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Linkage of the ubiquitin-conjugating system and the endocytic pathway in ligand-induced internalization of the growth hormone receptor

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The major function of the ubiquitin-conjugating system is the targeting of cytosolic and nuclear proteins for degradation by the proteasome. Recently, ubiquitin conjugation has been implicated in the downregulation of signalling receptors such as the mammalian growth hormone receptor (GHR) and the α-factor receptor in yeast. By examining truncated receptors, the internalization-deficient receptor mutant F327A and conditions under which clathrin-mediated GHR endocytosis is inhibited, we show here that GHR ubiquitination and ligand-induced GHR internalization are coupled events. Previously, we had shown that GHR endocytosis is dependent on an intact ubiquitination system. Here we present evidence that GHR ubiquitination depends on an intact endocytic pathway. Our data indicate that the ubiquitin-conjugating system and the endocytic pathway interact at the cytoplasmic tail of the GHR at the plasma membrane, where they cooperate to regulate internalization of the GHR.
Keywords: endocytosis/growth hormone receptor/ubiquitin

Introduction

Growth hormone (GH) regulates growth, differentiation and specific cellular metabolic functions (Carter Su *et al.*, 1996), which are mediated by the GH receptor (GHR). The GHR is a member of the cytokine receptor family (Finidori and Kelly, 1995; Ihle, 1995). In the extracellular domain, cytokine receptors contain four paired cysteine residues and a WSXWS motif, involved in ligand binding. Within the cytoplasmic tail they contain two short domains of homology, known as box1 and box2. One major characteristic of members of the cytokine receptor family is the absence of an intrinsic tyrosine kinase activity. GH binding to the GHR, followed by GHR dimerization (Cunningham *et al.*, 1991), induces recruitment and activation of the JAK2 tyrosine kinase (Argetsinger *et al.*, 1993) and the subsequent tyrosine phosphorylation of JAK2, the GHR (Argetsinger *et al.*, 1993) and signal transducers and activators of transcription (STATs). Upon activation, these STAT proteins dimerize and translocate to the nucleus (Finidori and Kelly, 1995), where they bind specific DNA sequence motifs and mediate GH-induced effects on gene transcription. GH also induces activation of the extracellular signal-regulated kinases ERK1 and ERK2, members of the mitogen-activated protein (MAP) kinase family (Winston and Bertics, 1992).

The GHR is a type I glycoprotein (M_r 130 000) consisting of 620 amino acid residues with a 350 cytoplasmic residue tail, a 24 amino acid transmembrane domain and 246 residues in the extracellular domain containing five potential *N*-glycosylation sites. The receptor is endocytosed rapidly in the presence of ligand (Roupas and Herington, 1988) and its degradation occurs within the lysosome (Murphy and Lazarus, 1984).

The GHR undergoes polyubiquitination, which is enhanced upon binding of GH (Leung *et al.*, 1987; Strous *et al.*, 1996). The ubiquitin pathway is responsible for non-lysosomal degradation of nuclear and cytosolic proteins (Jentsch, 1992; Jennissen, 1995). Proteins destined for degradation by the proteasome are tagged by the attachment of multiple ubiquitin moieties. While many cell surface proteins, such as the GHR, are now known to be ubiquitinated, it is unlikely that ubiquitin targets them for degradation by the proteasome. Moreover, for several of these proteins it has been shown that they are degraded in the lysosome (Murphy and Lazarus, 1984; Hicke and Riezman, 1996; Strous *et al.*, 1996). An important step in elucidating the role of the ubiquitin system in receptor regulation came from the study by Hicke and Riezman (1996) who showed that ubiquitination of the yeast α-factor receptor functions as a signal for ligand-induced receptor endocytosis. In mammalian cells, we recently have shown that the ubiquitin-conjugating system is required for ligand-induced GHR internalization and therefore indirectly mediates its lysosomal degradation (Strous *et al.*, 1996).

In the present study, we used eight cytosolic tail truncation mutants of the GHR varying in the number of cytoplasmic lysine residues to demonstrate the linkage of endocytosis and ubiquitination of the GHR. When internalization of the receptor was inhibited by mutating Phe327 in the GHR cytosolic tail or by cellular potassium depletion, hypertonic medium treatment or cellular cytosol acidification, GHR ubiquitination was abolished. Our results show that the ubiquitin system cooperates with the endocytic machinery in regulating its ligand-induced internalization.

Results

To examine whether ligand-induced GHR internalization is dependent on receptor ubiquitination, eight truncation mutants of the rabbit GHR were constructed (Figure 1). The wild-type GHR contains 19 cytoplasmic lysine residues to which ubiquitin may be attached. Each truncation

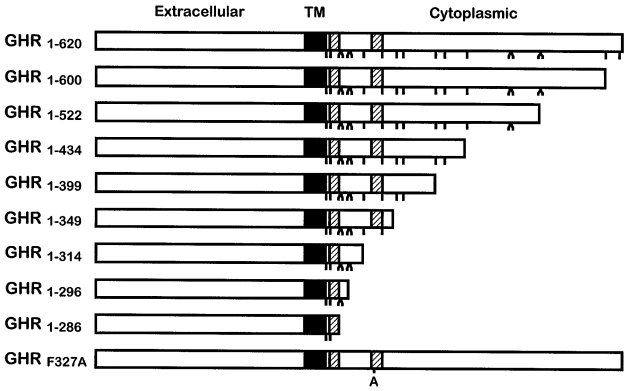

Fig. 1. Schematic representation of the wild-type GHR, GHR truncation mutants and GHR F327A. Cytoplasmic lysines are indicated for the wild-type GHR (GHR 1–620) and for the GHR truncations. Hatched squares represent box1 and box2 respectively. TM is the transmembrane domain of the GHR.

