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The ubiquitin conjugation system is required for ligand-induced endocytosis and degradation of the growth hormone receptor

Ger J.Strous[1], Peter van Kerkhof, Roland Govers, Aaron Ciechanover[2] and Alan L.Schwartz[3]

[1]Department of Cell Biology, Faculty of Medicine and Institute of Biomembranes, Utrecht University, Heidelberglaan 100, 3584CX Utrecht, The Netherlands, [2]Department of Biochemistry, Faculty of Medicine, Technion-Israel Institute Technology, Haifa 31096, Israel and [3]Departments of Molecular Biology, Pharmacology and Pediatrics, Washington University School of Medicine, St Louis, MO 63110, USA

[1]Corresponding author

The ubiquitin-dependent protein degradation system has recently been implicated in downregulation of signal transducing receptors. Growth hormone receptor (GHR) cDNA was transfected into Chinese hamster ovary cells, which exhibit a temperature-sensitive defect in ubiquitin conjugation (CHO-ts20), as well as into wild-type cells (CHO-E36). Upon binding of growth hormone (GH), two GHR polypeptides dimerize and initiate signal transduction. In CHO-E36 and in CHO-ts20 at the permissive temperature the GHR was ubiquitinated and degraded in a GH-dependent fashion. However, at the non-permissive temperature in CHO-ts20 cells, neither GH-dependent uptake nor degradation of the GHR was observed, while in CHO-E36 cells both GHR uptake and degradation were accelerated. Incubation of CHO-E36 cells with inhibitors of endosomal/lysosomal function (NH_4Cl, bafilomycin A1) markedly reduced ligand-induced GHR degradation. Our results indicate that a functional ubiquitin conjugating system is required for GH-induced endocytosis and that degradation of both the exoplasmic and cytoplasmic portions of the GHR occurs within the endosomal/lysosomal compartment.

Keywords: CHO-ts20 cells/downregulation/endocytosis/growth hormone receptor/ubiquitin

Introduction

Growth hormone receptor (GHR) is a prototype cytokine receptor. The rabbit receptor used in the present study is a glycoprotein (M_r 130 000) consisting of 620 amino acid residues with a 350 amino acid cytosolic portion, a 24 amino acid transmembrane domain, and 246 residues in the exoplasmic domain containing five N-linked oligosaccharides. Upon binding to its ligand (growth hormone, GH), two GHR polypeptide chains dimerize. This event initiates receptor downregulation as well as signal transduction.

Signal transduction via this receptor results in alterations in the metabolism in many cell types. These include stimulation of protein synthesis and amino acid transport as well as more specific effects, e.g. increased proliferation of germinal cells within the tibial growth plate, as well as of epithelial cells and T-lymphocytes (Ohlsson *et al.*, 1992; Young *et al.*, 1992; Murphy *et al.*, 1993).

The cellular fate of the GHR is cell type-dependent. For example, the receptor recycles constitutively in rat adipocytes (Roupas and Herington, 1988), whereas in IM-9 lymphocytes and mouse fibroblasts endocytosis of the receptor only occurs following ligand binding (Eshet *et al.*, 1984). Ligand-induced internalization also initiates receptor degradation. On the basis of the fate of ^{125}I-labelled GH and the pH-dependency of ligand degradation it is widely believed that the bulk of GHR degradation occurs within the vacuolar system (Lesniak and Roth, 1976; Gorin and Goodman, 1985; Roupas and Herington, 1988; Saito *et al.*, 1994). To date, however, no data are available as to the molecular mechanisms involved.

The GHR, as isolated from rabbit liver, is ubiquitinated (Leung *et al.*, 1987). Protein ubiquitination is a post-translational modification which plays a major role in regulated degradation of cellular proteins. In addition, a wide variety of basic cellular processes, including cell cycle progression, DNA repair, transcriptional control and antigen presentation, are affected by the activity of the ubiquitin system (reviewed in Ciechanover, 1994; Jentsch and Schlenker, 1995). In each case target proteins are specifically recognized and ubiquitinated by ubiquitin-conjugating (E2-family) and/or -ligating (E3-family) enzymes. These ubiquitin–protein conjugates are then recognized by the high molecular weight proteasome and degraded. As a consequence and in the course of this degradation process regulatory events are often initiated. Recent examples of the involvement of the ubiquitin/proteasome system in regulatory events include the transcription factors MATα2 from yeast (Chen *et al.*, 1993), c-jun product (Treier *et al.*, 1994), the p50 precursor subunit of NF-κB (Palombella *et al.*, 1994; Orian *et al.*, 1995), the tumour suppressor p53 (Ciechanover *et al.*, 1994) and mitotic cyclins (Glotzer *et al.*, 1991). Regulatory transmembrane proteins such as PDGF- (Mori *et al.*, 1992) and IgE receptors (Paolini and Kinet, 1993), c-kit (Miyazawa *et al.*, 1994), the ζ-chain of the T-cell receptor (Hou *et al.*, 1994), as well as yeast Ste6 (Kölling and Hollenberg, 1994) are also ubiquitinated. However, herein the conjugation reaction is generally dependent on ligand binding.

