Dimerization and Signal Transduction of the Growth Hormone Receptor

JÜRGEN GENT, MONIQUE VAN DEN EIJNDEN, PETER VAN KERKHOF, AND GER J. STROUS

Department of Cell Biology and Institute of Biomembranes, University Medical Center Utrecht, Heidelberglaan 100, 3584CX Utrecht, The Netherlands

GH binding to cell surface-localized GH receptors (GHRs) induces a conformational change of the dimerized receptors, resulting in activation of Janus kinase 2 and downstream signaling pathways. Interactions between the extracellular subdomain 2 of adjacent GHR polypeptides result in a 500-Å² contact interface, which has previously been suggested to stabilize the GH-(GHR)₂ complex. In this study, we investigated further the role of subdomain 2 in GHR function. Amino acids that participate in (e.g. aspartic acid 152, tyrosine 200, or serine 201) or lie close to (e.g. asparagine 143 or cysteine 241) the contact interface were mutated in rabbit GHR. Surprisingly, none of the mutations affected GHR dimerization, as demonstrated by coimmunoprecipitation of a truncated, epitope-tagged GHR. However, signal transduction of GHR(D152H), GHR(Y200D), and GHR(S201K) mutants was precluded. More insight into the molecular mechanism of the signaling defect was obtained when we examined the effect of the mutations on the integrity of the GH-(GHR)₂ complex in a protease-protection assay. In contrast to wild-type GHR, GHR(N143K), and GHR(C241S), the GHR(D152H), GHR(Y200D), and GHR(S201K) mutants were not protected against protease digestion by GH, indicating that a structural change is prevented. Together, we provide new evidence for a critical role of aspartic acid 152, tyrosine 200, and serine 201 of the GHR contact interface in the GH-induced conformational change to a signaling-competent complex rather than in GHR dimerization. (Molecular Endocrinology 17: 967–975, 2003)

HUMAN GH, a 191-amino acid polypeptide, is produced by the anterior pituitary. Besides regulating postnatal growth, GH functions throughout life in protein, lipid, and carbohydrate metabolism (1, 2). The release of GH is pulsatile and mainly controlled by two hypothalamic factors: somatostatin, which acts as an inhibitor, and GHRH, which stimulates secretion (3). Tight control of GH secretion is essential, because hypersecretion results in gigantism or acromegaly and hyposecretion causes dwarfism (4).

The effects of GH are mediated via the GH receptor (GHR), a 620-amino acid type I transmembrane glycoprotein (M, 130,000) that is ubiquitously expressed with high levels found in liver and adipose tissue (5, 6). On the basis of structural and amino acid homologies, GHR has been identified as a member of the cytokine-hematopoietin receptor superfamily (Ref. 7; for review, see Ref. 8). The GHR extracellular domain contains seven cysteine residues, of which six are paired. The unpaired cysteine at position 241 is intermediary in GH-dependent disulfide linkage of two adjacent GHRs but not essential for GHR internalization and signaling (9). Like all cytokine receptors, GHR lacks intrinsic kinase activity. GH binding induces the recruitment and activation of the tyrosine kinase Janus kinase 2 (JAK2; Ref. 10). Activated JAK2 phosphorylates the GHR and signal transducers and activators of transcription molecules, which dimerize and translocate to the nucleus to promote gene transcription (11). The MAPK and insulin-receptor substrate pathways are also activated by GH (for review, see Ref. 12). GH is constitutively internalized via clathrin-coated structures and subsequently transported via endosomes to lysosomes, where degradation occurs (13–15). Both internalization and transport from endosomes to lysosomes are regulated via the ubiquitin-proteasome system and depend on an intact ubiquitin-dependent endocytosis motif (UbE-motif, DSWVFIELD) in the GHR cytosolic domain (16–19).

