Identification of a novel ubiquitin conjugation motif, required for ligand-induced internalization of the growth hormone receptor

Roland Govers, Toine ten Broeke,
Peter van Kerkhof, Alan L.Schwartz¹ and
Ger J.Strous²

Department of Cell Biology, Faculty of Medicine and Institute of Biomembranes, Utrecht University, Utrecht, The Netherlands and
¹Departments of Molecular Biology, Pharmacology and Pediatrics, Washington University School of Medicine, St Louis, MO 63110, USA

²Corresponding author
e-mail: strous@med.uu.nl

In addition to its role in selective protein degradation, the conjugation of ubiquitin to proteins has also been implicated in the internalization of plasma membrane proteins, including the α -factor receptor Ste2p, uracil permease Fur4p, epithelial sodium channel ENaC and the growth hormone receptor (GHR). Binding of GH to its receptor induces receptor dimerization, resulting in the activation of signal transduction pathways and an increase of GHR ubiquitination. Previously, we have shown that the ubiquitin conjugation system mediates GH-induced GHR internalization. Here, we present evidence that a specific domain of the GHR regulates receptor endocytosis via the ubiquitin conjugation system. This ubiquitin-dependent endocytosis (UbE) motif consists of the amino acid sequence DSWVE-FIELD and is homologous to sequences in other proteins, several of which are known to be ubiquitinated. In addition, we show that GH internalization by a truncated GHR is independent of the presence of lysine residues in the cytosolic domain of this receptor, while internalization still depends on an intact ubiquitin conjugation system. Thus, GHR internalization requires the recruitment of the ubiquitin conjugation system to the GHR UbE motif rather than the conjugation of ubiquitin to the GHR itself.

Keywords: endocytosis/growth hormone receptor/internalization/ubiquitin

Introduction

Ubiquitin plays a central role in the degradation of short-lived and 'abnormal' soluble proteins (for review, see Hershko and Ciechanover, 1998). Multiple ubiquitin moieties are conjugated to the target protein, which then becomes a substrate for the 26S proteasome, a large cylindrical-like protein-degrading complex (Coux *et al.*, 1996). The specificity of proteasomal protein degradation is determined by the affinity of the proteasome for polyubiquitin chains (Deveraux *et al.*, 1994). Binding of the proteasome to the protein-ubiquitin complex ultimately results in protein degradation, while intact ubiquitin is removed from the substrate.

In 1986, Yarden *et al.* demonstrated that ubiquitin could be found conjugated to certain membrane proteins. Since then, numerous membrane proteins have been found to be ubiquitinated (Cenciarelli *et al.*, 1992; Paolini and Kinet, 1993; Kölling and Hollenberg, 1994). For most, it is unlikely that the proteasome is involved in their degradation, since the proteasome itself is not capable of removing proteins from the membrane. For several endoplasmic reticulum (ER) membrane proteins, however, the proteasome does appear to be involved in the degradation of the entire protein. Following translocation through the ER translocon in a reverse movement, these proteins are degraded by the proteasome in the cytoplasm (Wiertz *et al.*, 1996; Plemper *et al.*, 1997). For plasma membrane proteins this is not the case. Since no protein translocator has been defined in the plasma membrane, ubiquitination of these proteins does not lead to their complete degradation by the proteasome. Since the proteasome recognizes polyubiquitin chains, the cytoplasmic domains of these plasma membrane proteins may be degraded by this complex. Receptor ubiquitination often increases upon ligand binding (Mori *et al.*, 1992; Miyazawa *et al.*, 1994), wherein partial degradation by the proteasome may result in abrogation of signal transduction. Thus, the proteasome may be involved in modulation of signal transduction kinetics. However, for most ubiquitinated receptors, degradation occurs in the lysosome (Galcheva-Gargova *et al.*, 1995; Hicke and Riezman, 1996; Horak and Wolf, 1997).

We and others have shown that the ubiquitin conjugation system is involved in the endocytosis of several plasma membrane proteins. In yeast, receptor ubiquitination occurs at the plasma membrane and is required for internalization (Kölling and Hollenberg, 1994; Hicke and Riezman, 1996; Roth and Davis, 1996). In mammalian cells, one of the best studied ubiquitinated plasma membrane proteins is the growth hormone receptor (GHR) (Postel-Vinay and Finidori, 1995). The GHR has 19 potential ubiquitination sites, is polyubiquitinated on multiple lysines and its ubiquitination is increased upon binding of growth hormone (GH). Previously, we found that the GHR internalizes only in the presence of an intact ubiquitin conjugation system (Strous *et al.*, 1996). Furthermore, there is a strict correlation between GHR ubiquitination and GHR internalization, and Phe327 appears to be an important feature of both of these events (Govers *et al.*, 1997). Our data suggested that GHR internalization requires GHR ubiquitination.

