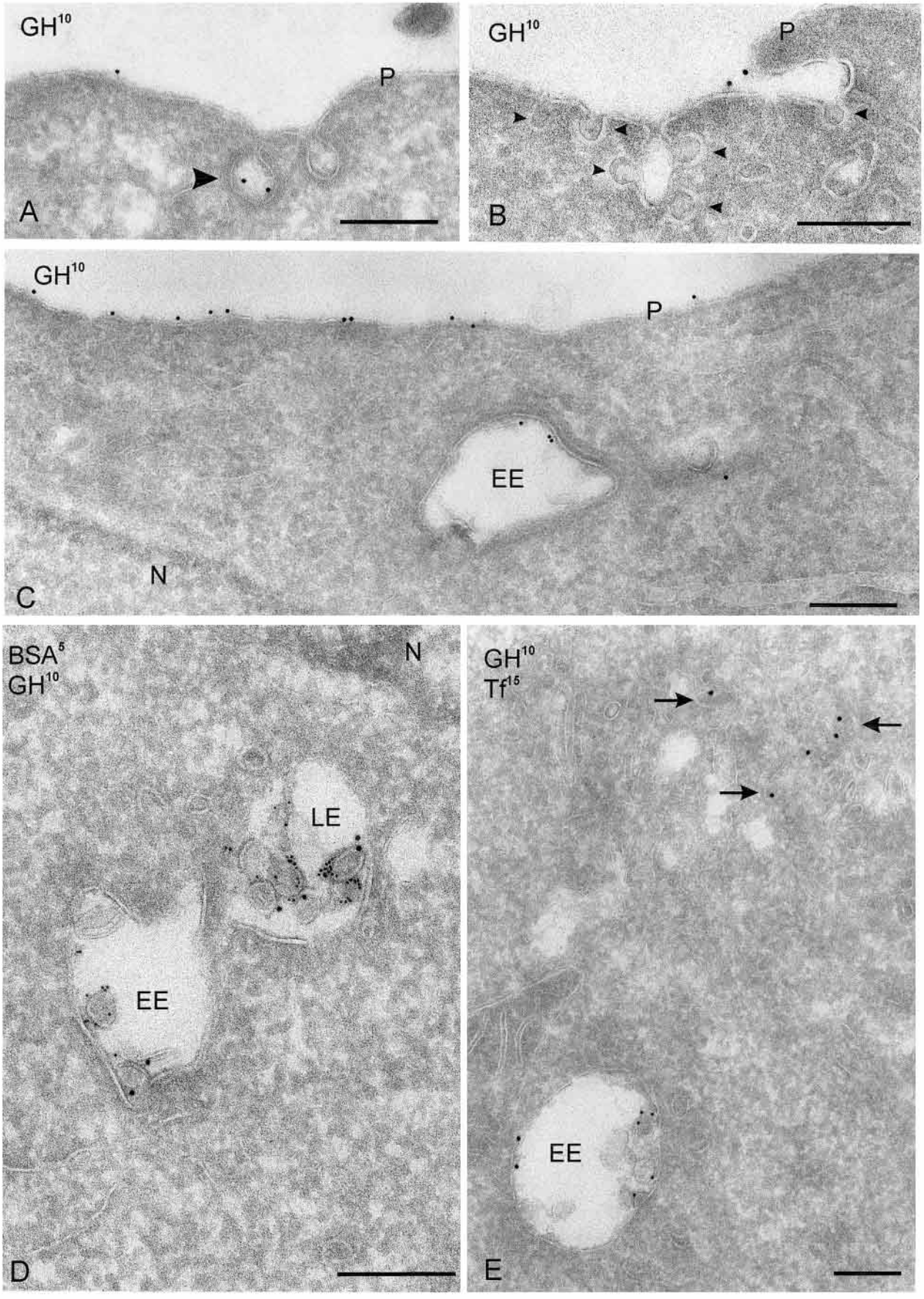
During maturation, endosomes form internal vesicles by inward budding from the limiting membrane, eventually resulting in late endosomes, which appear in the electron microscope as multivesicular bodies (MVBs) (Geuze, 1998). Incorporation of a protein into internal endosomal vesicles is a necessary step for subsequent degradation in lysosomes (Futter et al., 1996). Lysosomes, in contrast to late endosomes, have an electron dense content with only occasional internal membranes. In ts-20 cells, both late endosomes and lysosomes contained significant levels of the lysosomal enzyme cathepsin D (not shown). GH was consistently found on internal vesicles of early and late endosomes (Fig. 1D, Fig. 3A-C), but even after 1 hour's uptake could not be detected in dense lysosomes (Fig. 3B,D). To measure whether exogenous tracer can reach lysosomes at all within this time frame, cells were incubated with BSA-5 nm gold for 1 hour. This resulted in the labeling of early sorting endosomes, late endosomes and, most importantly in this respect, lysosomes (Fig. 1D, Fig. 3). Based on these data, the most likely explanation for lack of GH label in lysosomes is a rapid degradation of the antigenic epitope recognized by the antibody. This explanation is also consistent with the appearance of TCA-soluble counts in the medium, 1 hour after cells are incubated with [^{125}I]GH (Govers et al., 1998).

