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A Dominant Negative Human GHRH Receptor Splice Variant Inhibits GHRH Binding

Abbreviated title: Dominant Negative Human GHRH Receptor

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ABSTRACT

Growth Hormone-Releasing Hormone (GHRH) is a hypothalamic peptide that stimulates the synthesis and secretion of Growth Hormone from pituitary somatotroph cells. The GHRH receptor is a seven-transmembrane G-protein-coupled receptor that localizes to the surface of somatotroph cells and binds GHRH. Alternative splicing of the GHRH receptor primary transcript at the intron/exon boundary 3' of exon 11 results in inclusion of sequence that is normally intronic. In the human, this inclusion has an in-frame premature stop codon, and this variant mRNA encodes a protein truncated just before the sixth transmembrane domain. To identify the effects of the truncated receptor on signaling of the wild-type receptor and the mechanisms by which its effects are produced, the full-length and truncated receptor constructs were epitope-tagged and transfected into HeLa T4 cells to examine signaling and expression. Results show that the truncated GHRH receptor cannot signal through the cAMP pathway and acts as a dominant inhibitor of wild-type receptor signaling. The wild-type and truncated GHRH receptor proteins form a complex. Stably transfected cell lines were generated to examine the mechanism of signal inhibition by the truncated receptor. The data show that receptor cell surface expression is not altered when the wild-type and truncated receptors are co-transfected, but that truncated receptor co-expression substantially reduces GHRH binding by the wild-type receptor. The results support an important role for alternative splicing in mediating the effects of G-protein coupled receptors in general, and suggest that the GHRH receptor can form multimers, which may be important to its signaling properties.

INTRODUCTION

Growth hormone (GH) production and release are controlled through the actions of many peptide and steroid hormones, which function at different levels throughout the GH axis. The primary regulation of GH is achieved through regulated release of two hypothalamic peptides, Growth Hormone-Releasing Hormone (GHRH) and somatostatin. As its name suggests, GHRH is responsible for stimulating the synthesis (1, 2) and secretion (3, 4) of growth hormone from somatotroph cells of the anterior pituitary gland. Somatostatin is the hypothalamic signal to inhibit GH secretion (5). Additionally, negative feedback throughout the GH axis controls when GH is made and secreted, according to changing physiological states. Glucocorticoids (6-9), estrogen (9-11), thyroid hormone (9, 11, 12), and retinoic acid (9, 13) have been shown to influence GH production, primarily by regulating gene transcription of GH and of other genes in the GH axis.

Studies in transgenic mice overexpressing GHRH that exhibit gigantism (14-17), in addition to clinical cases of GHRH-expressing tumors that cause acromegaly in humans (3, 4, 18-20), show that GHRH is a potent stimulus for linear growth. As the major positive stimulus for GH synthesis and secretion, GHRH and its signaling pathways are essential to maintaining normal growth and development in vertebrates. When GHRH binds its receptor on the surface of pituitary somatotroph cells, G-protein coupling stimulates adenylate cyclase to produce cyclic AMP (21). Through the cAMP second messenger pathway, CREB is phosphorylated (22, 23) and stimulates the transcription of the pituitary-specific transcription factor Pit-1 gene, which in turn stimulates the transcription of the GH (24-26) and GHRH receptor (27, 28) genes. Binding of GHRH to its receptor also leads to an influx of calcium, which, through a pathway that is not completely understood, is involved in mediating GH secretion from secretory vesicles (29).

The GHRH receptor is a seven transmembrane G-protein coupled receptor (21) that, in rat and human, is 423 amino acids in length (21, 30). The GHRH receptor is a member of the B-III subfamily of G-protein coupled receptors, which includes the secretin/glucagon peptide receptors (31). Alternative splicing in G-protein coupled receptors is one of many emerging mechanisms by which this class of receptors diversifies its activities. Splice variants that result in changes in signaling or protein expression have been identified in many G-protein coupled receptors, such as the GnRH receptor (32, 33), the $GABA_B$ receptor (34), the angiotensin II type 1 receptor (35), and the luteinizing hormone receptor (36). In a particularly relevant example, several splice variants of the PACAP receptor, which is closely related to the GHRH receptor, have been identified that differ in their signal transduction properties (37, 38). These PACAP receptor splice variants differ in the third intracellular loop of the protein (37-39), which is important to G protein interactions (40) and consequently represents an excellent target for altering the signaling properties of the protein.

Alternative splicing of the GHRH receptor in the rat and human occurs at the intron/exon boundary 3' of exon 11, and results in distinct predicted protein products with differential signaling capacities. In the rat, the alternative splicing results in inclusion of 41 amino acids in the third intracellular loop (28). This long isoform of the rat receptor is capable of binding ligand, but incapable of signaling through cAMP production (28). The human splice variant that occurs at the same intron/exon junction leads to inclusion of intronic sequence that has an in-frame premature stop codon, and this mRNA encodes a protein truncated just before the sixth transmembrane domain (41, 42). This human splice variant was originally identified in GH-producing pituitary adenomas, though it is also present at lower levels in normal pituitaries (41, 42). The identification of a splice variant of the GHRH receptor present in acromegalic cancer

patients unveils a potential role for alternative splicing in response to changing physiological or pathophysiological conditions. One of the initial reports identifying this splice variant suggests that the truncated splice variant cannot signal through the cAMP pathway, but that its expression has no effect on wild-type receptor signaling (42). A later report suggests that the truncated receptor acts as a dominant negative repressor of the wild-type receptor, as measured by cAMP accumulation (43). In order to further investigate the role of this human splice variant, a FLAG epitope-tagged GHRH receptor was cloned. Expression, signaling, cellular localization and ligand binding of the truncated GHRH receptor alone, and of the HA-tagged wild-type GHRH receptor in the presence and absence of the truncated splice variant receptor were examined. The data show a novel mechanism for GHRH receptor splice variant function, in which the truncated receptor can form a complex with the wild-type receptor and inhibit normal GHRH binding, thereby altering the signaling activity of the wild-type receptor.

MATERIALS AND METHODS

Generation of the flag epitope-tagged truncated human GHRH receptor

The full-length human GHRH receptor plasmid was used as a template to PCR a FLAG epitope-tagged receptor with an engineered premature stop codon at amino acid 325, which corresponds to the predicted stop codon in the human splice variant that has been identified in pituitary adenomas, using oligonucleotide primers ((5'-GCT CTA GAC CTT GTC ATC GTC GTC CTT GTA GTC CCA ATA CTG AGA CTG-3'and 5'-GCG GTA CCC ATG GAC CGC CGG ATG-3') (Integrated DNA Technologies, Coralville, IA)). PCR products were cloned into pcDNA3 (Invitrogen, Carlsbad, CA) downstream of the T7 promoter using the Kpn I and Xba I sites. The full-length human GHRH receptor with an influenza hemagglutinin (HA) tag had been previously cloned in the laboratory (44).

Vaccinia Transfection System

HeLa T4 cells, maintained in DMEM with 4.5g/L glucose and L-glutamine (Mediatech, Inc., Herndon, VA) and 5 percent fetal bovine serum (Mediatech, Inc., Herndon, VA), were transfected with receptor constructs using the vaccinia virus-T7 polymerase expression system (obtained under license from Dr. Bernard Moss, NIH, Bethesda, MD), as described (45). For transfection, the cells were incubated with vaccinia at a multiplicity of infection of 10 in PBS/0.1% BSA for 30 minutes. Plasmid DNAs to be transfected were incubated with liposomes (46, 47) at 5μg lipid/μg DNA in OptiMEM media (Gibco BRL, Grand Island, NY) for 15-20 minutes at room temperature. After infection, the virus was aspirated, and the DNA/transfectAce was added. Cells were transfected for 12-15 hours, while at 37°C in 5 percent CO_2.