In order to define the cell biological consequences of ligand-induced receptor ubiquitination we have examined the role of the ubiquitin pathway in GHR degradation. Our studies have utilized CHO-ts20 cells which express a thermolabile ubiquitin-activating enzyme, E1 (Kulka *et al.*, 1988). At temperatures above 40°C, the ubiquitin system, and consequently its protein–ubiquitin conjugating capacity, is inactivated to <10%. We show that at non-

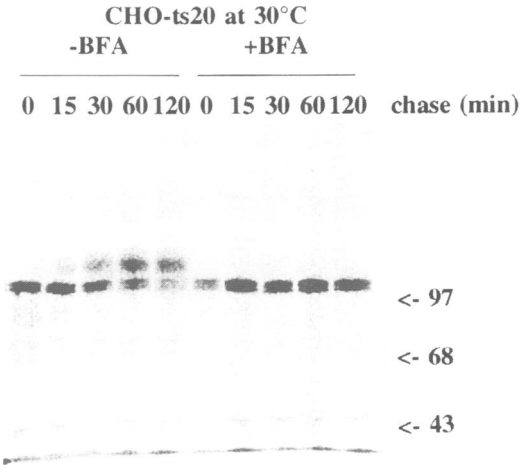

Fig. 1. Pulse–chase labelling of GHR. CHO-ts20 cells were labelled with [^{35}S]methionine for 10 min and chased in αMEM supplemented with 10% fetal bovine serum for the time periods indicated. GHR was immunoprecipitated using anti-GHR(B). Brefeldin A (2 μg/ml) was added at the start of the chase period. Relative molecular mass standards ($M_r \times 10^{-3}$) are shown at the right.

permissive temperatures endocytosis of GH–GHR complexes is inhibited.

Results

Effect of brefeldin A on GHR turnover

Initially, we investigated the life-cycle of the GHR at the permissive temperature (30°C) in CHO-ts20 cells transfected with GHR cDNA using pulse–chase labelling with [^{35}S]methionine (Figure 1). The receptor is initially synthesized as a 110-kDa glycoprotein precursor and thereafter converted to a 130-kDa mature species. Mature GHR is detectable by 15 min of chase and is maximal at 60 min, after which rapid degradation occurs. Recently, it has been shown that the cystic fibrosis transmembrane conductance regulator (CFTR) can be ubiquitinated and degraded within the rough endoplasmic reticulum (Jensen *et al.*, 1995; Ward *et al.*, 1995). To establish whether degradation of GHR occurs within the endoplasmic reticulum (ER), brefeldin A was used to prevent transport from the ER (Lippincott-Schwartz *et al.*, 1989). Under these conditions no degradation of GHR occurred during a 2-h chase period. Taken together, these results demonstrate that the mature GHR is turning over relatively rapid, even in the absence of ligand, while the GHR precursor is relatively stable.

GHR ubiquitination is ligand-dependent

As seen in Figure 2, ubiquitination of the GHR occurs in CHO-ts20 cells at 30°C upon GH addition. The ubiquitin-conjugated GHR appeared as a broad band, much of which being more than 200 kDa. Unlike multi-ubiquitinated forms of cytosolic proteins, there is no discrete ladder-like pattern of ubiquitinated GHR bands. This is a consequence of the presence of heterogeneity of N-linked oligosaccharides on the GHR. The appearance of the mature band on SDS gels remains the same in the presence or absence of ligand, indicating that other modifications such as phosphorylation do not induce changes in apparent molecular weight upon SDS–PAGE (Figure 2, lower