In summary, the data in Fig. 1, Fig. 2 and Fig. 3 are consistent with a clathrin-mediated uptake of GHR, followed by an efficient targeting to late endosomes and lysosomes, after which a rapid degradation of the ligand occurs. Notably, in contrast to what was previously reported (Lobie et al., 1999), caveolae consistently lacked GH (Fig. 1B), indicating clathrin-mediated endocytosis as the main pathway of GHR internalization.

A GHR mutant lacking an intact UbE-motif (GHR-F327A) is poorly internalized

We previously showed that ubiquitin-system dependent internalization of the GHR requires the so-called UbE-motif (Govers et al., 1999). Mutation of the phenylalanine at position 327 to alanine (GHR-F327A), which is part of this motif, impairs both internalization and ubiquitination of the full-length GHR (Govers et al., 1997). Several observations have indicated that ubiquitination is linked to the clathrin assembly machinery. When clathrin assembly at the plasma membrane is inhibited by K^+ depletion, no ubiquitination occurs. However, when clathrin assembly is allowed but formation of clathrin-coated vesicles is inhibited, by cholesterol depletion or a mutant dynamin, ubiquitination of GHR still occurs (van Kerkhof et al., 2001). To pin-point the block in endocytosis and address the question if the UbE-motif is necessary for incorporation into clathrin-coated pits, we compared the GH distribution in wild-type GHR and GHR-F327A cells by semi-quantitative immunoelectron microscopy.

In wild-type GHR cells, after 30 minutes incubation with GH, nearly 40% of GH was found in endocytic vesicles plus endosomes, whereas an additional 10% was found in clathrin-coated lattices, pits and vesicles near the plasma membrane (Table 1, Fig. 2). The remaining 50% of total GH label was localized to non-specialized areas of the plasma membrane. In GHR-F327A cells, 30 minutes incubation with GH resulted in its accumulation at the plasma membrane (Fig. 4A and Table 1). In these cells, only 10% of total GH label was present over endocytic compartments. Clathrin-coated pits and vesicles were largely devoid of GH (Table 1, Fig. 4C-E), but a notably 3% of the total GH label was still found in clathrin-coated


GH10
P
A
GH10
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B
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BSA5
GH10
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LE
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D
GH10
Tf15
EE
E

Fig. 1. Ultrathin cryosections of wild-type GHR cells showing the endocytic compartments involved in GH endocytosis. GH was added to cells for 30 minutes, after which cells were fixed and GH was visualized by immunogold labeling. (A) GH (10 nm gold) is internalized from the plasma membrane (P) via clathrin-coated pits. The electron dense layer (arrowhead) at the cytoplasmic site of the coated pit is characteristic for the presence of clathrin. (B) GH (10 nm gold) is present at the plasma membrane in close vicinity to caveolae (small arrowheads), which are always devoid of GH label. (C) Early after uptake, GH (10 nm gold) is transported to vacuolar early endosomes (EE). (D) At a later stage of endocytosis, GH (10 nm gold) is found on the intra-endosomal vesicles of early (EE) and late (LE) endosomes, indicative of targeting to lysosomes. The 5 nm gold represents internalized BSA-gold that was added for 1 hour. (E) Double-labeling of GH (10 nm gold) and internalized Tf (15 nm gold) shows co-localization in vacuolar early endosomes (EE), but not in the Tf-rich recycling endosomes (arrows). N, nucleus. Scale bars: 200 nm.

lattices (Table 1, Fig. 4B-C). To exclude that the decrease of GH-labeling in clathrin-coated pits was due to an overall decline in the number of clathrin-coated structures at the plasma membrane of GHR-F327A cells, we compared the number of clathrin-coated lattices and pits in wild-type GHR and GHR-F327A cells. As shown in Table 2, these numbers are very similar. In addition, the length of the plasma membrane in GHR and GHR-F327A cells was the same. Hence, the absolute number of clathrin-coated lattices and pits in wild-type GHR and GHR-F327A-expressing cells are equivalent, indicating that the decrease of GH labeling in clathrin-coated pits of GHR-F327A cells results from a decreased incorporation of the receptor in these structures.

