Di-leucine-mediated Internalization of Ligand by a Truncated Growth Hormone Receptor Is Independent of the Ubiquitin Conjugation System*

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The growth hormone receptor (GHR) is a member of the cytokine receptor family. Its function is to mediate cellular responses upon binding of growth hormone. Ligand binding induces dimerization and activation of the GHR. One mechanism by which the GHR is rapidly inactivated involves the ubiquitin conjugation system, a system implicated in the degradation of cytosolic and nuclear proteins. We have shown previously that the ubiquitin-conjugating system mediates the internalization of the GHR. Here, we present evidence that in addition to the ubiquitin-dependent endocytosis signal, the cytosolic tail of the GHR contains a di-leucine motif. Upon truncation of the GHR at amino acid residue 349, this di-leucine motif is activated and mediates ubiquitin-independent internalization of the receptor. Di-leucine-mediated GHR internalization requires functional clathrin-coated pits and results in GHR transport to the lysosome. Although the full-length GHR internalizes independently of the di-leucine motif, this motif may function in internalization of GHR isoforms.

Internalization of membrane proteins may be mediated via several amino acid sequences within the cytosolic domain (1, 2). The most common sequences consist of short stretches of amino acids, termed coated pit localization signals. The tyrosine-based motifs NPXY and YXXØ (where X is any amino acid and Ø is an amino acid with a bulky hydrophobic group) are well known internalization motifs involved in endocytosis of many transmembrane proteins (3). Receptors such as the LDL receptor are endocytosed via their NPXY motif (4), while receptors such as the transferrin receptor and the asialoglycoprotein receptor are sorted from the plasma membrane via a YXXØ motif (5, 6). These tyrosine-based motifs are thought to form a tight turn conformation. Another well known endocytosis motif is the di-leucine motif. Internalization of the insulin and β₂-adrenergic receptor is mediated by this motif (7, 8) and in case of the interleukin-6 receptor, GLUT4 and CD4, the di-leucine motif acts in cooperation with an upstream serine (9–11). CD3γ and invariant chain are internalized by a di-leucine motif and an upstream aspartic acid (12, 13). Other endocytosis motifs have been reported as well. The amino terminus of GLUT4 contains a FQQI internalization motif (14, 15). E-selectin internalization is also independent of di-leucine and tyrosine-based endocytosis motifs (16). Recently, it was shown that the attachment of ubiquitin moieties is involved in internalization of several plasma membrane proteins (17). Ubiquitination appears to be required for endocytosis of Ste2p (18), Ste6p, (19) and ENaC (20).

The growth hormone receptor (GHR) is a mammalian plasma membrane protein whose internalization is mediated by the ubiquitin conjugation system (21). In particular, the Phe272 residue within the GHR cytosolic tail is involved in both GHR ubiquitination and ligand-induced receptor endocytosis (22, 23). However, whether the GHR itself needs to be ubiquitinated in order to internalize ligand remains to be elucidated. The GHR initiates the cellular actions of growth hormone (GH) and belongs to the family of cytokine receptors (24, 25). The GHR is a type I glycoprotein (Mr, 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 has a short half-life (26–28), and the degradation occurs within the lysosome (26). In addition, it has been reported that the GHR is transported to the nucleus (29), to detergent-insoluble membrane domains (30) and back to the plasma membrane (31). GHR signaling is initiated at the plasma membrane when two receptors are dimerized by a single GH molecule (32). This dimerization induces recruitment and binding of the tyrosine kinase JAK2 (33), resulting in the activation of various signal transduction pathways (34, 35).

In the present study, we examined the internalization of GHR truncation mutant 1–349. Although the wild-type receptor internalized in a ubiquitin-dependent manner, this truncation mutant internalized independent of the ubiquitin conjugation system. Further analysis revealed that a carboxy-terminal di-leucine motif was responsible for internalization of truncation mutant 1–349. This di-leucine motif mediated clathrin-dependent internalization of the mutant, but not of the full-length wild type receptor.

EXPERIMENTAL PROCEDURES

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 Laboratories, Indianapolis, IN. GHR cDNA was kindly provided by Dr. William Wood, Genentech.

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Culture medium, fetal bovine serum, and Geneticin were purchased from Life Technologies, Inc.

**Mutagenesis, Transformation, and Cell Culture**—The CHO cell line CHO-ts20 (36) was used in this study. Due to a thermolabile ubiquitin-activating enzyme (E1), the ubiquitin conjugating system is inactive at the nonpermissive temperature of 42 °C.