Ligand binding to GHR represents the first step in GHR activation. A single GH molecule binds two GHRs sequentially (20, 21) and induces a conformational change in the GHR that is required for signal transduction (22, 23). A mutation (e.g. G120K or G120R) in human GH prevents the binding of a second GHR and creates a GH antagonist that interacts with one GHR (via site 1) but is unable to change the conformation and fails to initiate signal transduction (22, 24). The GHR extracellular domain comprises an N-terminal (subdomain 1) and C-terminal (subdomain 2) β-sandwich, which is linked by a four-amino acid hinge region. Interactions between amino acids of subdomain 2 of adjacent GH polypeptides create a 500-Å² contact interface that is thought to stabilize the GH-(GHR)₂ complex (20). Examination of the crystal structure of the complex and mutagenic analysis revealed the importance of serine 145, histidine 150, aspartic acid 152, tyrosine 200, and serine 201 of the GHR contact interface in the GH-induced conformational change to a signaling-competent complex rather than in GHR dimerization. (Molecular Endocrinology 17: 967–975, 2003)
acid 152, tyrosine 200, and serine 201 (20, 25). A D152H mutation has been identified in patients with Laron’s syndrome, a GH-insensitive short-stature condition caused by molecular defects in the GHR (26, 27). In general, Laron mutations either affect GH binding or result in GHR truncation mutants, which are unable to bind JAK2 (for review, see Ref. 28). However, GHR(D152H) is able to bind GH and contains an intact JAK2 binding region (26). Initially, the D152H mutation was proposed to affect receptor dimerization, but cross-linking of radiolabeled GH to GHR(D152H) revealed a GH-(GHR)_2 complex (29). Rather than affecting dimerization, the D152H mutation was suggested to alter the conformation of the GHR.

In the present study, we investigated the role of subdomain 2 in GHR function further. Recently, we have shown that GHR polypeptides occur as dimers in the absence of ligand in the endoplasmic reticulum and at the cell surface (30). Herein, we examined the effect of single amino acid mutations in subdomain 2 on ligand-independent GHR dimerization. In addition, we analyzed the effect on GH-induced signal transduction and the integrity of the GH-(GHR)_2 complex. The results demonstrate that aspartic acid 152, tyrosine 200, and serine 201 are critical for GH-induced signal transduction, apparently because the required conformational change is prevented rather than GHR dimerization. The data implicate an essential role for the contact interface of subdomain 2 in acquiring a signaling-competent conformation.

RESULTS

Characterization of the GHR Mutants

The amino acids aspartic acid 152, tyrosine 200, and serine 201 participate in the contact interface between adjacent GHR molecules in the GH-(GHR)_2 complex (20, 25). Previous studies reported the reduced signaling activity of GHR(D152H), GHR(Y200D), and GHR(S201K) but not of GHR(N143K) (Refs. 25 and 29). However, the mechanism for the signaling defect remained unclear. First, the mutations were suggested to destabilize or prevent the formation of the GH-(GHR)_2 complex, but Esposito et al. (29) demonstrated a trimeric complex for the GHR(D152H) mutant. Instead, the D152H mutation was proposed, but it was not experimentally shown to alter the conformation of the GHR. To study the role of subdomain 2 in GHR function, we introduced single D152H, Y200D, S201K, and N143K mutations in the rabbit GHR cDNA and generated Chinese hamster ts20 cell lines stably expressing the mutant GHRs. In addition, a GHR(C241S) mutant was created, because disulfide bridge formation could be important for stabilizing GHR complexes.