Table I. Binding analysis of mutant rabbit growth hormone receptors

GH receptor	K_d (nM)	Expression sites/cell ($\times 10^6$)
GHR 1–620	2.46 ± 1.20	0.98 ± 0.09
GHR 1–600	1.68 ± 0.49	0.79 ± 0.01
GHR 1–522	2.13 ± 0.50	0.96 ± 0.05
GHR 1–434	0.97 ± 0.12	1.31 ± 0.04
GHR 1–399	2.44 ± 0.74	0.81 ± 0.02
GHR 1–349	1.52 ± 0.81	0.96 ± 0.05
GHR 1–314	2.06 ± 1.04	0.92 ± 0.28
GHR 1–296	1.31 ± 0.90	1.38 ± 0.12
GHR 1–286	2.33 ± 1.05	1.02 ± 0.25
GHR F327A	2.78 ± 0.40	1.02 ± 0.08
ts20	n.d.[a]	n.d.

Binding of [^{125}I]hGH to CHO-ts20 cells expressing wild-type GHR (GHR 1–620), mutant GHR or no GHR (ts20) was measured in the absence or presence of unlabelled hGH for 2 h at 4°C as described in Materials and methods.
[a]Specific binding not detectable

mutant contains two or three cytoplasmic lysines fewer than the former. Of all truncation mutants, stable clones were selected with approximately equal numbers of GH-binding sites at the cell surface (Table I). Ligand binding affinity of the various mutants was comparable. No specific binding of [^{125}I]GH could be detected when untransfected were examined.

Ligand internalization by GHR truncation mutants

We examined the mutant receptors for their ability to internalize GH, using fluorescence-labelled GH and confocal microscopy (Figure 2). Cells were incubated at 30°C with Cy3-labelled GH. Untransfected CHO-ts20 cells showed neither Cy3-GH binding nor internalization (not shown). The wild-type GHR-expressing cells were also incubated with Cy3-GH at 42°C. The CHO-ts20 cells used in this study contain a thermolabile ubiquitin-activating enzyme (E1), which is inactive at this non-permissive temperature (Kulka *et al.*, 1988). As can be seen in Figure 2B, Cy3-GH internalization is inhibited following inactivation of the ubiquitin conjugation system, compared with internalization at the permissive temperature

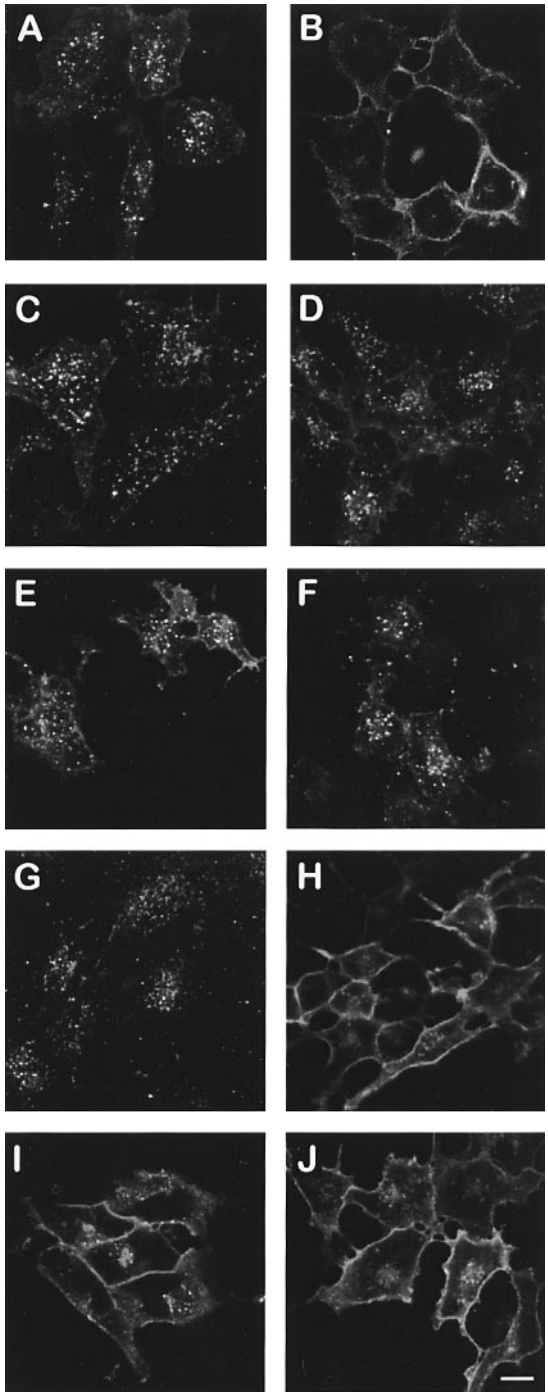

Fig. 2. Internalization of Cy3-GH by wild-type GHR and GHR truncation mutants. CHO-ts20 cells expressing (**A** and **B**) wild-type GHR or GHR truncations (**C**) 1–600, (**D**) 1–522, (**E**) 1–434, (**F**) 1–399, (**G**) 1–349, (**H**) 1–314, (**I**) 1–296 or (**J**) 1–286 were incubated with Cy3-GH for 1 h at 30 (A and C–J) or 42°C (B). Cy3-GH was visualized by confocal microscopy. Bar = 10 μm.

(Figure 2A). These findings are in agreement with our previous observations (Strous *et al.*, 1996). As for the truncation mutants, it is obvious that the three shortest truncations, 1–314, 1–296 and 1–286 (Figure 2H–J), do not internalize ligand at 30°C, while the longer truncations, 1–600 to 1–349 (Figure 2C–G), internalize GH. These results indicate that amino acid residues 314–349 are necessary for GH internalization.