In addition, to rule out that the accumulation of GH at the plasma membrane in GHR-F327A cells was due to a general effect on clathrin-mediated endocytosis, we performed a quantitative analysis of the uptake of Tf. After 30 minutes of incubation, comparable amounts of Tf, 22% in wild-type GHR and 19% in GHR-F327A cells, were found at the plasma membrane. This observation confirms that the F327A mutation in the GHR results in a specific block of internalization for the receptor, which does not affect endocytosis in general.

A GHR mutant, which can not be ubiquitinated, is incorporated into clathrin-coated pits with the same efficiency as wild-type GHR

To address the role of ubiquitination of GHR itself, we visualized GH-uptake by a mutant GHR of which the cytoplasmic tail was truncated after amino acid residue 399 and all lysines were replaced by arginines (GHR-399K$^-$). Because lysine residues are attachment sites for ubiquitin, GHR-399K$^-$ cannot be ubiquitinated. Nevertheless, uptake studies have shown that GHR-399K$^-$ internalizes GH (Govers et al., 1999; van Kerkhof et al., 2000). It should be emphasized that the UbE-motif in GHR-399K$^-$ is still intact and that internalization depends on an active ubiquitin-system. By quantitative analysis of the GH labeling pattern after 30 minutes uptake, we found that approximately 50% of GH was present at the plasma membrane, which is similar as observed in wild-type GHR cells (Table 1). Additionally, later transport stages were not influenced. GH was frequently found on internal vesicles of MVBs, indicating targeting to lysosomes. Similar to wild-type GHR cells, in GHR-399K$^-$ cells GH was not found in caveolae and was distributed equally between clathrin-coated lattices, pits and vesicles (Table 1). These data unequivocally demonstrate that GHR-399K$^-$, which contains no

Table 1. Relative distributions of GH in wild-type GHR, GHR-F327A and GHR-399K$^-$ cells

	Plasma membrane	Endocytic vesicles	Endosomes	Clathrin-coated lattices	Clathrin-coated pits	Clathrin-coated vesicles	Caveolae
Wild-type GHR	50.5±1.4	14.4±0.6	24.8±0.9	3.4±0.2	2.2±0.7	4.2±1.1	0.5±0.0
GHR-F327A	84.8±1.2	4.2±0.4	5.9±0.4	3.1±0.5	1.1±0.7	0.6±0.3	0.3±0.3
GHR-399K$^-$	44.7±0.8	20.6±1.0	28.9±1.1	2.4±0.3	2.6±0.2	1.8±0.5	0.5±0.3

Cells were allowed to take up GH for 30 minutes and processed for immunogold labeling of GH. Numbers represent the percentage of total gold label (±s.e.m.) found over the indicated compartments.