First, the GHR mutants were characterized by analyzing the uptake of fluorescence-labeled ligand. Recently, we have shown that GHR dimerization occurs already in the absence of ligand in the endoplasmic reticulum and at the cell surface (30). The introduction of a mutation in the extracellular domain could prevent ligand-independent GHR dimer formation. Because normal divalent GH has the potential to dimerize monomeric receptors, the effect of the mutation could be overcome. Therefore, we used Cy3-labeled B2036, a GH antagonist containing a G120K mutation in binding site 2 and eight additional mutations that enhance the binding affinity of site 1 (31, 32). As demonstrated in Fig. 1A, uptake of Cy3-labeled B2036 in wild-type (wt)GHR-expressing cells resulted in a vesicular staining pattern corresponding to endosomal and lysosomal localization of the ligand as described before (33). The staining was acid-resistant (data not shown) and therefore considered to be intracellular. The involvement of the ubiquitin system in GHR endocytosis was demonstrated by incubating the cells at 41.5 °C. The ubiquitin system is inactivated at this temperature because of a temperature-sensitive mutation in the ubiquitin-activating enzyme (E1) (34). As seen in Fig. 1B, internalization of Cy3-labeled B2036 was inhibited. To exclude a general inhibition of clathrin-mediated endocytosis, internalization of Alexa594-labeled transferrin (Alexa594-Tf) was examined. Internalization of transferrin via endogenous transferrin receptors is clathrin-dependent but ubiquitin system-independent (16). Indeed, at both the permissive and restrictive temperatures, Alexa594-Tf was internalized (Fig. 1, C and D). The GHR mutants behaved like wtGHR; all GHR mutants internalized Cy3-labeled B2036, and the internalization was ubiquitin system-dependent (Fig. 1, E–N). Nontransfected cells showed neither ligand binding nor uptake at 30 °C (Fig. 1O) and 41.5 °C (data not shown), although light microscopy confirmed the presence of cells (Fig. 1P). The level of expression of the GHR mutants varied among the cell lines, but the affinity for GH, as determined by Scatchard analysis of 125I-labeled GH binding curves, was similar for all mutants and comparable with wtGHR (data not shown; and Ref. 14).

Previously, GH was shown to stimulate GHR ubiquitylation (14, 16). To examine whether the mutant GHRs could be ubiquitylated, cells were incubated in the presence or absence of GH. GHR polypeptides were immunoprecipitated with anti-C, an antibody directed against the distal part of the cytosolic domain (see Materials and Methods), and the immunoblot was stained with an antibody recognizing poly-ubiquitylated proteins (anti-Ubi). As shown in Fig. 2A, GH increased the amount of ubiquitylated wtGHR molecules that appeared as high molecular weight species in the upper part of the gel. For all GHR mutants, a GH-dependent increase in GHR ubiquitylation was observed, but the extent of receptor ubiquitylation varied. Reprobing the same blot with anti-T, an antibody recognizing the membrane-proximal part of the GHR cytosolic domain, revealed comparable amounts of the high-mannose glycosylated precursor and complex-glycosylated mature receptor in the absence and pres-
ence of GH (Fig. 2B). Furthermore, the appearance of a 110-kDa precursor species for the N143K mutant confirmed that this asparagine is not involved in N-linked glycosylation as was also shown for porcine GHR (35). Together, these results demonstrate that the mutations of the extracellular domain do not prevent GHR biosynthesis, GH binding, or GHR ubiquitylation and internalization.