Immunofluorescence localization of epitope-tagged receptors

HeLa T4 cells were cultured on 12mm round glass coverslips in 24-well plates and transfected as described with 500ng/well DNA. Cells were washed with 1X PBS and fixed in 1% paraformaldehyde for 30 minutes at 4°C. The cells were washed twice in PBS and incubated for 4-6 hours at 4°C with 1μg/ml of the HA-specific 12CA5 ascites fluid (a gift from Dr. Robert Lamb, Northwestern University) or the anti-M2 monoclonal antibody against the flag epitope (Sigma Co., St. Louis, MO) in PBS/0.1% BSA containing 0.1% saponin to permeabilize cells. After extensive washing in PBS, the cells were incubated for 1 hour at room temperature with 2μg/ml fluorescein isothiocyanate-conjugated goat anti-mouse secondary antibody (Jackson ImmunoResearch Laboratories, Inc, West Grove, PA) in PBS/0.1% BSA containing 0.1% saponin. Coverslips were mounted in VectaShield mounting media with DAPI (Vector Laboratories, Burlingame, CA), and images were taken at 40X with a Leica DM5000 fluorescence microscope, using OpenLab software (Improvision, Lexington, MA).

Measurement of Intracellular Cyclic AMP Levels

HeLa T4 cells were transfected using the vaccinia infection/transfection system with 2μg DNA per well in 12-well plates, or stably transfected cells were plated to confluency in 12-well plates. Cells were washed twice with 1X PBS and incubated in serum-free media with 0.1mM isobutylmethylxanthine (IBMX) for 20 minutes at 37°C to inhibit phosphodiesterase. Cells were then treated with 10^{-7} M hormone, or incubated with media alone for unstimulated control conditions, at 37°C for 20 minutes. Cells were lysed in 150μl cold 0.1N HCl. The lysates were collected and neutralized in an equal volume of 50mM Tris-HCl, pH 8.0 with 4mM EDTA. 25μl of neutralized lysates were used in a competitive protein-binding assay to measure intracellular cAMP levels (48). [8-^{3}H] cAMP (Amersham, Piscataway, NJ) was used as a tracer in this assay.

The assays were performed with triplicate samples, and a linear standard curve was performed in each experiment. Statistical analysis was performed using a two-way ANOVA (GraphPad PRISM 4.0, GraphPad Software, Inc., San Diego, CA).

Metabolic labeling of transfected cells and immunoprecipitation of epitope-tagged receptors

HeLa T4 cells were grown in 6-well plates, transfected with 4μg total DNA per well for 12-15 hours using the vaccinia transfection system, starved in cysteine/methionine-deficient DMEM (Gibco BRL, Grand Island, NY) for 30 minutes, and labeled with 50μCi/well Trans [^{35}S] Label (ICN Biomedical Inc., Irvine, CA) for 3 hours at 37°C in 5% CO_2. The cells were harvested in 1X PBS, pelleted, and resuspended in 400μl RIPA buffer (150mM NaCl, 50mM Tris-HCl, pH 7.5, 1% Igepal CA-630 (Nonidet P 40), 0.5% deoxycholic acid, 0.1% sodium dodecyl sulfate) containing 0.1mM phenylmethylsulfonylfluoride, 1μg/ml leupeptin, and 1μg/ml aprotinin. Cells were lysed by a series of five freeze-thaw cycles in a dry ice-ethanol bath. The lysates were centrifuged for 10 minutes to pellet cellular debris, and the supernatant was divided into two fractions. 1μg/ml of the HA-specific 12CA5 ascites fluid or the anti-M2 monoclonal antibody against the FLAG epitope was added. Immunoprecipitation went overnight at 4°C on a hematology mixer. 30μl of a 50% suspension of protein A-Sepharose beads (Amersham Biosciences, Piscataway, NJ) in PBS was added to the tubes, and the incubation was continued for 1 hour. The beads were washed eight times with 500μl cold RIPA buffer and resuspended in 30μl 2X SDS-PAGE sample buffer (50mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 100mM dithiothreitol, 0.1% bromophenol blue). The samples were boiled for 5 minutes, then separated by SDS-PAGE using a Tris-glycine buffer with the Benchmark Pre-stained Protein Ladder (Invitrogen, Carlsbad, CA) as a size marker. Gels were fixed in 20% methanol/7% acetic acid for 30 minutes, saturated with glacial acetic acid (two five-minute washes), impregnated with

22% (w/v) 2, 5 diphenyl-ox-axole in acetic acid for 45 minutes, dried, and exposed to Kodak X-OMAT AR film (Rochester, NY). Quantification of expression was performed using the Image J program provided by the National Institutes of Health (http://rsb.info.nih.gov/ij/).

Generation of Stable Cell Lines

Human embryonic kidney 293 cells were transfected with the FLAG tagged truncated hGHRH receptor construct alone or with equivalent amounts of the HA tagged full-length hGHRH receptor construct, both of which are cloned in pcDNA3, which is neomycin-resistant. Transfections were performed using lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA), and 10μg DNA per 10cm plate. Transfected cells were selected in 400mg/L G418 (Gibco BRL, Grand Island, NY), and individual clones were isolated and proliferated for analysis. Previously generated HPR9B cells were used for full-length receptor-expressing cells (21).

Detection of RNA Expression in Stable Cell Lines

RNA was isolated from stable cell lines using the RNeasy kit (Qiagen, Valencia, CA). RNA was reverse transcribed using avian myeloblastosis virus reverse transcriptase in the presence of 1mM deoxynucleosidyltriphosphates and random hexameric oligonucleotides. Complementary DNA was amplified by PCR incorporating [^{32}P] radiolabeled-dCTP. Human ribosomal protein L19 primers were used as an internal control (5'-CTG AAG GTG AAG GGG AAT GTG-3' and 5'-GGA TAA AGT CTT GAT GAT CTC-3'). Full-length and truncated hGHRH receptor expression were detected by RT PCR with a shared 5' primer (5'-CGT GGG TGA GCT GCA AAC TGG-3') and one of two 3' primers specific to each sequence (5'-CTC ACC TCT TGG TTG AGGG AAG-3' or 5'-GTC CTT GTA GTC CCA ATA CTG-3'). PCR products were separated on 5 percent polyacrylamide gels by electrophoresis. Dried gels were exposed to

Kodak X-OMAT AR film (Rochester, NY) and PCR products were quantified using a PhosphorImager and ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Fluorescence Activated Cell Sorting

Stable cells were grown to confluency in 6cm plates. Cells were fixed in 1% paraformaldehyde in PBS for 30 minutes at 4°C. Cells were blocked in 1%BSA/0.02% sodium azide in PBS for 30 minutes at 4°C for 30 minutes. Cells were washed three to five times in PBS containing 0.02% sodium azide. Primary antibody incubation was performed at 4°C for 4 hours using a polyclonal antibody to the GHRH receptor (1:750 dilution). Cells were washed three to five times in PBS containing 0.02% sodium azide followed by secondary antibody incubation (donkey anti-rabbit conjugated to fluorescein isothiocyanate at a 1:500 dilution) for 30 minutes at 4°C. Following three washes in PBS containing 0.02% sodium azide, cells were scrape-collected in 50mM EDTA/1X PBS and run through a flow cytometer (FACSCalibur, Becton Dickinson, Franklin Lakes, NJ). 10,000 cells were counted and analysis was performed using the Cell Quest program (BD Biosciences). Statistical analysis was performed using a two-way ANOVA (GraphPad PRISM 4.0, GraphPad Software, Inc., San Diego, CA).

