Four Missense Mutations in the Ghrelin Receptor Result in Distinct Pharmacological Abnormalities

Guang Liu, Jean-Philippe Fortin, Martin Beinborn, and Alan S. Kopin

Molecular Pharmacology Research Center, Molecular Cardiology Research Institute, Tufts-New England Medical Center, Boston, Massachusetts

Received March 20, 2007; accepted June 26, 2007

ABSTRACT

The growth hormone secretagogue receptor (GHSR) plays an important role in regulating food intake and energy homeostasis. In this study, we compared the pharmacological properties of four reported variants of the human GHSR (I134T, V160M, A204E, and F279L) with those of the wild-type receptor. Corresponding recombinant receptors were transiently expressed in either human embryonic kidney 293 or COS-7 cells. Basal as well as ligand-induced signaling was assessed by luciferase reporter gene assays and measurement of inositol phosphate production. In addition, receptor expression levels were monitored by whole-cell enzyme-linked immunosorbent assay. Ligand-independent signaling of the wild-type GHSR is significantly reduced with introduction of either the V160M or F279L substitutions, whereas basal activity of the A204E mutant is not detectable. Ghrelin potency is markedly increased at the V160M mutant, whereas the I134T variant is unresponsive to this endogenous agonist. In contrast, the I134T mutant responds to a known GHSR inverse agonist, [d-Arg¹, d-Phe⁵, d-Trp⁷,⁹, Leu¹¹]-substance P (SP-analog), albeit with reduced efficacy. Activity of the SP-analog at the V160M and F279L mutants is comparable to the wild type (WT) value. The overall expression level of each of the four GHSR variants is reduced relative to WT; however, the ratio between the intracellular and plasma membrane receptor density remains comparable. Treatment with the SP-analog significantly increases cell surface expression of each receptor with the exception of the A204E variant. Taken together, our studies reveal that naturally occurring GHSR mutations affect a wide range of pharmacologic properties. The physiological impact of these alterations within selected populations (e.g., obese, lean individuals) as well as the pharmacogenomic consequences of corresponding mutations remain to be further investigated.

The growth hormone secretagogue receptor (GHSR), also referred to as the ghrelin receptor, modulates physiological effects, including pituitary growth hormone secretion, food intake, and energy expenditure (Shuto et al., 2002; Lall et al., 2004; Sun et al., 2004). Targeted disruption of the ghrelin receptor gene confirmed the link between this G protein-coupled receptor (GPCR) and metabolic function. Receptor null mice show reduced food intake, decreased body weight, improved blood glucose homeostasis, as well as resistance to diet-induced obesity (Zigman et al., 2005).

The GHSR is a seven-transmembrane domain receptor coupled to \( \Gamma_q \)-mediated activation of phospholipase C, which in turn leads to an elevation of intracellular calcium (Holst et al., 2003). In addition to the functionally intact isoform of this receptor (GHSR1a), an inactive splice variant (GHSR1b) has been reported that lacks the carboxyl-terminal portion of the receptor including transmembrane domains 6 and 7 (Kojima and Kangawa, 2005). Stimulation of GHSR1a with the orexigenic hormone, ghrelin, triggers second messenger signaling. In addition, it is well established that the ghrelin receptor shows a considerable degree of constitutive activity (i.e., basal function is greater than 50% of the ghrelin-induced maximum) (Holst et al., 2003; Holst and Schwartz, 2006). It has been postulated that both ligand-induced and basal activity of this GPCR play an important role in controlling appetite and energy expenditure (Holst and Schwartz, 2006; Pantel et al., 2006). As a result, there has been much interest in developing selective nonpeptide inverse agonist drugs to inhibit ghrelin receptor function (Holst and Schwartz, 2004).
A synthetic peptide, \([\text{d-Arg}^1, \text{d-Phe}^5, \text{d-Trp}^7,\text{Leu}^{11}]\)-substance P (SP-analog), which acts as an inverse agonist at this receptor, has been utilized to pharmacologically probe the consequences of blocking GHSR activity (Holst et al., 2003).