CHO-ts20 cells were transfected with a pc66 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 the GHR truncations 1–399, 1–349, and 1–314 without additional mutations, polymerase chain reactions were performed, using a 5′-oligonucleotide containing a 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 with NcoI and KpnI and ligated into a PGM62-Z-GHR construct. The truncated GHR cDNAs were subcloned into the CMV-NEO expression plasmid pcDNA3 (Invitrogen).

For the other GHR truncations, PCRs were performed, using a 5′-oligonucleotide containing a PflM I site, corresponding to the PflM I site in the cDNA of the luminal part of the GHR and the 3′-oligonucleotides containing a KpnI restriction site, a stop codon, and overlapping sequences within the cDNA, encoding the intracellular domain of the GHR. For construct GHR L347,348A a 5′-oligonucleotide was used that contained the mutations and a ClaI site, corresponding to the second ClaI site in the cDNA of the intracellular domain of the GHR, while the 3′-oligonucleotide contained a KpnI restriction site, a stop codon, and a sequence overlapping the cDNA, encoding the intracellular domain of the GHR up to amino acid residue 399. For construct 1–349 L347,348A a 5′-oligonucleotide was used which contained a PflM I site, corresponding to the PflM I site in the cDNA encoding the luminal domain of the GHR. The 5′-oligonucleotide used for this construct contained a KpnI restriction site, a stop codon, and a sequence overlapping the cDNA, encoding the intracellular domain of the GHR up to amino acid residue 349, including the appropriate mutations. These PCR products were digested by the indicated restriction enzymes and ligated directly into a 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 cDNA constructs were transfected into CHO-ts20 cells using the calcium phosphate transfection method. For truncations 1–399, 1–349, and 1–314 and for GHR 1–349 L347,348A, clonal cell lines were obtained that expressed the receptor stably. For the other mutated receptors, cells were grown for 2 weeks in Geneticin after transfection, whereafter the cells were used for experiments. Stably Geneticin-resistant transfectants were grown in Eagle’s minimal essential medium (MEM-a) 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 approximate 75% confluence. In the experiments described herein, 10 mM sodium butyrate was added to the cells 18 h before use to increase GHR expression (23). Treatment of transfected CHO-ts20 cells with sodium butyrate did not alter the behavior 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 analyzed by binding of 125I-GH. 125I-GH was prepared using chloramine T (26). Cells were grown in six-well culture dishes, washed with ice-cold phosphate-buffered saline (PBS), and incubated for 2 h on ice with 0.2–18 nM 125I-GH in PBS containing 0.1% bovine serum albumin, in the absence or presence of excess unlabeled GH. After extensive washing, 1 ml NaOH was added, and the cell extracts were counted in an LKB γ counter. Plasma membrane receptor numbers and binding affinities were calculated by Scatchard analysis. For experiments, cells were grown in 30-mm dishes, washed with MEM-a, supplemented with 20 mM HEPES, incubated for 1 h at 30 °C or 42 °C in MEM-a/HEPES and for 0–120 min with 8 nM 125I-GH (700,000 cpm), in the absence or presence of excess unlabeled 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) (37), and internalized GH was determined by measuring the radioactivity after solubilization of the acid-treated cells by 1 M NaOH. To measure intracellular 125I-GH degradation, the incubation medium was trichloroacetic acid-precipitated after incubation of the cells with 125I-GH for different time periods. Briefly, part of the incubation medium was mixed with an equal volume of 20% ice-cold trichloroacetic acid. After incubation for 30 min on ice, the sample was centrifuged for 10 min at 14,000 rpm at 4 °C, and the radioactivity in the supernatant was determined.

**Microscopy**—Cy3-GH was prepared using a Fluorolink-Cy3 label kit according to the supplier’s instructions (Amersham Pharmacia Biotech). Transfected CHO cells, grown on coverslips, were incubated for 60 min in MEM-a, supplemented with 20 mM HEPES at 30 °C or at the nonpermissive temperature of 42 °C and for 60 min with Cy3-GH (0.7 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**—Cells used for immunoprecipitations expressed wild-type GHR, GHR truncation 1–349, or GHR 1–349 L347,348A and contained 0.98 ± 0.09 × 106, 0.96 ± 0.05 × 106, and 1.79 ± 0.15 × 106 GH binding sites at the plasma membrane, respectively. These cells displayed the same binding affinity for hGH.