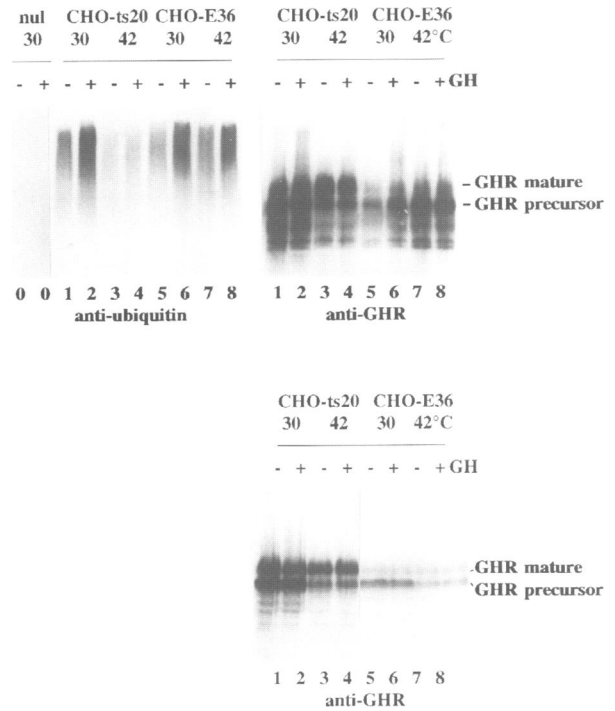

Fig. 2. Ubiquitination of the GHR. CHO-ts20 and CHO-E36 cells were cultured in serum-free medium overnight and the incubation was continued at the temperatures indicated in αMEM supplemented with 10 mM HEPES pH 7.2. After 60 min GH (8 nM) was added and the incubation was continued for 30 min. The cells were then lysed in hot lysis buffer, subjected to SDS–PAGE and immunoblotted using anti-GHR (lower panel); based on the quantitation of the direct immunoblot, equal amounts of GHR were immunoprecipitated with anti-GHR and immunoblotted with anti-ubiquitin conjugate antibody (left panel); after stripping of the blot, the immune reaction was repeated with anti-GHR (right panel). In lane 5 part of the sample was lost. Untransfected CHO-ts20 cells were used to determine the anti-ubiquitin signal if no GHR was present (lanes 0.0 in upper left panel).

panel). The amount of ubiquitinated GHR is relatively small as inferred from the distribution of immune reactivity using anti-GHR (right panel). Judging the percentage of ubiquitinated GHR is risky, as de-ubiquitination during immunoprecipitation can occur and the efficiency of electrophoretic transfer of high molecular weight ubiquitinated protein is different from that of non-ubiquitinated GHR. A guess based on the overexposed immunoblot (Figure 2, right upper panel) would be that no more than a few percent is ubiquitinated. At the non-permissive temperature (42°C) little or no ubiquitination of GHR occurred; this can also be concluded from the anti-GHR blot (upper right panel) where a clear smear pattern is present towards the top in lanes 2, 6 and 8, which is absent in the CHO-ts20 cell immunoprecipitation after incubation at the non-permissive temperature (lane 4). As expected, incubation of wild-type CHO-E36 cells at 42°C in the presence of GH resulted in ubiquitinated GHR. Use of untransfected CHO-ts20 in place of transfected cells, resulted in a complete absence in ubiquitin signal (lanes 0.0). Immunoblotting of cellular extracts, immediately after boiling in SDS-sample buffer using anti-GHR serum illustrates the protease sensitivity of GHR during immunoprecipitation (compare the right panels in Figure 2). It also demonstrates that during incubation at

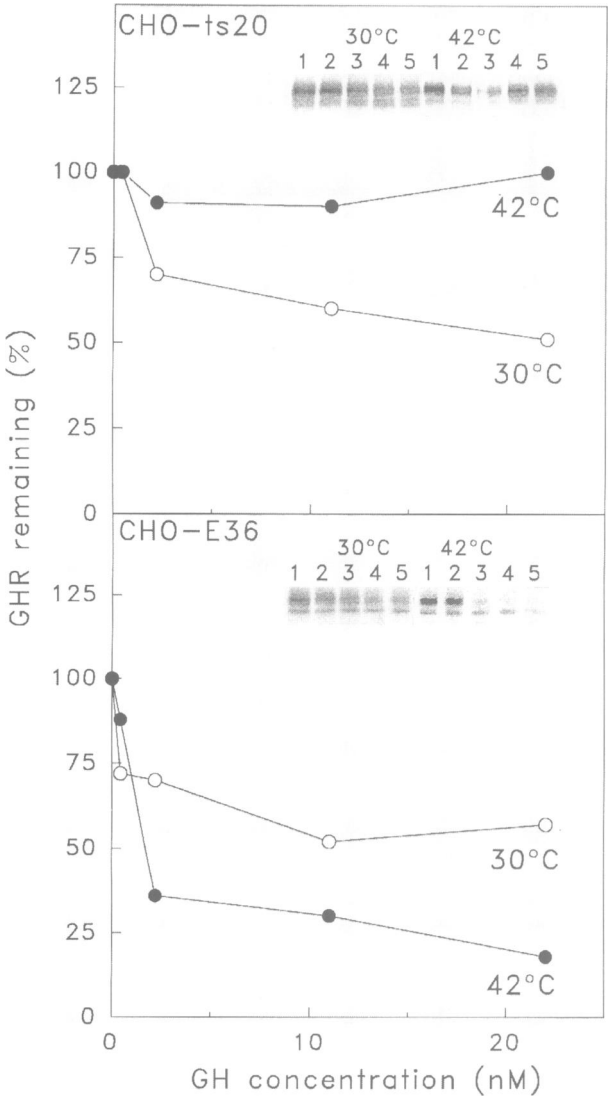

Fig. 3. Effect of GH on GHR degradation. Cells were cultured in serum-free medium overnight and the incubation was continued at the temperatures indicated in αMEM supplemented with 10 mM HEPES pH 7.2 for 60 min, followed by a 15-min period in methionine-free medium. Cells were radiolabelled with [^{35}S]methionine for 30 min and chased for 2 h in the presence of various concentrations GH as indicated. GHR was immunoprecipitated using anti-GHR(B), analysed by SDS–PAGE (insets) and the amounts of GHR (including GHR precursor) were quantitated by PhosphorImager and ImageQuant (Molecular Dynamics) and expressed as the percentage of labelled GHR present without GH. The upper bands (insets) represent the 130 kDa mature GHR.