The pharmacological properties of GPCRs can be markedly altered by missense mutations. This variability may lead to physiologic consequences and/or have pharmacogenomic implications. Four missense mutations have been documented in either the National Center for Biotechnology Information single nucleotide polymorphism database or the literature (Holst and Schwartz, 2006; Pantel et al., 2006). These naturally occurring variants of the human GHSR1a include the I134T, V160M, A204E, and F279L substitutions. The objective of the current study was to assess the pharmacological properties of these ghrelin receptor isoforms. Our results indicate that mutations in the GHSR may affect: 1) basal activity, 2) agonist-induced receptor signaling, 3) inverse agonist signaling, and 4) receptor expression. Given our findings, it will be of interest to examine the extent to which naturally occurring variability in the ghrelin receptor has an impact on disease susceptibility (e.g., obesity) and/or the response to drugs targeting this GPCR.

Materials and Methods

Materials. Ghrelin and SP-analog were purchased from Bachem (Bubendorf, Switzerland). Myo-[\text{3}H]inositol (specific activity, 65 Ci/mmol) was obtained from NEN Life Science Products (Boston, MA). Cell culture media, fetal bovine serum, and lipofectamine reagent were obtained from Invitrogen (Carlsbad, CA). Peroxidase-conjugated, anti-hemagglutinin (HA) monoclonal antibody (3F10), and BM-blue, a peroxidase substrate, were purchased from Roche Applied Science (Indianapolis, IN). The plasmid encoding the serum response element (SRE)-luciferase reporter gene has been described previously (Hearn et al., 2002).

Construction of Human GHSR Plasmids. The wild-type human GHSR cDNA (isoform 1a) was generously provided by Dr. Michael Bown (National Institutes of Health, Bethesda, MD). After subcloning into pcDNA1.1, missense mutations were introduced into the cDNA using oligonucleotide-directed site-specific mutagenesis as described previously (Beinborn et al., 1993; Bläker et al., 1998). To enable assessment of receptor expression, an HA epitope (YPYDYPDYA) was introduced at the N terminus following the initiator methionine of each receptor isoform. For each mutant, the presence of only the indicated amino acid change was confirmed by full sequence analysis of each construct.

Cell Culture. Human embryonic kidney (HEK) 293 and COS-7 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen, Carlsbad, CA; catalog no. 12100-048 for HEK293 cells and 12100-058 for COS-7 cells) supplemented with 10% fetal bovine serum, 100 U/ml penicillin G, and 100 \(\mu\)g/ml streptomycin. The cells were maintained at 37°C in a humidified environment containing 5% CO2.

Luciferase Reporter Gene Assay. Receptor-mediated signaling was assessed using a luciferase assay as described previously (Hearn et al., 2002; Mukherjee et al., 2006; Al-Fulaij et al., 2007). In brief, HEK293 cells were plated at a density of 2000 to 3000 cells/well onto 96-well Primaria plates (BD Biosciences, Bedford, MA) and grown for 2 days to ~80% confluence. Cells were then transiently transfected using Lipofectamine reagent with 1\%) pcDNA1.1 or the relevant receptor plasmid and 2\%) an SRE-luciferase construct. Twenty-four hours following transfection, cells were stimulated for 18 h with ligand in serum-free medium. Unless otherwise noted, the final concentration of ghrelin (agonist) or substance-P analog (inverse agonist) was 1 \(\mu\)M. Ligand potencies were determined by stimulating receptor-expressing cells with increasing concentrations of these peptides. After ligand treatment, the cells were lysed, and luciferase activity was quantified using Luciferase reagent (PerkinElmer Life and Analytical Sciences, Boston, MA). Luminescence was measured using the Topcount NXT counter (PerkinElmer Life and Analytical Sciences).