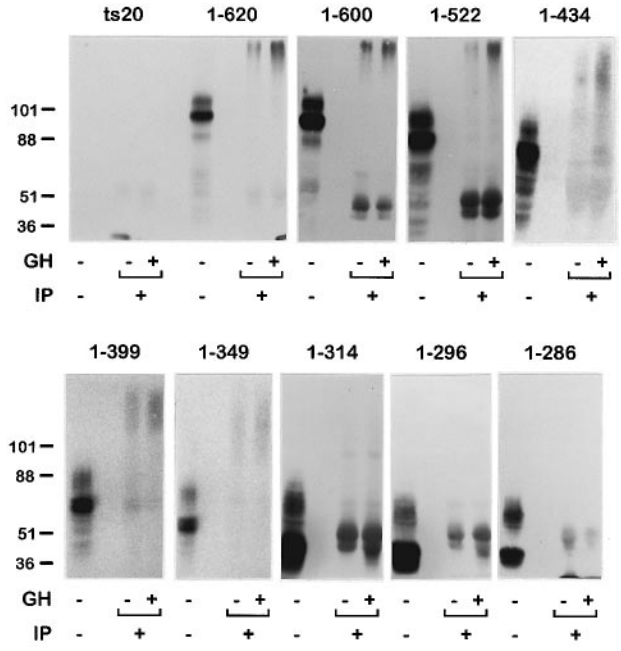

Fig. 3. Ubiquitination of wild-type GHR and GHR truncation mutants. CHO-ts20 cells, expressing wild-type GHR (GHR 1–620) or GHR truncations, and untransfected cells (ts20) were incubated for 30 min in MEMα, supplemented with 20 mM HEPES in the absence or presence of GH. Ubiquitinated proteins were immunoprecipitated using an anti-ubiquitin conjugate antiserum and Western blotted, using an anti-GHR antibody, directed against the luminal domain of the GHR. The left lane in each panel contains total cellular lysate and represents the receptor: upper band, mature species; lower band, precursor species.

Ubiquitination of GHR truncation mutants

To determine the relationship between ligand-induced GHR internalization and GHR ubiquitination, all GHR truncation mutant-expressing cells, wild-type GHR-expressing cells and untransfected cells were incubated with 8 nM hGH at 30°C for 30 min. After GH incubation, the cells were lysed and protein–ubiquitin conjugates were immunoprecipitated by an antibody specific for conjugated ubiquitin. The immunoprecipitates and the cellular lysates were analysed by Western blotting using an antibody directed against the luminal part of the GHR (Figure 3). The left lane in each panel contains total cellular lysate and shows a sequential decrease in molecular weight of the different GHR truncations compared with the wild-type GHR (panel 1–620). In all panels, this lane shows a fast migrating band, which is the receptor precursor, and a slower migrating band, representing the mature receptor (see Strous *et al.*, 1996). Cell extracts containing approximately equal amounts of GHR were used for the anti-ubiquitin immunoprecipitation. The 40–55 kDa bands seen in most immunoprecipitations originated from IgG, and the intensity of these background bands varied among experiments. The high molecular weight bands visible in the upper part of these lanes represent ubiquitinated receptors. To obtain signal from ubiquitinated GHRs, the immunoblots of truncations 1–434 to 1–286 were exposed to film three to five times longer than the other blots, indicating a dramatic decrease in the amount of ubiquitin signal of truncations 1–434, 1–399 and 1–349 compared with wild-type or truncations 1–600 and 1–522. Note that the left lanes on the blots of truncations 1–434 to 1–286 contained four times less cellular lysate compared with the other blots. The molecular weight of ubiquitinated truncation 1–434 compared with that of truncation 1–522 also indicates that the three lysine residues within domain 434–522 are major ubiquitination sites. The truncations 1–314, 1–296 and 1–286 showed no detectable ubiquitin signal, while ubiquitination was induced by ligand in all of the other truncations. Truncation 1–349 demonstrates that multi-ubiquitin chains, and not simply monomeric ubiquitin, are attached to lysine residues in the receptor tail. The mature receptor species of this truncation migrates at 70 kDa, while the ubiquitinated receptor truncation containing only eight lysine residues exists as a multiple species up to 170 kDa. Thus, we conclude that the GHR is polyubiquitinated on multiple lysines and that the three shortest receptor truncations are not ubiquitinated. Together with the data from Figure 2, these experiments show that ligand-induced GHR internalization and GHR ubiquitination are linked. Figures 2 and 3 also show that the relative decrease in ubiquitin signal of GHR 1–620 to 1–349 is not accompanied by an apparent decrease in internalization of fluorescent GH. Moreover, quantification of ligand internalization using iodinated GH showed that truncations 1–399 and 1–349 internalized ligand to a greater extent than did wild-type GHR (not shown), indicating that receptor-mediated ligand internalization is dependent on the presence of conjugated ubiquitin and not on the amount of ubiquitin conjugated to the receptor.

Role of Phe327 in GHR ubiquitination and ligand internalization

Since GHR domain 314–349 is essential for both GHR ubiquitination and endocytosis, we focused on the role of the previously described internalization mutant GHR F327A (Allevato *et al.*, 1995) in this process. This mutant GHR is incapable of internalization, while GH-mediated signal transduction is unaffected (Allevato *et al.*, 1995; Strous *et al.*, 1997). GHR F327A cDNA-transfected CHO-ts20 cells displayed the same amount of surface receptors as wild-type GHR-expressing cells (Table I). Incubation of these cells with Cy3-GH (Figure 4A) as well as quantitative [^{125}I]GH uptake (Figure 4B) at 30°C confirmed that this GHR mutant is unable to internalize ligand. The GHR F327A mutant bound [^{125}I]GH to the same extent as did the wild-type GHR, but ligand internalization was almost fully impaired and reached the same background levels as the wild-type GHR at the non-permissive temperature (not shown). Wild-type GHR- and GHR F327A-expressing cells were incubated with GH, whereafter the cells were lysed and the GHR was immunoprecipitated by an anti-GHR tail antibody and analysed by Western blotting (Figure 4C). GHR F327A was not ubiquitinated in the presence or absence of ligand (left panel), while GH-induced tyrosine phosphorylation was minimally affected (middle panel). That the GHR cytoplasmic tail is still tyrosine phosphorylated while internalization is inhibited is in agreement with the findings of Allevato (1995) and Strous (1997). If, following GH treatment, protein–ubiquitin conjugates from GHR F327A-expressing cells were immunoprecipitated by an anti-ubiquitin antibody and immunoblotted using an anti-GHR antibody, no ubiquitinated receptor was detected (not shown). That ubiquitination of the wild-type GHR cannot