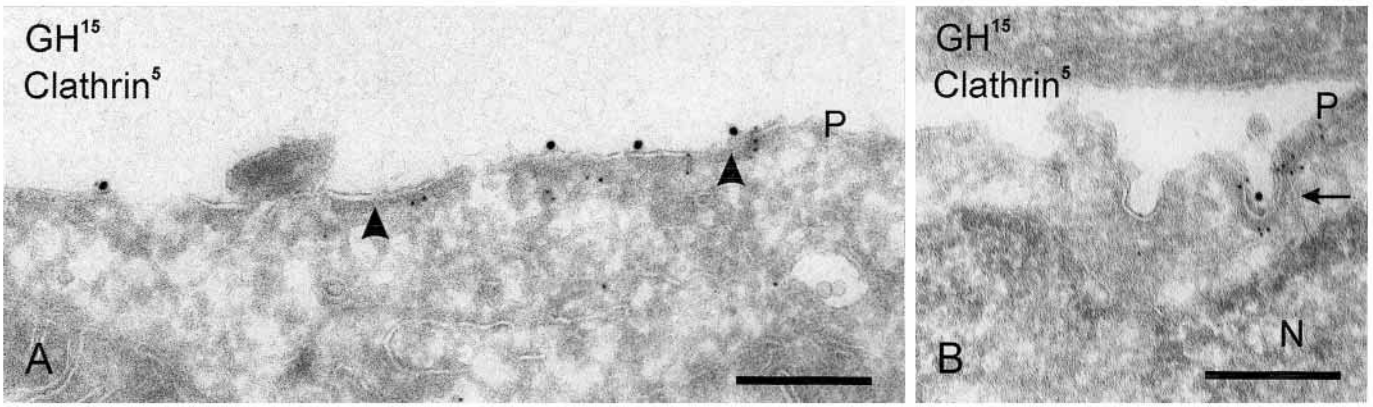

Fig. 2. Wild-type GHR cells incubated for 30 minutes with GH were double immunolabeled for GH (15 nm gold) and clathrin (5 nm gold) to unequivocally identify coated areas. (A) GH was found at non-coated areas of the plasma membrane as well as in flat clathrin-coated lattices (arrowheads). (B) In addition, GH was regularly seen in clathrin-coated pits (arrow). P, plasma membrane. Scale bars: 200 nm.