Ligand-Independent Dimerization of the GHR Mutants

Recently, we have shown that GHR dimerization is required for ubiquitin system-dependent endocytosis (30). Therefore, the results from Figs. 1 and 2 suggested that none of the mutations affected dimerization. To study GHR dimer formation directly, coimmunoprecipitation experiments were performed as previously described (30). Cells stably expressing wt-GHR or GHR mutants were used to transiently express a truncated, epitope-tagged GHR(1–369; HA-His6-Myc) mutant. After lysis, immunoprecipitations were performed with anti-C, recognizing only full-length GHRs. Lysates and immunoprecipitates were immunoblotted with an anti-hemagglutinin (HA) antibody. Figure 3A demonstrates the comparable expression of high-mannose glycosylated precursor and complex-glycosylated mature GHR (1–369; HA-His6-Myc) in the different cell lines. Coimmunoprecipitation of the epitope-tagged GHR species with anti-C (Fig. 3B) indicates heterodimer formation with full-length wtGHR or GHR mutants. In addition, the coprecipitation of both precursor and mature species shows that the dimerization occurs in the early biosynthetic compartments and at the plasma membrane, as previously described (30). The specificity of the communoprecipitation was determined with the use of ts20 cells expressing only GHR(1–369; HA-His6-Myc; Fig. 3B, lane 1). To exclude that the interaction occurs after...
lysis, lysates from ts20 cells expressing GHR(1–369; HA-His6-Myc) were mixed with lysates from cells expressing wtGHR or GHR extracellular domain mutants. Under those conditions, no interaction was observed (Fig. 3B, M lanes). Reprobing the same blot with the GHR antibody anti-T, revealed similar amounts of precipitated full-length GHR species (Fig. 3C, compare H11001 and M lanes). Although the experiment suggested more efficient heterodimer formation between epitope-tagged GHR (1–369) and GHR(C241S) than the other GHRs, this conclusion could not be safely drawn from repeated experiments. In addition, there is a remarkable difference in the ratio of precursor vs. mature GHR species between transiently and stably expressed GHRs (Fig. 3, compare A and C). The reason for this difference is yet unclear. Together, none of the mutations in the GHR extracellular subdomain 2 prevented dimerization with an epitope-tagged GHR containing an intact extracellular domain. To examine the effect on dimerization when a mutation is

**Fig. 2.** Mutations in the Extracellular Domain Do Not Prevent GHR Ubiquitylation

Nontransfected ts20 cells (none) or cells expressing wt-GHR or the GHR mutants were preincubated for 1 h at 30 C. After incubation for 30 min in the absence or presence of (8 nM) GH, cells were lysed. GHR molecules were immunoprecipitated with anti-C, an antiserum against the distal part of the GHR cytosolic tail, and separated by SDS-PAGE. A, Ubiquitylated GHR species (+) were detected by immunoblotting with an antibody against ubiquitin (anti-Ubi). B, Efficiency of immunoprecipitation as determined by reprobing the blot shown in A with an anti-T antibody, recognizing a membrane-proximal region of the GHR cytosolic tail. Similar results were obtained in three independent experiments. m, Mature GHR; p, precursor GHR; IP, immunoprecipitation; IB, immunoblotting. Relative molecular weight standards (M, × 10^3) are shown at the left.

**Fig. 3.** Ligand-Independent Dimerization Is Not Affected by the GHR Mutations

Nontransfected ts20 cells (none) or cells stably expressing wt-GHR or mutant GHR were used to transiently coexpress GHR(1–369; HA-His6-Myc) (369-HA). After lysis, full-length receptors were precipitated with anti-C antiserum, which is directed against a region of the GHR cytosolic tail that is deleted in the truncation mutant. Immune complexes were separated by SDS-PAGE. A, Immunoblotting of lysates with anti-HA antibody. B, Immunoblotting of anti-C immunoprecipitates with anti-HA antibody. C, Efficiency of immunoprecipitation as determined by reprobing the blot shown in panel B with anti-T antibody against a membrane-proximal region of the GHR cytosolic tail. D, The presence of the D152H or Y200D mutation in both full-length and epitope-tagged GHRs does not prevent dimerization. Transfection was performed as above, except that epitope-tagged GHR(1–369; HA-His6-Myc) (369-HA) or GHR(1–369; Y200D) (Y200D-HA) mutants were used. Lysates (left) or immunoprecipitates (right) were immunoblotted with anti-HA antibody. M, Mix of lysate from ts20 cells expressing GHR(1–369; HA-His6-Myc) with lysate of wtGHR or GHR mutant expressing ts20 cells. Similar results were obtained in two independent experiments. m, mature GHR; p, precursor GHR; IP, immunoprecipitation; IB, immunoblotting. Relative molecular weight standards (M, × 10^3) are shown at the left.
present in both full-length and truncated GHRs, we created epitope-tagged GHR(1–369; D152H) and GHR(1–369; Y200D) mutants. Coimmunoprecipitation experiments were performed as described above. Both D152H and Y200D epitope-tagged GHRs were expressed to similar extents in the cell lines (Fig. 3D, left). Despite the presence of either the D152H or Y200D mutation in full-length and truncated GHR species, specific coimmunoprecipitation, and thus dimerization, was observed (Fig. 3D, right).