42°C the biosynthesis of GHR is affected, as is concluded from the changed precursor/mature ratios after incubation at the different temperatures (Figure 2, lower panel). Thus, under basal conditions ubiquitination of the GHR is stimulated several-fold upon ligand binding, but is not seen upon incubation of CHO-ts20 cells at the non-permissive temperature where E1 is inactive.

GHR degradation depends on an intact ubiquitin conjugation system

We next assessed the processes affecting degradation of GHR following biosynthetic labelling with [^{35}S]methionine. Cells were chased for 2 h in the presence of increasing concentrations of GH (Figure 3). At 30°C, GH induced a 50% decrease of total GHR both in CHO-E36 and CHO-ts20 cells. At 42°C in CHO-E36 cells, addition of GH caused virtually complete disappearance of the GHR, while in CHO-ts20 cells no degradation was observed when compared with cells without GH addition. Thus, inactivation of the ubiquitin pathway abrogated the GH-dependent degradation of the GHR.

Ligand-induced GHR endocytosis

Endocytosis is the initial step in the downregulation of cell surface signalling receptors. To examine whether the ubiquitin conjugation system is involved in this early event, cells were incubated for 1 h in the presence of Cy3-labelled GH. Following fixation, the distribution of fluorescent label was examined by confocal microscopy. When CHO-ts20 cells were incubated at 30°C (Figure 4A) the label was present at the cell surface and in vacuoles, probably endosomes and/or lysosomes. Following an acidic wash, label at the cell surface disappeared, while the vacuoles remained fluorescent (Figure 4C). Incubation of cells in the presence of excess unlabelled GH abolished fluorescent labelling under both conditions (not shown). When Cy3-GH was added to CHO-ts20 cells following preincubation at 42°C no uptake of fluorescent label was detectable (Figure 4B). Virtually all the fluorescent Cy3-GH was bound at the cell surface after incubation at 42°C. Furthermore, the vast majority of this label was removed by an acidic wash (Figure 4D). When CHO-E36 cells were incubated at 42°C in the presence of Cy3-GH a punctate intracellular distribution of label was present as in Figure 4A (not shown). These results indicate that ligand-induced GHR endocytosis is dependent upon an intact ubiquitin-conjugating system.

To ascertain the specificity of this observation we compared the endocytosis of GH with that of a constitutively recycling receptor, the transferrin receptor (TfR). TfR recycles between the cell surface and endosomes, thereby providing the cells with ferric iron. Depending on the number of receptors and the recycling time, it takes ~15 min to obtain steady-state conditions for intracellular ligand-bound TfRs (Ciechanover *et al.*, 1983). Figure 4E and F shows that the cellular distribution of transferrin is similar in CHO-ts20 cells at 30 and 42°C. Transferrin labelling is virtually absent at the plasma membrane, probably because uptake times are very short and recycled apotransferrin rapidly dissociates from the TfR once it reappears at the cell surface. These data thus demonstrate that the ubiquitin-conjugating system is involved in the endocytosis of GHR without interfering with the endocytosis of other membrane receptors.

Next, in order to examine a potential role for the endosomal/lysosomal system in this process, cells were labelled with [^{35}S]methionine and chased at 42°C in the presence of increasing concentrations of GH and the presence or absence of 10 mM NH_4Cl (Figure 5). GHR was not degraded in CHO-ts20 cells in the absence or presence of NH_4Cl. As seen in Figure 3, GHR disappeared rapidly in CHO-E36 in the absence of NH_4Cl. However, in the presence of the weak base, GHR degradation was inhibited to a considerable extent, resulting in the accumulation of intermediates (Figure 5). Furthermore, identical results were seen when the analysis was performed with antibodies specific to the carboxy-terminal

