0013-7227/99/$03.00/0
Endocrinology

Vol. 140, No. 11
Printed in U.S.A.

The Mutant Growth Hormone-Releasing Hormone (GHRH) Receptor of the *Little* Mouse Does Not Bind GHRH*

BRUCE D. GAYLINN, VENITA I. DEALMEIDA, CHARLES E. LYONS JR., KENNETH C. WU, KELLY E. MAYO, AND MICHAEL O. THORNER

Department of Medicine (B.D.G., C.E.L., M.O.T.), University of Virginia, Charlottesville, Virginia 22908; and Department of Biochemistry, Molecular Biology & Cell Biology (V.I.D., K.C.W., K.E.M.), Northwestern University, Evanston, Illinois 60208

ABSTRACT

The *little* mouse is a dwarf strain characterized by low levels of GH, pituitary hypoplasia, and an unresponsiveness to treatment with exogenous GHRH. The defect has been mapped to a missense mutation in the GHRH receptor gene that abolishes the function of the receptor, but the mechanism of this inactivation is unknown. Receptor function might be affected at the level of protein expression, maturation and processing, localization to the cell surface, ligand binding, or signaling. In this study, Western blots, using antiserum raised against the GHRH receptor and immunoprecipitation analysis of epitope-tagged receptors, demonstrate that both wild-type and mutant receptor proteins are expressed at equivalent levels in transfected cells. Immunofluorescence analysis of intact and permeabilized cells expressing the epitope-tagged receptors suggests that wild-type and *little* mouse receptors are similarly localized to the cell surface. A species homologous binding assay was developed and used to show that ^{125}I-mouse GHRH binds with high affinity to the wild-type mouse receptor but not to the *little* mutant receptor. Consistent with this, the mutant receptor fails to stimulate intracellular cAMP accumulation. Our results demonstrate that the *little* mutation does not dramatically affect the expression level, glycosylation, or cellular localization of the receptor protein but that it blocks specific GHRH binding, and therefore, signaling does not take place. (*Endocrinology* **140:** 5066–5074, 1999)

GROWTH IN VERTEBRATES is controlled by the hypophyseotropic peptides somatostatin and GHRH, which suppress and stimulate, respectively, the secretion of GH from the somatotroph cells of the anterior pituitary (1, 2). GHRH is a peptide hormone produced by the neurosecretory cells of the hypothalamic arcuate nuclei that acts through its receptor on pituitary somatotroph cells, resulting in G protein coupling, adenylyl cyclase activation and cAMP production, Ca^{2+} influx, and increased expression and secretion of GH.

The *little* mouse is a dwarf strain with an autosomal recessive defect characterized by substantially reduced levels of circulating GH and GH messenger RNA (mRNA) and anterior pituitary hypoplasia, with fewer pituitary somatotroph cells and sparse granulation (3–7). Cultured somatotroph cells from these animals do not release GH upon GHRH treatment but secrete GH upon treatment with cAMP or agents that increase cAMP levels, indicating that the defect is in the GHRH signaling pathway (5, 6). Genetic mapping and cloning studies have localized this defect to a point mutation in the N-terminal extracellular domain of the GHRH receptor (8, 9), where an aspartic acid residue at position 60 is mutated to glycine. Consistent with the dwarf phenotype, the mutant receptor is inactive, and cells expressing the mutant receptor do not accumulate cAMP in response to GHRH (8, 9). Mutations in the GHRH receptor have recently been identified in heritable isolated GH deficiencies in humans (10–13), and thus, establishment of the molecular and cellular basis of these inactivating mutations promises to enhance our understanding not only of normal regulation of the GH axis but also of diseases of GH secretion.

The GHRH receptor belongs to family B-III of the G protein-coupled receptor (GPCR) superfamily (14), whose members include the receptors for secretin, vasoactive intestinal peptide (VIP), pituitary adenylate cyclase-activating peptide (PACAP), GLP-1, glucagon, and glucose-dependent insulinotropic polypeptide (GIP, also known as gastric inhibitory peptide). All these receptors share a high degree of homology and share several conserved residues, including the aspartic acid residue that is mutated in the *little* mouse. In all receptors of family B-III, the N-terminus has been shown to be important for ligand binding (15–24). Studies on the GHRH receptor have indicated that the N-terminus is essential for ligand binding (24–26) but that the N-terminus from VIP or secretin receptors can substitute for the GHRH receptor N-terminus, resulting in chimeric receptors that bind GHRH (24). This demonstrates that residues of the transmembrane domains and/or the connecting extracellular loops are important in determining the specificity of ligand binding.

There are multiple levels at which the mutation of the aspartate 60 to glycine in the GHRH receptor might impact receptor function. It could influence the folding of the receptor protein, resulting in protein instability and thus decreased levels of receptor expression. It could affect transport of the receptor through the endoplasmic reticulum-Golgi network, resulting in lower cell surface expression of the

Received March 29, 1999.
Address all correspondence and requests for reprints to: Bruce D. Gaylinn, Box 511, Department of Medicine, University of Virginia, Charlottesville, Virginia 22908. E-mail: bg2g@virginia.edu.
* This work was supported by NIH Grants DK-45350 (to B.D.G.) and DK-48071 (to K.E.M.).

receptor protein. The mutation could also alter receptor glycosylation, because it is only 10 residues from the single site for N-linked glycosylation at amino acid 50. Alternatively, the GHRH receptor protein might be normally expressed, but the *little* mutation might impact the ability of the receptor to bind its ligand, GHRH. Finally, the mutation might interfere with an appropriate hormone-induced conformational change affecting G protein coupling and signaling. To characterize the defect in the GHRH receptor of the *little* mouse, we used protein blot analysis and immunoprecipitation to compare expression and glycosylation of the wild-type and mutant receptor proteins and immunofluorescence analysis to compare the subcellular localization of the 2 receptors. We also developed a homologous binding assay for the mouse GHRH receptor and compared the ability of the wild-type and mutant GHRH receptors to bind GHRH and to activate adenylyl cyclase. Our results point to a selective defect in GHRH binding by the *little* mutant GHRH receptor.

Materials and Methods

Generation of stable cell lines

Full-length complementary DNA (cDNA) clones for the mouse GHRH receptors were isolated by RT-PCR amplification of pituitary RNA from homozygous wild-type and *little* mice, as described (8). The cDNAs were subcloned into the eukaryotic expression vector pcDNA-1 and used for the generation of stable cell lines. HEK293 cells were transfected using lipofectin reagent (Life Technologies, Inc., Gaithersburg, MD) and 10 μg DNA/100 mm plate at a 9:1 ratio of the cDNA expression construct to pSV2neo. Transfected cells were selected in the presence of 400 μg/ml G418 (Sigma, St. Louis, MO), and individual clones were isolated and expanded for analysis. Cells were tested for expression of the receptor mRNAs by Northern analysis, and clones expressing comparable levels of mRNA for the two receptors were maintained in 400 μg/ml G418 and used for Western analysis and binding and signaling studies. The cells stably expressing the human GHRH receptor were generated previously (27).