**Signal Transduction of the GHR Mutants**

The above-mentioned experiments demonstrated that none of the mutations affected the GHR functions tested. Previously, species specificity has been observed for the D152H mutation, which prevents signal transduction via the human GHR, but it is silent in rat (29). Therefore, we examined the tyrosine phosphorylation of the GHR mutants in our particular cell system. Ts20 cells expressing wtGHR or the GHR mutants were incubated in the absence or presence of GH. After lysis, GHR molecules were immunoprecipitated with anti-C, an antibody against the GHR cytosolic domain, and immunoblotted with an antibody that specifically recognizes phosphorytrosine residues (anti-PY; Fig. 4A, top). Incubation with GH induced tyrosine phosphorylation of the mature wtGHR. When the same blot was reprobed with the GHR antibody anti-T, the precursor species was also observed (Fig. 4A, bottom). In addition, this blot revealed comparable amounts of precipitated GHRs in the absence or presence of GH. Like wtGHR, the GHR(N143K) and GHR(C241S) mutants were also phosphorylated on GH treatment, albeit to lesser extents. The reduced amounts of precipitated GHR were comparable to wtGHR, GHR(C241S), and to a much lower extent of GHR(N143K), but not in cells expressing GHR(D152H), GHR(Y200D), or GHR(S201K). Immunoblotting of the lysates with anti-JAK2 antibody showed similar amounts of JAK2 in the different cell lines (Fig. 4B, middle). However, due to different expression levels of the GHR mutants, the amount of GHRs in the lysates as determined by immunoblotting with GHR anti-C antibody (Fig. 4B, bottom) varied. Strikingly, although the amount of GHR(C241S) phosphorylation was low,
there was substantial activation of JAK2. Together, both the GHR and JAK2 phosphorylation experiments implicate an essential role of amino acids aspartic acid 152, tyrosine 200, and serine 201 in GHR signal transduction.

**GH Fails to Protect the Signaling-Deficient Mutants from Protease Digestion**

Because the mutations do not affect receptor dimerization, the question remains why signaling is affected. Recently, GH binding has been shown to induce a conformational change in the GHR, rendering the receptor protease-resistant (33, 36). To examine whether the mutations had an effect on the GH-induced structure of the GHR, a protease-protection assay was performed. Cells were treated with proteinase K after GH binding on ice. Proteinase K is a non-specific protease that digests all accessible proteins but is membrane-impermeable. After lysis, GHR molecules were immunoprecipitated with anti-C and immunoblotted with anti-B antibody, recognizing the middle part of the GHR cytosolic domain (see Materials and Methods). Figure 5A shows the results of a typical experiment. In the absence of GH, the mature wtGHR species disappeared almost completely after protease treatment, whereas the precursor species remained visible (Fig. 5A, compare lanes 1 and 2). At the same time, a 70-kDa fragment corresponding to the membrane-bound remnant of the mature GHR appeared. When cells were incubated with GH on ice before proteinase K digestion, the mature species was (partly) protected and less of the 70-kDa fragment was observed (Fig. 5A, lane 3). The amount of remnant formation was determined by densitometric scanning of blots from four different experiments. Subsequently, the percentage of GH protection was determined (Fig. 5B). GH binding protected the GHR(N143K) and GHR(C241S) mutant to a similar extent as wtGHR. However, remnant formation was hardly prevented by binding of GH to the GHR(D152H), GHR(Y200D), or GHR(S201K) mutants. This failure to protect the GHRs was not due to dissociation of GH from the GHRs during the experiment because no release of $^{125}$I-labeled GH was observed under similar conditions (data not shown). Because the same three mutants were also signaling-deficient, the data show a correlation between the ability to change conformation and GHR signaling.