Immunoprecipitations were performed as described previously (21). Cells, grown in 6-cm dishes, were incubated for 1 h in MEM-a, supplemented with 20 mM HEPES and for 15 or 30 min with 8 nM hGH. After this time, cells were solubilized in 1% Triton X-100, 0.5% SDS, 0.25% sodium deoxycholate, 0.5% bovine serum albumin, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 2 mM Na3VO4, 20 mM NaF, 10 μg/ml leupeptin, 10 μg/ml aprotinin in PBS. The lysate was incubated with anti-ubiquitin antiserum for 2 h on ice. Immunocomplexes 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. Immunocomplexes were subjected to SDS-polyacrylamide gel electrophoresis together with total cellular lysate and transferred to polyvinylidene difluoride paper. Blots were immunostained using anti-GHR antiserum for 2 h on ice. After washing the cells once with 0.14 M KCl, 20 mM HEPES, 2 mM CaCl2, 1 mM MgCl2, 1 mM amiloride, 1 glitier glucose, 0.1% BSA pH 7.0, cells were incubated in the same buffer for 5 min, whereafter Cy3-GH or unlabeled GH was added to the dishes. Control cells were subjected to the same incubations, except that they were incubated in the absence of NH4Cl. All incubation steps were performed at 30 °C.

**RESULTS**

**Ubiquitin-independent Internalization of a GHR Truncation Mutant**—The GHR consists of 620 amino acid residues, of which 350 residues are located intracellularly (Fig. 1). This intracellular domain contains all of the information required for signal transduction as well as for down-regulation. Previously, we characterized the internalization and ubiquitination of several GHR truncation mutants (23). We showed that GHR truncation 1–349 internalized ligand and was ubiquitinated, while truncation mutants shorter than 314 amino acids residues were neither ubiquitinated nor able to internalize after binding of ligand.

In the present study we examined whether the ubiquitin conjugation system was involved in endocytosis of these GHR mutants. Therefore we used the Chinese hamster ovary cell line CHO-ts20, which contains a thermolabile ubiquitin-activating enzyme (E1). This temperature-sensitive CHO-ts20 is active when the CHO-ts20 are incubated at the nonpermissive temperature of 42 °C, while at 30 °C the enzyme retains its full activity. We transfected the cDNAs of the truncation mutants into these cells and examined whether internalization of fluorescence-labeled GH via these receptors was abrogated at the nonpermissive temperature (Fig. 2A). As we have shown before...
(21,23), wild-type GHR (panels 1–620) internalized in a ubiquitin-independent manner. GH was internalized by the GHR at 30 °C, while the ligand remained at the plasma membrane at 42 °C, when the ubiquitin conjugation system is inactive. GHR truncations 1–600, 1–522, and 1–434 showed the same phenotype (not shown). Internalization of these receptors was also dependent on an intact ubiquitin-conjugating system, as was the case for GHR truncation 1–349 (Fig. 2A). GHR truncation 1–349 showed vesicular Cy3-GH staining at 30 °C as well as at 42 °C (Fig. 2A, panels 1–349), indicating that this truncation was still able to internalize ligand in the absence of an intact ubiquitin conjugation system. GHR truncation 1–349 internalizes independent of the ubiquitin conjugation system. Previously, we have shown that internalization of the wild type GHR is both ubiquitin-dependent and clathrin-mediated (23). To examine whether truncation 1–349 was internalized via clathrin-coated pits, we subjected the cells to cellular cytosol acidification (Fig. 3A), which retains clathrin lattices at the membrane but prevents coated vesicle formation. Cells were preincubated in 20 mM NH4Cl, whereafter they were incubated with fluorescence-labeled GH in a KCl/amiloride buffer. This treatment inhibited ligand uptake both for the wild type GHR (1–620) as well as for GHR truncation 1–349 (Fig. 3A, right panels). Control incubations of the cells in the KCl/amiloride buffer without NH4Cl preincubation did show GH internalization (Fig. 3A, left panels). This indicates that internalization of the GHR truncated at amino acid residue 349 is ubiquitin-independent but still dependent upon the clathrin-mediated endocytic pathway.