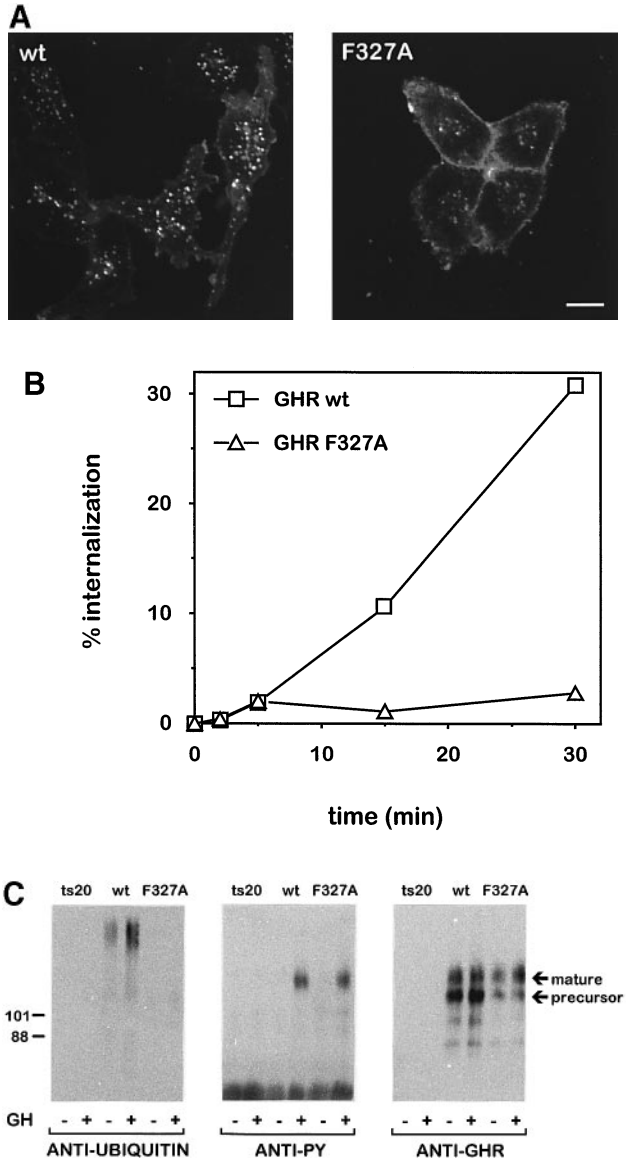

Fig. 4. Effect of mutation F327A on GHR endocytosis, ubiquitination and tyrosine phosphorylation. (**A**) GH internalization was examined by immunofluorescence confocal microscopy using Cy3-GH. Transfected CHO-ts20 cells expressing wild-type GHR (left panel) or GHR F327A (right panel) were incubated with Cy3-GH for 30 min at 30°C. (**B**) GH internalization was quantitated by [^{125}I]GH uptake. Cells were incubated with [^{125}I]GH in the absence or presence of excess unlabelled GH for the indicated periods of time. Specific internalized [^{125}I]GH was expressed as the percentage of total label at 30 min. (**C**) Cells were incubated with or without unlabelled GH for 30 min at 30°C. The GHR was immunoprecipitated by an anti-GHR tail antibody and analysed by Western blotting using the indicated antibodies. As a control, untransfected cells (ts20) were subjected to the same procedure.

be detected after anti-GHR immunoblotting (Figure 4C, right panel) is in accordance with our previous findings (Strous *et al.*, 1996) and indicates that only a few percent of the cellular GHR content is ubiquitinated. Moreover, during lysis and immunoprecipitation, GHR–ubiquitin conjugates are very unstable due to a high deubiquitination activity in cellular lysates.

Inhibition of endocytosis and GHR ubiquitination

To examine whether GHR ubiquitination is dependent upon GHR endocytosis, the clathrin-mediated endocytic

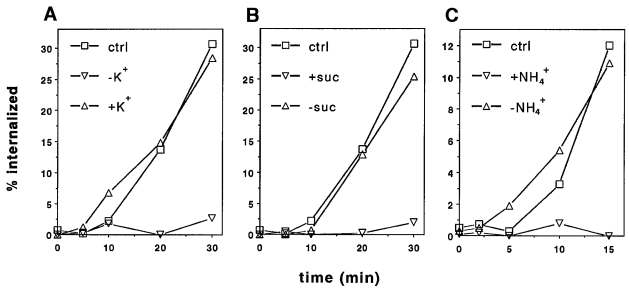

Fig. 5. Effect of cellular potassium depletion, hypertonic medium treatment and cellular cytosol acidification on GHR internalization. (**A**) Cellular potassium depletion; cells were hypotonically shocked for 5 min and incubated in the absence (–K^+, ▽) or presence (+K^+, △) of KCl. (**B**) Hypertonic medium treatment; cells were incubated in 0.45 M sucrose (+suc, ▽) or incubated in 0.45 M sucrose followed by an incubation without sucrose (–suc, △). (**C**) Cellular cytosol acidification; cells were incubated in KCl/amiloride buffer after NH_4Cl pre-incubation (+NH_4^+, ▽) or without NH_4Cl pre-incubation (–NH_4^+, △). All cells were incubated with [^{125}I]GH in the absence or presence of excess unlabelled GH for the indicated periods of time. Specific internalized [^{125}I]GH was expressed as a percentage of total label at 30 min (A and B) or 15 min (C). Control cells (ctrl, □) were incubated with [^{125}I]GH without additional incubations.