In the present study, we have identified the domain involved in ubiquitination and internalization of the GHR and determined many of its characteristics. Since mutations in this domain affected both receptor ubiquitination and receptor internalization, we have named this domain the ubiquitin-dependent endocytosis (UbE) motif. This domain

Fig. 1. Schematic representation of the wild-type and mutant GHRs. Of all GHR mutants, in which one amino acid residue surrounding Phe327 (between Asn320 and Asp334) was mutated in the full-length receptor, only the first and last mutant is shown (N320A and D334A, respectively). For GHR mutants truncated at residue 399, all cytoplasmic lysines, lysine mutations and Phe327 or Ala327 are indicated. For GHR mutants, truncated at residue 349, only Phe327 or Ala327 is indicated. A black square represents the transmembrane domain of the GHR. Hatched squares represent box1 and box2, respectively, corresponding to the conserved homology domains within members of the cytokine receptor family.

serves as the binding site for the ubiquitin conjugation system. Furthermore, examination of the internalization of a truncated GHR, in which all cytoplasmic lysines were mutated in arginines, revealed that GHR internalization is dependent on the UbE motif, yet independent of the conjugation of ubiquitin to lysine residues in the cytosolic domain of the GHR.

Results

GHR Phe327 is part of a ubiquitination domain

The GHR consists of 620 amino acid residues, of which 350 residues are located intracellularly. Part of this intracellular domain is involved in internalization of the ligand-receptor complex. Previously, we examined the internalization and ubiquitination of several GHR mutants (Govers *et al.*, 1998). We showed that the cytoplasmic tail of the receptor contains two independent internalization signals. A di-leucine motif in the GHR (Leu347,348) mediates ubiquitin-independent GHR internalization. This motif is only active upon truncation of the receptor at position 349 (Govers *et al.*, 1998). The second motif (including Phe327) is active in the full-length GHR as well as in receptor truncation mutants, and links endocytosis to the ubiquitin conjugation system.

To determine the role of Phe327 in ubiquitin-dependent GHR internalization, Phe327 was mutated to alanine in GHR truncation mutant 1-349 (Figure 1). This truncation mutant internalizes via the di-leucine motif, independently of the ubiquitin conjugation system (Govers *et al.*, 1998). We transfected the cDNAs encoding wild-type GHR, GHR mutant F327A and receptor truncations 1-349 and 1-349 F327A into the Chinese hamster ovary cell line CHO-ts20, which contains a thermolabile ubiquitin-activating enzyme (E1) (Kulka *et al.*, 1988). This temperature-sensitive enzyme is fully active when the cells are incubated at 30°C. However, when these cells are incubated at the non-permissive temperature of 42°C, the enzyme is inactive and ubiquitination of proteins can no longer occur.

Fig. 2. Effect of GHR mutation F327A on internalization and ubiquitination of a GHR, which internalizes independently of the ubiquitin conjugation system. **(A)** CHO-ts20 cells expressing wild-type GHR, GHR F327A, GHR truncation 1-349 or truncation 1-349 F327A were incubated with Cy3-GH for 1 h at 30 or 42°C. Cy3-GH was visualized by confocal microscopy. Untransfected CHO-ts20 cells showed neither Cy3-GH binding nor internalization (not shown). Bar = 10 µm. **(B)** Untransfected cells (ts20) and cells expressing the indicated GHRs were incubated for 30 min with or without unlabelled 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. The 40–55 kDa bands seen in the immunoprecipitations originated from IgG.

Internalization of fluorescence-labelled GH by wild-type and mutant receptors was inhibited at the non-permissive temperature (Figure 2A). As we have shown previously, wild-type GHR (Figure 2A, panels 1-620) internalized in a ubiquitin-dependent manner (Strous *et al.*, 1996). Cy3-GH was internalized by the GHR at 30°C, while the

Fig. 3. Alanine-scanning analysis of the GHR ubiquitination domain. CHO-ts20 cells expressing the indicated GHRs were incubated with Cy3-GH for 1 h at 30°C. Cy3-GH was visualized by confocal microscopy. Bar = 10 μ m.