Generation of antibodies to the GHRH receptor and Western blotting

The synthetic peptide Ac-HMHPEADFITQLREDESAALQAAC-COOH, modeled after the proposed N-terminus of the mature human GHRH receptor after signal peptide cleavage (28) and with two internal cysteines replaced with alanines, was prepared by solid-phase synthesis and purified by HPLC, and its identity was confirmed by mass spectrometry (University of Virginia Biomolecular Research Facility). This peptide was then coupled through a C-terminal cysteine to maleimide-activated keyhole limpet hemocyanin (Pierce Chemical Co., Rockford, IL) and used to raise antiserum in rabbits (HRP Antiserum Services, Denver, PA). This crude antiserum was used directly for the detection of mouse or human GHRH receptor by Western analysis.

Crude membranes from HEK293 cells expressing the various receptors were extracted in 5 mM 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) and 11.4 μg of total protein, as measured using the bicinchoninic acid assay (Pierce Chemical Co.), was electrophoresed on SDS-polyacrylamide gels with prestained low-range molecular weight standards (Life Technologies, Inc.), as previously described (29). Deglycosylation was performed by treatment with peptide-*N*-glycosidase F (PNGase F) (Roche Molecular Biochemicals, Indianapolis, IN), as described (29). The separated proteins were then electroblotted to nitrocellulose membrane (Protran, Schleicher & Schuell, Inc., Keene, NH), and electrophoretic transfer to nitrocellulose was confirmed by ponceau S staining of the blot. Crude rabbit sera, before (preimmune) or after immunization, were used at a 1:10,000 dilution and incubated with blots at 4 C overnight. The antibody-antigen complexes were detected by incubation with a 1:10,000 dilution of horseradish peroxidase-conjugated goat antirabbit antibody and visualized using the enhanced chemiluminescence method (Amersham Pharmacia Biotech, Arlington Heights, IL).

Generation and expression of epitope-tagged receptor constructs

An oligonucleotide primer complementary to the 3′ end of the mouse GHRH receptor and encoding the sequence for the hemagglutinin (HA) epitope (30) was synthesized. Recognition sites for the enzymes *Kpn*I and *Xba*I were engineered on either side of the HA epitope with the stop codon within the *Xba*I site. This primer, together with an upstream primer within the third intracellular loop of the receptor cDNA was used to amplify a 700-bp fragment from the 3′ region of the receptor. The PCR product was cloned in context with the 5′ end of the wild-type or mutant receptor into the expression vector pcDNA-3 (Invitrogen, San Diego, CA) to generate full-length constructs for the wild-type and mutant receptor, having the HA epitope at the C-terminus [mGHRHRwt.HA (wild-type mouse GHRH receptor) and mGHRHR*lit*.HA (*little* mouse GHRH receptor)].

An oligonucleotide primer complementary to the 5′ end of the mouse GHRH receptor and encoding the sequence for the FLAG epitope (31) was synthesized and used along with a downstream primer in the third transmembrane domain of the receptor, to amplify a 700-bp fragment from the 5′ end of the cDNAs of the wild-type and *little* mouse GHRH receptors. The PCR products from the wild-type and *little* mouse receptor were cloned in context with the 3′ end of the receptor into the expression vector pcDNA-3, to generate full-length constructs for the respective receptors having the FLAG epitope at the N-terminus (mGHRHRwt.FLAG and mGHRHR*lit*.FLAG).

To assess the activity of the epitope-tagged wild-type mouse GHRH receptors, ligand binding and signaling were measured, and it was observed that the presence of the HA tag did not affect the activity of the receptor. The introduction of the FLAG tag at the N-terminus of the wild-type mouse GHRH receptor disrupted hormone binding in a manner similar to the FLAG-tagged human GHRH receptor (24); however, the receptor was expressed at levels comparable with the HA-tagged wild-type receptor and was present at the cell surface of intact cells.

The epitope-tagged constructs of the wild-type and mutant mouse GHRH receptors were expressed in HeLa T4 cells, as described (24), using the Vaccinia virus-T7 RNA polymerase system (obtained under license from Dr. Bernard Moss, National Institutes of Health, Bethesda, MD) (32). Cells were transfected using plasmid DNAs that were complexed with liposomes at a ratio of 4–5 μg lipid/μg DNA (33).

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

Transfected cells expressing the epitope-tagged receptor were labeled with Pro-mix *in vivo* cell labeling mix (L-[^{35}S]Methionine and L-[^{35}S]Cysteine, Amersham Pharmacia Biotech) and immunoprecipitated, as described (24), using the anti-HA monoclonal antibody 12CA5 (a gift from Dr. R. A. Lamb, Northwestern University). The samples were separated by SDS-PAGE using a Tris-glycine buffer with See-Blue Pre-Stained Standard (Novex, San Diego, CA) as size markers. The gels were fixed in 20% methanol/7% acetic acid, saturated with glacial acetic acid, impregnated with 22% wt/vol of 2,5-diphenyl-oxazole in acetic acid, dried, and exposed to Kodak X-OMAT AR film (Eastman Kodak Co., Rochester, NY).

Immunofluorescence localization of the wild-type and mutant epitope-tagged receptors

Subconfluent monolayers of HeLa T4 cells, cultured on glass coverslips, were transfected, the coverslips were washed twice in PBS, and immunofluorescense analysis was performed as described (24). For permeabilized cells, the coverslips were incubated for 2 h at 4 C with 3 μg/ml of the anti-FLAG M2 monoclonal antibody (Kodak IBI Division, New Haven, CT) or 1 μg/ml of the HA specific 12CA5 ascites fluid in PBS containing 0.1% saponin. After washing, the coverslips were incubated at 4 C for 30 min with 2 μg/ml fluorescein isothiocyanate-conjugated goat antimouse secondary antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) in PBS containing 0.1% saponin and 0.2% whole goat serum, washed with PBS, and mounted using FITC-Guard (Testog Inc., Chicago, IL). For nonpermeabilized cells, incubation was carried out using similar conditions with primary and secondary antibodies diluted in PBS (without saponin). The images of cells are

optical sections obtained using confocal laser scanning microscopy with a Bio-Rad Laboratories, Inc. MRC 600 connected to a Nikon microscope using a 40× objective. All samples were scanned using the same contrast settings for equivalent times, and optical sections were taken through the central plane of the cell.

Iodination of GHRH analogs and assay of GHRH binding and cAMP accumulation

Mouse GHRH and [His^1, Nle^{27}]human GHRH (1–32)NH_2 were purchased from Peninsula Laboratories, Inc. (Belmont, CA). These were iodinated to low stoichiometry using the Iodobead method (Pierce Chemical Co.) and purified by reverse-phase HPLC, as previously described (29).

GHRH binding was measured in crude membrane pellets permeabilized with alamethicin, as described (29). Each tube contained approximately 100,000 cpm of probe corresponding to approximately 3.5×10^{-11} M ^{125}I-GHRH and was incubated for 1 h at room temperature. The crude membranes were pelleted, detergent extracted with 5 mM CHAPS, and free GHRH was removed with charcoal-dextran. Soluble, receptor-bound GHRH was then assayed using a γ-counter (29). The binding data were analyzed by nonlinear least-squares fitting to model curves using the computer program Ligand (34). Intracellular cAMP was assayed by RIA of extracts from cells grown in 24-well cluster plates and pretreated with isobutylmethylxanthine, as described (28).