pathway was inhibited by cellular potassium depletion, treatment with hypertonic medium or cellular cytosol acidification. Potassium depletion was performed as described by Hansen *et al.* (1993). The effect on continuous ligand internalization is shown in Figure 5A. Under standard conditions, 30% of total cellular [^{125}I]GH was internalized after 30 min of incubation. When the cells were subjected to potassium depletion, internalization was undetectable, while the addition of potassium during the experiment restored the internalization efficiency to standard levels. In addition, incubation of the cells in hypertonic medium (Hansen *et al.*, 1993) inhibited [^{125}I]GH internalization (Figure 5B). This effect was fully reversible. When cells were incubated for 30 min in MEMα/HEPES supplemented with 0.45 M sucrose, followed by a 30 min incubation in MEMα/HEPES without sucrose, uptake occurred as in control cells. The third method used to inhibit clathrin-mediated endocytosis was cellular cytosol acidification via NH_4Cl/amiloride treatment (Sandvig *et al.*, 1987). Figure 5C shows that following a 30 min pre-incubation in 20 mM NH_4Cl, incubation in KCl/amiloride buffer completely inhibited [^{125}I]GH internalization. However, incubation of cells in the KCl/amiloride buffer without NH_4Cl pre-incubation resulted in control rates of ligand internalization. Inhibition of ligand internalization by all three methods was confirmed independently by Cy3-GH uptake and confocal microscopy (not shown).

To examine the effect of inhibition of clathrin-mediated endocytosis on GHR ubiquitination, cells were incubated as described above, including an incubation with GH for 30 min (potassium depletion and hypertonic medium) or 15 min (cytosol acidification). After the GH incubation, cells were lysed and the GHR was immunoprecipitated and analysed by Western blotting, using the antibodies indicated in Figures 6 and 7. Potassium depletion inhibited GHR ubiquitination almost completely (Figure 6B), while total cellular ubiquitination was not affected (Figure 6A). To confirm that this effect of potassium depletion on GHR ubiquitination was due primarily to the absence of

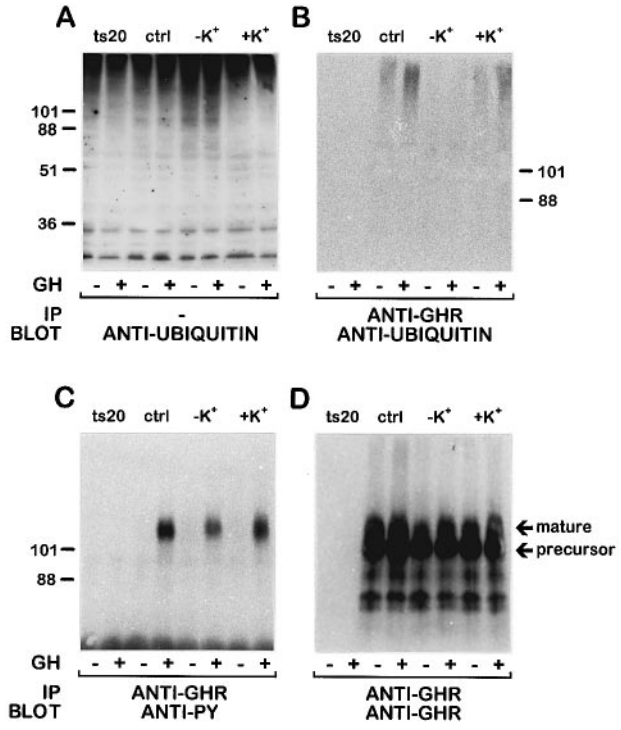

Fig. 6. Effect of cellular potassium depletion on ubiquitination and tyrosine phosphorylation of the GHR. Cells were hypotonically shocked for 5 min, incubated in the absence ($-K^+$) or presence ($+K^+$) of KCl and incubated for 30 min with or without GH. Control cells (ctrl) and untransfected cells (ts20) were incubated with GH without additional incubations. The GHR was immunoprecipitated, subjected to polyacrylamide gel electrophoresis, transferred to PVDF paper and analysed using the indicated antibodies. Total cellular lysate was also included (**A**) to examine the activity of the ubiquitin-conjugating system in cells which had been potassium depleted.

potassium, cells were incubated under identical conditions except for the presence of 10 mM potassium. In this case, there was only a minimal decrease in GHR ubiquitination, compared with control cells incubated in MEMα/HEPES. Cellular potassium depletion had virtually no effect on GHR tyrosine phosphorylation (Figure 6C), in accordance with the GHR F327A experiments (Figure 4C), and confirms that tyrosine phosphorylation occurs at the plasma membrane. When internalization of GH by the GHR was inhibited by hypertonic medium treatment, GHR ubiquitination was also abolished (Figure 7B), while total cellular ubiquitination was not impaired (Figure 7A). The high molecular weight bands at the top of Figure 7C are background bands which were also present when untransfected cells were subjected to immunoprecipitation in that experiment (not shown) and do not represent ubiquitinated GHR. Incubating the cells in MEMα/HEPES without sucrose after the hypertonic medium treatment restored GHR ubiquitination. Cytosol acidification also inhibited GHR ubiquitination (Figure 7E) without affecting total cellular ubiquitination (Figure 7D). When cells were incubated in the KCl/amiloride buffer without the NH_4Cl pre-incubation, the GHR was ubiquitinated to the same extent as in control incubations.

Discussion

The ubiquitin-conjugating system plays an essential role in many cellular regulatory processes. A major function of this system is the degradation of short-lived and 'abnormal' proteins (Hiller *et al.*, 1996; Qu *et al.*, 1996). Many of these proteins have regulatory functions, such as the cyclins (Seufert *et al.*, 1995; Sudakin *et al.*, 1995; Won and Reed, 1996), protein kinase C (Lee *et al.*, 1996), IκBα (Li *et al.*, 1995; Roff *et al.*, 1996) and p53 (Scheffner *et al.*, 1990). Although it has been 11 years since the first mammalian receptors were found to be ubiquitinated (Siegelman *et al.*, 1986; Yarden *et al.*, 1986), only recently has it become apparent that the conjugation of ubiquitin to cell surface receptors plays a role in receptor down-regulation. Using CHO cells, exhibiting a temperature-sensitive defect in ubiquitin conjugation (Kulka *et al.*, 1988), it was shown that GH-induced GHR internalization was dependent on an intact ubiquitin conjugation system, while endocytosis of the transferrin receptor was not impaired after inactivation of the ubiquitin conjugation system (Strous *et al.*, 1996). These observations suggested that ubiquitination of the GHR must occur prior to endocytosis of the receptor. Similarly, Hicke and Riezman (1996) showed that Ste2p ubiquitination in yeast is a prerequisite for ligand-stimulated Ste2p endocytosis.