ligand remained at the plasma membrane when the cells were incubated at 42°C. Internalization of the full-length receptor was dependent on Phe327 (Figure 2A, panel 1–620 F327A) (Govers *et al.*, 1997). Internalization of GHR truncation 1–349 was mediated by the di-leucine motif and occurred in the presence (30°C) as well in the absence (42°C) of an intact ubiquitin conjugation system. As expected, mutation F327A did not affect internalization of this short GHR, indicating that Phe327 is only involved in the ubiquitin-dependent GHR internalization. Furthermore, we examined the effect of mutation F327A on the ubiquitination of GHR truncation 1–349 (Figure 2B). Cells expressing the indicated receptors and untransfected cells (ts20) were incubated in the absence or presence of GH. Upon cell lysis, protein–ubiquitin conjugates were immunoprecipitated and analysed by Western blotting. The left lane in each panel contains total cellular lysate and shows the apparent molecular weight of the receptor precursor (faster migrating band) and mature receptor (slower migrating band). In these lanes, no ubiquitinated GHR can be detected, which is in accordance with our previous findings (Strous *et al.*, 1996) and indicates that only a small portion of the GHR is ubiquitinated. Cell extracts containing approximately equal amounts of GHRs were used for the anti-ubiquitin immunoprecipitation. The high molecular weight bands visible in the upper part of these lanes represent ubiquitinated receptors. Cells expressing wild-type GHR or GHR truncation 1–349 showed ubiquitination of the GHR. Previously, we have shown that mutation F327A abolishes ubiquitination of the full-length GHR (Govers *et al.*, 1997). Mutation of Phe327 in truncation 1–349 also resulted in complete inhibition of receptor ubiquitination, although this mutant

still internalized GH. This implies that Phe327 is part of a ubiquitination domain and that the effect of the F327A mutation on ligand internalization by the full-length GHR is due primarily to the defective ubiquitination domain.

Characteristics of the ubiquitin-dependent endocytosis motif

Previously, we have shown that GHR truncation mutant 1–334 internalizes GH (dependent on the ubiquitin conjugation system), while GHR truncations shorter than 1–331 do not internalize ligand (Govers *et al.*, 1998). This suggested that the GHR ubiquitination domain comprises at least five amino acid residues (Phe327–Asp331) and is situated upstream of amino acid residue 334. In order to elucidate the extent of the ubiquitination domain, we sequentially mutated amino acid residues between Asn320 and Asp334 to alanines in the full-length receptor (Figure 1). We analysed internalization of ligand by the mutated GHRs to examine the role of the individual amino acid residues in the ubiquitin-dependent endocytosis motif (Figure 3). Cells stably expressing the indicated GHRs were incubated with Cy3-GH for 1 h at 30°C. Wild-type GHR (wt) showed a vesicular pattern of internalized GH, while GH internalization by GHR mutants S323A, W324A, E326A, F327A, I328A, L330A, D331A and I332A was clearly impaired. These data suggest that the GHR ubiquitination domain comprises ~10 amino acid residues. To examine the precise characteristics, we studied [¹²⁵I]GH internalization in the alanine mutants and in additional GHR mutants, in which amino acid residues involved in ligand internalization (as indicated in Figure 3) were mutated to other amino acid residues (Figure 4). Cells expressing the indicated GHRs were incubated with

Fig. 4. Effect of mutations in the GHR surrounding Phe327 on [125 I]GH internalization. CHO-ts20 cells expressing the indicated GHRs were incubated with [125 I]GH for 1 h at 30°C in the presence or absence of excess unlabelled GH. Specifically internalized [125 I]GH was expressed as a percentage of total cell-associated label.

8 nM [125 I]GH for 1 h at 30°C and the percentage of internalized label was determined. Untransfected CHO-ts20 cells showed neither specific [125 I]GH binding nor internalization (not shown). Figure 4 shows that the GHR ubiquitination domain consists of 10 consecutive amino acid residues (Asp322–Asp331). Mutation of a single residue within this domain resulted in at least 50% inhibition of ligand uptake. GH internalization by GHR mutants D322A, S323A, W324A, V325G, E326A, F327A, F327L, F327V, I328A, D331A and D331N was severely impaired. Phe327 could be substituted by a tyrosine without affecting ligand internalization. The most inhibitory GHR mutants still show some ligand internalization (15%). We attribute this to constitutive GHR endocytosis independent of its cytoplasmic tail, since GHRs with only 15 residues in their cytoplasmic domain already show some internalization (Govers *et al.*, 1997). These findings are in agreement with data on the transferrin receptor which also internalizes to a small extent in the absence of its cytoplasmic domain (Jing *et al.*, 1990). This type of internalization is thought to reflect bulk membrane flow.

To confirm that these amino acid residues are indeed part of the ubiquitination domain, we studied ubiquitination of the most inhibitory mutants (E326A, I328A and D331A) as well as mutant F327Y, which internalized ligand to the same extent as wild-type GHR (Figure 5). Protein-ubiquitin conjugates were immunoprecipitated and analysed as described for Figure 2B. The wild-type GHR was ubiquitinated (panel wt). Its ubiquitination was enhanced upon incubation with GH. All alanine mutants showed severely impaired ubiquitination, indicating that in addition to Phe327, Glu326, Ile328 and Asp331 are also primarily involved in GHR ubiquitination. Mutation of Phe327 to

Fig. 5. Effect of mutations in the GHR ubiquitination domain on GHR ubiquitination. CHO-ts20 cells expressing the indicated GHRs were incubated for 30 min with or without unlabelled 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.

tyrosine did not interfere with receptor ubiquitination. In order to confirm that most of the mature (130 kDa) GHR species resided at the plasma membrane, we performed proteinase K digestions on the cells. These experiments showed that the majority of the mature mutant GHR species were localized at the plasma membrane, as is the case for the wild-type receptor (data not shown). Our findings with the GHR mutants showed a strict correlation between GHR ubiquitination and GHR-mediated GH internalization, and indicated that amino acid residues Asp322–Asp331 are part of the GHR ubiquitination domain.