Measurement of Ligand Binding

Assays to measure binding to membrane fractions were performed on stably transfected cells grown in 10cm plates. Cells were washed with PBS and homogenized by 20 strokes with a Teflon-glass homogenizer on ice in 50mM Tris-HCl, pH 7.4, 5mM $MgCl_2$, 2mM EGTA, and 0.1mM phenylmethylsulfonyl fluoride. Homogenates were centrifuged for 5 min at 100 x *g*, and the supernatant was recentrifuged at 4000 x *g* for 10 min. Membrane pellets were resuspended in binding buffer (25mM HEPES, pH 7.4, 50mM NaCl, 5mM $MgCl_2$, 1mM EGTA, 0.1 mM phenylmethylsulfonyl fluoride, 0.1 mg/ml leupeptin, 1mg/ml bacitracin, and 0.1% BSA).

Approximately 50μg membrane protein was used for each reaction in a volume of 300μl with 75pM [^{125}I] hGHRH (1-44)-amide (Amersham Biosciences, Piscataway, NJ) in the presence or absence of unlabeled hormone at a concentration of 10^{-6}M. Binding reactions were performed at 25°C for 60 min and were terminated by centrifugation for 10 min at 4°C. Membrane pellets were washed with binding buffer and the bound radioligand was measured using a Micromedic 4/600 Plus Automatic Gamma Counter (Micromedic, Horsham, PA). Statistical analysis was performed using a two-way ANOVA (GraphPad PRISM 4.0, GraphPad Software, Inc., San Diego, CA).

RESULTS

Expression of the full-length and truncated GHRH receptor splice variants in transiently transfected cells

Alternative splicing in the human GHRH receptor produces a receptor protein truncated at amino acid 325. The truncated receptor is identical to the full-length receptor up to amino acid 325, where a premature in-frame stop codon results in a protein terminated just before the sixth transmembrane domain. In order to develop a system in which expression of the wild-type and truncated GHRH receptors could be independently examined, the truncated GHRH receptor was cloned with a carboxyl-terminal FLAG epitope tag (DYKDDDDK). The full-length GHRH receptor was previously cloned with a carboxyl-terminal influenza virus hemagglutinin (HA) epitope tag (YPYDVPDYA). A schematic depicts the structures of the full-length and truncated receptor proteins, and the relative location of the epitope tags (Fig. 1).

In immunoprecipitation of metabolically labeled transfected cells, a specific band corresponding to the size of the full-length GHRH receptor is pulled down with the antibody recognizing the HA epitope tag (Fig. 2A, lane 3), and a specific band corresponding to the predicted size of the truncated GHRH receptor is pulled down with the anti-FLAG antibody (Fig. 2A, lane 6). Furthermore, both constructs are localized similarly in transfected HeLa T4 cells, as shown by immunofluorescence (Fig. 2B). In permeabilized cells, diffuse expression of the receptors on the cell surface and in intracellular compartments, including the endoplasmic reticulum and Golgi apparatus, is detected.

Effect of the truncated receptor on GHRH receptor signaling

To determine the signaling properties of the truncated GHRH receptor splice variant, basal and GHRH-stimulated cAMP production were measured in cells transfected with the full-

length GHRH receptor, the truncated GHRH receptor, or both receptors. Cells transfected with the full-length GHRH receptor have measurable cAMP that increases approximately six-fold upon stimulation with 10^{-7}M GHRH (Fig.3A). In contrast, cells transfected with the truncated GHRH receptor show no increase in cAMP levels when stimulated with GHRH, indicating that the truncated GHRH receptor is not capable of signal transduction through the cAMP pathway (Fig. 3A). When the truncated GHRH receptor is co-transfected with the full-length GHRH receptor, GHRH-stimulated cAMP signaling by the full-length receptor is repressed by about 60 percent, indicating that the truncated GHRH receptor acts as a dominant negative inhibitor (Fig. 3A). The effect on signaling of the full-length receptor is dependent on the dose of co-transfected truncated receptor (Fig. 3B). The dominant negative signaling effect is lost when full-length receptor is expressed at a ten-to-one ratio with truncated receptor. Further, the reduction in cAMP signaling is greater with increasing ratios of truncated receptor expression (Fig. 3B).

In order to determine the specificity of the dominant negative effect of the truncated GHRH receptor, signaling of the VIP receptor, a member of the same family of G-protein coupled receptors with 42 percent identity to the GHRH receptor, was examined when co-expressed with the truncated GHRH receptor. The wild-type VIP receptor produces about a thirteen-fold increase in cAMP levels when stimulated with 10^{-7}M VIP (Fig. 4). When the truncated GHRH receptor is co-transfected with the wild-type VIP receptor, cAMP levels are increased about twelve-fold upon 10^{-7}M VIP stimulation (Fig. 4), which is not significantly different from the wild-type VIP receptor alone. These results suggest that the signaling effect of the truncated GHRH receptor is specific to the full-length GHRH receptor.

Co-expression of full-length and truncated GHRH receptors

To investigate the mechanism of the dominant negative signaling effect, lysates from metabolically labeled cells transfected with the full-length and truncated GHRH receptor constructs were immunoprecipitated, using both anti-HA and anti-FLAG antibodies. To examine the hypothesis that decreased expression of the full-length receptor is responsible for the observed reduction in cAMP signaling, the expression level of the full-length GHRH receptor was examined when the truncated GHRH receptor is co-expressed. The bands corresponding to the full-length receptor are approximately of equal intensity, whether the full-length receptor is transfected alone, or co-transfected with the truncated GHRH receptor (Fig. 5, lanes 1 & 5). When the truncated GHRH receptor is co-transfected, the band corresponding to the full-length receptor is 94 percent the intensity of the band that is pulled down from cells transfected with the full-length receptor alone, indicating that the dominant negative signaling effect is not caused by a change in the amount of full-length GHRH receptor protein expression.

In order to test whether the truncated GHRH receptor interacts with the full-length GHRH receptor, co-immunoprecipitation experiments were performed. Immunoprecipitation of one receptor also brings down the second when both are co-expressed, indicating that the two receptors form a complex. When co-transfected lysates are immunoprecipitated with the HA antibody, 29 percent of the truncated receptor is pulled down with the full-length receptor. Similarly, when co-transfected lysates are immunoprecipitated with the FLAG antibody, 31.6 percent of the full-length receptor is pulled down with the truncated receptor (Fig. 5, lanes 5 & 6). Formation of the complex is ligand-independent, as the cells in the experiment were unstimulated. To test whether ligand stimulation alters the extent of complex formation, immunoprecipitation of lysates from stimulated versus unstimulated cells was performed. When

cells are stimulated with GHRH, there is no significant difference in the amount of receptor involved in complex formation. In unstimulated cells 31.8 percent of the full-length GHRH receptor is pulled down with the antibody to the truncated receptor. In GHRH stimulated cells 28.8 percent of the full-length GHRH receptor is pulled down with the antibody to the truncated receptor (data not shown). Similarly, in unstimulated cells 29 percent of the truncated GHRH receptor is pulled down with the antibody to the full-length receptor, while in stimulated cells 29.3 percent of the truncated receptor is pulled down with the antibody to the full-length receptor (data not shown).

As a control, immunoprecipitations on lysates from metabolically labeled cells transfected with the truncated GHRH receptor and the VIP receptor, which is epitope tagged with HA, were performed. Bands corresponding to multiple glycosylation states of the VIP receptor are immunoprecipitated with the anti-HA antibody (Fig. 6, lane 1), and a specific band corresponding to the size of the truncated GHRH receptor is detected by immunoprecipitation with the anti-FLAG antibody (Fig. 6, lane 4). In co-expressing cells, no complex formation is detected between the wild-type VIP receptor and the truncated GHRH receptor (Fig. 6, lanes 5 & 6). These data are consistent with the signaling studies and again suggest a specific interaction between the truncated GHRH receptor and the full-length GHRH receptor.