Assessment of Receptor Expression Using ELISA. The expression levels of the GHSR variants were determined using a procedure described by Shinyama et al. (2003). In brief, HEK293 cells grown in 96-well plates were transiently transfected with either pcDNA1.1 or a plasmid encoding an HA-tagged wild-type (WT) or mutant ghrelin receptor. Forty-eight hours post-transfection, cells were washed once with phosphate-buffered saline (PBS), pH 7.4, and fixed with 4% paraformaldehyde in PBS for 10 min at room temperature. For assessment of total receptor expression, cells were permeabilized with 0.1% Triton-X100 in PBS for 2 min after fixation. For the measurement of surface expression, cells were not permeabilized. After washing with 100 mM glycine in PBS, the cells were incubated for 30 min in blocking solution (PBS containing 20% bovine serum). A horseradish peroxidase-conjugated monoclonal antibody (3F10) directed against the HA-epitope (1.500 dilution in blocking solution) was then added to the cells. After 1 h, the cells were washed five times with PBS, and BM-blue solution (50 \(\mu\)l per well) was added. After incubation for 30 min at room temperature, conversion of this substrate by antibody-linked horseradish peroxidase was determined by adding 2.0 M sulfuric acid (50 \(\mu\)l well). Converted substrate (indicating the amount of receptor-bound antibody) was assessed by measuring light absorbance at 450 nm using a SpectraMax microplate reader (Molecular Devices, Sunnyvale, CA).

Measurement of Inositol Phosphate Production. Ligand-induced inositol phosphate (IP) formation in COS-7 cells expressing either the WT or a mutant GHSR was assayed as described previously (Beinborn et al., 1998). COS-7 cells were grown for 24 h (1,000,000 per 10-cm plate). Using the DEAE-dextran method, cells were transfected with 5 \(\mu\)g of either pcDNA1.1 or a receptor-encoding plasmid. Twenty-four hours post-transfection, cells were split into 24-well plates (Falcon Primaria; BD Biosciences) at a density of 200,000 cells/well. After an overnight incubation with 3 \(\mu\)Ci/ml myo-[\text{3}H]inositol in serum-free Dulbecco’s modified Eagle’s medium, labeled cells were incubated with increasing concentrations of ligand in PBS containing 10 mM LiCl. After an additional 1-h incubation at 37°C, the cells were lysed and extracted with methanol/chloroform. The upper aqueous phase was analyzed for IP content by strong anion exchange chromatography. IP production was expressed as a percentage of the total cellular tritium activity that had been incorporated into the cells during overnight exposure to myo-[\text{3}H]inositol.

Data Analysis. GraphPad Prism software version 3.0 (GraphPad, San Diego, CA) was used for sigmoidal curve fitting of ligand concentration response curves. The same program was utilized to calculate half-maximal effective concentrations (EC50 values) as an index of ligand potency. Results are presented as mean \(\pm\) S.E.M.

Results

Ghrelin Receptor Variants. Four missense mutations, I134T, V160M, A204E, or F279L, in the GHSR were the focus of this study. The altered amino acids are located either in the extracellular loop of GHSR (A204E, second extracellular loop), or in the transmembrane domains (I134T, TM3; V160M, TM4; F279L, TM6) (Fig. 1A). Each of the substituted residues is otherwise completely conserved among all receptor species orthologs studied to date (Fig. 1B). This high degree of conservation suggests that amino acid substitutions in these positions have the potential to affect receptor structure and/or function.
V160M, A204E, and F279L variants of the GHSR each show a significant decrease in basal activity relative to the wild-type protein. With the exception of the A204E variant that lacks constitutive activity, each of these mutants retained some degree of ligand-independent signaling. The I134T variant shows constitutive activity comparable with the WT value.

Stimulation of the WT receptor with ghrelin (1 μM) results in a significant elevation (~2-fold) in signaling relative to basal. The I134T mutant, despite its similar level of constitutive activity compared with WT, fails to respond to ghrelin stimulation. With each of the other variants, ghrelin shows significant agonist function.