GHR-mediated GH internalization is independent of its cytoplasmic lysine residues

Our data thus far suggest that ubiquitination of the GHR is linked to ligand-induced GHR internalization (except for truncation 1–349). To establish whether ubiquitination of the GHR is indeed required for receptor internalization, we constructed a GHR in which all cytoplasmic lysine residues were mutated to arginines. Since internalization of ligand by GHR truncation mutant 1–399 is as dependent on an intact ubiquitin conjugation system as the wild-type GHR (Govers *et al.*, 1998), we mutated the cytoplasmic lysine residues in this truncation (Figure 1). Unexpectedly, incubation of CHO-ts20 cells expressing this GHR mutant (GHR 1–399 K271–362R) with Cy3-GH resulted in internalization of ligand (Figure 6D). Quantification of ligand internalization using radiolabelled GH (as described for Figure 4A) demonstrated that GHR 1–399 and GHR 1–399 K271–362R internalized ligand to the same extent (not shown). Internalization of ligand by this mutant receptor was dependent on an intact conjugation system, since incubation at the non-permissive temperature inhibited ligand internalization (Figure 6E). Identical incubations of cells expressing GHR truncation 1–399 in which all lysines were present resulted in similar internalization patterns (Figure 6A and B). To confirm that ligand internalization by GHR mutant 1–399 K271–362R required the UbE motif, we mutated Phe327 in this mutant (Figure 1). Internalization experiments indicated that for both GHR 1–399 and GHR 1–399 K271–362R the ubiquitination domain is required for receptor internalization (Figure 6C and F, respectively). Thus, these data show that GHR-mediated GH internalization does not require conjugation of ubiquitin to lysine residues in the intracellular domain of the GHR, although internalization is controlled by the ubiquitination domain.

Fig. 6. Internalization of ligand by a GHR that contains no potential ubiquitin conjugation sites. CHO-ts20 cells expressing (A and B) GHR truncation mutant 1–399 or (D and E) truncation 1–399 K271–362R were incubated with Cy3-GH for 1 h at 30°C (A and D) or 42°C (B and E). CHO-ts20 cells expressing GHR truncation mutants (C) 1–399 F327A or (F) 1–399 K271–362R F327A, were incubated with Cy3-GH for 1 h at 30°C. Cy3-GH was visualized by confocal microscopy. Bar = 10 µm.

Discussion

Ubiquitin conjugation has been implicated in the regulation of many cellular processes, such as signal transduction (Lee *et al.*, 1996; Strous *et al.*, 1997), cell-cycle progression (Hershko, 1997), stress response (Gropper *et al.*, 1991; Jungmann *et al.*, 1993) and receptor internalization (Hicke and Riezman, 1996). For most of these processes, ubiquitin is thought to function via targeting regulatory proteins for degradation by the proteasome. Many receptors and other plasma membrane proteins have been found ubiquitinated, and for several of them ubiquitin conjugation was shown to be involved in endocytosis. Activation of the T cell antigen receptor (Cenciarelli *et al.*, 1992), c-kit (Miyazawa *et al.*, 1994), IgE receptor (Paolini and Kinet, 1993), epidermal growth factor (EGF) receptor (Galcheva-Gargova *et al.*, 1995) and colony-stimulating factor-1 (CSF-1) receptor (Mori *et al.*, 1995a) induce receptor ubiquitination. The actual function of ubiquitin conjugation to these receptor species has not been characterized. However, ubiquitin conjugation has been implicated in proteasomal degradation of the platelet-derived growth factor (PDGF) receptor (Mori *et al.*, 1995b), Met receptor (Jeffers *et al.*, 1997) and c-erbB-2 (Mimnaugh *et al.*, 1996). For the mammalian GHR (Strous *et al.*, 1996; Govers *et al.*, 1997) and epithelial sodium channel ENaC (Staub *et al.*, 1996, 1997) as well as for the yeast plasma membrane proteins Ste6p (Kölling and Hollenberg, 1994; Kölling and Losko, 1997), Ste2p (Hicke and Riezman, 1996), Ste3p (Roth and Davis, 1996), Pdr5 (Egner and Kuchler, 1996) and uracil permease (Galan *et al.*, 1994, 1996), ubiquitin conjugation is clearly involved in endocytosis.