Expression and Signaling of Full-length and Truncated GHRH Receptors in Stably Transfected Cell Lines

To investigate the specific mechanism by which this complex exerts its dominant negative effect on signaling of the full-length GHRH receptor, cell surface expression was examined quantitatively by fluorescence-activated cell sorting (FACS). Because the FACS experiments were not sensitive enough to detect expression in transiently transfected cells, stable

cell lines were generated to examine cell surface localization and ligand binding. Cells expressing full-length, truncated, or both receptor constructs were analyzed for RNA expression (Fig. 7). The clones used for analysis express the receptors at high levels, and the co-expressing FTGR10 cells express equivalent levels of both receptor mRNAs. To confirm the dominant negative signaling effect of the truncated receptor, stable cell lines were used in a cAMP measurement assay. Untransfected HEK293 cells do not produce cAMP in response to GHRH stimulation (Fig. 8). HPR9B cells, which express only the full-length receptor, show a four-fold increase in cAMP upon GHRH stimulation (Fig. 8). The truncated receptor does not signal through the cAMP pathway in response to GHRH in stably transfected TGR4 cells, and acts as a dominant inhibitor of full-length receptor signaling in co-expressing FTGR10 cells (Fig. 8). The stable cell lines were subsequently used to examine the mechanism of the dominant negative signaling effect of the truncated GHRH receptor.

Cell Surface Localization of the Full-length and Truncated GHRH Receptors

Cell surface localization of the variant receptor expressed alone and co-expressed with the wild-type receptor was examined in stably transfected cell lines using FACS. The full-length GHRH receptor is expressed on the cell-surface, as shown by a shift in fluorescence in HPR9B cells compared to untransfected HEK293 cells (light gray line) using an antibody that recognizes the N-terminus of the GHRH receptor (Fig. 9A). The same antibody recognizes the truncated GHRH receptor, which is also detected on the cell-surface of TGR4 cells (Fig. 9B). When the full-length and truncated GHRH receptors were co-transfected in FTGR10 cells, cell-surface expression was also detectable (Fig. 9C), as expected, though it is not possible to differentiate full-length from truncated receptors on the cell surface because it is not possible to place epitope tags in the N-terminus, as N-terminal tags disrupt receptor function (44). Quantification of

FACS experiments shows that the percent of cells expressing surface receptors is not significantly different for TGR4 cells expressing the splice variant or FTGR10 cells co-expressing both receptors (Fig. 9D). Similarly, there is no significant difference in mean fluorescence per cell (Fig. 9D), which represents an index of receptor density. These data indicate that equivalent numbers of receptors reach the cell surface of cells expressing the full-length receptor and cells expressing the truncated receptor or both receptors. Because the antibody detects all receptors in the FTGR10 co-expressing cells and the truncated and full-length receptors are expressed at equivalent levels in these cells, it is not possible to determine which species are on the cell surface. It is possible that approximately half of each receptor species reaches the cell surface, or that only the truncated receptor reaches the cell surface. To differentiate the possibilities, ligand binding in stable cell lines was examined.

Ligand Binding to Full-length and Truncated GHRH Receptors

Membrane fractions were isolated from each stable cell line and incubated with iodinated GHRH in the presence or absence of 10^{-6}M unlabeled GHRH. Untransfected HEK293 cells bind a small amount GHRH non-specifically, probably through the VIP receptor (44) (Fig. 10). HPR9B cells expressing the full-length receptor bind GHRH, which is fully competed in the presence cold GHRH (Fig. 10). The TGR4 cell line, expressing the truncated receptor, shows significantly higher binding levels in the absence of competitor than those seen in the HEK293 cells, but binding is not competed with cold GHRH (Fig. 10). These results are perhaps indicative of variable non-specific binding. The FTGR10 cell line that co-expresses the wild-type and truncated GHRH receptors clearly shows reduced binding compared to HPR9B cells expressing the full-length receptor, though cold GHRH still significantly competes iodinated ligand binding in FTGR10 cells (Fig. 10). The effective abolishment of GHRH binding in co-

expressing cells indicates that the predominant mechanism for the dominant negative signaling effect of the truncated GHRH receptor involves an inability to bind ligand.

DISCUSSION

The discovery of splice variant receptors that have diverse signaling activities indicates further complexity in the way that GHRH regulates the production and release of growth hormone. The characterization of a major splice variant of the human GHRH receptor lends insight into the role that this isoform of the receptor may play in normal development and in pathological conditions. In addition, elucidating the mechanism of how this splice variant receptor affects signaling of the wild-type receptor has revealed a possible new role for oligomerization in normal functioning of the wild-type GHRH receptor.

Examination of the signaling properties of the truncated GHRH receptor indicates that the splice variant receptor is unable to signal through the cAMP pathway. That the truncated GHRH receptor is incapable of cAMP signaling is not surprising, given that the splice variant receptor has an incomplete third intracellular loop and lacks the cytoplasmic tail, which have been shown to be essential for proper G-protein coupling in other G-protein coupled receptors (40), and lacks the third extracellular loop, which is involved in high-affinity GHRH binding and receptor activation (49). Additionally, the complete lack of the last two transmembrane domains may disrupt the structure or topology of the truncated receptor, which may be the cause for its inability to effectively bind ligand and signal. The dominant negative activity that the truncated GHRH receptor exerts on the wild-type receptor is intriguing. To establish whether the effect was specific to the GHRH receptor, signaling of a closely related G-protein coupled receptor, the VIP receptor, was examined, and it was determined that the truncated GHRH receptor has no effect on signaling of this close family member, which is 42 percent identical to the GHRH receptor. These data indicate that the truncated GHRH receptor has some specific effect on the full-length GHRH receptor, and is not acting by competing for G-proteins, for example. The

specificity of the effect alluded to a mechanism for dominant negative activity that involved an interaction between the truncated and full-length GHRH receptors, which was confirmed by co-immunoprecipitation studies. The proposed model for the dominant negative activity of the truncated receptor is that the truncated receptor forms a complex with the wild-type receptor. These truncated receptor containing complexes reach the cell surface, but do not bind GHRH at wild-type levels, perhaps due to a conformational change in the structure of the complex. The truncated receptor may bind GHRH at lower levels, but cannot signal through the cAMP pathway, and likely competes for dimerization with the wild-type receptor, downregulating GHRH signaling of the wild-type receptor, changing the predominant species on the cell surface.

Studies in several GPCR's suggest the existence of multiple activation conformations for the receptors, which impart altered ligand affinities (37, 50-53). Even if the truncated receptor binds GHRH at low levels, its inability to activate signaling is not surprising, given the lack of TM6 and TM7 and the third extracellular loop. Chimeric receptor studies in the GHRH receptor indicate that the third extracellular loop, along with transmembrane domains 6 and 7, which are all lacking in the truncated splice variant, are important for ligand binding and signaling activity of the GHRH receptor (49). In addition, studies in the rhodopsin receptor show that ligand binding induces movements of TM3 and TM6, which may be involved in achieving appropriate conformation of the intracellular loops required for G-protein coupling (54-57). In another GPCR, the M3 muscarinic receptor, data suggest that TM5 and TM6 movement occurs as a result of ligand binding (58). Studies in the β_2-adrenergic receptor suggest that multiple intermediate conformations of GPCR's are required for each step of signal activation (59). The different conformation requirements indicate that structural integrity of GPCR's is essential to proper signal activation, in addition to high affinity ligand binding.