**DISCUSSION**

The crystal structure of the GH-(GHR)$_2$ complex has revealed a 500-Å$^2$ contact area between the membrane-proximal subdomain 2 of adjacent GHR polypeptides. Initially, this region of interaction was proposed to stabilize the complex. In this study, we have investigated further the role of this domain in GHR function. Amino acids that participate in the interaction (e.g. aspartic acid 152, tyrosine 200, and serine 201) or are located close to this region (e.g. asparagine 143 and cysteine 241) were mutated in rabbit GHR. In agreement with the literature, the signaling capacity of the GHR(D152H), GHR(Y200D), and GHR(S201K) mutants was severely reduced, as determined by both GHR and JAK2 tyrosine phosphorylation (Fig. 4). Because these amino acids all belong to the contact interface, impaired GHR dimerization was previously proposed to cause the signaling defect (20, 25, 26). More recently, however, GH was shown to
dimerize the GHR(D152H) mutant, and an altered conformation was proposed (but not experimentally shown) instead. Herein, we have provided novel evidence demonstrating that none of the mutations affected (ligand-independent) dimerization. First, an epitope-tagged GHR truncation mutant coimmunoprecipitated with the mutated GHRs, even if the mutation was present in both receptor species as was demonstrated for the D152H and Y200D mutation (Fig. 3). Secondly, internalization of GHR mutants was ubiquitin system-dependent (Fig. 1), and GH increased the ubiquitylation of the mutant GHRs (Fig. 2). The latter observations are consistent with our previous finding that dimerization and ubiquitin system-dependent endocytosis are correlated (30).

Because the D152H, Y200D, and S201K mutations are all located in the contact region, identification of the molecular defect that precludes GHR signaling might clarify the function of this domain. We assumed that the mutations cause local structural perturbations, as was proposed for the D152H mutant (29). Because crystallographic data of these mutant GHRs are not available, we applied our protease-protection assay to investigate the effect of the mutations on the integrity of the GHR-(GHR)2 complex (Fig. 5). The assay revealed a strict correlation between signaling deficiency and absence of GH-induced protease protection. Therefore, it is conceivable that GH fails to organize the extracellular domain of these mutants such that activation of JAK2 at the cytosolic side can occur (22). Apparently, mutating a single amino acid in the contact region can alter the conformation of subdomain 2, changing its rigidity. How this works mechanistically remains unclear and probably requires crystallographic analysis to be solved. Together, the data demonstrate a critical role for the contact region of the GHR extracellular subdomain 2 in GH-induced signaling. A role in dimer formation is also likely and not excluded by our data. Multiple hydrogen bond interactions occur between amino acids of adjacent GHRs (20). Therefore, single mutations could have local effects, whereas dimerization is maintained via the architecture of the whole domain. Expression of a GHR mutant that lacks subdomain 2 or contains multiple amino acids mutations therein might be useful to address its role in dimer formation.

In addition to subdomain 2, other regions of the GHR could be involved in dimerization. The unpaired cysteine 241 in the extracellular domain has been reported to be critical for GH-induced disulfide linkage of GHR molecules but is not essential for GH internalization, signaling, and GH-induced protease protection (9). Also in our cell system, the C241S mutation had no effect on GHR dimerization, internalization, and signaling. The relevance of disulfide bridge formation is therefore still unclear, but it might stabilize the complex transiently, e.g., during protein folding in the endoplasmic reticulum.

Recently, the transmembrane domain of an increasing number of membrane proteins has been demonstrated to mediate their dimerization (for review, see Ref. 37). Accordingly, the transmembrane domain of the GHR-homologous erythropoietin receptor has been suggested to mediate ligand-independent oligomerization (38). Whether the GHR transmembrane domain is involved in receptor dimerization is currently under investigation.

Our data clarify another mechanism by which some mutations can cause Laron-type dwarfism. In addition to preventing GH or JAK2 binding, apparently single mutations in the extracellular domain can induce subtle changes in the structure of the GHR that allow dimer formation but prevent the switch to a signaling-competent complex.