Previously, we have shown that the ubiquitin conjugation system is involved in GHR-mediated GH internalization (Strous *et al.*, 1996). Allevato and colleagues had shown that Phe327 is essential for GHR internalization (Allevato *et al.*, 1995). We then demonstrated that Phe327 is also involved in GHR ubiquitination (Govers *et al.*, 1997). Using GHR truncation mutant 1–349, which internalizes via a di-leucine motif, independently of the ubiquitin

conjugation system (Govers *et al.*, 1998), we characterized the function of this particular phenylalanine. Mutation of Phe327 in GHR truncation 1–349 resulted in impaired GHR ubiquitination, while ligand internalization via the di-leucine motif was unaffected. This indicated that Phe327 is involved primarily in GHR ubiquitination and that this phenylalanine is likely to be part of a ubiquitination motif. Since ubiquitin cannot be conjugated to an internal phenylalanine residue (i.e. it has no free amino groups), Phe327 is probably part of a domain involved in recognition and binding of components of the ubiquitin conjugation system, perhaps one of the ubiquitin ligases (E3s). Recently, a similar phenomenon has been described for the PY motif of ENaC, which interacts with ubiquitin ligase Nedd4 (Staub *et al.*, 1996). Since GHR internalization is dependent on an intact ubiquitination domain (including Phe327), we define this domain as the ubiquitin-dependent endocytosis (UbE) motif. Thus, the F327A mutation probably results in impaired docking of the ubiquitin conjugation system to the receptor and in decreased ligand-induced GHR internalization.

To define the GHR UbE motif, we studied GHRs, in which single amino acid residues surrounding Phe327 were mutated. Our data indicated that GH internalization by GHR mutants D322A, S323A, W324A, V325G, E326A, F327A, F327L, F327V, I328A, D331A and D331N was severely impaired and suggest that the UbE motif comprises 10 amino acid residues (D322–D331). That Asp331 is involved in receptor internalization is in accordance with our previous work, in which we showed that GHR truncation 1–334 internalized, while truncation 1–330 did not (Govers *et al.*, 1998). Lack of ubiquitination was observed for the E326A, F327A, I328A and D331A GHR mutants. Mutation F327Y affected neither GHR internalization nor GHR ubiquitination. The main characteristics of the UbE motif are the presence of three negatively charged residues, the absence of positively charged residues and the requirement for a phenylalanine or tyrosine at position 327. In general, the acidic residues within the motif are interchangeable, while large hydrophobic residues can be replaced by homologous residues. Although the UbE motif regulates GHR down-regulation, GH-induced signal transduction pathways, initiated by the recruitment of JAK2, are not affected by mutations in this motif (Allevato *et al.*, 1995; Strous *et al.*, 1997).

The UbE motif does not resemble any other known ubiquitination domain. It is not homologous to the destruction box of cyclin A, cyclin B and uracil permease (Glötzer *et al.*, 1991; Galan *et al.*, 1994), nor to the F-box of cyclin F (Bai *et al.*, 1996). In addition, the UbE motif does not resemble the SINNDKSS motif in Ste2p (Hicke and Riezman, 1996), the DAKTI motif in Ste6p (Kölling and Losko, 1997), the PY motif in ENaC (Staub *et al.*, 1996), the delta domain of c-Jun (Treier *et al.*, 1994), or the DSGXXS motif in IκBα (Didonato *et al.*, 1996) and β-catenin (Aberle *et al.*, 1997). The GHR does contain a DSGXXS sequence, but this sequence has no apparent function in ubiquitin-dependent GHR internalization since GHR truncation mutants, which do not contain this sequence, still internalize (Govers *et al.*, 1998). Database searches have identified a number of proteins that contain a stretch of amino acid residues homologous to the GHR UbE motif, including the prolactin receptor, which contains

Fig. 7. Models for GHR internalization, mediated by the ubiquitin conjugation system. Model A: GH induces GHR dimerization and the attachment of the ubiquitin conjugation system (E2/E3) to the GHR UbE motif (1). Bound E2/E3 ubiquitinates the GHR (2), while the endocytic machinery recognizes the GHR-associated E2/E3, via interaction of an endocytic adaptor protein with the enzyme complex (3). GHR-adaptor protein interaction results in GHR internalization (4). Model B: GH induces dimerization of its receptor. GHR dimerization results in the association of a regulatory protein and components of the ubiquitin conjugation system to the dimerized receptor (1). GHR-bound E2/E3 ubiquitinates the GHR and the regulatory protein (3), leading to internalization of the GH-GHR complex (4). Model C: the cytoplasmic domain of the GHR contains an internalization sequence, which is shielded by an associated regulatory protein when the receptor is in its monomeric state. GH-GHR interaction leads to dimerization of the GHR and to the interaction of E2/E3 with the GHR UbE motif (1). Receptor-bound E2/E3 ubiquitinates the GHR and the regulatory protein (2). Once the regulatory protein is ubiquitinated, it detaches from the GHR and/or is degraded (3). The internalization sequence becomes available and serves as a binding partner for an endocytic adaptor protein (4), resulting in the internalization of the GHR (5).

