Naturally-occurring missense mutations in the human growth hormone-releasing hormone receptor alter ligand binding

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Abstract

Growth hormone (GH) releasing hormone (GHRH) is a hypothalamic factor that stimulates GH secretion. It acts by activating a seven transmembrane domain G protein-coupled receptor of 423 amino acids expressed by the somatotroph cells of the pituitary gland (GHRH receptor, GHRH-R). Familial isolated growth hormone deficiency (IGHD) can be caused by mutations in the GHRH-R gene both in humans and mice. We have described six disease-causing missense mutations in this gene in IGHD patients (H137L, L144H, A176V, A222E, F242C, K329E). These mutations are inherited as autosomal recessive traits, and cause impairment of the receptor to transmit GHRH signalling. The aim of this study is to investigate the mechanisms through which these mutations cause receptor malfunction. To this end, we

transiently expressed each mutated receptor into Chinese hamster ovary cells. Cells expressing each of the mutated receptors did not show an increase in intracellular cyclic AMP in response to GHRH. Immunoprecipitation and immunofluorescence studies indicated that the amino acid changes do not cause protein degradation, and do not alter the proper insertion of the receptor into the cell membrane. Binding studies with human ¹²⁵I-GHRH showed that the lack of response to GHRH is due to inability of all the mutated receptors to bind the ligand. These studies demonstrate that abnormal ligand binding is a common mechanism by which naturally occurring missense mutation alter GHRH-R function.

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Introduction

The production and secretion of growth hormone (GH) from the anterior pituitary is stimulated by the hypothalamic growth hormone releasing hormone (GHRH) and inhibited by somatostatin (Muller *et al.* 1999). GHRH is secreted by the arcuate nucleus into the portal hypophyseal circulation, and causes somatotroph cells proliferation and GH synthesis and secretion by acting on a G protein-coupled receptor (GPCR), the GHRH receptor (GHRH-R). This is a 423 amino acid protein (an N-terminal 22 amino acid signal peptide is likely cleaved after translation), with one extracellular N-terminal domain (containing a potential N-glycosylation site at amino acid 50), seven hydrophobic transmembrane α -helices, and one intracellular C-terminal domain with multiple potential phosphorylation sites.

Interaction between the GHRH and its receptor leads to activation of the G_s α subunit, stimulation of adenylyl-cyclase, and increase in intracellular cyclic AMP (cAMP), which in turn promotes the phosphorylation of protein-kinase A, thereby causing cellular proliferation and GH secretion. Activation of the GHRH-R also triggers the

mitogen-activated protein kinase pathway, which may in part contribute to the proliferation of the somatotroph cells (Pombo *et al.* 2000).

The first evidence that mutations in the GHRH-R gene cause growth hormone deficiency (GHD) came from the *little* mouse, a naturally occurring murine model for human isolated GHD (IGHD), characterized by autosomal recessive dwarfism and pituitary hypoplasia (Eicher & Beamer 1976). The *little* mouse carries a point mutation in the extracellular domain of the receptor that causes a single amino acid substitution (D60G) (Godfrey *et al.* 1993, Lin *et al.* 1993). This change impairs the ability of the receptor to bind GHRH (Gaylinn *et al.* 1999). The first described mutation in the human GHRH-R gene (*GHRH-R*) was a non-sense mutation in the extracellular domain (codon 72) (Wajnrajch *et al.* 1996). Since then, several other naturally occurring *GHRH-R* mutations have been described, including a promoter mutation (Salvatori *et al.* 2002a), six missense mutations (Salvatori *et al.* 2001a, Salvatori *et al.* 2001b, Salvatori *et al.* 2002a, Salvatori *et al.* 2002b, Carakushansky *et al.* 2003), four splice-donor mutations (Salvatori *et al.* 1999, Roelfsema *et al.* 2001, Salvatori *et al.* 2002c, Alba *et al.* 2004), a second non-sense

mutations (Salvatori *et al.* 2002c), and two small deletions (Salvatori *et al.* 2001b, Horikawa 2002). All these mutations are transmitted as autosomal recessive traits, with the possible exception of one of the small deletions that has been reported to have an *in vitro* dominant negative effect (Horikawa 2002). All but the missense mutations are expected to cause the receptor to be prematurely truncated or its translation to be out of frame.

The mechanism by which the six single amino acid substitutions (H137L, L144H, A176V, A222E, F242C, and K329E) alter receptor function is not yet determined. In this study, we have examined the *in vitro* effect of these mutations on receptor expression and function. We demonstrate that the mutations do not alter proper surface expression of the receptor, but they do alter ligand binding.

Materials and Methods

Plasmid constructs

Using site-directed mutagenesis technique (Kunkel 1985) we introduced each missense mutation (H137L, L144H, A176V, A222E, F242C, K329E) into individual GHRH-R cDNA clones. Plasmids with cDNA's with different epitopes were used in different experiments:

1) Due to the lack of a functioning antibody to the human GHRH-R, for immunoprecipitation experiments we used wildtype (WT) and mutant receptor cDNAs containing the *Aequorea victoria* green fluorescent protein (GFP) tag (Tarasova *et al.* 2000) at the carboxy-terminus of the receptor in the pEGFP-N2 plasmid (Clontech, Palo Alto, CA, USA).