Photoaffinity cross-linking

The UV-activatable heterobifunctional cross-linking reagent ANB-NOS (Pierce Chemical Co.) was coupled at low stoichiometry to the lysines of [His^1, Nle^{27}]human GHRH (1–32)NH_2, and the crude products were iodinated as above. The iodinated photoprobe was then purified from this mix by HPLC, and used in the GHRH binding protocol, as described above. After 60-min binding, the GHRH-receptor complexes were cross-linked with long-wave UV, SDS denatured, analyzed by gel electrophoresis as described for Western blots, and then autoradiographed as described (29). Endoglycosidase H was obtained from Roche Molecular Biochemicals (Indianapolis, IN)

Results

Immunological detection of the mouse GHRH receptor

A polyclonal antiserum against the GHRH receptor was raised in rabbits using a synthetic peptide corresponding to the first 23 amino acids of the predicted N-terminus of the human GHRH receptor as the antigen. This region is highly conserved among GHRH receptors from different species, but is not conserved in related receptors that bind other ligands. Because 19 of these 23 amino acids are identical in the human and mouse GHRH receptors, this strategy allowed the production of an antiserum found to recognize human, mouse, and also rat GHRH receptors.

The specificity of the antiserum for the GHRH receptor was confirmed by using it to probe Western blots of CHAPS-extracted crude membranes from nontransfected HEK293 cells and from cells transfected with the mouse, *little* mouse, and human GHRH receptors (Fig. 1). Consistent with previous GHRH cross-linking studies (29), a band of approximately 52 kDa was detected in cell lines transfected with the human GHRH receptor cDNA (Fig. 1D, lane 2). A similar band of slightly greater mobility was seen in cell lines transfected with either wild-type mouse or *little* mouse GHRH receptors (Fig. 1, B and C). This band was not observed in control HEK293 cells, and it was not detected by preimmune serum in the control or receptor-expressing cell lines. As a further proof of specificity, inclusion of excess of the synthetic peptide immunogen blocked the labeling of this band by GHRH receptor antibody. After treatment with PNGase F (Fig. 1, lane 3), the receptor-specific band shifted to a greater apparent mobility, as expected from our previous observations of receptor glycosylation (29). Because deglycosylation with endoglycosidase H (not shown) gave the same results as the PNGase F treatments in Fig. 1, this suggests that the glycosylation of both wild-type and *little* GHRH receptors seen in these Western blots is of the high mannose, core-glycosylated type.

UV cross-linking, using iodinated GHRH photoprobe and electroblotting under the same conditions as the Western blots, shows both complex and core-glycosylated forms of the GHRH receptor in cell lines expressing the wild-type receptor (Fig. 2). To better resolve receptor glycosylation forms, these gels were run in 10% polyacrylamide *vs.* 7.5% polyacrylamide in Fig. 1. In Fig. 2, lane 2 demonstrates three distinct receptor glycosylation forms, each of which bind GHRH, because they are detected by cross-linking of the labeled ligand. Lane 3 shows that all of these forms can be deglycosylated to a higher mobility form by PNGase F. Lane 4 shows that endoglycosidase H, which can cleave high mannose (core glycosylated) but not complex carbohydrates, shifts the lowest of the glycosylated forms to the deglycosylated position but does not shift the upper 2 bands. This cross-linking data demonstrates that both core glycosylated

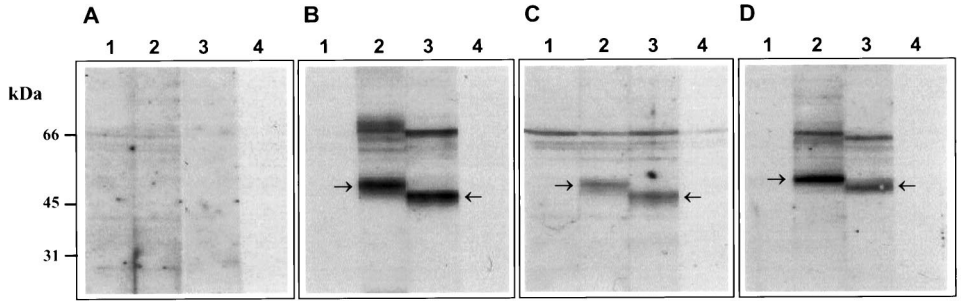

FIG. 1. Western protein blot analysis with GHRH receptor antiserum. Immunoblots of membrane extracts from nontransfected HEK293 cells (A), or stable HEK293 cell lines transfected with the following constructs: mGHRHRwt (B); mGHRHR*lit* (C), and wild-type human GHRH receptor (hGHRHRwt) (D). The samples in the lanes were probed as follows; 1) preimmune serum; 2) receptor antiserum; 3) receptor antiserum after deglycosylation with PNGase F; and 4) receptor antiserum after preincubation with 100 pg/ml of the synthetic peptide used as antigen for the generation of the antibody. The positions of the receptor, before and after deglycosylation, are marked with *arrows*. Matched amounts of protein were loaded in A, B, and C.

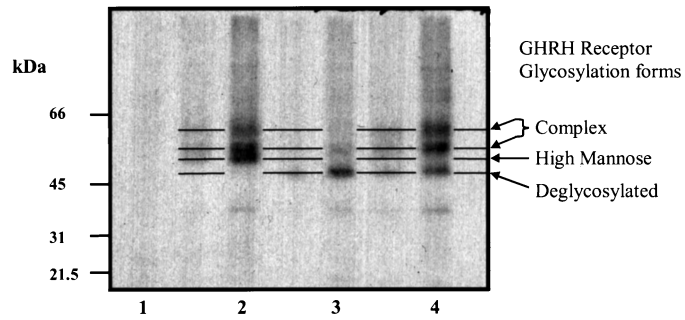

FIG. 2. Photoaffinity cross-linking to GHRH receptor. Autoradiograph of a 10% PAGE separation of GHRH receptor from homogenized crude membranes of HEK293 cells stably transfected with wild-type human receptor and photoaffinity cross-linked with His^1, $^{125}I\text{-}Tyr^{10}$, ANBNOS-Lys^{12}, Nle^{27} hGHRH (1–32)NH_2. Lane 1 shows cross-linking in the presence of 10 nM unlabeled competing GHRH. A sample, cross-linked without competing GHRH, was split into thirds and incubated for 72 min, at 37 C, with no enzyme (lane 2), PNGase F (lane 3), or endoglycosidase H (lane 4) (each unmarked lane contains a lesser loading of the sample to its *right*). This experiment was repeated three times, with the same result.

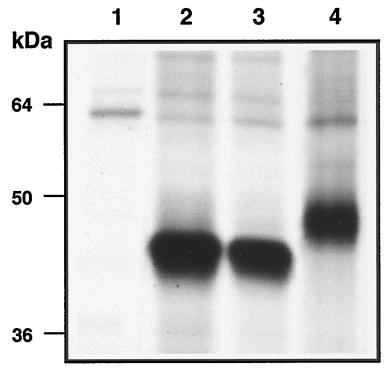

FIG. 3. Immunoprecipitation of the epitope-tagged wild-type and *little* mouse GHRH receptors. Immunoprecipitation of the epitope-tagged mouse GHRH receptor from HeLa T4 cells, transfected with the pcDNA-3 vector or the various receptor constructs in pcDNA-3, was performed using the monoclonal antibody 12CA5 against the HA epitope, followed by precipitation with protein A-Sepharose beads. The lanes on the SDS gel show proteins immunoprecipitated from cells transfected with: 1) pcDNA-3; 2) mGHRHRwt.HA; 3) mGHRHR-*lit*.HA; and 4) hGHRHR.HA. The apparent difference in the receptor mobility, relative to Figs. 1 and 2, is likely attributable to differences in the gel systems and size standards used.

and complex glycosylated forms of the wild-type GHRH receptor are accessible in our permeabilized binding assay and bind GHRH. Thus, complex glycosylation is not required for GHRH binding. It is not clear why the complex glycosylated receptor was not detected by Western analysis.