In addition to agonist-induced modulation of basal activity, it is well established that the SP-analog is an efficacious inverse agonist at the WT ghrelin receptor (Holst et al., 2003). The effect of this ligand (1 μM) was explored on each of the GHSR variants. As anticipated with a receptor lacking constitutive activity, there was no effect of the SP-analog on the A204E variant. Each of the other receptors, despite variability in the level of basal signaling, showed a significant decrease in constitutive activity after treatment with the SP-analog.

**GHSR Missense Mutations Show Altered Receptor Expression.** Cell surface expression of the GHSR variants was compared with that of the WT receptor after HEK293 cells had been transfected with increasing concentrations of corresponding cDNAs (0–8 ng per well) (Fig. 3A). For each receptor, transfection of increasing amounts of cDNA led to an elevation in cell surface expression. When compared at equal cDNA amounts, each mutant is expressed at ~40 to 60% of the corresponding WT level. To explore whether the mutation-induced alterations in expression levels correlated with observed differences in basal signaling, we performed a parallel series of experiments where constitutive activity of the GHSR variants was also assessed with increasing concentrations of transfected cDNA. Figure 3B illustrates that both the WT receptor and the I134T mutant display a similar cDNA dose-dependent increase in constitutive activity. In contrast, the A204E variant failed to show appreciable basal signaling at any amount of transfected cDNA, consistent with the premise that this variant lacks constitutive activity. The other two variants, V160M and F279L, showed a minor elevation in constitutive activity with increasing expression; corresponding levels of basal signaling were markedly lower than those of the WT receptor.

**GHSR Missense Mutations Alter Ghrelin Potency.** Stimulation of the GHSR variants with increasing concentrations of ghrelin demonstrates that the endogenous agonist has comparable potency at the WT, A204E, and F279L receptors (Fig. 4; Table 1). In contrast, the V160M variant shows an almost 20-fold increase in ghrelin potency despite its reduced level of basal activity. The ghrelin EC₅₀ values at this mutant and at the WT receptor are 3.8 and 75.2 nM, respectively. Finally, although the I134T variant shows constitutive activity, ghrelin stimulation does not lead to a detectable increase in signaling.

To complement our studies of agonist induced activity using a luciferase reporter gene construct, we also assessed GHSR-mediated inositol phosphate production (Fig. 5). In response to ghrelin, the wild-type GHSR showed a concentration-dependent increase in IP production (EC₅₀ = 13.4
GHSR Missense Mutations Alter Inverse Agonist Potency. Further comparison of wild-type and mutant GHSR isoforms focused on the response of these receptors to an inverse agonist, the SP-analog. When assessed by luciferase reporter gene assay, this compound inhibits the basal activity of the wild-type, V160M, and F279L receptors with comparable potency (Fig. 6; Table 1). In contrast, the SP-analog has no appreciable effect on the A204E mutant, which lacks basal activity. It is noteworthy that although the constitutively active I134T variant cannot be further stimulated by ghrelin, this mutant shows a significant decrease in basal signaling when treated with the SP-analog (Fig. 6). Despite conserved inverse agonist function, both the potency and efficacy of this compound are reduced.

In a complementary series of experiments, inverse agonist effects on wild-type and mutant GHSR were also examined using IP production as an alternative readout of receptor-mediated function. Again, mutation-induced alterations in the response to the SP-analog were consistent when assessed by either luciferase reporter gene assay (described above) or by measurement of IP production (Fig. 7). With the latter readout, mutation-induced changes in inverse agonist function at the wild-type versus mutant receptors were marked by 1) a detectable yet attenuated ability of the SP-analog to inhibit basal signaling of the I134T variant and 2) no appreciable effect of the compound on A204E activity.