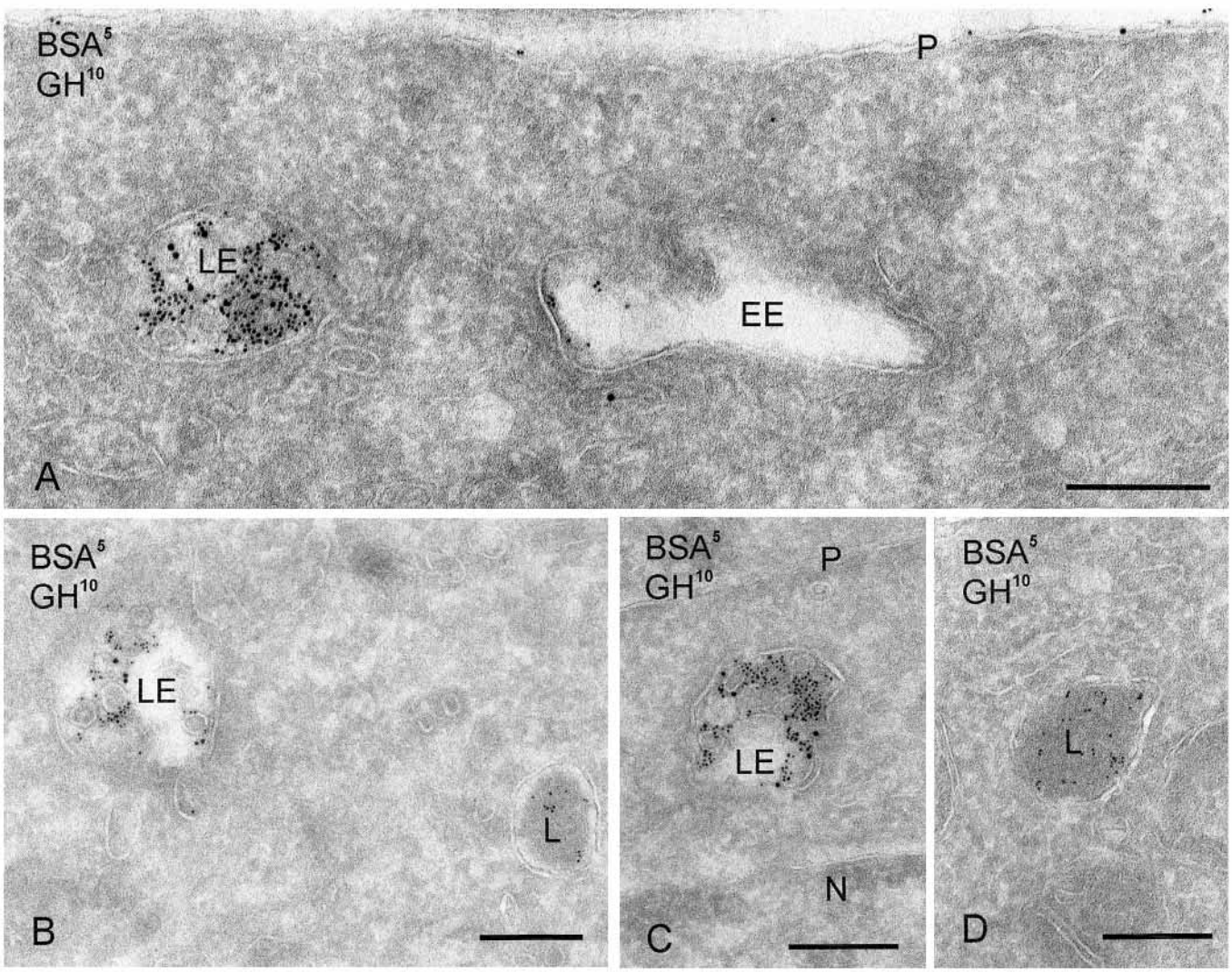

Fig. 3. Cryosections of wild-type GHR cells co-incubated with BSA-5 nm gold and GH for 1 hour. (A-C) BSA-gold (5 nm) and GH (10 nm gold) co-localize extensively in late endosomes (LE). (B,D) Lysosomes (L) are reached by BSA gold but lack GH staining, which is probably due to rapid degradation. EE, early endosome; N, nucleus; P, plasma membrane. Scale bars: 200 nm.

ubiquitination sites, is taken up by the same pathway and to the same extent as the wild-type GHR. Thus, abolishing ubiquitination of the GHR itself does not interfere with its internalization.

GHR-F327A is concentrated in clathrin-coated lattices and excluded from clathrin-coated pits when compared with non-coated plasma membrane domains

The data in Table 1 show that in GHR-F327A cells, clathrin-coated lattices are still regularly labeled for GH (Fig. 4B-C). This prompted us to study the relative distribution of GH over distinct clathrin-coated structures in more detail (Table 3). In these quantitations we also included GHR399K$^-$ cells. In both wild-type GHR and GHR399K$^-$ cells, about equal numbers of GH-positive clathrin-coated lattices, pits and vesicles were found. By contrast, in GHR-F327A cells, the vast majority (56%) of GH-positive clathrin-coated structures were lattices. To measure GH concentrations over the distinct plasma membrane domains, we also determined the labeling densities for GH in wild-type GHR and GHR-F327A cells (Table 4). In wild-type GHR cells, GH was at least twice as concentrated in clathrin-coated lattices and pits when compared with non-coated domains of the plasma membrane. By contrast, in GHR-F327A cells, GH concentrations in clathrin-coated pits were even lower than on the non-coated plasma membrane,

Table 2. Wild-type GHR and GHR-F327A cells display equivalent numbers of clathrin-coated lattices and pits

		Wild-type GHR	GHR-F327A
A	Clathrin-coated lattices	55	49
	Clathrin-coated pits	86	88
B	Membrane length	38.2±2.2	37.9±2.2

(A) The figures represent the total number of clathrin-coated lattices and pits found in 45 cell profiles of each cell type.

(B) The membrane length (arbitrary units±s.e.m.) was measured by counting the number of intersections with a transparent overlay displaying squared lines at 1 cm distance (n=27 cell profiles).