We have shown previously that ubiquitination of the GHR is dependent upon clathrin-mediated endocytosis and that GHR truncation 1–349 can be ubiquitinated (23). Thus, we next determined whether this GHR truncation, which does not require an intact ubiquitin conjugation system for internalization, remains ubiquitinated upon inhibition of the clathrin-mediated endocytic pathway. Therefore, we examined ubiquitination of wild type GHR and GHR truncation 1–349 when clathrin-mediated endocytosis was inhibited by cellular cytosol acidification (Fig. 3B). Cells expressing wild-type GHR or GHR 1–349 were preincubated for 30 min in 20 mM NH4Cl and incubated for 5 min in a KCl/amiloride buffer and for 15 min in a KCl/amiloride buffer with or without 8 nM hGH. Control cells were incubated with or without 8 nM GH in the KCl/amiloride buffer without the NH4Cl preincubation. After the incubations, the cells were lysed, and protein-ubiquitin conjugates were immunoprecipitated and immunoblotted (Fig. 3B). The left lane in the two panels contains total cellular lysate and shows the apparent molecular masses of the two receptors (130 and 75 kDa, respectively). In each panel, the faster migrating band represents the receptor precursor and the slower migrating band represents the mature receptor. As only a few percent of the GHR is ubiquitinated, ubiquitination of the GHR cannot be detected in this lane, in accordance with our previous findings (21). Cell extracts containing approximately equal amounts of GHR 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, which was enhanced upon incubation of the cells with GH. However, when clathrin-mediated internalization was inhibited by cellular cytosol acidification, ubiquitination of both receptors was almost completely abrogated. This effect on GHR ubiquitination is not caused by a general inactivation of the ubiquitin conjugation system, since cellular cytosol acidification does not affect total cellular ubiquitination (23). These data indicate that, while ubiquitination of GHR truncation 1–349 is not involved in internalization, inhibition of receptor internalization results in a marked reduction of ubiquitination of this mutant.

Characterization of Intracellular Domains Involved in Ubiquitin-independent GHR Internalization—To determine which
domains are involved in the ubiquitin-independent internalization of GHR 1–349, we examined internalization of fluorescence-labeled GH by various GHR truncation mutants (Fig. 1) in the presence (30 °C) or absence (42 °C) of an active ubiquitin-conjugating system (Fig. 4). Cy3-GH internalization by truncations 1–399 to 1–359 was dependent upon an intact ubiquitin-conjugating system. At 30 °C these truncations showed a vesicular pattern, but at the nonpermissive temperature Cy3-GH remained at the plasma membrane. This indicated that amino acid residues 350–359 restored the ubiquitin-dependent internalization of the GHR. Ubiquitin-dependent GHR internalization was also restored by truncation of the receptor at amino acid residue 346, suggesting that the amino acid residues 347, 348, and 349 were involved in the ubiquitin-independent internalization of GHR truncation 1–349. Since this amino acid residue triplet comprised two sequential leucine residues and a serine residue, it likely represented a di-leucine endocytosis motif, responsible for ubiquitin-independent internalization when the receptor was truncated at amino acid residue 349. The full-length receptor, however, was internalized only when the ubiquitin conjugation system was active, indicating that the di-leucine motif at position 347–348 was not an active endocytosis signal in the full-length GHR. GHR truncation mutants shorter than 330 amino acid residues did not internalize ligand at 30 °C nor at 42 °C. This finding demonstrated that the ubiquitin-dependent endocytosis motif was located upstream of amino acid residue 334. For these experiments we used a mixed culture of stable transfectants, of which only a small percentage expressed the GHR to a considerable extent. Therefore, we were unable to determine whether these GHRs were ubiquitinated. We expect that GHR truncation 1–334 was ubiquitinated, while the internalization-deficient truncation 1–330 was not ubiquitinated.

Involvement of Di-leucine Motif in GHR Internalization and Ubiquitination—To investigate the role of the di-leucine motif at position 347–348 in internalization of the wild-type GHR, we mutated this leucine doublet in the full-length receptor cDNA and in the cDNA encoding the GHR truncation mutants 1–399 and 1–349 (Fig. 1). We transfected the cDNAs into the CHO-ts20 cells and studied the uptake of Cy3-GH (Fig. 5A). Mutating the leucine doublet in the full-length receptor did not influence internalization of Cy3-GH (panels 1–620 L347,348A). The same was observed when the leucine doublet was mutated in GHR truncation 1–399 (panels 1–399 L347,348A). As expected, mutating the leucine doublet in GHR 1–349 restored the ubiquitin-dependent ligand internalization (panels 1–349 L347,348A).