Though the GHRH receptor has not yet been shown to homodimerize, this report shows that it can form a complex with a splice variant receptor that is identical to the wild-type receptor for the first 325 amino acids. While we do not know if this complex includes directly interacting GHRH receptors, this would be the simplest explanation, given the wealth of evidence supporting such interactions in other G-protein coupled receptors (60-66). The fact that the truncated GHRH receptor, missing the last 98 amino acids of the full-length GHRH receptor, can form a complex with the wild-type GHRH receptor indicates that the interaction motif is located somewhere within the first 325 amino acids of the protein. Alternatively, a domain inhibiting interaction may be located within the last 98 amino acids of the wild-type GHRH receptor, and, when removed, the receptor may be allowed to interact. Interestingly, the VIP receptor, which is 42 percent identical to the GHRH receptor, does not interact with the truncated GHRH receptor in co-transfected cells.

Oligomerization of G-protein coupled receptors is increasingly recognized as a mechanism by which this class of receptors, classically thought to act as monomers, alters its functions under different physiological conditions. In several examples of G-protein coupled receptor oligomerization, differences in complex formation are linked to changes in physiological or pathophysiological conditions (67). Ligands that bind G-protein coupled receptors have different effects on the oligomer state of different receptors, in some cases increasing the formation of oligomers, as with the somatostatin receptor (65), the dopamine and adenosine receptors (62), and the β_2-adrenergic receptor (60, 68). In other examples, ligand binding appears to decrease the formation of oligomers, such as opioid δ and κ receptors, which produce monomers from dimers in the presence of a ligand for the δ receptor (61). Several

instances of ligand-independent dimerization have also been reported (69-71), indicating many different potential roles for oligomerization in G-protein coupled receptors.

The interaction between the truncated and full-length GHRH receptors is ligand-independent. This result conflicts with previous computer models that suggested that the GHRH receptor might form a dimer when bound to its ligand, when an α-helical region of the N-terminus of a family B G-protein coupled receptor interacts with an α-helical region in the C-terminus of the hormone to form a coiled-coil (72). Oligomerization of GPCR's in general has been suggested to provide a mechanism for masking hydrophobic patches or retention signals that would keep receptors in the ER (73). Given that the truncated GHRH receptor is identical to the wild-type receptor for the first 325 amino acids, it is reasonable to suggest that a truncated receptor could mask ER retention signals of the wild-type receptor, allowing the nonfunctional complex to be transported to the cell surface. Many mutants and splice variants of G-protein-coupled receptors that exert dominant negative effects on wild-type receptors act by decreasing or preventing cell-surface localization (74-78), often retaining the receptor complex in the ER, probably through an inability to mask ER retention signals; however, a model exists for allowing heterodimers to reach the cell surface and differentially regulate signaling (71). Dimers can form in the ER and be trafficked to the cell surface, where ligands might alter oligomerization state or bind differentially to heterodimers. A truncated splice variant of the $GABA_{B(1)}$ receptor acts similarly to the truncated GHRH receptor described here, heterodimerizing with the wild-type receptor, forming a complex with reduced ability to bind hormone and signal (34).

The human GHRH receptor splice variant examined here differs from known isoforms in other species both in protein structure and in its signaling properties, which may be informative to dissecting how the normal receptor functions. The long isoform of the rat receptor results

from alternative splicing at the same intron/exon boundary as the truncated human receptor (28). The long rat receptor can bind GHRH, not surprisingly, considering the intact extracellular domains, but cannot signal through the cAMP pathway (28). The insertion in the third cytoplasmic loop of the long rat receptor may disrupt G-protein coupling, as the third intracellular loop is important to G-protein activation (40). In addition, a carboxyl terminal isoform of the rat GHRH receptor was identified in both normal and dwarf animals (79). This isoform has a substitution of the last 5 amino acids and an addition of 17 amino acids in the carboxyl terminus, and is functional in response to GHRH. The differences of the composition of these variant receptors, taken together with different ligand binding and signaling abilities divulge functional roles for the different domains of the GHRH receptor. For example, receptor isoforms with intact extracellular domains bind GHRH normally, but the third extracellular loop, which is lacking in the truncated GHRH receptor, is important to high affinity binding, as suggested by chimeric receptor studies (49). Both the C-terminus of the third intracellular loop and the entire C-terminal tail are missing in the truncated receptor, which cannot signal through the cAMP pathway. Combined with the lack of signaling of the long isoform of the rat receptor, these data support the importance of these domains in G-protein activation. Because the extension of the C-terminus in the rat GHRHβ receptor isoform does not affect receptor function, it is clear that full function can be achieved with an intact receptor through the N-terminal part of the intracellular tail.

The effect of the truncated GHRH receptor on signaling of the full-length GHRH receptor and the fact that it is expressed in both normal pituitary and GH-secreting pituitary adenoma raises the question of its potential roles in physiological and pathological conditions. One hypothesis involves a preferential expression of the truncated splice variant receptor during

physiological and pathological states that require a dampening of GH signal, such as in the excess GH secretion observed in pituitary adenomas. In this view, the truncated splice variant receptor would be preferentially expressed in response to excess GH secretion, representing a mechanism to downregulate GHRH signaling, and, thereby, GH production. Understanding the regulation of splice variant expression and determining the interaction domains of interacting G-protein coupled receptors will prove essential for learning the structures of the complexes, and for potentially manipulating the oligomerization states of G-protein coupled receptors, such as the GHRH receptor.

This report examines the dominant negative activity of a truncated GHRH receptor splice variant, and determines a specific mechanism by which the dominant negative action is achieved. In addition, the discovery of complex formation between the splice variant receptor and the wild-type receptor supports a role for dimerization or higher order oligomerization in GHRH signaling, and elucidates a new mechanism by which this receptor can modify its activity, forming complexes with other isoforms of the GHRH receptor, and possibly with different G-protein coupled receptors. The data in this report add to the quickly expanding field of GPCR oligomerization, supporting the idea that interactions between receptors of this class represents an important means by which a single type of receptor can diversify its signaling properties.

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FIGURE LEGENDS

Figure 1 Schematic Representation of Full-Length *(A)* and Truncated *(B)* GHRH Receptors, The full-length GHRH receptor is epitope tagged with influenza hemagglutinin (HA) on the C-terminus. The truncated GHRH receptor is tagged with a C-terminal FLAG-epitope tag. Conserved residues of the closely related VIP receptor are shaded in black.

Figure 2 Expression of Full-length and Truncated GHRH Receptors, *(A)* Immunoprecipitation of metabolically labeled vaccinia infected/transfected HeLa T4 lysates. Cells were either mock transfected (*lanes 1 and 2*), transfected with the HA-tagged full-length GHRH receptor (*lanes 3 and 4*) or transfected with the FLAG-tagged truncated GHRH receptor (*lanes 5 and 6*) and immunoprecipitated using either the 12CA5 monoclonal antibody to the HA epitope tag or the anti-M2 antibody to the FLAG epitope tag and separated by SDS-PAGE on a 10% gel. ***(B)*** Immunofluorescence localization of epitope-tagged full-length and truncated human GHRH receptors. Because the receptor constructs are C-terminally tagged, the cells were permeabilized with 0.1% saponin to detect the receptors using the αHA or αFLAG antibodies. Data are representative of three independent experiments.