In conclusion, this study provides novel information on the molecular interactions that are required to achieve a functional GHR. The finding that single amino acid mutations affect signaling without disturbing dimerization adds another layer of complexity to the GHR activation mechanism, which we are just beginning to unravel.

**MATERIALS AND METHODS**

**Materials and Antibodies**

Rabbit antisera directed against the cytosolic GHR amino acids 271–318 (anti-T), 327–493 (anti-B), and 493–620 (anti-C) were as described before (16, 18). Monoclonal anti-HA antibody 16B12 was purchased from Babco (Richmond, CA). Rabbit antiserum against JAK2 was generated as previously described (39). Antibody 4G10, recognizing PY residues, and anti-JAK2 antibody were obtained from Upstate Biotechnologies Inc. (Lake Placid, NY). Mab5, a mouse monoclonal antibody directed against the GHR extracellular domain was from AGEN Inc. ( Parsippany, NJ). Antiserum specific for protein-ubiquitin conjugates was a gift from Aaron Ciechanover (Techinon-Israel Institute of Technology, Haifa, Israel). LipofectAMINE was from Invitrogen (Carlsbad, CA). Human GH was kindly provided by Eli Lilly & Co. Research Labs (Indianapolis, IN). Human GH antagonist B2036, containing a G120K mutation in binding site 2 and eight additional mutations that enhance the binding affinity of site 1 (31, 32), was a generous gift from William F. Bennett of Sensus Drug Development Corporation (Austin, TX).

**GHR Mutants and Cell Lines**

Single amino acid mutations of rabbit GHR extracellular domain were created by QuikChange Site-directed Mutagenesis (Stratagene, La Jolla, CA). Briefly, cDNA3 plasmids (In-vitrogen, San Diego, CA) encoding wtGHR were used as a template in a PCR with 3’ and 5’ oligonucleotides encoding the N143K, D152H, or Y200D mutations. The oligonucleotides also introduced a silent mutation that either created or disrupted a restriction site. The construction of a double epitope-tagged truncation mutant GHR(1–369; HA-His6-Myc) was described before (30). Epitope-tagged GHR(1–369; D152H) and GHR(1–369; Y200D) mutants were created with the same oligonucleotides as described above and the GHR(1–369; HA-His6-Myc) cDNA3 construct as a template. All constructs were verified by restriction analysis, in vitro transcription/translation assays, and sequencing.

Chinese hamster ts20 cells, bearing a thermolabile ubiquitin-activating (E1) enzyme, were used (34). Clonal cell lines
stably expressing wtGHR or GHR mutants were obtained with the calcium phosphate transfection method. Transient transfections were performed with lipofectAMINE according to the manufacturer’s description. Forty-eight hours after the transfection, cells were used for experiments. Cells were cultured at 30°C in MEM supplemented with 10% fetal calf serum, 4.5 g/liter glucose, 100 U/ml penicillin, 100 µg/ml streptomycin, and 0.45 mg/ml geneticin. For experiments, cells were cultured in the absence of geneticin and treated for 16 h with 10 mU butyrate to increase the GHR expression (16).

Cell Lysis, Immunoprecipitations, and Western Blotting

Coimmunoprecipitations were performed as previously described (30). Briefly, ts20 cells stably expressing wtGHR or GHR mutants were used to transiently express epitope-tagged GHR (1–369), GHR(1–386; D152H), or GHR(1–369; Y200D). Cells were lysed in lysis buffer containing 0.3% Triton X-100, 1 mM EDTA, 50 mM NaF, 1 mM Na3VO4, 100 µM H9262, and 0.45 mg/ml geneticin. For experiments, cells were cultured in the absence of geneticin and treated for 16 h with 10 mU butyrate to increase the GHR expression (16).

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Address all correspondence and requests for reprints to: Ger J. Strous, Department of Cell Biology, University Medical Center Utrecht, Heidelberglaan 100, Room 302.525, 3584 CX Utrecht, The Netherlands. E-mail: strous@med.uu.nl.

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