high overall sequence similarity to the GHR and has been reported ubiquitinated (Cahoreau *et al.*, 1994). Several other plasma membrane proteins which are known to be ubiquitinated and contain a potential UbE motif include c-erbB-2 (Mimnaugh *et al.*, 1996) and the PDGF receptor (β subunit) (Mori *et al.*, 1992). For the PDGF receptor, it had already been demonstrated that Tyr579 is involved in PDGF internalization (Mori *et al.*, 1994). Interestingly, the potential UbE motif in the PDGF receptor includes Tyr579 (corresponding to Phe327 in the GHR). Other potential candidate membrane proteins are the vascular endothelial growth factor (VEGF) receptor, the insulin-responsive glucose transporter Glut 4 and several chloride, potassium and calcium channels. A ubiquitinated cytosolic protein which contains a putative UbE motif is protein kinase C (α , δ and ϵ subtype). A similar domain is found in subunits of cAMP-dependent kinase. The serine/threonine kinase PCTAIRE, which belongs to the CDC2/CDC28 subfamily, also contains a UbE motif-like domain (Meyerson *et al.*, 1992). Further studies will be necessary to determine whether the putative UbE motifs within these proteins are involved in their ubiquitination as well as in internalization or proteasomal degradation.

Our data thus far had shown a strict correlation between GHR ubiquitination and GHR-mediated GH internalization (Govers *et al.*, 1997). The UbE motif is involved in GHR ubiquitination and internalization. Our current results with the lysine-less GHR mutant 1-399 show that the conjugation of ubiquitin to lysine residues in the cytoplasmic

domain of the GHR is not required for endocytosis. However, Western blotting revealed that this GHR is ubiquitinated, although to a minor extent. We attribute this effect to ER-associated degradation and not to ubiquitination at the plasma membrane, since we have found that the GHR precursor (~100 kDa) is localized in the cytosol as well as associated with membranes (unpublished results). We speculate that a portion of the GHR precursor is removed from the ER membrane through the translocon into the cytosol, where it is ubiquitinated on lysine residues in the extracytoplasmic domain, in analogy to mutant CPY (Hiller *et al.*, 1996), apolipoprotein B (Fisher *et al.*, 1997) and mutant Sec61 (Biederer *et al.*, 1996). Since this GHR mutant still contains 12 lysine residues in its extracellular and transmembrane domains, it may well be that these residues are ubiquitinated upon translocation into the cytosol. Therefore, we suggest that GHR internalization requires the recruitment of the ubiquitin conjugation system to the GHR UbE motif, rather than the conjugation of ubiquitin to the receptor. Such a mechanism has not been described previously for plasma membrane proteins. For several plasma membrane proteins, it has been suggested that ubiquitination of the protein itself is required for endocytosis (Staub *et al.*, 1997; Terrell *et al.*, 1998). Analogously to the recruitment of JAK2 to the GHR box1 domain, the UbE motif may serve, directly or via adaptor proteins, as an anchoring site for the ubiquitination machinery. The most likely scenarios for GHR internalization are shown in Figure 7. In each, binding of the

ubiquitin conjugation system to the GHR UbE motif results in the (indirect) interaction of an endocytic adaptor protein with the GHR, leading to coated pit localization and subsequently to internalization of the GHR. In model A, the endocytic machinery internalizes the receptor via interaction with enzymes of the ubiquitin conjugation system associated with the GHR. E3 is a likely candidate to play such an adaptor role. In this model, ubiquitin conjugation has no direct role in receptor internalization. Furthermore, incubation of the CHO-ts20 cells at the non-permissive temperature would not only result in inhibition of ubiquitin activation and protein ubiquitination but also in inhibition of the binding of E3 to the GHR. Since certain E3s form a ubiquitin thioester linkage, binding of these E3s to their specific substrates probably depends on the presence of E1-activated ubiquitin. In model B, a regulatory protein is associated with the receptor. Upon receptor dimerization, this associated protein is ubiquitinated, resulting in recognition by a component of the endocytic machinery and in receptor internalization. In model C, a regulatory protein is shielding an internalization signal on the GHR, retaining the GHR at the plasma membrane. GH-induced ubiquitination of this associated protein results in the release/degradation of this protein, whereupon the internalization motif becomes available. The cytoplasmic domain of the GHR does contain two potential di-leucine internalization motifs, upstream of the UbE motif. One of those motifs may be exposed upon ubiquitination of a receptor-associated protein. In models B and C, the UbE motif is required though not sufficient for GHR internalization. At present, we cannot exclude the possibility that a second domain in the GHR cytoplasmic tail is required for GHR internalization. Studies in which the GHR UbE motif is transferred to another plasma membrane protein will reveal whether this is indeed the case. Which enzymes of the ubiquitin conjugation system bind to the GHR UbE motif also remains to be determined. Should a GHR-associated regulatory protein mediate GHR internalization, it may well have a more general role in receptor internalization. One potential candidate is Eps15, which is associated with receptors (Fazioli *et al.*, 1993), involved in endocytosis (Carbone *et al.*, 1997; van Delft *et al.*, 1997b) and ubiquitinated upon receptor activation (van Delft *et al.*, 1997a).