In the present study we show by examining truncated GH receptors, an internalization-deficient GHR mutant and conditions under which clathrin-mediated endocytosis was inhibited by cellular potassium depletion, hypertonic medium treatment or cellular cytosol acidification, that GHR ubiquitination and ligand-induced GHR internalization are linked. Moreover, we show that the GHR is ubiquitinated on multiple lysines and that these lysines are polyubiquitinated.

Together, these data show that the minimal requirements for both GHR ubiquitination and endocytosis are present in GHR truncation 1–349, but not in truncation 1–314, and that Phe327 is a critical element for both events. This phenylalanine residue is situated within box2 of the GHR cytoplasmic tail but is not conserved in other members of the cytokine receptor family. Recently, the motif mediating internalization of another member of the cytokine receptor family was determined. This internalization motif is a di-leucine motif in the transmembrane glycoprotein gp130, which mediates internalization of the interleukin-6 receptor (Dittrich *et al.*, 1996). Di-leucine motifs are well known to be involved in the internalization of many membrane proteins (Trowbridge, 1991; Haft *et al.*, 1994; Garippa *et al.*, 1996). The di-leucine motif in gp130 is situated 85 amino acid residues C-terminal to box2. GHR domain 314–349, which is necessary for GHR internalization, contains both the Phe327 residue required for internalization and a leucine doublet at positions 347 and 348. Whether this leucine doublet is involved in GHR internalization remains to be determined. The SINNDAKSS motif, involved in both internalization and ubiquitination of the yeast receptor Ste2p (Hicke and Riezman, 1996), is not found within the cytoplasmic tail of the GHR.

Since GH internalization is dependent upon an intact ubiquitin-conjugating sytem and since the conjugation of ubiquitin to the GHR mutants strictly correlates with the ability of these mutants to internalize ligand, it is tempting to speculate that GHR ubiquitination is a prerequisite for GHR-mediated GH internalization. Thus, inhibition of GHR endocytosis could lead to an accumulation of ubiquitinated receptors at the plasma membrane, as was

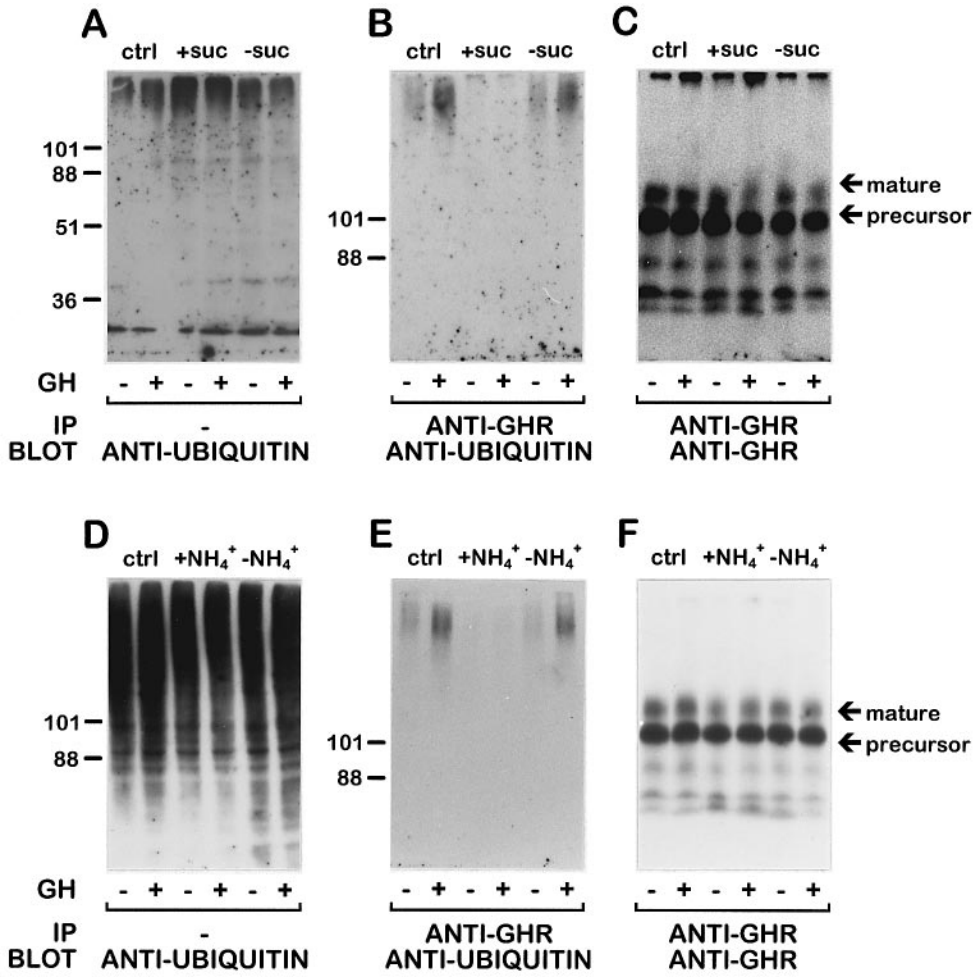

Fig. 7. Effect of hypertonic medium treatment and cellular cytosol acidification on ubiquitination of the GHR. (**A**, **B** and **C**) Hypertonic medium treatment; cells were incubated in 0.45 M sucrose (+suc) or incubated in 0.45 M sucrose followed by an incubation without sucrose (–suc). (**D**, **E** and **F**) Cellular cytosol acidification; cells were incubated in KCl/amiloride buffer after NH_4Cl pre-incubation ($+NH_4^+$) or without NH_4Cl pre-incubation ($–NH_4^+$). After hypertonic medium treatment or cellular cytosol acidification, the cells were incubated for 30 min in the absence or presence of GH. Control cells (ctrl) were incubated with GH without additional incubations. The GHR was immunoprecipitated, subjected to polyacrylamide gel electrophoresis, transferred to PVDF paper and analysed using the indicated antibodies. Total cellular lysate was also included (A and D) to examine the effect of hypertonic medium treatment and cellular cytosol acidification on the activity of the ubiquitin-conjugating system.