GHSR Mutations Result in Decreased Receptor Expression. To explore whether GHSR mutations affect the cellular distribution of receptors, we compared both total and cell surface expression of WT and mutant ghrelin receptors. As shown in Fig. 8, each of the GHSR mutants shows a reduced level of both total and cell surface receptor expression compared with WT. However, despite these changes, the percentage of receptors that were found at the cell surface (70–85% of total expression) was close to the corresponding WT value (88%).

Missense Mutations May Alter Inverse Agonist Induced Up-Regulation of Receptor Expression. Inverse agonists have been shown to increase surface expression of GPCRs (Shinjumiya et al., 2003). In the current study, the effects of the SP-analog on both GHSR cell surface and total expression were examined. After exposure to 1 μM SP-analog for 18 h, surface expression of the wild-type receptor as well as the I134T and F279L variants was markedly increased as assessed by ELISA. In contrast, the SP-analog only slightly increased surface expression of the V160M variant and had no significant effect on the A204E isoform. For each of the
receptor variants, total GHSR expression showed no significant change after stimulation with the SP-analog (Fig. 9). Immunocytochemical analysis qualitatively confirmed that cell surface receptor density of each of the mutants was reduced relative to WT (Supplemental Fig. 1). Also consistent with results obtained by ELISA, immunocytochemistry revealed a readily apparent increase in surface receptor expression of the WT, I134T, and F279L receptors after treatment with the SP-analog.

Discussion

In this study, we explored the pharmacological consequences of known missense mutations in the GHSR. Our

| Ligand potencies at the wild type and mutant ghrelin receptors |
|--------------------|------------------|
| **Ligand**        | **Wild Type**    | **I134T** | **V160M** | **A204E** | **F279L** |
| Ghrelin           | \(10^{-7}\) | 34.9      | 7.04      | 3.8       | 6.69       |
| SP analog         | 34.9             | 7.04      | 3.8       | 6.69      | 36.5       |

Data represent the mean ± S.E.M. from at least four independent experiments using a luciferase reporter gene assay; each experiment was performed in triplicate.

**Fig. 5.** GHSR missense mutations alter ghrelin-stimulated inositol phosphate production. COS-7 cells were transiently transfected with either the empty vector pcDNA1.1 or plasmid encoding the WT or a mutant ghrelin receptor. Cells were stimulated for 1 h with increasing concentrations of ghrelin. Ligand-induced IP production was measured as described under Materials and Methods. In contrast to each of the other receptors, data obtained with the I134T variant did not fit a sigmoidal curve and were instead analyzed by linear regression (dashed line). The slope of the line was not significantly different from zero. Respective EC\(_{50}\) values are as follows: WT, 13.4 nM; V160M, 1.2 nM; A204E, 26.0 nM; and F279L, 63.5 nM. Data represent the mean ± S.E.M. from at least three independent experiments, each performed in duplicate.

**Fig. 6.** SP-analog-mediated inhibition of ligand-independent signaling is affected by GHSR mutations. HEK293 cells were transiently transfected with plasmid encoding either the wild-type or a mutant GHSR receptor, together with an SRE-luciferase reporter gene construct. Cells were stimulated with increasing concentrations of the SP-analog. After 18 h, luciferase activity was quantified and expressed relative to the wild-type basal value (100%). Data represent the mean ± S.E.M. from at least four independent experiments, each performed in triplicate. Basal, without ligand.
investigations showed that single amino acid changes in the GHSR can result in a wide range of pharmacological alterations, including shifts in basal activity, ligand-induced signaling, and receptor expression. Such variation in receptor pharmacology has the potential to influence disease susceptibility as well as the response to drug treatment. The impact of missense mutations on individual aspects of ghrelin receptor function will be discussed and interpreted against the background of related observations with other GPCRs.