Figure 3 Stimulation of cAMP Production by GHRH in Cells Expressing Full-length and Truncated GHRH Receptors, *(A)* Lysates from vaccinia infected/transfected HeLa T4 lysates were used to measure basal and GHRH-stimulated cAMP levels in cells expressing either the full-length receptor, the truncated receptor, or co-expressing equivalent amounts of the full-length and truncated receptors. Shown is the average fold increase in cAMP production from nine experiments with triplicate samples in each experiment. ***(B)*** Cells were transfected with varying doses of full-length or truncated receptors, and cAMP assays were performed. Shown is the average fold increase in cAMP production from four experiments with triplicate samples in

each experiment. For both panels, the values from unstimulated cells transfected with the full-length GHRH receptor were used as basal, which was set equal to one. Error bars represent the SEM. Statistical analysis was performed using a two-way ANOVA analyzing receptor expression and GHRH stimulation (***, $p<0.001$; F (interaction)=11.6, F (stimulation)=18.54, F (receptor)=29.25).

Figure 4 Stimulation of cAMP Production by VIP in Cells Expressing VIP and Truncated GHRH Receptors, Lysates from vaccinia infected/transfected HeLa T4 cells were used to measure basal and VIP-stimulated cAMP levels in cells expressing either the VIP receptor or equivalent amounts of the VIP receptor and truncated GHRH receptor. Data represent the average fold increase in cAMP production from nine experiments with triplicate samples in each experiment. The values from unstimulated cells transfected with the VIP receptor were used as basal, which was set equal to one. Error bars represent the SEM. Statistical analysis was performed using a two-way ANOVA analyzing receptor expression and VIP stimulation. For stimulated cAMP levels in the presence and absence of co-transfected truncated GHRH receptor, $p>0.05$ (F (interaction)=0.04311, F (receptor)=0.06005, F (stimulation)=23.96).

Figure 5 Expression of Full-length and Truncated GHRH Receptors in Co-transfection Experiments, Lysates from metabolically labeled vaccinia infected/transfected HeLa T4 cells were immunoprecipitated with either the 12CA5 monoclonal antibody to the HA epitope tag or the anti-M2 antibody to the FLAG epitope tag, and separated by SDS-PAGE on a 10% gel. Lanes 1 and 2 are lysates from cells transfected with the full-length GHRH receptor. Lanes 3 and 4 are lysates from cells transfected with the truncated GHRH receptor. Lanes 5 and 6 are lysates from cells co-transfected with equal amounts of full-length and truncated GHRH receptors. Data are representative of three independent experiments. Statistical analysis was

performed using a two-way ANOVA analyzing receptor expression and GHRH stimulation. For each comparison, there were no significant differences ($p>0.05$).

Figure 6 Expression of Wild-type VIP and Truncated GHRH Receptors in Co-transfection Experiments, Using metabolic labeling of vaccinia infected/transfected HeLa T4 cells and SDS-PAGE as described, lanes 1 and 2 are lysates from cells transfected with the wild-type VIP receptor. Lanes 3 and 4 are lysates from cells transfected with the truncated GHRH receptor. Lanes 5 and 6 are lysates from cells co-transfected with the wild-type VIP receptor and the truncated GHRH receptor at a two-to-one ratio to normalize protein expression. Data are representative of three independent experiments.

Figure 7 Expression of Full-length and Truncated GHRH Receptors in Stably Transfected Cells, RNA was isolated from stable cell lines and used in RT-PCR to examine expression of GHRH receptor constructs. HPR9B cells express the full-length GHRH receptor, TGR4 cells express the truncated GHRH receptor, and FTGR10 cells express both full-length and truncated GHRH receptors. Primers amplifying either the full-length receptor (Full) or the truncated receptor (Trunc) or control primers to ribosomal protein L19 (RPL19) were used, as described in Materials and Methods. Data are representative of three independent experiments.

Figure 8 Stimulation of cAMP Production by GHRH in Stably Transfected Cells Expressing Full-length and Truncated GHRH Receptors, Lysates from stably transfected cells were used to measure basal and GHRH-stimulated cAMP levels in cells expressing either the full-length receptor alone, the truncated receptor alone or equivalent amounts of the full-length and truncated GHRH receptor. Data are representative of three independent experiments with triplicate samples in each experiment. Error bars represent the SEM. Statistical analysis was performed using a two-way ANOVA analyzing receptor expression and GHRH stimulation

(**, $p<0.01$; ***, $p<0.001$; F (interaction)=6.323, F (receptor)=7.162, F (stimulation)=15.64).

Figure 9 Cell Surface Localization of Truncated and Full-length Receptors in Stably Transfected Cells, ***(A-C)*** Stably transfected cells were fixed in 0.1% paraformaldehyde and incubated with a primary polyclonal antibody to the GHRH receptor followed by secondary antibody conjugated to FITC. Cells were then run through a flow cytometer for fluorescence activated cell sorting. Data are representative of three independent experiments. The x-axis represents intensity of the fluorescein fluorophore for each cell. ***(D)*** Quantification of cells expressing surface receptors and average fluorescence is shown. Data represent the average of three independent experiments. Statistical analysis was performed using a two-way ANOVA analyzing receptor expression and either percent cells expressing surface receptor or average mean fluorescence. For percent cells expressing surface receptor as well as fluorescence intensity, there is no significant difference for all conditions ($p>0.05$) (F (interaction)=1.229, F (receptor)=2.176).

Figure 10 GHRH Binding to Full-length and Truncated GHRH receptors in Stably Transfected Cells, Cells were incubated with 70pM ^{125}I-GRF in the presence or absence of 10^{-6}M cold GHRH competitor and collected for counting on a gamma counter. In cells expressing only the full-length receptor, cold GHRH significantly reduces iodinated GHRH binding ($p<0.001$). In cells co-expressing full-length and truncated receptors, cold GHRH significantly reduces iodinated GHRH binding ($p<0.05$), but overall binding levels are significantly reduced with respect to full-length receptor alone ($p<0.001$). Data represent the average of three independent experiments. Error bars represent the SEM. Statistical analysis was performed using a two-way ANOVA analyzing receptor expression and cold GHRH

competition (ns, $p>0.05$; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; F(interaction)=67.2, F (receptor)=77.31, F(competitor)=160.1).