observed by Hicke and Riezman (1996) for the Ste2p receptor in the yeast *end4*Δ mutant. We approached this issue by examining the fate of the GHR under conditions in which clathrin-mediated endocytosis was inhibited, since the GHR enters the cell via clathrin-coated pits (Ilondo *et al.*, 1986, 1991). Inhibition of GHR endocytosis was achieved by three different methods. All methods inhibited GHR-mediated GH uptake as well as ligand-dependent and ligand-independent GHR ubiquitination, while total cellular ubiquitin conjugation was not affected. This finding is in contrast to that observed for the Ste2p receptor in yeast (Hicke and Riezman, 1996), but is similar to observations with the epidermal growth factor receptor (Galcheva-Gargova *et al.*, 1995), in which mutation of the tyrosine kinase domain, essential for internalization and cellular potassium depletion, inhibited both receptor internalization and receptor ubiquitination.

To date, we have found a strict correlation between GHR internalization and GHR ubiquitination. We have shown that GHR endocytosis is dependent upon an intact ubiquitination system and that GHR ubiquitination is dependent upon an intact endocytic pathway. We speculate that, in the absence of ligand, the GHR is constitutively ubiquitinated and internalized prior to its degradation within the lysosome. GH-induced GHR dimerization results in an increase in ubiquitination as well as in internalization, resulting in the disappearance of GHR molecules from the plasma membrane. Inhibition of the clathrin-mediated endocytic pathway inhibits ligand-dependent and ligand-independent GHR internalization and therefore inhibits GHR ubiquitination both in the presence and absence of ligand.

We propose that the ubiquitination and endocytic pathways interact at the cytoplasmic tail of the GHR at the plasma membrane, where they cooperate in regulation of GHR internalization, by directing the GHR into the coated pit. If ubiquitination of the GHR is disturbed, the endocytic machinery is unable to select the GHR for internalization. If GHR internalization is disturbed, the ubiquitin-conjugating system has no access to the GHR cytoplasmic tail or the GHR is deubiquitinated rapidly because of its inability to enter the endocytic pathway. On the other hand,

constitutive recycling receptors such as the transferrin receptor do not require interaction with the ubiquitin system for recognition by the endocytic machinery, as we have shown previously (Strous *et al.*, 1996).

Materials and methods

Materials and antisera

Antibody to GHR was raised against amino acid residues 327–493 as described by Strous *et al.* (1996) and recognizes part of the cytoplasmic tail of the GHR. Antibody (Mab5) recognizing the luminal part of the GHR was from AGEN Inc, Parsippany, NJ. Antiserum specific for protein–ubiquitin conjugates was a generous gift from Dr A.Ciechanover (Technion-Israel Institute of Technology, Haifa, Israel). Antibody 4G10, recognizing phosphotyrosine residues, was obtained from Upstate Biotechnologies Inc. (Lake Placid, NY). hGH was a gift of Lilly Research Labs, Indianapolis, IN. GHR cDNA was kindly provided by Dr William Wood. Culture medium, fetal bovine serum (FBS) and geneticin were purchased from Gibco.

Mutagenesis, transfection and cell culture

The CHO cell line CHO-ts20 (Kulka *et al.*, 1988) was used in this study. Due to a thermolabile ubiquitin-activating enzyme (E1), the ubiquitin-conjugating system is inactive at the non-permissive temperature of 42°C.

CHO-ts20 cells were transfected with a pCB6 construct containing the full-length rabbit GHR cDNA sequence using the calcium phosphate transfection method. Truncated receptors were constructed by introducing stop codons at various positions within the cDNA of the rabbit GHR. For this purpose, polymerase chain reactions (PCRs) were performed, using a 5′ oligonucleotide containing an *Nco*I restriction site, corresponding to the *Nco*I site in the cDNA of the transmembrane region of the GHR, together with 3′ oligonucleotides containing a *Kpn*I restriction site, a stop codon and overlapping sequences at various positions within the cDNA, encoding the intracellular domain of the GHR. PCR products were cut by *Nco*I and *Kpn*I and ligated into a PGEM3Z-GHR construct. Truncated GHR cDNAs were subcloned into the CMV-NEO expression plasmid pcDNA3 (Invitrogen) and verified by *in vitro* transcription–translation assays and by sequencing. These constructs were transfected into CHO-ts20 cells, resulting in cell lines stably expressing receptors truncated at amino acid residues 600, 522, 434, 399, 349, 314, 296 and 286.

The internalization-deficient mutant GHR F327A, also described as GHR F346A (Allevato *et al.*, 1995), was constructed by site-directed mutagenesis, using the unique restriction site *Cla*I (Strous *et al.*, 1997). This GHR mutant cDNA was also cloned into pcDNA3, sequenced and transfected into CHO-ts20 cells.

Stable, geneticin-resistant transfectants were selected and grown in Eagle's minimal essential medium (MEMα) supplemented with 10% FBS, penicillin and streptomycin and 0.45 μg/ml geneticin. For experiments, cells were grown in 60 mm dishes in the absence of geneticin to ~75% confluence. In the experiments described in this study, 10 mM sodium butyrate was added to the cells 18 h before use to increase GHR expression (Gorman and Howard, 1983; Strous *et al.*, 1996). Treatment of transfected CHO-ts20 cells with sodium butyrate did not alter the behaviour of the GHR in any of the parameters examined in this study.