The experiments with untagged receptors used stable cell lines expressing mouse and *little* mouse receptors that had been selected from multiple clones for matched levels of receptor mRNA using Northern analysis. For the work with epitope-tagged receptors, we used a transient expression system that allowed us to obtain higher and more consistently matched receptor expression levels. The cDNAs for the wild-type and *little* mouse GHRH receptors were modified to include the sequence for an HA epitope at the C-terminus of the expressed protein. Immunoprecipitation of the epitope-tagged mouse GHRH receptors transiently transfected in HeLa T4 cells (Fig. 3), using anti-HA monoclonal antibody 12CA5, confirmed that the wild-type and mutant receptor proteins were produced in similar abundance.

Cellular localization of the wild-type and mutant mouse GHRH receptors

The cDNAs for the wild-type and *little* mouse GHRH receptors were also modified to insert the sequence for the FLAG epitope at the N-terminus of the expressed receptor so that an extracellular epitope could be used to detect the protein. These constructs were used to determine the cellular localization of the GHRH receptors in intact cells. The previously described human GHRH receptor, having an N-terminal FLAG-tag (hGHRHR.FLAG), was used as a control in these experiments (24). Indirect immunofluorescence detection of both the FLAG- and HA-tagged mouse GHRH receptors was performed using monoclonal antibodies against the epitope-tags, and the localization of the receptors was recorded using confocal microscopy (Fig. 4). In intact cells expressing the FLAG-tagged receptors, it was observed that the mutant GHRH receptor was localized on the cell surface in a manner indistinguishable from that of the wild-type receptor, whereas in permeabilized cells expressing either the FLAG- or HA-tagged receptors, the receptors were present both on the cell surface and intracellularly. Again, no differences in the cellular distribution of the wild-type and *little* receptors were observed. Thus, aspartate 60 is not required for receptor protein expression, core glycosylation, or appropriate transport to the cell surface, although it may be required for attaining a receptor conformation necessary for high-affinity GHRH binding and subsequent activation of G_S α. To test this possibility, we developed a species homologous ligand-binding assay for the mouse GHRH receptor.

Ligand binding by the mouse GHRH receptor

In initial attempts to use ^{125}I-human GHRH to analyze ligand interaction with the mouse GHRH receptor from membranes of transfected HEK293 cells, we were unable to detect any specific binding to the wild-type mouse receptor. We therefore iodinated mouse GHRH and used the homologous ligand to establish a GHRH-binding assay for the mouse GHRH receptor. Saturation binding revealed that the binding affinities of the wild-type mouse and human receptors for their homologous ligand were not statistically different, with dissociation constant (K_d) values of 0.162 nM and 0.136 nM respectively (Fig. 5), with the receptor protein expressed at 210 and 18.9 fmol/mg protein, respectively. Competition binding of both mouse and human GHRH to membranes from cell lines expressing the mouse or human receptor was performed, and the data were normalized to the amount of receptor expressed. As shown in Fig. 6, each receptor bound better to the species-homologous ligand. Under these assay conditions, the specific binding of human GHRH to the mouse GHRH receptor was less than 10% of that seen with mouse GHRH in the same membrane preparation, explaining our previous inability to detect specific binding of human GHRH to the mouse receptor.

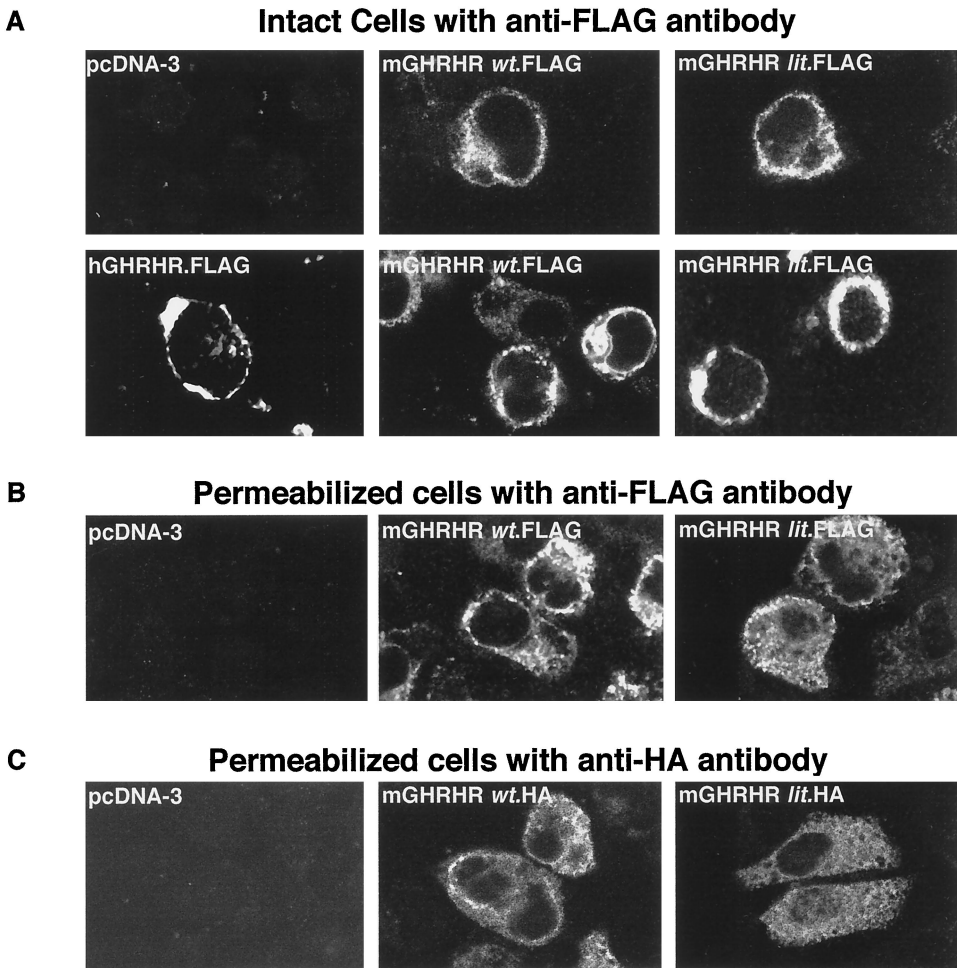

FIG. 4. Immunological localization of the epitope-tagged mouse GHRH receptor. The cellular localization of the FLAG- or HA-epitope-tagged wild-type and *little* mouse GHRH receptors was determined using indirect immunofluorescence. A, Images of intact cells transfected with the indicated constructs and detected using the monoclonal antibody to the FLAG-epitope tag. Two different fields have been shown for cells expressing the wild-type and the *little* mouse GHRH receptors. B and C, Images of cells expressing the indicated constructs that have been permeabilized with 0.1% saponin and detected using the monoclonal antibody against either the FLAG (B) or HA (C) epitope tags. All images were scanned using a confocal microscope, under the same contrast settings for equivalent times, and they are representative of at least two independent experiments.