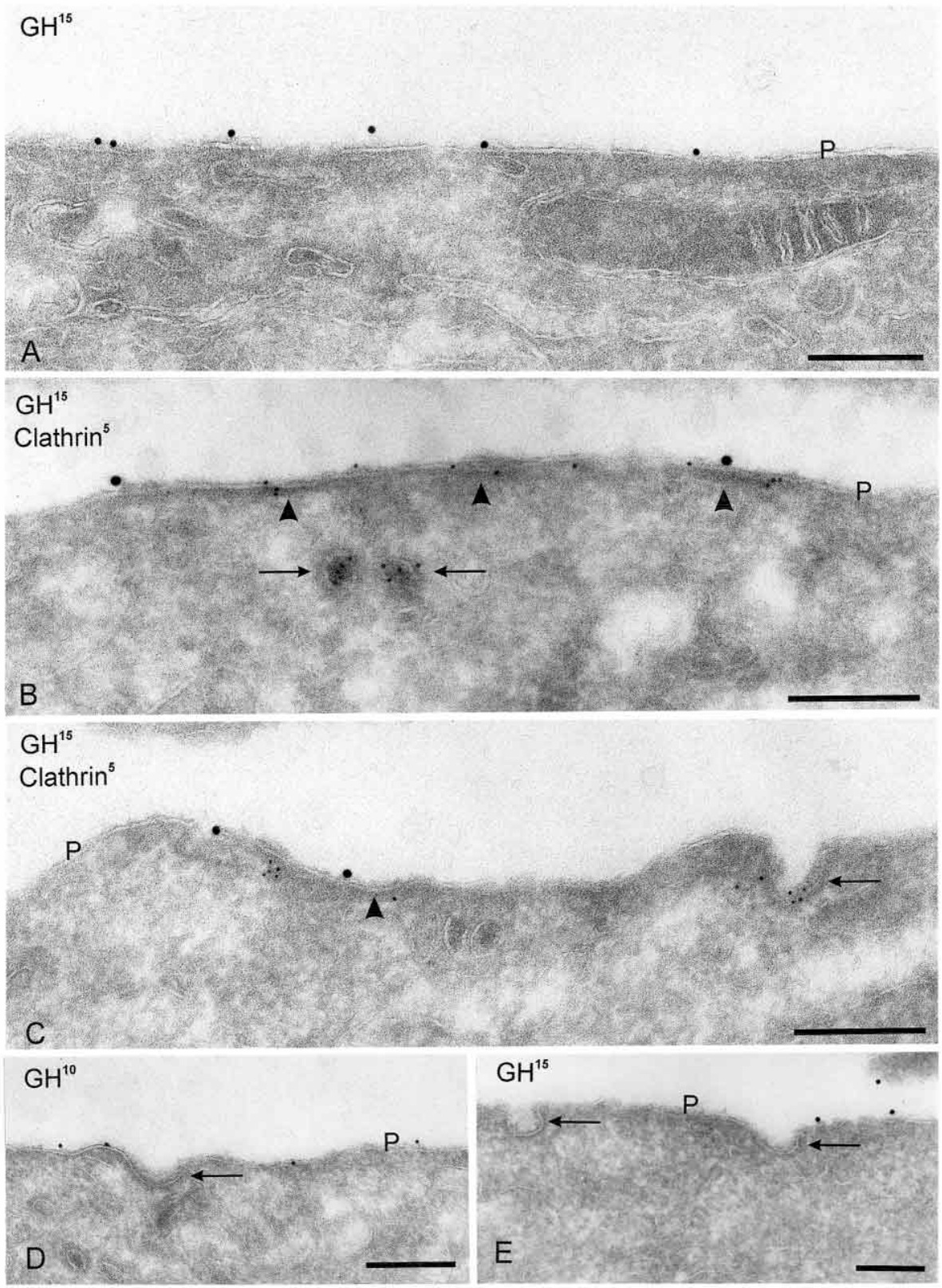

Fig. 4. Cryosections of GHR-F327A cells incubated for 30 minutes with GH, showing the exclusion of GH from clathrin-coated pits and vesicles. (A) GH (15 nm gold) accumulates at the plasma membrane (P). (B) GH (15 nm gold) can also be found associated with clathrin-coated lattices (arrowheads) at the plasma membrane, which positively label for clathrin (5 nm gold). The arrows point to clathrin-coated vesicles. (C) GH (15 nm gold) is found in a clathrin (5 nm gold) -coated lattice (arrowhead), but not in the clathrin-coated pit (arrow). (D) GH (10 nm gold) and (E) GH (15 nm gold) are two more examples, showing that GH is excluded from clathrin-coated pits (arrow). Scale bars: 200 nm.

Table 3. Distribution of GH over different categories of clathrin-coated structures at the plasma membrane of wild-type GHR, GHR-F327A and GHR-399K⁻ cells

	Wild-type GHR	GHR-399K⁻	GHR-F327A
Clathrin-coated lattices	30	33	56
Clathrin-coated pits	32	35	28
Clathrin-coated vesicles	38	31	16

GH was taken up for 30 minutes. For each cell type, a random scan was performed until 50 GH-positive clathrin-coated structures at or near the plasma membrane were encountered. Clathrin-coated structures were categorized as lattices, pits or vesicles. Numbers represent the relative frequency of a clathrin-coated structure positive for GH.

Table 4. Labeling densities of GH in wild-type GHR- and GHR-F327A-expressing cells

	Plasma membrane	Clathrin-coated lattice	Clathrin-coated pit
Wild-type GHR	0.59±0.09	1.15±0.54	1.35±0.33
GHR-F327A	0.57±0.08	0.99±0.38	0.33±0.19

GH was taken up for 30 minutes in wild-type GHR and GHR-F327A cells. Labeling densities are expressed as gold particles per μm±s.e.m.

consistent with an exclusion of GHR-F327A from clathrin-coated pits. Notably, the labeling density of GH in clathrin-coated lattices of GHR-F327A cells, as in wild-type GHR cells, was increased compared with non-coated areas of the plasma membrane. Together, the data in Table 3 and Table 4 show that access to lattices is differentially regulated, as for clathrin-coated pits and vesicles, to which entrance of GHR-F327A is clearly inhibited. They suggest that the UbE-motif is not involved in recruitment of GHR in clathrin-coated lattices but is required for retention of the receptor in the forming clathrin-coated pit.