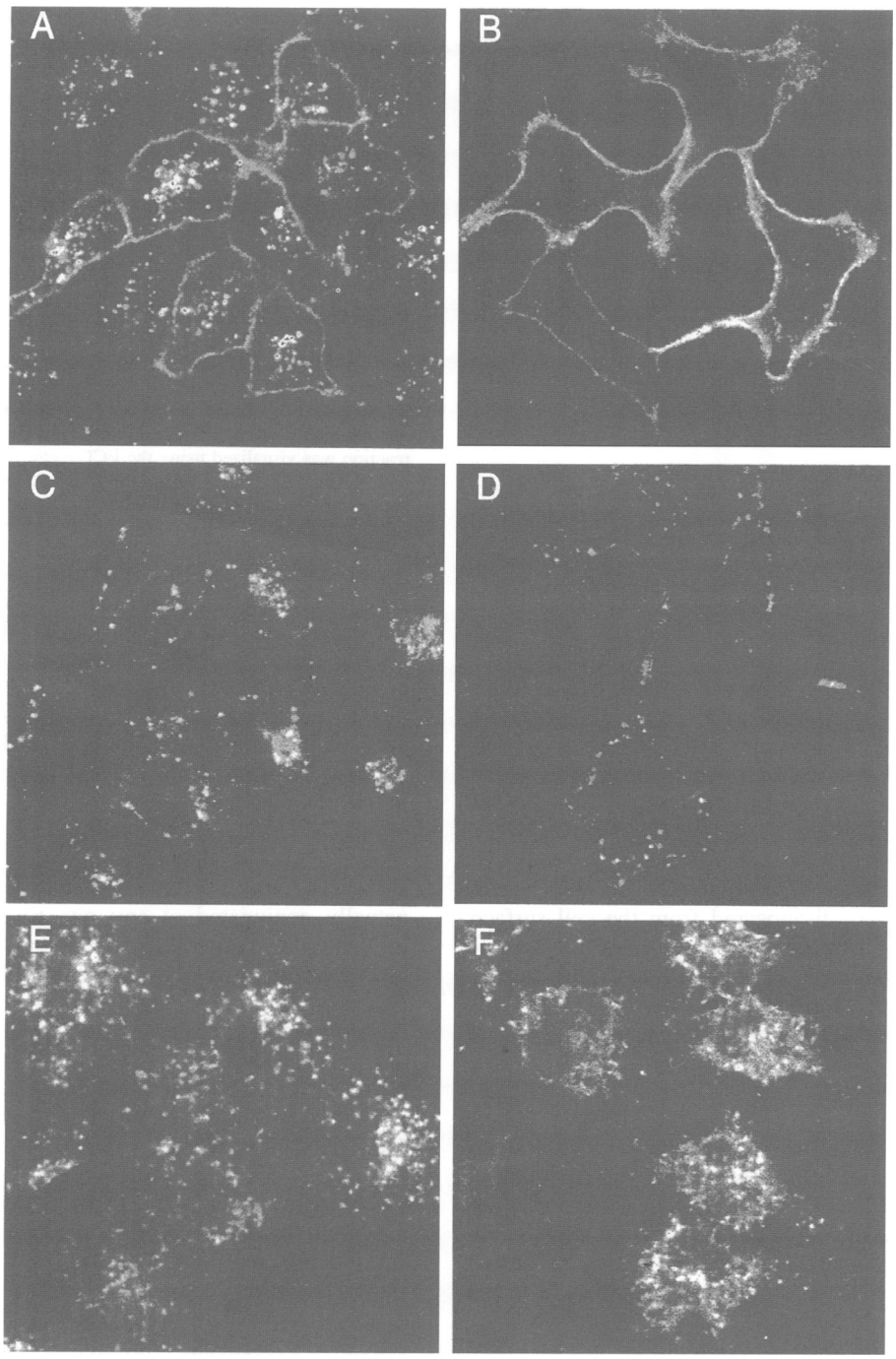

Fig. 4. Distribution of Cy3-GH and Tf-HRP in CHO-ts20 cells visualized by confocal scanning laser microscopy. Cells were incubated at 30°C (**A**, **C** and **E**) or 42°C (**B**, **D** and **F**) for 60 min after which Cy3-GH (A–D) or Tf-HRP (E–F) was added. The incubations were then continued for 60 min (A–D) or 30 min (E–F). Before fixation, the cells were either washed with PBS (A, B, E and F) or treated with acidic buffer and washed with PBS (C and D). The Tf-HRP label was specific, as no signal was visible if a 50-fold excess of non-conjugated Tf was added together with Tf-HRP (not shown).

120 amino acid cytoplasmic tail (not shown). Thus, the data suggest that GH-induced degradation of GHR is initiated at the amino-terminal, exoplasmic portion since NH_4Cl inhibits degradation exclusively within the endosomal/lysosomal compartment.