Binding and signaling by the little mouse GHRH receptor

Mouse GHRH binding to crude membrane preparations was measured for HEK293 cells that were transfected with the wild-type or *little* mouse GHRH receptors and for nontransfected controls (Fig 7). Specific binding of GHRH to the wild-type receptor was competed by GHRH and decreased by the G protein uncoupling agent guanosine 5′-O-(3-thiotriphosphate) (GTPγS). Because some types of receptor mutations can cause inactivation resulting from intracellular accumulation of protein (35), this binding assay employed homogenized and permeabilized cell membranes to detect binding by both cell surface and intracellular membranes. Though binding to wild-type receptor could be detected on intact live cells, more apparent binding sites were seen in permeabilized membrane preparations, consistent with the presence of functional receptor at both surface and internal sites. GHRH binding to the *little* mouse receptor containing permeabilized membranes was no greater than to membranes from nontransfected cells, indicating that the mutant receptor was unable to bind GHRH.

Intracellular cAMP accumulation in response to mouse GHRH was measured, and it was observed that cells expressing the wild-type receptor accumulated cAMP in a dose-dependent manner. As expected (based on the binding data), cells expressing the *little* mouse GHRH receptor did not accumulate cAMP at significantly higher levels than the nontransfected controls (Fig. 8), confirming our previous findings in a different cell background (8).

Discussion

GHRH and its receptor are key proteins for the proliferation and differentiation of pituitary somatotroph cells, for the stimulation of GH synthesis and secretion, and the regulation of somatic growth (2, 36). The high-affinity binding of GHRH to its receptor on pituitary somatotroph cells is therefore a critical event for normal functioning of the GH axis. The *little* mouse mutation has been studied extensively as a model for human isolated GH deficiency type 1B, and recently profound growth failure analogous to that seen in the *little* mouse has been observed in humans homozygous for mutations in the GHRH receptor gene (10–13). Three studies involving distantly related kindreds identified a mutation in the human GHRH receptor gene that introduced a premature stop codon into the mRNA, 12 bp downstream from the site where the *little* mouse mutation occurs, thus encoding a truncated receptor predicted to be nonfunctional (10–12). Data from another study revealed a mutation in a donor splice site that causes the gene's first intron to be retained in the mRNA, resulting in a disruption of the translational reading frame of the protein (13). It is anticipated that

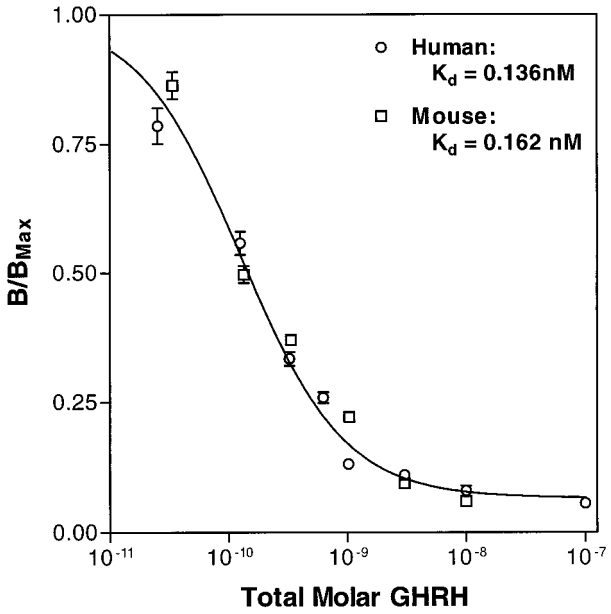

FIG. 5. Ligand binding by the mouse and human GHRH receptors. Saturation binding of the species-specific ligand by the wild-type mouse and human GHRH receptors stably expressed in HEK293 cells. The iodinated ligand was competed with the homologous cold ligand and binding graphed as the fraction of the maximum bound, which was determined by computer fitting. Binding parameters were estimated by statistically weighted nonlinear least squares fitting to a single binding site model using the program LIGAND. Each *point* is the average of four to six replicates, with the SEM shown when it is larger than the symbol. The calculated K_d values for each receptor with its homologous ligand were not statistically different.

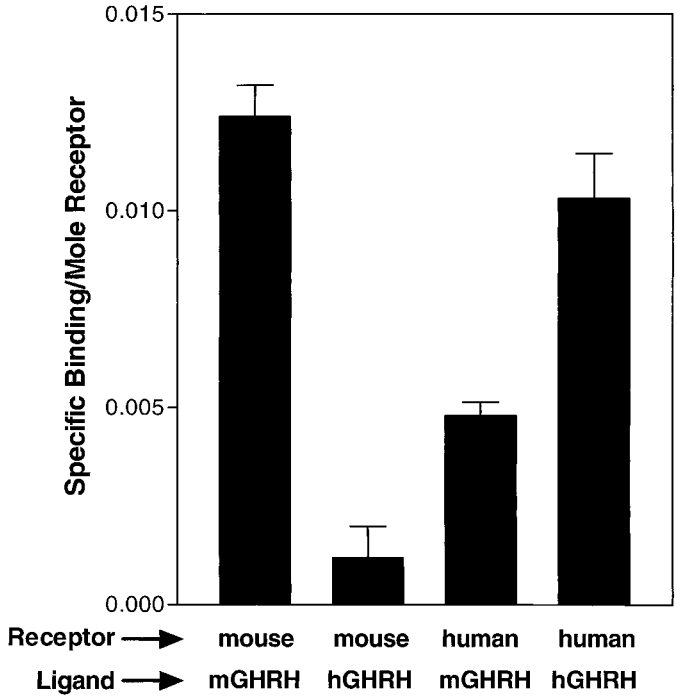

FIG. 6. Homologous and heterologous ligand binding by the mouse and human GHRH receptors. Competition binding of the mouse and human GHRH receptors was performed using ^{125}I-mouse or ^{125}I-human GHRH, in the absence of competitor or in the presence of 10 nM GHRH of the indicated species. The *vertical axis* shows the total moles of GHRH bound per specific site, as calculated from computer fitting of the saturation binding data from Fig. 1. Each *bar* is the average of four replicates ± SEM.

other GHRH receptor mutations will be discovered in patients with abnormalities of GH secretion, and studies on the *little* mouse should thus prove useful as a model of human disease.

Several inherited human diseases have been attributed to single amino acid substitutions in membrane proteins that either affect protein folding, resulting in the degradation of the protein with a reduction in the level of mature protein (37–39); or affect glycosylation, processing, and transport, resulting in a decrease in the functional protein at the cell surface (35, 38, 40–42). The most striking example of such a phenomenon is the ΔF508 mutant of the cystic fibrosis transmembrane conductance regulator. The mutant protein is misfolded, contains only core carbohydrates, and is rapidly degraded within a pre-Golgi compartment, resulting in a decrease in the level of mature protein in the cell (37–39). In another example, a point mutation in the extracellular domain of the LH and FSH receptors resulted in core-glycosylated receptors that are retained in the endoplasmic reticulum of transfected cells (35). The mutant LH receptor was unresponsive to its ligand because of its localization but bound its ligand in a permeabilized membrane binding assay. The analogous mutation in the closely related FSH receptor resulted in both retention in the endoplasmic reticulum and loss of ability to bind ligand, even in permeabilized cells (35). In several other diseases, single-point mutations in GPCRs have been shown to abolish the ligand-binding ability of the receptor (40, 43, 44). In the type-2 vasopressin receptor, a point mutation in the first extracellular loop decreases the cell surface expression of the receptor as well as the affinity of the receptor for its ligand (40), whereas the mutation of a conserved proline residue in the N-terminus of the PTH receptor affects the binding and, consequently, the signaling ability of the receptor, without affecting the cell surface localization of the receptor (44).