Materials and methods

Materials and antisera

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). hGH was a generous gift of Lilly Research Labs, Indianapolis, IN. GHR cDNA was kindly provided by Dr William Wood, Genentech. Culture medium, fetal bovine serum and geneticin were purchased from Gibco-BRL.

Mutagenesis, transfection and cell culture

Wild-type rabbit GHR cDNA was cloned into the CMV/neo expression plasmid pcDNA3 (Invitrogen). In order to mutate single amino acid residues around Phe327, a GHR cDNA construct was prepared, which contained two silent mutations, one introducing a unique *HpaI* site around Asn305 and the other deleting the first *Clal* site. For this construct, a PCR was performed, using a 5' oligonucleotide containing a *PfI*MI site, corresponding to the *PfI*MI site in the cDNA encoding the luminal part of the GHR, and a 3' oligonucleotide containing a *Clal* site, corresponding to the second *Clal* site in the GHR cDNA and the

described silent mutations. This PCR product was digested by *PfI*MI and *Clal*I and ligated directly into a pcDNA3-GHR construct, which was digested by the same enzymes.

This construct was used as the template to generate GHRs containing single point mutations around Phe327. For these GHRs, PCRs were performed using either a 5' oligonucleotide containing a *PfI*MI site and deleting an *NciI* site together with a 3' oligonucleotide containing a *Clal* site and the desired mutations, or a 5' oligonucleotide containing an *HpaI* site and the desired mutations together with a 3' oligonucleotide containing a *KpnI* site, an *Afl*III site and a stop codon. These PCR products were digested by *PfI*MI and *Clal*I or by *HpaI* and *KpnI* and ligated directly into the pcDNA3-GHR construct, containing the silent mutations.

cDNA encoding GHR truncation mutants GHR 1–399 and GHR 1–349 was constructed by introducing stop codons at the proper positions within the GHR cDNA. For these GHR truncations, PCRs were performed, using a 5' oligonucleotide containing an *NcoI* restriction site, corresponding to the *NcoI* site in the cDNA of the transmembrane region of the GHR, together with 3' oligonucleotides containing a *KpnI* 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 *NcoI* and *KpnI* and ligated into a PGEM3Z-GHR construct. The truncated GHR cDNAs were subcloned into the pcDNA3 vector. cDNA of truncation mutants GHR 1–399 F327A and GHR 1–349 F327A was constructed in a similar way, except that GHR F327A cDNA was used as template for PCR.

GHR 1–399 K271–362R cDNA was constructed in a four-step procedure. cDNA encoding the wild-type GHR was used as template for all PCRs. In each step, a cDNA construct was made, which was used to clone the PCR product of the next step. In the first construct, lysines 338, 354 and 362 were mutated to arginines and the receptor was truncated at amino acid residue 399, by performing PCR, using a 5' oligonucleotide containing a *Clal*I site, corresponding to the second *Clal*I site in the GHR cDNA and the Lys to Arg mutations and a 3' oligonucleotide containing a *KpnI* restriction site, a stop codon and overlapping sequences encoding the GHR up to amino acid residue 399. In the second construct, a unique *HpaI* site was introduced around Asn305, the second *Clal*I site was deleted and Lys315 was mutated to arginine. For this, a PCR was performed, using a 5' oligonucleotide containing a *PfI*MI site and a 3' oligonucleotide containing a *Clal*I site, corresponding to the second *Clal*I site in the GHR cDNA, silent mutations introducing an *HpaI* site and deleting a *Clal*I site, and the Lys to Arg mutation. In the third construct, a unique *SacII* site was introduced around Pro286, and lysines 287, 289, 297 and 300 were mutated to arginines. For this, a PCR was performed, using a 5' oligonucleotide containing a *PfI*MI site and a 3' oligonucleotide containing an *HpaI* site, a silent mutation introducing a *SacII* site and the Lys to Arg mutations. In the fourth construct, lysines 271 and 276 were mutated to arginines, by performing PCR, using a 5' oligonucleotide containing a *PfI*MI site and deleting a *NciI* site and a 3' oligonucleotide containing a *SacII* site and the Lys to Arg mutations.