Figure 1

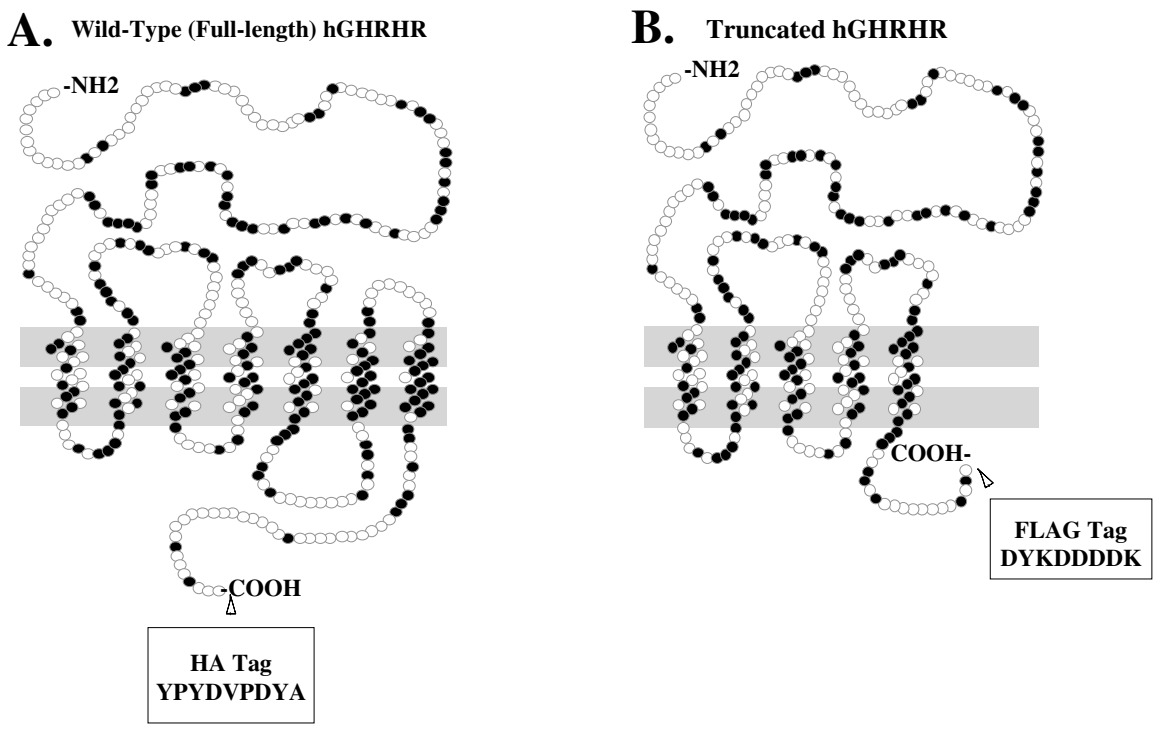
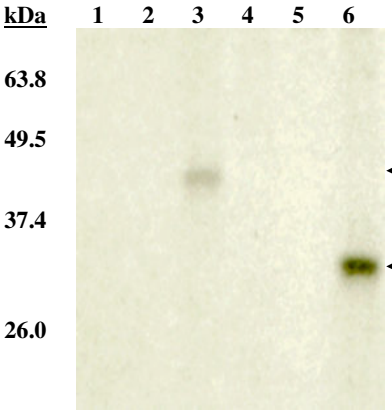

A.
Wild-Type (Full-length) hGHRHR
-NH2
-COOH
HA Tag
YPYDVPDYA
B.
Truncated hGHRHR
-NH2
COOH-
FLAG Tag
DYKDDDDK

Figure 2

A.



Full hGHRH-R			+	+		
Trunc hGHRH-R					+	+
αHA Ab	+		+		+	
αFLAG Ab		+		+		+

B.

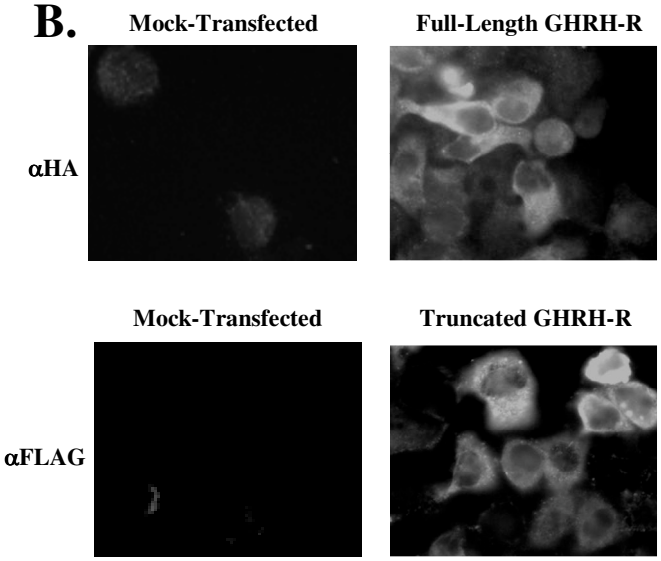


Figure 3

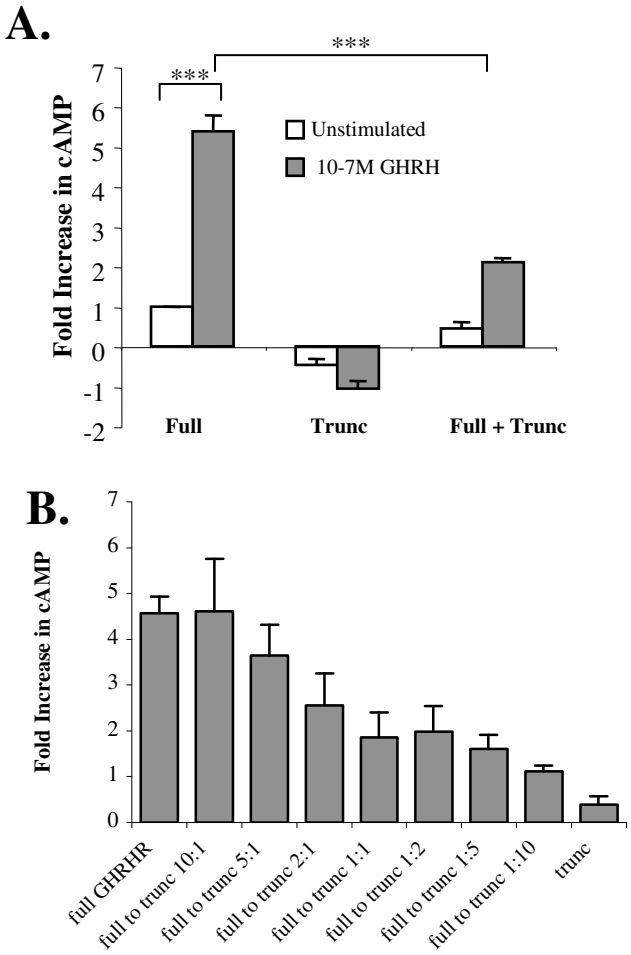

A.
***
***
Unstimulated
10-7M GHRH
Fold Increase in cAMP
7
6
5
4
3
2
1
0
-1
-2
Full
Trunc
Full + Trunc
B.
Fold Increase in cAMP
7
6
5
4
3
2
1
0
full GHRHR
full to trunc 10:1
full to trunc 5:1
full to trunc 2:1
full to trunc 1:1
full to trunc 1:2
full to trunc 1:5
full to trunc 1:10
trunc

Figure 4

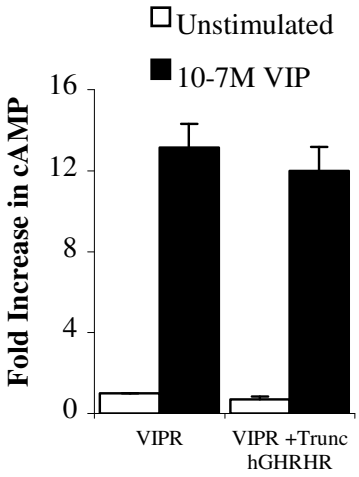
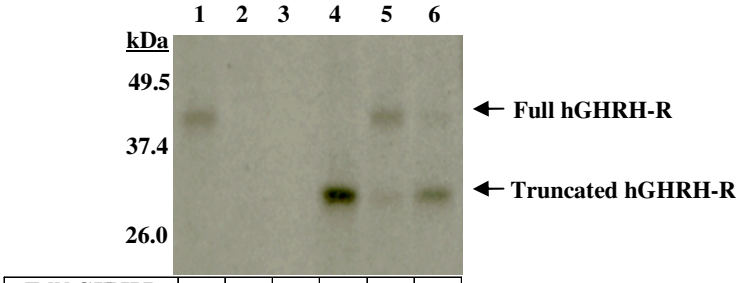
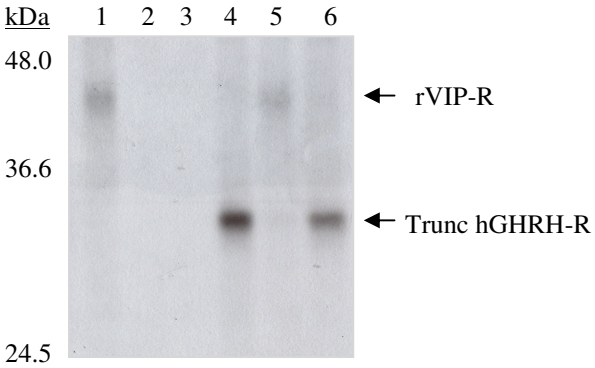