To examine this issue further, cells were incubated in the presence of bafilomycin A1, a macrolide antibiotic which is a highly potent, specific inhibitor of the vacuolar proton pump. Bafilomycin A1 inhibits acidification of endosomes, lysosomes and phagosomes, without causing the morphological changes in vacuolar compartments which are characteristic of weak bases and ionophores (Bowman *et al.*, 1988; Yoshimori *et al.*, 1991). Before analysis, cells were treated with proteinase K specifically to remove the extracellular portion of GHR at the cell surface. This procedure enables simultaneous monitoring of GHR at the cell surface (i.e. outside) and within intracellular compartments (i.e. inside) of the cells, as anti-GHR(B) antibody detects both the complete molecule if protected from the protease and the truncated GHR

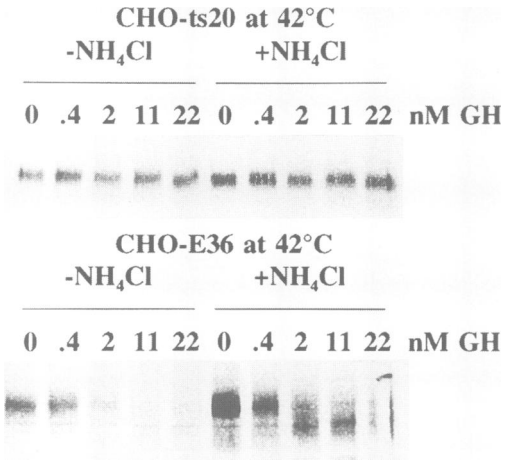

Fig. 5. Effect of NH_4Cl on GHR degradation. Cells were pre-treated and radiolabelled as in Figure 3, except that 10 mM NH_4Cl was present during the chase period in two series of samples (right panels). [^{35}S]methionine-labelled GHR is visualized after SDS–PAGE and phosphorimage analysis. The upper band represents the 130 kDa mature GHR.

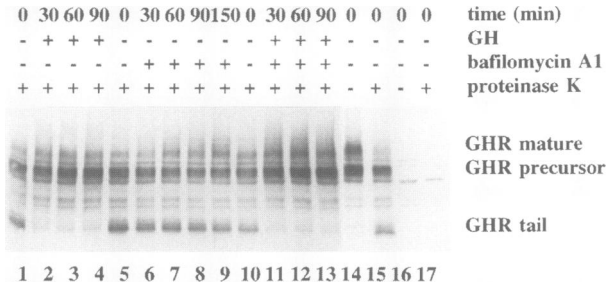

Fig. 6. Effect of bafilomycin A1 and ligand on GHR degradation. Cells were incubated at 30°C in 5% CO_2; bafilomycin A1 (1 mM) with and without GH (8 nM) was added at time 0 min. At the times indicated the cells were treated with proteinase K on ice, lysed in Triton X-100 containing the protease inhibitor mix and aliquots were immunoblotted after SDS–PAGE using anti-GHR(B); the immunoreaction was visualized using the ECL method. In the right two lanes untransfected CHO-ts20 cells were used to demonstrate that the minor bands represent background reaction.

which remains following removal of the extracellular portion by the protease. As seen in Figure 6 (lanes 14–15) most mature GHR molecules were present at the cell surface. When GH was added, GHR disappeared rapidly from the cell surface. When bafilomycin A1 was present the amount of intracellular GHR increased (lanes 5–9). This suggests that there is a baseline, bafilomycin-sensitive degradation of receptors. When both GH and bafilomycin were added, receptors disappeared from the cell surface with a concomitant increase in the amount of intact GHR (protected from proteinase K) (lanes 10–13). This implies that ligand-induced receptor degradation is dependent upon acidification of the vacuolar system.

Discussion

Ubiquitin, a highly conserved 76 amino acid residue protein, is present in all eukaryotes where its conjugation to target proteins plays a pivotal role in a diverse array of regulatory events including cell cycle progression, DNA repair and transcriptional control. Many of these processes are dependent upon both ubiquitin conjugation to target proteins and the subsequent degradation of the protein–ubiquitin moieties via the proteasome. In the present study we demonstrate that the ubiquitin-conjugating system serves a central role in the downregulation of GHR. In yeast a similar mechanism has been described for the endocytosis of Ste2p, the α-factor receptor (Hicke and Riezman, 1996). The initial step in GHR downregulation is the dimerization of two polypeptides by one GH molecule (deVos *et al.*, 1992). This event triggers signal transduction and rapid degradation of the GHR. Our major finding is that a functional ubiquitin conjugation system is necessary for endocytosis of the GHR. Ubiquitination of the GHR cytoplasmic tail is likely the molecular event which triggers receptor endocytosis. This possibility is strengthened by the observation that GHR is ubiquitinated upon ligand binding. The studies described above, which demonstrate that endocytosis of the transferrin receptor proceeds normally even at the non-permissive temperature, eliminates the possibility that there is a global effect on the process of endocytosis *per se*. This is consistent with our earlier observations that early events of endocytosis are unaltered in CHO-ts20 cells in which the ubiquitin-conjugating process is inactivated (Schwartz *et al.*, 1992). One attractive hypothesis would imply that polyubiquitination of all receptors occurs upon GH binding at the cell surface. As most of the cell's GHRs are at the cell surface at steady state and enter the cell within 30 minutes upon GH binding, the process of ubiquitination and subsequently, de-ubiquitination must be extremely rapid such that only a relatively small fraction of GHR is actually conjugated at any one time. This scenario is in fact quite reasonable in that multi-ubiquitination of immunoglobulin E receptor occurs at the cell surface upon ligand binding and is rapidly reversed upon disengagement of the receptor from the ligand (Paolini and Kinet, 1993). The most likely sequence of events is that, following transport to endosomes and interaction with an acidic milieu, GH uncouples from its receptor, after which GHR is transported onto multivesicular endosomes and lysosomes. Such a process may occur within minutes (Stoorvogel *et al.*, 1987). Thus, ubiquitination may act as a switch for the onset of downregulation of the signal-transducing receptors (similar to the role of protein phosphorylation in certain examples of signal transduction). However, as many basic cellular processes are regulated subsequent to ubiquitin conjugation, use of the E1 mutant cell line at the non-permissive temperature does not exclude the possibility that another E1-dependent event is the critical factor in the control of GHR endocytosis.