To investigate the expression, glycosylation, and cellular localization of the GHRH receptor protein, and to determine whether differences in these processes might explain the loss of function of the *little* mutant receptor, we generated a receptor-specific polyclonal serum and used it to detect GHRH receptor protein in stably transfected HEK293 cells. Western blots of cells stably transfected with the wild-type and mutant GHRH receptor constructs indicate that both proteins are expressed and are of a comparable size and that the *little* mutation, which is only 10 residues away from the site of N-linked glycosylation of the receptor, does not inhibit core glycosylation of the receptor protein (Fig. 1). This core glycosylation is comparable in both the wild-type and mutant receptors and is therefore unlikely to be a consequence of the mutation. Complex glycosylation could only be seen by cross-linking (Fig. 2), which only shows receptors functional in binding and so could give no information about the *little* mutant.

Using the alternative detection strategy of epitope-tagging the receptor proteins, we observed that using equivalent

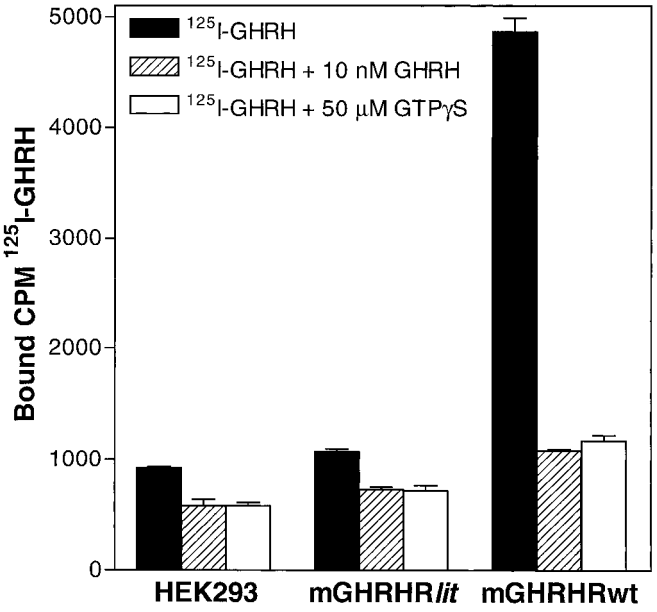

FIG. 7. Binding of GHRH to the wild-type or *little* mouse GHRH receptor. Binding of ^{125}I-mouse GHRH to membranes from nontransfected HEK293 cells or stable lines expressing the mGHRHRwt or mGHRHR*lit* was performed. Each reaction represents binding to crude membranes prepared from 3.0×10^5 cells. *Bars* show the total counts bound in the absence of competitor or in the presence of 10 nM mouse GHRH or 50 μM GTPγS. Each *bar* is the average of four replicates ± SEM.

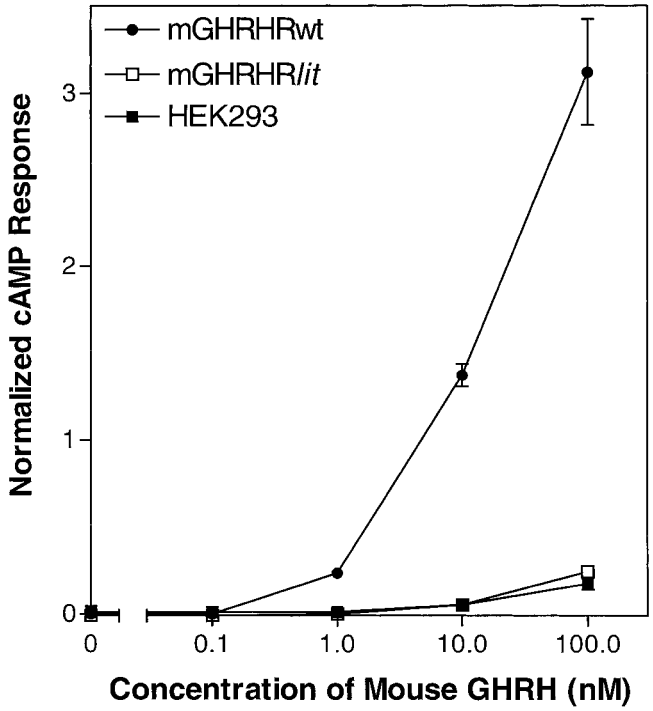

FIG. 8. GHRH-stimulated cAMP accumulation in cell lines expressing the wild-type or *little* mouse GHRH receptor. Dose-dependent accumulation of cAMP in response to mouse GHRH in nontransfected HEK293 cells, or stable cell lines expressing the mGHRHRwt or mGHRHR*lit*, was measured. Data were normalized to the response seen with 1 μM isoproterenol in matching wells (typically 100–120 pmol cAMP/well), to adjust for variations in cell density. Each *point* is the average of four replicates, with the SEM shown when it is larger than the symbol.

amounts of wild-type and *little* DNA in transient transfection experiments resulted in equivalent levels of expressed receptors, suggesting that the *little* mutant protein does not have a significantly reduced stability. Interestingly, both Western protein blot analysis of the native receptors and immunoprecipitation of the epitope-tagged receptors indicate that the human GHRH receptor migrates more slowly than the mouse GHRH receptor, although the cDNAs from both species encode an identical number of amino acids and the proteins are predicted to have similar masses. This difference persists, even after complete deglycosylation with PNGase F, suggesting that the difference in mobilities between the two receptors is attributable to a higher negative charge in the human receptor (six more negative charges are expected, based on differences in protein sequence).

We were able to use the epitope-tagged GHRH receptors for immunofluorescence experiments designed to investigate cellular localization of the receptor proteins. Our studies, using both intact and permeabilized cells expressing the wild-type and mutant forms of the epitope-tagged mouse GHRH receptor, indicate that both receptors have a similar cellular distribution. Both receptors could be visualized on the cell surface in intact cells using confocal microscopy. Importantly, the mutant GHRH receptor does not seem to be trapped intracellularly to any extent. Overall, these results indicate that the wild-type and mutant GHRH receptor proteins have similar expression levels, glycosylation pattern, and cellular distribution, implying that the defect is at the level of binding or signaling.

To determine whether the *little* mouse GHRH receptor is defective in ligand binding, we found it necessary to develop a species homologous assay using iodinated mouse GHRH. Binding of human GHRH to the human, ovine, bovine, porcine, rat, and mouse receptors has been previously examined, and the mouse receptor was found to be the only one that did not bind human GHRH at appreciable levels. GHRH binding to mouse pituitary membranes displayed the same species-specificity as was seen with the recombinant mouse receptor (data not shown). This is most likely because, of all the characterized mammalian GHRH peptides, mouse GHRH has the least sequence similarity to human GHRH, with 11 substitutions or deletions in the biologically active region of the peptide, which includes residues 1–29 (45–47). The decreased ability of the mouse GHRH receptor to bind human GHRH, combined with the relatively high nonspecific binding of GHRH to HEK293 cells, makes specific binding of iodinated human GHRH to the mouse receptor difficult to detect and explains previous difficulties in studying GHRH binding by the mouse receptor using the human peptide (48).