DISCUSSION

The main focus of this study was to gain insight into the role of the ubiquitin system in the early steps of GH endocytosis. GHR endocytosis was studied at the subcellular level by visualizing its stably bound ligand GH, in cells overexpressing wild-type or mutant GHR. This ultrastructural approach enabled us to discriminate between clathrin-coated lattices, pits and vesicles. Previous studies had indicated that GHR-399K⁻, a lysine-less mutant which cannot be ubiquitinated, was still endocytosed. Using a quantitative approach, we show that in GHR-399K⁻ cells, GH is recruited to clathrin-coated lattices, pits and vesicles with precisely the same efficiency as in cells expressing wild-type GHR. These data unequivocally show that although internalization of GHR-399K⁻ depends on the ubiquitin-conjugation system (Govers et al., 1999), ubiquitination of the receptor itself has no role in its recruitment to clathrin-coated pits and subsequent endocytosis. By contrast, GH uptake in GHR-F327A cells, with an inactivated UbE endocytosis motif, was largely inhibited. The minor level of GH endocytosis observed in these cells is in the same range as earlier observations with iodinated GH, and most probably reflects constitutive uptake of GHR, independent of the ubiquitin system (Govers et al., 1997).

Quantitative analysis of GH distribution in GHR-F327A cells indicated the exclusion of GHR-F327A from clathrin-coated pits and vesicles. Unexpectedly, the occurrence of GHR-F327A in clathrin-coated lattices was only mildly affected when compared with wild-type GHR cells. This observation pinpoints the endocytosis defect of GHR-F327A to clathrin-coated pits rather than lattices. Proteins bearing a signal for clathrin-mediated internalization are recruited to clathrin-coated pits by direct or indirect interactions with coat components (Kirchhausen et al., 1997), ensuring that vesicles are not formed without cargo proteins. The ability of GHR-F327A to enter clathrin-coated lattices but not pits and vesicles suggests not only morphological, but also regulational and functional differences between clathrin-coated lattices and pits. This notion is in agreement with a previous study by Miller et al. (Miller et al., 1991), in which it was shown that overexpression of TfR induces formation of TfR-positive clathrin-coated lattices but not of clathrin-coated pits, suggesting that lattice versus pit/vesicle formation are separately regulated events. It is tempting to view these and our data in light of recent insights into the mode of clathrin-coated pit formation. A clathrin-coated lattice consists of clathrin-triskelions which are assembled as hexagons, whereas coated pits consist of both hexagons and pentagons (Heuser, 1980). Recent structural analysis of the clathrin heavy chain has suggested that to transform a lattice into a curved pit, a rapid disassembly and re-assembly of clathrin triskelions must occur (Musacchio et al., 1999). Thus, our observation that GHR-F327A has access to clathrin-coated lattices but is excluded from pits and vesicles, indicates that high-affinity recruitment of GHR occurs in clathrin-coated pits, after breakdown and re-assembly of clathrin.

In case of stimulated endocytosis, entry into the clathrin coated pit must be regulated. Indeed, it has been found that some internalization signals are activated by phosphorylation (Dietrich et al., 1994; Pitcher et al., 1999). Phosphorylation of the CD4 serine residues S408 and S415 increases the affinity of CD4 towards the clathrin-adaptor complex AP2 which is necessary for internalization (Pitcher et al., 1999). In addition, in vitro interactions between clathrin and AP2 increase the affinity of the μ2 subunit of AP2 for a tyrosine-based internalization motif (Rapoport et al., 1997). Hence, formation of clathrin coats and cargo recruitment depend on interactions between cargo and coat-components, as well as between coat-components itself. This network of interactions provides multiple layers of regulation in the formation of a clathrin-coated vesicle. Our finding that wild-type GHR and the endocytosis-deficient GHR-F327A show a higher concentration in clathrin-coated lattices when compared with non-coated areas of the plasma membrane, suggests that both receptors are recruited to the lattices. The relative absence of GH-label in clathrin-coated pits of GHR-F327A cells demonstrates that despite its initial concentration in the lattices, GHR-F327A is excluded from clathrin-coated pits. Therefore, we propose that the incorporation of GHR in clathrin-coated lattices is differentially regulated from incorporation in clathrin-coated pits and that the UbE-motif only acts in the last process.