Unstimulated
10-7M VIP
Fold Increase in cAMP
16
12
8
4
0
VIPR
VIPR +Trunc hGHRHR

Figure 5



Full hGHRH-R	+	+			+	+
Trunc hGHRH-R			+	+	+	+
αHA Ab	+		+		+	
αFLAG Ab		+		+		+

Figure 6



rVIP-R	+	+			+	+
Trunc hGHRH-R			+	+	+	+
αHA Ab	+		+		+	
αFLAG Ab		+		+		+

Figure 7

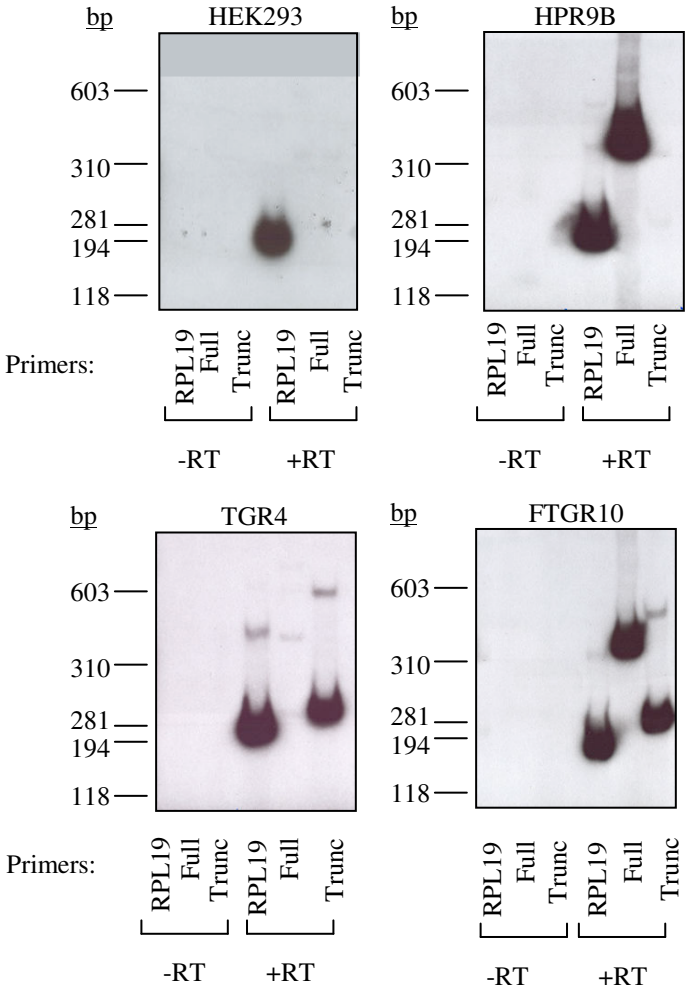

bp
HEK293
603
310
281
194
118
Primers:
RPL19
Full
Trunc
RPL19
Full
Trunc
-RT
+RT
bp
HPR9B
603
310
281
194
118
RPL19
Full
Trunc
RPL19
Full
Trunc
-RT
+RT
bp
TGR4
603
310
281
194
118
Primers:
RPL19
Full
Trunc
RPL19
Full
Trunc
-RT
+RT
bp
FTGR10
603
310
281
194
118
RPL19
Full
Trunc
RPL19
Full
Trunc
-RT
+RT

Figure 8

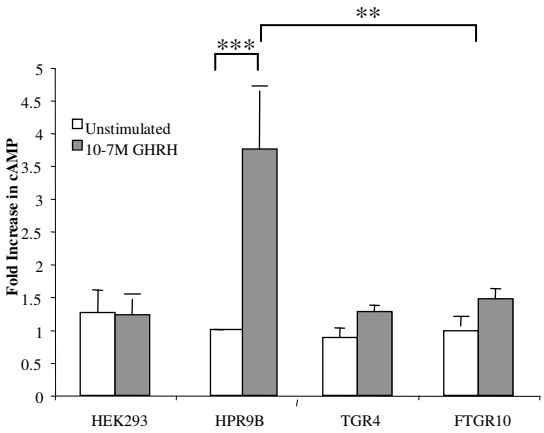
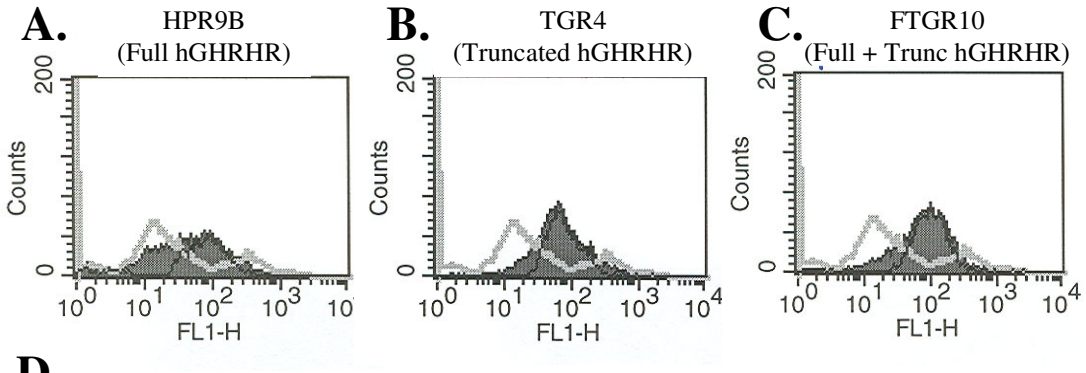

**
***
Unstimulated
10-7M GHRH
Fold Increase in cAMP
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
HEK293
HPR9B
TGR4
FTGR10

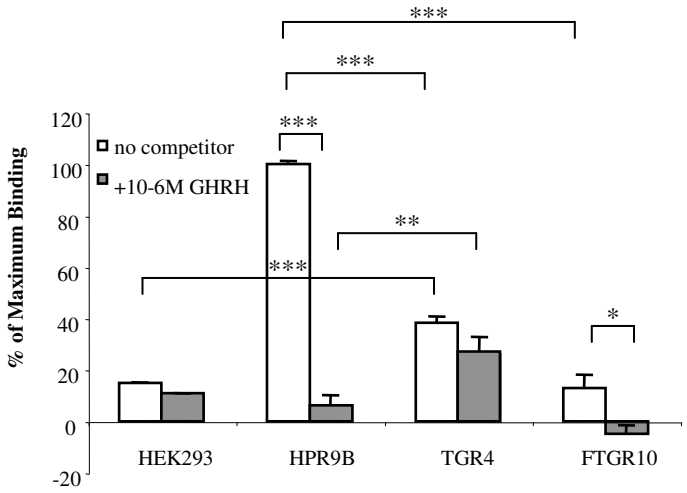
Figure 9



D.

	HPR9B	**TGR4**	**FTGR10**
Average % Cells Expressing Surface Receptor (±SEM):	43.64 ± 2.93	58.2 ±2.3	45.02 ±9.48
Average Mean Fluorescence (±SEM):	109.47 ±11.68	108.42 ±16.06	74.6 ±17.57

Figure 10


***
***
***
**
***
*
120
100
80
60
40
20
0
-20
% of Maximum Binding
no competitor
+10-6M GHRH
HEK293
HPR9B
TGR4
FTGR10