cDNA encoding GHR 1–399 K271–362R F327A was constructed using cDNA of GHR 1–399 K271–362R as template for PCR. For PCR, a 5' oligonucleotide was used containing an *HpaI* site and mutations encoding K315R and F327A, and a 3' oligonucleotide containing a *KpnI* site, an *Afl*III site, a stop codon and overlapping sequences encoding the GHR up to amino acid residue 399. All PCR products were digested by the indicated restriction enzymes and ligated directly into the indicated pcDNA3-GHR construct, which was digested by the same enzymes. All constructs were verified by *in vitro* transcription-translation assays (Promega) and by sequencing.

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. cDNA constructs were transfected into CHO-ts20 cells using the calcium phosphate transfection method. For all GHR mutants, clonal cell lines were obtained which expressed the receptor stably, except for GHR 1–399 K271–362R F327A. For this mutant, cells were grown for 2 weeks in geneticin after transfection, after which the cells were used for experiments. On the mRNA of CHO-ts20 cells, transfected with cDNA encoding GHR 1–399 K271–362R, RT-PCR was performed. This RT-PCR product was sequenced, to ensure that these cells expressed a GHR without cytoplasmic lysines. Stable geneticin-resistant transfectants were grown in Eagle's minimal essential medium (MEM- α) supplemented with 10% fetal bovine serum, penicillin and streptomycin, and 0.45 mg/ml geneticin. For experiments, cells were grown in 30 or 60 mm dishes in the absence of geneticin to ~75% confluence. In the

experiments described herein, 10 mM sodium butyrate was added to the cells 18 h before use to increase GHR expression (Govers *et al.*, 1997). 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 internalization

For internalization studies, cells were grown in 30 mm dishes, washed with MEM- α , supplemented with 20 mM HEPES, incubated for 1 h at 30°C in MEM- α /HEPES and for 1 h at 30°C with 8 nM [¹²⁵I]GH (700 000 c.p.m.), in the absence or presence of excess unlabelled GH. The cells were washed three times with ice-cold phosphate-buffered saline (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. Internalization efficiency was expressed as the percentage of total specific cell-associated radioactivity that was not removable by acid wash.

Microscopy

Cy3-GH was prepared using a Fluorolink-Cy3 label kit according to the supplier's instructions (Amersham). Transfected CHO cells, grown on coverslips, were incubated for 60 min in MEM- α , supplemented with 20 mM HEPES at 30°C or at the non-permissive temperature (42°C) and for 60 min with Cy3-GH (0.7 μ g/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

Cells used for immunoprecipitations were stably transfected with GHR cDNAs and expressed wild-type GHR, GHR truncation 1–349, GHR 1–349 F327A, GHR E326A, GHR F327A, GHR F327Y, GHR I328A or GHR D331A.

Immunoprecipitations were performed as described previously (Strous *et al.*, 1996). Cells grown in 6 cm dishes were incubated for 1 h in MEM- α , supplemented with 20 mM HEPES and for 30 min with 8 nM hGH. After GH incubation, cells were lysed 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% bovine serum albumin, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 2 mM Na₃VO₄, 20 mM NaF, 10 μ g/ml leupeptin, 10 μ g/ml aprotinin in PBS. The lysate was incubated with anti-ubiquitin antiserum for 2 h on ice. Immune complexes were isolated by the use of protein A-agarose beads (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 together with total cellular lysate and transferred to polyvinylidene difluoride (PVDF) paper. Blots were immunostained using anti-GHR (Govers *et al.*, 1997). After incubating the blots with rabbit anti-mouse IgG conjugated to horseradish peroxidase, antigens were visualized using the ECL system (Boehringer Mannheim).

Proteinase K digestion

To establish whether the mature (130 kDa) species of the studied GHRs were localized predominantly at the plasma membrane, cells were subjected to proteinase K digestion. Cells were incubated without GH and put on ice. They were washed three times with ice-cold PBS and incubated for 20 min on ice in PBS in the absence or presence of 0.5 mg/ml proteinase K (Boehringer Mannheim) and 1 mM EDTA. After extensive washing of the cells with cold PBS containing 1 mM PMSF, cells were lysed and lysates were subjected to SDS-PAGE and transferred to PVDF paper. Blots were immunostained with an antibody directed against the cytosolic domain of the GHR. Plasma membrane receptor species (130 kDa) showed a substantial decrease in molecular weight in the presence of proteinase K, while that of the GHR precursor (100 kDa), localized in ER/Golgi, was unaffected.

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