GHSR Missense Mutations Alter Receptor Expression. Each of the missense mutations in the GHSR that were characterized in this study results in a decrease in receptor expression compared with the WT value. However, these mutations do not significantly affect the ratio between total and cell surface receptor densities, suggesting that despite decreased expression, each of the variants is efficiently transported to the plasma membrane. This observation contrasts with reports on other GPCR mutants that show decreased expression. Lubrano-Berthelier et al. (2003) reported that the majority of naturally occurring MC4R mutations that result in loss of function (an alteration that is associated with childhood obesity) lead to intracellular retention of the receptor. A similar defect has been reported with the characterization of mutant human vasopressin type 2 receptors. These naturally occurring variants show defective processing to the cell membrane (Oksche and Rosenthal, 1998). The absence of ligand-induced signaling results in insufficient tubular concentration of the renal filtrate, causing nephrogenic diabetes insipidus. Although reduced surface expression of mutant ghrelin receptors occurs by a different mechanism (i.e., without intracellular receptor trapping), individuals who express these variants may also show diminished receptor-mediated function.

Basal Activity of the GHSR Variants. Our study, as well as reports by others (Holst et al., 2003), have demonstrated that the WT ghrelin receptor has a high level of
ligand-independent signaling. Basal activity exceeds 50% of the ghrelin-induced maximum. Of the four mutant receptors characterized in this study, the V160M, A204E, and F279L variants show reduced or completely abolished basal function. It is possible that the observed decrease in ligand-independent signaling may, at least in part, reflect reduced expression levels of the mutant receptors. The latter mechanism, however, cannot fully explain the loss of basal activity. This is suggested by the observation that transfection of 4 ng/well mutant receptor cDNA results in expression levels of corresponding GHSR variants similar to those observed with transfection of 1 ng/well WT receptor cDNA (Fig. 3). Despite comparable expression under these experimental conditions, basal activities of the variants versus the WT receptor are markedly different. It is possible that in addition to expression levels, ligand-independent signaling of the mutant receptors is affected by a structural change that shifts the putative equilibrium between active and inactive receptor conformations (Lefkowitz et al., 1993). For the F279L mutant, a structural basis for this shift in receptor equilibrium is suggested by the observation that this amino acid substitution falls within a cluster of GHSR residues that is known to be critical for the receptor to adopt an active conformation (Holst et al., 2004). Introduction of the F279L mutation may partially disrupt this structural domain, thus contributing to reduced basal signaling. A similar mechanism may apply to the V160M and A204E mutations, although the underlying structural correlate is not yet known. Conversely, mutations in GHSR may shift the receptor toward a conformation that is more active than wild type. This scenario may apply to the I134T variant, which despite decreased expression retains constitutive activity comparable with the WT receptor. The enhanced basal function of this mutant (relative to cell surface expression) may reflect a shift toward a more active receptor state (Lefkowitz et al., 1993), thereby compensating for decreased receptor expression.

It is increasingly appreciated that constitutive GPCR activity has physiologic significance. For ghrelin receptors, the high level of basal activity is postulated to play a role in regulating both food intake and growth (Holst and Schwartz, 2006; Pantel et al., 2006). On this basis, efforts are in progress to develop drugs that inhibit GHSR basal signaling. In addition, the observation that receptors display the highest levels of basal activity (WT GHSR, I134T, and F279L) respond to the SP-analog treatment with the strongest increase in surface expression suggests that their up-regulation is related to the ability of this ligand to induce a shift in the protein conformation toward an inactive state. Variants V160M and A204E, which show reduced basal activity, seem to be affected to a much lesser degree by the SP-analog. One may speculate that the SP-analog reduces constitutive receptor internalization and in addition enhances protein stability by inducing a conformational change. Regardless of the molecular mechanism underlying
the ligand-induced increase in receptor surface expression, variations in this pharmacologic parameter resulting from missense mutations may alter the therapeutic response to inverse agonists acting on the GHSR.