GH binding and internalization

The expression of wild-type and mutant receptors was analysed by binding of [^{125}I]GH. [^{125}I]hGH was prepared using chloramine T (Murphy and Lazarus, 1984). Cells were grown in 6-well culture dishes, washed with ice-cold phosphate-buffered saline (PBS) and incubated for 2 h on ice with 0.2–18 nM [^{125}I]GH in PBS containing 0.1% bovine serum albumin (BSA), in the absence or presence of excess unlabelled GH. After extensive washing, 1 M NaOH was added and the cell extracts were counted in an LKB gamma counter. Plasma membrane receptor numbers and binding affinities were calculated by Scatchard analysis (Chamness and McGuire, 1975).

For internalization studies, cells were grown in 30 mm dishes, washed with MEMα, supplemented with 20 mM HEPES, incubated for 1 h at 30 or 42°C in MEMα/HEPES and for 0–30 min with 8 nM [^{125}I]GH (700 000 c.p.m.), in the absence or presence of excess unlabelled GH. The cells were washed three times with ice-cold PBS, membrane-associated GH was removed by acid wash (0.15 M NaCl, 0.05 M glycine, pH 2.5) (Roupas and Herington, 1986) and internalized GH was determined by measuring the radioactivity after solubilization of the acid-treated cells by 1 M NaOH.

Microscopy

Cy3-GH was prepared using a Fluorolink-Cy3 label kit according to the supplier's instructions (Amersham, UK). Transfected CHO cells, grown on coverslips, were incubated for 60 min in MEMα, supplemented with 20 mM HEPES at 30°C or at the non-permissive temperature (42°C) and for 30 or 60 min with Cy3-GH (1 μg/ml). Cells were washed with PBS to remove unbound label and fixed for 2 h in 3% paraformaldehyde in PBS. After fixation, the cells were embedded in Mowiol, and confocal laser scanning microscopy was performed using a Leica TCS 4D system.

Immunoprecipitation and Western blotting

Immunoprecipitations were performed as described previously (Strous *et al.*, 1996). Cells, grown in 60 mm dishes, were incubated for 1 h in MEMα, supplemented with 20 mM HEPES and for 30 min with 8 nM hGH, unless indicated otherwise. After GH incubation, cells were lysed immediately in 0.25 ml of boiling buffer, containing 1% SDS in PBS to avoid isopeptidase activity. After heating the lysate for 5 min at 100°C and shearing the DNA, immunoprecipitations were performed in 1% Triton X-100, 0.5% SDS, 0.25% sodium deoxycholate, 0.5% BSA, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 2 mM Na_3VO_4, 20 mM NaF, 10 μg/ml leupeptin, 10 μg/ml aprotinin in PBS. The lysate was incubated with the indicated antibodies for 2 h on ice. Immune complexes were isolated by the use of protein A–agarose (Repligen Co, Cambridge, MA). The immunoprecipitates were washed twice with the same buffer and twice with 10-fold diluted PBS. Immune complexes were subjected to SDS–PAGE and transferred to polyvinylidene difluoride (PVDF) paper. In some experiments, an aliquot of the total cellular lysate was also run on the gel. In the case of the experiments using the GHR truncation mutants, four times less total cellular lysate was run on the gel for the six shortest truncations (1–434 to 1–286), compared with that used for the two other truncation mutants and for the wild-type GHR. Blots were immunostained using the indicated antibodies. For anti-PY and anti-GHR immunostaining, low-fat milk powder in PBS was used, while gelatin in Tris-buffered saline (TBS) was used for anti-ubiquitin immunostaining. After incubating the blots with protein A or rabbit anti-mouse IgG conjugated to horseradish peroxidase, antigens were visualized using the ECL system (Amersham Corp., UK). To reprobe blots, blots were incubated for 30 min in 2% SDS, 0.1 M β-mercaptoethanol, 60 mM Tris pH 6.8 at 55°C.

Inhibition of clathrin-mediated endocytosis

All incubation steps were performed at 30°C. Cells were subjected to potassium depletion as described by Hansen *et al.* (1993). After a 30 min incubation in MEMα, supplemented with 20 mM HEPES, cells were washed twice with isotonic K^+-free buffer A, containing 0.14 M NaCl, 20 mM HEPES, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 g/l glucose, 0.1% BSA, pH 7.4, washed once with hypotonic buffer B (buffer A/H_2O, 1:1) and subjected to a hypotonic shock, by incubation in buffer B for 5 min. Cells were washed three times with buffer A and incubated in buffer A for 30 min, whereafter the cells were incubated with Cy3-GH or [^{125}I]GH in buffer A to measure internalization or with 8 nM unlabelled GH in buffer A for Western blotting. Parallel cultures, which had also been hypotonically shocked, were incubated in buffer A, supplemented with 10 mM KCl.

For inhibition of receptor internalization by hypertonic medium treatment (Hansen *et al.*, 1993), cells were incubated for 30 min in MEMα/HEPES and for 30 min in MEMα/HEPES supplemented with 0.45 M sucrose, whereafter either Cy3-GH, [^{125}I]GH or unlabelled GH was added to the dishes. To measure reversibility of the hypertonic medium treatment, cells were incubated for 30 min in MEMα/HEPES supplemented with 0.45 M sucrose, for 30 min in MEMα/HEPES without sucrose and for the indicated periods of time with ligand.

Inhibition of clathrin-mediated endocytosis by cellular cytosol acidification was performed as described by Sandvig *et al.* (1987). Cells were incubated for 30 min in MEMα/HEPES and for 30 min in MEMα/HEPES containing 20 mM NH_4Cl. After washing the cells once with 0.14 M KCl, 20 mM HEPES, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM amiloride, 1 g/l glucose, 0.1% BSA pH 7.0, cells were incubated in the same buffer for 5 min, whereafter Cy3-GH, [^{125}I]GH or unlabelled GH was added to the dishes. Parallel cultures were subjected to the same incubations, except that they were incubated in the absence of NH_4Cl.

Control incubations were included in all experiments described above, in which GHR-expressing and non-transfected cells were incubated for

60 min in MEMα/HEPES and for the indicated periods of time with either Cy3-GH, [^{125}I]GH or unlabelled GH.

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