We observed a low level of GHRH-displaceable binding (Fig. 7) and GHRH-induced stimulation of cAMP (Fig. 8) in nontransfected HEK293 cells, even though GHRH receptor mRNA was not detectable in Northern blots of these cells. This trace binding to nontransfected cells could be significantly displaced by 50 μM GTPγS (Fig. 7) or 10 nM VIP (data not shown). The GTPγS effect suggests that the observed binding is to a GPCR that can be uncoupled by the nonhydrolyzable GTP analog. As in our assay, 10 nM VIP does not

displace GHRH binding to GHRH receptors (not shown); this suggests that the trace GHRH binding is caused by the ability of GHRH to cross-react with some closely related endogenously expressed GPCR, such as that for VIP or PACAP (49, 50). Consistent with this idea, RT-PCR analysis, using degenerate primers designed to detect related receptors of this family, demonstrate that HEK293 cells express the type I PACAP receptor but not the GHRH receptor (unpublished results).

Our data indicate that the GHRH receptor bearing the *little* mutation is defective at the level of GHRH binding. When the homologous conserved aspartic acid residue was mutated in the related receptors for glucagon (51) and VIP (17), the mutant receptors did not bind their respective ligands. This aspartic acid is conserved in nearly all known members of GPCR family B. Because these receptors bind diverse nonhomologous ligands (14) it is unlikely that it plays a role in the determination of ligand specificity. It is not anticipated that this aspartate interacts directly with GHRH; instead, this residue probably provides an acidic charge crucial for the structure or conformation required for hormone binding in all receptors of this family. Studies on the GHRH receptor have indicated that the N-terminus is essential for ligand binding (24–26) and deletion of this domain or alterations, such as the introduction of an epitope-tag into this domain, can result in inactivation of the receptor (24). Our current study demonstrates that the conserved aspartic acid at position 60 in the N-terminus of the GHRH receptor is essential for the interaction with the ligand, further supporting the role of the N-terminal domain in ligand recognition.

The discovery that the *little* mutation in mice is in the gene encoding the GHRH receptor (8, 9) provides support for the importance of the GHRH signaling system in normal growth. Our findings provide a cellular and molecular basis for understanding the defect in GHRH signal transduction in the *little* mouse and provide a foundation for further studies on the interaction of GHRH with its receptor.

Acknowledgments

We are grateful to Kevin W. Huff for technical help, to the University of Virginia Biomolecular Research Facility and Pratt Bequest for peptide synthesis work, and to Dr. Robert Lamb (Northwestern University) for the 12CA5 monoclonal antibody against the HA epitope.

References

1. **Gelato MC, Merriam GR** 1986 Growth hormone releasing hormone. Annu Rev Physiol 48:569–591
2. **Frohman LA, Jansson JO** 1986 Growth hormone-releasing hormone. Endocr Rev 7:223–253
3. **Eicher EM, Beamer WG** 1976 Inherited ateliotic dwarfism in mice. Characteristics of the mutation, *little*, on chromosome 6. J Hered 67:87–91
4. **Cheng TC, Beamer WG, Phillips JA, Bartke A, Mallonee RL, Dowling C** 1983 Etiology of growth hormone deficiency in *little*, Ames and Snell dwarf mice. Endocrinology 133:1669–1678
5. **Clark RG, Robinson IC** 1985 Effects of a fragment of human growth hormone-releasing factor in normal and '*little*' mice. J Endocrinol 106:1–5
6. **Jansson JO, Downs TR, Beamer WG, Frohman LA** 1986 Receptor-associated resistance to growth hormone-releasing factor in dwarf "*little*" mice. Science 232:511–512
7. **Wilson DB, Wyatt DP** 1992 Histopathology of the pituitary gland in neonatal *little* (*lit*) mutant mice. Histol Histopathol 7:451–455
8. **Godfrey P, Rahal JO, Beamer WG, Copeland NG, Jenkins NA, Mayo KE** 1993 GHRH receptor of *little* mice contains a missense mutation in the extracellular domain that disrupts receptor function. Nat Genet 4:227–232
9. **Lin SC, Lin CR, Gukovsky I, Lusis AJ, Sawchenko PE, Rosenfeld MG** 1993 Molecular basis of the *little* mouse phenotype and implications for cell type-specific growth. Nature 364:208–213
10. **Wajnrajch MP, Gertner JM, Harbison MD, Chua Jr SC, Leibel RL** 1996 Nonsense mutation in the human growth hormone-releasing hormone receptor causes growth failure analogous to the *little* (*lit*) mouse. Nat Genet 12:88–90
11. **Baumann G, Maheshwari H** 1997 The dwarfs of Sindh: severe growth hormone (GH) deficiency caused by a mutation in the GH-releasing hormone receptor gene. Acta Paediatr Suppl 423:33–38
12. **Netchine I, Talon P, Dastot F, Vitaux F, Goossens M, Amselem S** 1998 Extensive phenotypic analysis of a family with growth hormone (GH) deficiency caused by a mutation in the GH-releasing hormone receptor gene. J Clin Endocrinol Metab 83:432–436
13. **Salvatori R, Hayashida CY, Augiar-Olivera MH, Phillips III JA, Souza AH, Gondo RG, Toledo SP, Conceicao MM, Prince M, Maheshwari HG, Baumann G, Levine MA** 1999 Familial dwarfism due to a novel mutation of the growth hormone-releasing hormone receptor gene. J Clin Endocrinol Metab 84:917–923
14. **Kolakowski LF, Zhuang J** 1997 G protein-coupled receptor family B, in the G protein-coupled receptor database, World Wide Web site. URL: http://www.gcrdb.uthscsa.edu/FB_intro.html
15. **Holtmann MH, Hadac EM, Miller LJ** 1995 Critical contributions of amino-terminal extracellular domains in agonist binding and activation of secretin and vasoactive intestinal polypeptide receptors. Studies of chimeric receptors. J Biol Chem 270:14394–14398
16. **Cao YJ, Gimpl G, Fahrenholz F** 1995 The amino-terminal fragment of the adenylate cyclase activating polypeptide (PACAP) receptor functions as a high affinity PACAP binding domain. Biochem Biophys Res Commun 212:673–680
17. **Couvineau A, Gaudin P, Maoret JJ, Rouyer FC, Nicole P, Laburthe M** 1995 Highly conserved aspartate 68, tryptophane 73 and glycine 109 in the N-terminal extracellular domain of the human VIP receptor are essential for its ability to bind VIP. Biochem Biophys Res Commun 206:246–252
18. **Vilardaga JP, De NP, Di PE, Bollen A, Waelbroeck M, Robberecht P** 1995 Properties of chimeric secretin and VIP receptor proteins indicate the importance of the N-terminal domain for ligand discrimination. Biochem Biophys Res Commun 211:885–891
19. **Buggy JJ, Livingston JN, Rabin DU, Yoo-Warren H** 1995 Glucagon.glucagon-like peptide I receptor chimeras reveal domains that determine specificity of glucagon binding. J Biol Chem 270:7474–7478
20. **Gaudin P, Couvineau A, Maoret JJ, Rouyer-Fessard C, Laburthe M** 1996 Role of cysteine residues in the N-terminal extracellular domain of the human VIP 1 receptor for ligand binding. A site-directed mutagenesis study. Ann NY Acad Sci 805:585–589
21. **Unson CG, Cypess AM, Wu CR, Goldsmith PK, Merrifield RB, Sakmar TP** 1996 Antibodies against specific extracellular epitopes of the glucagon receptor block glucagon binding. Proc Natl Acad Sci USA 93:310–315
22. **Wilmen A, Goke B, Goke R** 1996 The isolated N-terminal extracellular domain of the glucagon-like peptide-1 (GLP)-1 receptor has intrinsic binding activity. FEBS Lett 398:43–47
23. **Gelling RW, Wheeler MB, Xue J, Gyomorey S, Nian C, Pederson RA, McIntosh CHS** 1997 Localization of the domains involved in ligand binding and activaton of the glucose-dependent insulinotropic polypeptide receptor. Endocrinology 138:2640–2643
24. **DeAlmeida VI, Mayo KE** 1998 Identification of binding domains of the growth hormone-releasing hormone receptor by analysis of mutant and chimeric receptor proteins. Mol Endocrinol 12:750–765
25. **Gaylinn BD, Lyons Jr CE, Thorner MO,** Proteolytic cleavage of photoaffinity cross-linked GHRH receptor-ligand complex identifies fragments of receptor adjacent to bound ligand. Program of the 77th Annual Meeting of The Endocrine Society, Washington, DC, 1995, p 500
26. **Kajkowski EM, Price LA, Pausch MH, Young KH, Ozenberger BA** 1997 Investigation of growth hormone releasing hormone receptor structure and activity using yeast expression technologies. J Recept Signal Transduct Res 17:293–303
27. **Mayo KE** 1992 Molecular cloning and expression of a pituitary-specific receptor for growth hormone-releasing hormone. Mol Endocrinol 6:1734–1744
28. **Gaylinn BD, Harrison JK, Zysk JR, Lyons Jr CE, Lynch KR, Thorner MO** 1993 Molecular cloning and expression of a human anterior pituitary receptor for growth hormone-releasing hormone. Mol Endocrinol 7:77–84
29. **Gaylinn BD, Lyons Jr CE, Zysk JR, Clarke IJ, Thorner MO** 1994 Photoaffinity cross-linking to the pituitary receptor for growth hormone-releasing factor. Endocrinology 135:950–955
30. **Kolodzeij PA, Young RA** 1991 Epitope tagging and protein surveillance. Methods Enzymol 194:508–519
31. **Hopp TP, Prickett KS, Price VL, Libby RT, March CJ, Cerretti DP, Urdal DL, Conlon PJ** 1988 A short polypeptide marker sequence useful for recombinant protein identification and purification. Biotechnology 6:1204–1210
32. **Fuerst TR, Earl PL, Moss B** 1987 Use of a hybrid vaccina virus-T7 RNA polymerase system for expression of target genes. Mol Cell Biol 7:2538–2544
33. **Campbell MJ** 1995 Lipofection reagents prepared by a simple ethanol injection technique. Biotechniques 18:1027–1032