The endogenous agonist for the GHSR is ghrelin, a well established orexigenic hormone (Kojima and Kangawa, 2005; Hosoda et al., 2006). Inverse agonists have the potential to block both ghrelin-induced signaling as well as basal receptor activity. Such compounds targeting GHSR are thus considered promising drugs for the treatment of obesity. Despite evidence supporting this strategy, it should be noted that the therapeutic consequences of altering basal activity remain difficult to predict. It is now appreciated that inverse agonists can either enhance or diminish GPCR-mediated signaling acting through two different mechanisms. Such drugs can directly attenuate basal activity while at the same time increasing receptor expression, an effect that enhances ligand-independent signaling. The net effect of an inverse agonist on the WT receptor is thus defined by an interplay of these properties. The possibility that naturally occurring GHSR mutations may alter both inverse agonist-mediated inhibition of constitutive activity as well as receptor expression adds yet another layer of complexity to the development of such drugs as a treatment for obesity.

Clinical Implications of GHSR Missense Mutations.
The physiologic consequences of amino acid substitutions in the GHSR are not fully understood. In a recent screen, the A204E and F279L mutations were found in children with obesity and short stature, respectively (Wang et al., 2004). Subsequently, mutation A204E was also reported in two Moroccan families with short stature (Pantel et al., 2006). These reports have raised additional speculation regarding the physiologic role of the GHSR as a determinant of height and body weight (Holst and Schwartz, 2006). Further clinical studies are needed to better define the frequency and the etiologic role of GHSR isoforms in populations with variability in these traits.

Conclusion
We illustrate in this study that each of the naturally occurring missense mutations in the GHSR results in receptors with considerable functional alterations. These include shifts in basal signaling, the response to endogenous agonist and/or a synthetic inverse agonist, as well as the level of receptor expression. The identification of pharmacologically significant GHSR isoforms provides a foundation on which to explore the possible impact of corresponding mutations on disease susceptibility (e.g., obesity, anorexia) and/or variability in the response to future therapeutics.

References
Address correspondence to: Dr. Alan S. Kopin, 750 Washington Street, P.O. Box 7703, Boston, MA 02111. E-mail: akopin@tufts-nemc.org
Supplemental Figure

<table>
<thead>
<tr>
<th></th>
<th>-</th>
<th>+</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>GHSR WT</strong></td>
<td><img src="image1" alt="Image 1" /></td>
<td><img src="image2" alt="Image 2" /></td>
</tr>
<tr>
<td><strong>I134T</strong></td>
<td><img src="image3" alt="Image 3" /></td>
<td><img src="image4" alt="Image 4" /></td>
</tr>
<tr>
<td><strong>V160M</strong></td>
<td><img src="image5" alt="Image 5" /></td>
<td><img src="image6" alt="Image 6" /></td>
</tr>
<tr>
<td><strong>A204E</strong></td>
<td><img src="image7" alt="Image 7" /></td>
<td><img src="image8" alt="Image 8" /></td>
</tr>
<tr>
<td><strong>F279L</strong></td>
<td><img src="image9" alt="Image 9" /></td>
<td><img src="image10" alt="Image 10" /></td>
</tr>
</tbody>
</table>
Supplemental Figure Legend

HEK293 cells were plated at a density of 12,000 cells per well onto 8 well Labtek Chambered Coverglass (Nalge Nunc International, Rochester, NY) and grown for 1 day to ~ 80% confluency. Cells were then transfected with cDNA encoding an N-terminally HA-tagged GHSR (either WT or mutant) using Lipofectamine® reagent (Invitrogen, Carlsbad, CA). Twenty-four hours following transfection, cells were incubated in the presence or absence of SP-analog for an additional 18 hours. Cells were then washed once with PBS and fixed with 4% paraformaldehyde in PBS for 10 minutes. After an additional two washes with PBS, cells were incubated for 30 min at room temperature in PBS containing 10% FBS. Cells were then incubated in this buffer in the presence of a fluorescein isothiocyanate (FITC)-conjugated monoclonal antibody directed against the HA-epitope (Sigma, clone HA-7) at 1 µg/ml final concentration. After 1 hour, the cells were washed three times with PBS and kept in this buffer. Fluorescence images were obtained by a Zeiss Axiovert 200M fluorescence microscope.