A second important result from the present study concerns the degradation of GHR within the vacuolar system. Evidence along these lines has been published previously and derives primarily from studies in which the vacuolar protein degradation system has been inhibited pharmacologically (Lesniak and Roth 1976; Gorin and Goodman, 1985; Roupas and Herington, 1988; Saito *et al.*, 1994). Ubiquitination of the GHR is linked to its ligand-induced degradation. Pulse–chase metabolic labelling studies demonstrate that weak bases such as NH_4Cl inhibit GH-induced degradation of the receptor almost completely. In addition, bafilomycin A1, a specific inhibitor of the

vacuolar proton pump, abrogates the decrease in cellular GHR content, following GH addition. These observations are consistent with observations by others, as cited above. The experiments employing bafilomycin also demonstrate an accumulation of GHR within the cell even in the absence of GH. This suggests that GH-independent degradation also takes place within the vacuolar system. As there is a small degree of ligand-independent ubiquitination of the GHR, this 'constitutive' GHR degradation may well be similar to that seen upon GH binding; namely (monomeric) GHR may undergo ubiquitination, albeit slowly, after which endocytosis and degradation occur.

Several other cell surface receptors, including the T-cell receptor ζ-chain (Hou *et al.*, 1994), the IgE- (Paolini and Kinet, 1993), the platelet-derived growth factor (Mori *et al.*, 1992) and c-kit receptor (Miyazawa *et al.*, 1994), are ubiquitinated. One general feature of the ubiquitination of these signalling receptors is the dependence on ligand binding. At present, it is not known whether the attachment of ubiquitin to these receptor proteins signals their degradation, or whether it serves other, as yet unknown, non-proteolytic functions. Our data demonstrate that, although ubiquitin is attached to the GHR and inactivation of the ubiquitin conjugation system abolishes receptor degradation, the initial degradative step(s) takes place within the endosomal/lysosomal system. Whether the 26S proteasome is involved in later steps of degradation is unknown, but unlikely, because the process of multivesicular vesicle formation during endosome maturation likely segregates the carboxy-terminal GHR tail from the cytoplasm, as has been described for the epidermal growth factor receptor (Felder *et al.*, 1990).

The involvement of the ubiquitin system in selective membrane transport has been observed earlier; maturation of autolysosomes into residual bodies is inhibited in CHO-ts20 cells at the non-permissive temperature (Lenk *et al.*, 1992), and in yeast mutants, defective in endocytosis, the ubiquitinated transporter Ste6 accumulates at the plasma membrane (Kölling and Hollenberg, 1994). Our present findings are in full agreement with these observations; yeast Ste6 is ubiquitinated at the cell surface and its degradation occurs within the vacuole. Moreover, when ubiquitination is inhibited, the half-life of Ste6 is prolonged, similar to the case for GHR. Recently, studies by Hicke and Riezman (1996) demonstrate that ligand-induced endocytosis of the Ste2 protein, a G-protein-coupled plasma membrane receptor for yeast mating pheromone α-factor, depends upon an intact ubiquitination system. Their results clearly show that ubiquitination of Ste2p itself is required for ligand-stimulated endocytosis. The amino acid sequence motif involved in ubiquitination of Ste2p (SINNDAKSS) does not occur in the GHR tail. However, in GHR the DEKT sequence at the end of box-2 could possibly substitute perfectly well for the DAKS motif. Recently, Allevato and co-workers (1995) have reported that a phenylalanine residue within the rat GHR tail is critical for ligand-induced internalization. Further analysis will determine whether this residue is also important in GHR ubiquitination and intravacuolar degradation.