34. **Munson PJ, Rodbard D** 1980 Ligand: a versatile computerized approach for characterization of ligand-binding systems. Anal Biochem 107:220–239
35. **Rozzell TG, Wang H, Liu X, Segaloff DL** 1995 Intracellular retention of mutant gonadotropin receptors results in loss of hormone binding activity of the follitropin receptor but not of the lutropin/choriogonadotropin receptor. Mol Endocrinol 9:1727–1736
36. **Mayo KE, Godfrey PA, Suhr ST, Kulik DJ, Rahal JO** 1995 Growth hormone-releasing hormone: synthesis and signaling. In: Bardin CW (ed) Recent Progress in Hormone Research. Academic Press, Inc., pp 35–73
37. **Ward CL, Kopito RR** 1994 Intracellular turnover of cystic fibrosis transmembrane conductance regulator. Inefficient processing and rapid degradation of wild-type and mutant proteins. J Biol Chem 269:25710–25718
38. **Oksche A, Schulein R, Rutz C, Liebenhoff U, Dickson J, Muller H, Birnbaumer M, Rosenthal W** 1996 Vasopressin V2 receptor mutants that cause X-linked nephrogenic diabetes insipidus: analysis of expression, processing, and function. Mol Pharmacol 50:820–828
39. **Qu BH, Strickland EH, Thomas PJ** 1997 Localization and suppression of a kinetic defect in cystic fibrosis transmembrane conductance regulator folding. J Biol Chem 272:15739–15744
40. **Birnbaumer M, Gilbert S, Rosenthal W** 1994 An extracellular congenital nephrogenic diabetes insipidus mutation of the vasopressin receptor reduces cell surface expression, affinity for ligand, and coupling to the Gs/adenylyl cyclase system. Mol Endocrinol 8:886–894
41. **Tsukaguchi H, Matsubara H, Taketani S, Mori Y, Seido T, Inada M** 1995 Binding-, intracellular transport-, and biosynthesis-defective mutants of vasopressin type 2 receptor in patients with X-linked nephrogenic diabetes insipidus. J Clinical Invest 96:2043–2050
42. **Sadeghi H, Robertson GL, Bichet DG, Innamorati G, Birnbaumer M** 1997 Biochemical basis of partial nephrogenic diabetes insipidus phenotypes. Mol Endocrinol 11:1806–1813
43. **Pan Y, Wilson P, Gitschier J** 1994 The effect of eight V2 vasopressin receptor mutations on stimulation of adenylyl cyclase and binding to vasopressin. J Biol Chem 269:31933–31937
44. **Karaplis AC, He B, Nguyen MTA, Young ID, Semeraro D, Ozawa H, Amizuka N** 1998 Inactivating mutation in the human parathyroid hormone receptor type 1 gene in Bloomstrand chondryoplasia. Endocrinology 139: 5255–5288
45. **Frohman MA, Downs TR, Chomczynski P, Frohman LA** 1989 Cloning and characterization of mouse growth hormone-releasing hormone (GRH) complementary DNA: increased GRH messenger RNA levels in the growth hormone-deficient lit/lit mouse. Mol Endocrinol 3:1529–1536
46. **Suhr ST, Rahal JO, Mayo KE** 1989 Mouse growth-hormone-releasing hormone: precursor structure and expression in brain and placenta. Mol Endocrinol 3:1693–1700
47. **Campbell RM, Bongers J, Felix AM** 1995 Rational design, synthesis, and biological evaluation of novel growth hormone releasing factor analogues. Biopolymers 37:67–88
48. **Lin C, Lin SC, Chang CP, Rosenfeld MG** 1992 Pit-1-dependent expression of the receptor for growth hormone releasing factor mediates pituitary cell growth. Nature 360:765–768
49. **Rosselin G** 1986 The receptors of the VIP family peptides (VIP, secretin, GRF, PHI, PHM, GIP, glucagon and oxyntomodulin). Specificities and identity. Peptides 1:89–100
50. **Lutz EM, Sheward WJ, West KM, Morrow JA, Fink G, Harmar AJ** 1993 The VIP2 receptor: molecular characterisation of a cDNA encoding a novel receptor for vasoactive intestinal peptide. FEBS Lett 334:3–8
51. **Carruthers CJ, Unson CG, Kim HN, Sakmar TP** 1994 Synthesis and expression of a gene for the rat glucagon receptor. Replacement of an aspartic acid in the extracellular domain prevents glucagon binding. J Biol Chem 269:29321–29328