Having established that GHR truncation 1–349 is internalized by a di-leucine motif, which acts independent of the ubiquitin conjugation system, we investigated the role of this di-leucine motif on GHR ubiquitination (Fig. 5B). Cells expressing the indicated receptors and untransfected cells (ts20) were incubated in the absence or presence of 8 nM GH for 30 min at 30 °C. The cells were lysed, and protein-ubiquitin conjugates were immunoprecipitated. The immunoprecipitates and the total cellular lysates were analyzed as described for Fig. 3. The high molecular weight bands in the upper part of these lanes represent ubiquitinated receptors. Untransfected cells (ts20)
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The ubiquitin conjugation system has an important function in many regulatory processes within the cell (for review see Hershko and Ciechanover (39)). The way by which the ubiquitin conjugation system acts on the vast majority of these processes is thought to be via the targeting of regulatory proteins for degradation by the proteasome. Therefore, a regulatory protein becomes polyubiquitinated by specific enzymes catalyzing a cascade of reactions leading to the covalent attachment of ubiquitin to free ε-amino groups within the protein. The 26 S proteasome recognizes the polyubiquitin chains on the substrate and degrades the protein, thereby releasing free ubiquitin molecules, which can enter a new ubiquitin cycle. Ubiquitin conjugation has been implicated in the degradation of many regulatory proteins, such as cyclins (40, 41), IκBα (42), p53 (43), Eps15 (44), and STAT1 (45). Recent studies have indicated that ubiquitin is a key player in several endocytic events (46, 47). Leung et al. showed that the GHR is ubiquitinated in rabbit liver (48). We have shown that the ubiquitin conjugation system is required for GH-induced internalization (down-regulation) of the GHR (21) and that ubiquitination of the GHR itself is linked to ligand-mediated GHR endocytosis (23). Furthermore, Hicke and Riezman (18) demonstrated that ligand-stimulated Ste2p endocytosis in yeast is preceded by Ste2p ubiquitination. For several plasma membrane proteins it has been suggested that ubiquitination is involved in (partial) degradation by the proteasome (49–51).

In the present study we show that truncation of the GHR at amino acid residue 349 results in a receptor which is capable of internalizing ligand independent of the ubiquitin-conjugating system. This GHR truncation mutant is able to internalize ligand at the nonpermissive temperature in the CHO-ts20 cell line, which contains a temperature-sensitive ubiquitin-activating enzyme (E1) (36). Using this cellular system, we previously showed that the recycling transferrin receptor is internalized independent of the ubiquitin conjugation system (21). Our present data indicate that GH internalized by GHR truncation 1–349 is degraded in the lysosome. Since dissociation of ligand from the GHR requires a low pH (28), we conclude that the GHR truncation mutant itself is also transported to the lysosome. Moreover, we found that GHR truncation mutant 1–349 is internalized via the clathrin-mediated endocytic pathway as is the wild type GHR. Although GHR truncation 1–349 is internalized independent of the ubiquitin-conjugating system, this receptor is still ubiquitinated. Apparently, ubiquitination of this truncation mutant serves no function, although ubiquitination still depends upon clathrin-mediated endocytosis.

Using various GHR mutants we show that the ubiquitin-independent internalization of GHR truncation 1–349 is mediated by a di-leucine motif at the carboxyl-terminal end of the truncation (positions 347 and 348). This motif is known to be involved in internalization of many membrane proteins (2). Recently, it was shown that a di-leucine motif mediates internalization of the interleukin-6 receptor, another member of the cytokine receptor family (9). Since mutating the di-leucine motif in the full-length GHR did not inhibit internalization, the di-leucine motif has no apparent function in endocytosis of the wild type GHR. Mutating the di-leucine motif in truncation 1–349 restored the ubiquitin-dependent internalization, most probably mediated via a motif containing Phe-327 (23). Ubiquitin-dependent internalization was also restored by truncating the amount of ubiquitin conjugated to the mutated truncation (1–349 L347,348A) does not differ appreciably from the amount of ubiquitin on GHR truncation 1–349 with the intact di-leucine motif. Therefore we conclude that the di-leucine motif affects GHR internalization but not GHR ubiquitination.