Materials and methods

Plasmids and cells

CHO-ts20 and CHO-E36 cells were transfected with a pCB6 construct containing the full-length rabbit GHR cDNA sequence using the calcium phosphate method (Brewer and Roth, 1991). Stable, geneticin-resistant transfectants were selected after screening with anti-GHR antiserum using immunofluorescence and immunoblotting. Cells were grown in Eagle's minimal essential medium (αMEM) supplemented with 10% fetal bovine serum, penicillin and streptomycin, and 0.4 mg/ml geneticin. For experiments the cells were grown on 35 mm plates and used at 75% confluence. The CHO-ts20 clone, used in this study, expressed ~10-fold more receptor than the CHO-E36 clone. To compensate for this, 10 mM sodium butyrate was added to CHO-E36 cells 18 h before use, increasing GHR expression ~5-fold (see Brown *et al.*, 1989). Treatment of CHO-ts20 cells with sodium butyrate did not alter the behaviour of GHR in any of the parameters examined in this study. Bafilomycin A1 (1 μM) was added from a 100× stock in DMSO. DMSO alone had no effect on transport or on degradation kinetics.

Antibodies and materials

Anti-GHR antisera were raised in rabbits against fusion proteins of glutathione *S*-transferase and GHR peptides consisting of amino acids 327–493 [anti-GHR(B)] or 493–621 [anti-GHR(C)]. Fusion proteins were expressed in *Escherichia coli* after transformation with pGEX vectors (Pharmacia, Sweden). In most experiments anti-GHR(B) was used. GH was a gift of Lilly Research Labs, Indianapolis, IN.

Cell lysis, immunoprecipitation, and immunoblotting

The cells were lysed on ice in 0.3 ml of lysis mix containing 2 mM Na_3VO_4, 1% Triton X-100, 1 mM EDTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin and 1 mM phenylmethylsulfonyl fluoride in PBS. In ubiquitin blotting experiments the cells were lysed in 0.3 ml of boiling lysis buffer containing 1% SDS in PBS in order to avoid isopeptidase activity. The lysate was heated at 100°C for 5 min, sheared to break DNA and centrifuged for 5 min at 10 000 *g*. Immunoprecipitations were carried out in 1% Triton X-100, 0.5% SDS, 0.25% sodium deoxycholate, 0.5% bovine serum albumin, 1 mM EDTA, 2 mM Na_3VO_4, 0.1 M NaF, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 1 mM PMSF in PBS. The reactions were incubated for 2 h at 0°C with 5 μl of specific rabbit anti-GHR(B) antiserum; protein A–agarose (Repligen Co, Cambridge, MA) was used to isolate the immune complexes. The immunoprecipitates were washed twice with the same buffer and once with 10-fold diluted PBS; immune complexes were analysed by 7.5% polyacrylamide gel electrophoresis in the presence of SDS (SDS–PAGE). For proteinase K treatment cells (35 mm plates) were washed in ice-cold PBS containing 1 mM EDTA and then incubated in 0.5 ml of a 0.5 mg/ml solution of proteinase K (Boehringer, Mannheim) for 60 min. The resulting cell suspension was then washed twice in PBS–EDTA containing 2 mM phenylmethylsulfonyl fluoride, and lysed as above.

For immunoblotting, the cells were lysed, GHR was immunoprecipitated, analysed by gel electrophoresis and transferred to PVDF paper. The blots were immunostained using anti-ubiquitin conjugate antibody, anti-GHR Mab antibody (Mab5, AGEN, Brisbane, Australia) or anti-GHR(B) (after stripping of the blots), and the antigens were visualized using the ECL system (Amersham, UK).

Metabolic labelling

For metabolic labelling, the cells were incubated in methionine-free MEM for 20 min and then [^{35}S]methionine (TRAN-^{35}S label, 1.85 MBq/ml, 40 TBq/mmol, ICN, CA, USA) was added and the incubation was continued for 30 min; the radioactivity was chased in the presence of αMEM containing 0.05 mM unlabelled methionine. The radioactivity was determined using a Molecular Dynamics PhosphorImager.

Microscopy

Cells, grown on cover slips, were cultured in αMEM supplemented with 10 mM HEPES pH 7.2 at 30 or 42°C in a water bath. After 60 min, Cy3-GH (1 μg/ml) was added and the incubations were continued for 1 h. Thereafter, the cells were washed to remove unbound label in PBS and fixed overnight in 3% paraformaldehyde. To remove ligand from the plasma membrane, cells were washed in 50 mM glycine, 150 mM NaCl pH 2.5 for 5 min on ice before fixation. The cells were embedded in Mowiol and confocal laser scanning microscopy was performed using a Leica TCS 4D system. The Cy3-GH was prepared using a Fluorolink-Cy3 label kit according to the supplier's recommendations (Amersham, UK). To visualize the uptake via the transferrin receptor, cells (grown on cover slips) were incubated in the presence of 5 μg/ml transferrin–horseradish peroxidase for 30 min, washed and fixed in 2% paraformaldehyde. Cells were permeabilized in 0.05% saponin and incubated with biotinyl tyramide (Bobrow *et al.*, 1991) using the Renaissance TSA-indirect kit (DuPont NEN) and optimized for cytochemistry (A.van

Weert and W.Stoorvogel, manuscript in preparation). A streptavidin–fluorescein conjugate was used as a marker for deposited biotin. Control cells were incubated in the presence of a 50-fold excess concentration of transferrin.

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