In case of epidermal growth factor receptor (EGFR), blockage of clathrin-mediated endocytosis resulted in a ligand-

induced transient localization of the receptor to clathrin-coated pits, in parallel with a reversible ubiquitination of the receptor. These data can be interpreted as that ubiquitination may function as a signal to either recruit or retain the EGFR in clathrin-coated pits (Stang et al., 2000). Our data on GHR-F327A and the GHR-399K$^-$ indicate that interaction with the ubiquitin-machinery is necessary for retention in forming clathrin-coated vesicles, rather than recruitment to clathrin-coated lattices. An alternative or additional explanation for our findings is that clathrin-coated lattices may function as clathrin reservoirs and not necessarily be precursors of coated pits (Kirchausen, 2000b).

The mode of interaction between GHR and the ubiquitin-conjugation machinery remains to be established. In yeast it was suggested that ubiquitin itself serves as an internalization signal that directly interacts with the endocytic machinery. Replacement of the cytoplasmic tail of a chimeric receptor (Ste2p) by a single ubiquitin, resulted in efficient endocytosis (Shih et al., 2000). Likewise, attachment of mono-ubiquitin to the a-factor receptor Ste3p, of which the PEST-like internalization signal was deleted, initiated endocytosis (Roth and Davis, 2000). However, as our present and previous data (Govers et al., 1999) show that ubiquitination of the receptor itself is not necessary for efficient incorporation into clathrin-coated vesicles and subsequent endocytosis, the mechanism responsible for incorporation of GHR in clathrin-coated pits must be different. It has been suggested that the E2/E3 enzyme complex, which initiates ubiquitination, could function directly as an adaptor-like complex, analogous to arrestin in the internalization of β2AR (Goodman et al., 1996; Strous and Govers, 1999). Possibly therefore, the UbE-motif serves as a docking site for the E2/E3 enzyme-complex. Alternatively, the findings that an active ubiquitin-system but not ubiquitination of the GHR itself is required for uptake, might imply the existence of an as yet unidentified protein that needs to be ubiquitinated in order to allow GHR endocytosis. The internalization of wild-type GHR is dependent on the proteasomal-system, while the truncated receptor GHR-369 and shorter truncations enter the cell independent of the proteasomal system (van Kerkhof et al., 2000). It is therefore well possible that a cut of the GHR tail is necessary for incorporation of the receptor into clathrin-coated pits. The UbE-motif could recruit the proteasome onto the GHR or act upstream of this proteasomal action. Taking this scenario one step further, the F327A mutation within the UbE-motif could disrupt this interaction, so that the tail is not cut and GHR-F327A is excluded from clathrin-coated pits and vesicles. Our present studies are dedicated to distinguish between these different possibilities.

In addition to a detailed analysis of the first steps of GHR endocytosis, our study provides the first detailed ultrastructural analysis of the endocytic pathway of the GHR. After uptake via clathrin-coated vesicles, GH-GHR complexes were delivered to early endosomes. No evidence for a significant recycling of internalized GHR to the plasma membrane was obtained. Instead, the absence of GH-GHR from Tf-rich recycling endosomes and the presence in intra-endosomal vesicles indicated an efficient sorting away from recycling Tf-TfR complexes. Consistent herewith, GH was regularly found in the internal vesicles of late endosomes or MVBs, which is indicative for targeting of GH-GHR to lysosomes (Futter et al., 1996). Lysosomes contained high labeling densities of the lysosomal hydrolase cathepsin D, but even after 1 hour of internalization they lacked GH labeling, which is in agreement with biochemical studies showing a rapid degradation of internalized GH (Govers et al., 1998).

It should be noted that in none of the cell types under study GH was found in caveolae. This is in contrast with a recent study by Lobie et al. (Lobie et al., 1999), who by immunogold labeling of CHO cells transfected with GHR, found GH in both clathrin-coated vesicles and caveolae. An explanation for this discrepancy might be that in these studies a sixfold higher concentration of GH was added to the cells. Our data indicate that at more physiological concentrations, uptake of GH exclusively occurs via the clathrin-mediated pathway.

In summary, our data are consistent with uptake of GH-GHR via clathrin-coated vesicles and a rapid breakdown in lysosomes. They show that the UbE-motif but not ubiquitination of the GHR is important for incorporation in clathrin-coated pits but not required for localization to lattices. Whether the ubiquitin-system is also involved in targeting to lysosomes at the level of late endosomes, as was suggested for EGFR, will be topic of future research.

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