**FIG. 3.** Effect of cellular cytosol acidification on internalization and ubiquitination of wild-type GHR and GHR truncation mutant 1–349. Cells expressing wild-type GHR or GHR truncation 1–349 were incubated in KCl/amiloride buffer after NH₄Cl preincubation (acidif) or without NH₄Cl preincubation (ctrl). A, cells were incubated for 15 min with Cy3-GH in KCl/amiloride buffer. Cy3-GH was visualized by confocal microscopy. Bar = 10 μm. B, cells were incubated for 15 min with or without unlabeled GH in KCl/amiloride buffer. 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 25- and 40–55-kDa bands seen in the immunoprecipitations of the right panel originated from IgG.

showed no (ubiquitinated) receptor. Wild-type as well as mutant receptors showed ubiquitination. The amount of ubiquitinated GHR in the absence of ligand varied between experiments as can be seen in Figs. 3 and 5. In general, GH-induced ubiquitination of truncation 1–349 did not differ much from basal (non-GH-induced) receptor ubiquitination. Presuming that GHR ubiquitination is a plasma membrane event, the time for GH-induced ubiquitination of GHR 1–349 might be limited, since this mutant internalizes at an increased rate (Fig. 2B), resulting in a reduced GH-dependent receptor ubiquitination. The molecular weight of the ubiquitinated truncations 1–349 and 1–349 L347,348A is significantly lower than the molecular weight of the ubiquitinated wild type receptor (1–620), partly due to a decreased amount of ubiquitination. Interestingly, the
receptor only 10 amino acid residues behind residue 349. This indicates that amino acid residues 350 to 359 inhibit receptor internalization by the di-leucine motif, probably by masking this motif. Truncating the GHR between amino acid residues 334 and 346 resulted in ubiquitin-dependent internalization of the GHR and indicates that the ubiquitin-dependent internalization motif is located between amino acid residues 271 and 334. Moreover, the last four amino acid residues in this region are likely part of the ubiquitin-dependent internalization signal together with Phe$^{327}$. At present, we cannot exclude that additional amino acid residues between residues 271 and 334 are needed for GHR internalization. Furthermore, the DSGXXS sequence between amino acid residue 365 and 370 does not seem to represent a ubiquitination domain, although a DSGXXS motif has been implicated in ubiquitination of β-catenin and IκBα (52, 53). Previously, we have shown that GHR truncation mutant 1–314 is neither internalized nor ubiquitinated (23). Since Lys-315 is the only lysine residue between amino acid residues 314 and 334, this particular lysine might be involved in both of these processes.

Our data show that truncation of the GHR at amino acid residue 349 activates a ubiquitin-independent di-leucine internalization motif. This motif is extremely well conserved within the GHRs of different species (54) and is identical to the DXXXLL internalization motif found in CD3γ (12). Internalization of receptor truncation 1–349 may be mediated by a ubiquitin-dependent mechanism as well as by a ubiquitin-independent mechanism, while the full-length receptor is internalized exclusively by the ubiquitin-dependent mechanism. The physiological role of the di-leucine motif is thus not yet
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FIG. 5. Effect of mutations L347,348A on internalization and ubiquitination of wild type GHR and GHR truncation mutants 1–399 and 1–349. A, CHO-ts20 cells expressing the indicated GHR were incubated with Cy3-GH for 1 h at 30 or 42 °C. Cy3-GH was visualized by confocal microscopy. B, CHO-ts20 cells expressing the indicated GHR and untransfected cells (ts20) were incubated for 30 min with or without unlabeled GH. Ubiquitinated proteins were immunoprecipitated using an anti-ubiquitin conjugate antibody directed against the luminal domain of the receptor. Bar was visualized by confocal microscopy.

clear. It may well be that, under certain conditions, the GHR di-leucine motif is activated due to conformational changes within or partial degradation of the GHR. Alternatively, the di-leucine motif may be functional in a GHR isoform, as many receptors are present as alternative spliced isoforms that differ in their COOH-terminal intracellular domains (e.g. G protein-coupled 5-HT_{2} receptor (55), dopamine D_{2} receptor (56), angiotensin II receptor (57), tyrosine kinase receptor TrkB (58)). Several GHR isoforms have been described. One lacks exon 3, resulting in a 22-amino acid deletion in the extracellular domain of the receptor (59). Two GHR isoforms have an intracellular domain of only 7 and 9 amino acid residues (60, 61). Other GHR isoforms have been located within the brain (62, 63) and liver (64, 65). Whether any of these GHR isoforms is internalized via the di-leucine motif remains to be determined. Finally, ubiquitin itself or associated proteins may possess structural elements, which mimic the di-leucine motif, such that a common feature underlies these two apparently independent internalization signals.

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