2) For the immunofluorescence experiments, WT cDNA receptor containing the FLAG tag in the N-terminal extracellular domain was kindly donated by Dr Kelly Mayo (Northwestern University, Chicago, IL, USA), and subcloned into pcDNA1.0 expression plasmid (Invitrogen, Carlsbad, CA, USA). cDNA fragments containing each mutation were then inserted in substitution of WT sequences (utilizing an *EcoRI* site) to obtain all 6 mutant cDNAs containing the extracellular FLAG tag.

3) For cAMP assay and for binding experiments, WT and mutant receptor cDNA cloned in pcDNA 1.0 AMP were used.

Cyclic AMP assay

In order to compare the response to GHRH of all six mutated receptors, we measured cAMP response to GHRH in Chinese Hamster Ovary (CHO) cells transiently transfected with either the WT or mutant receptor cDNA. Briefly, cells were trypsinized and seeded in 24-well plates at 2×10^5 cells/well in minimum essential medium (MEM)+10% fetal bovine serum (FBS) (Life Technologies, Gaithersburg, MD, USA) and transfected

immediately using FuGENE 6 transfection reagent (Roche, Indianapolis, IN, USA). Not transfected CHO cells (MOCK) and cells transfected with vector alone served as negative controls. Forty-eight h after transfection, cells were washed with serum free MEM containing 0.5 mmol/L isobutylmethylxanthine (IBMX) and treated with MEM+IBMX (baseline) or with MEM+IBMX+ forskolin 10^{-5} mol/L (Sigma-Aldrich, St Louis, MO, USA) or challenged with MEM+IBMX+[Nle²⁷]-GHRH-(1–29) 10^{-8} M or 10^{-9} M or 10^{-10} M (Peninsula Laboratories, Inc, Belmont, CA, USA). After 15 min incubation at 37 °C, total cAMP was extracted by addition of HCL to a final concentration of 0.1 mol/l and a cycle of freeze-thawing. Cellular cAMP in the acid extracts was measured by RIA as previously described (Levine *et al.* 1983). The results are the mean of three separate experiments, each performed in duplicate wells and each well assayed in duplicate using 100 µl cell extracts. Results have been normalized to the cAMP response to forskolin and are expressed as picomoles of cAMP produced per well.

Immunoprecipitation

CHO cells, which do not express endogenous GHRH-R, were grown in 6 well/plates in MEM+10% FBS (Invitrogen) and transfected at 60–70% confluence with 1 µg of plasmid GFP-tagged GHRH-R cDNA using FuGENE 6 transfection reagent following manufacturer's recommendations. After 48 h, cells were washed twice with ice-cold PBS, harvested and lysed on ice in RIPA buffer (1 xPBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1%SDS, 10µg/ml PMSF, protease inhibitor cocktail) (Sigma-Aldrich). The lysate was clarified by centrifugation (10 min at 15 000 g at 4 °C) and incubated overnight at 4 °C with 10 µg of mouse anti-GFP agarose-conjugated antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). Immunoprecipitates were then washed 3 times with ice cold PBS and resuspended in protein sample buffer. Samples were boiled for 5 min and protein separated on a 10% SDS-PAGE gel, followed by transfer onto polyvinylidene difluoride membrane (PVDF) (Immobilon-P, Millipore Corporation, Billerica, MA, USA). The filter was blocked for 1 h in Tris-buffered saline+0.05% Tween 20 (TBS-T) (Sigma-Aldrich)+3% non-fat dry milk (Bio Rad Lab, Hercules, CA, USA), incubated for 1 h with mouse anti-GFP antibody (1:5000) (Santa Cruz Biotechnology Inc.), washed extensively in TBS-T. After 1 h incubation with HRP-conjugated goat anti mouse IgG (1:3000), and washing in TBS-T, immunoreactivity was detected by enhanced chemiluminescence (ECL kit, Amersham Biosciences, Chalfont St Giles, Buckinghamshire, England) and band size compared with pre-stained protein weight marker (Full Range Rainbow, Amersham Biosciences). All steps were performed at room temperature.

Immunofluorescence

In order to detect whether the missense mutation could alter proper receptor expression on the cell surface we performed immunofluorescence experiments with the goal of detecting the presence of extracellular FLAG epitope in non-permeabilized transfected cells.

CHO cells were grown on glass cover slips and at 50–70% confluence were transfected with FLAG-tagged GHRH-R cDNA using FuGENE 6 transfection reagent. As control, we used CHO cells transfected with WT GHRH-R cDNA with an hemoagglutinin (HA)-tag, kindly donated by Dr Kelly Mayo, in the intracellular C-terminus. At 100% confluence cells were washed three times with PBS and fixed with fresh 2% paraformaldehyde in PBS pH 7.4 for 15 min at room temperature. After four washing steps with PBS, cells were incubated with fluorescein isothiocyanate (FITC)-conjugated anti-FLAG monoclonal antibody (1:100) (Sigma-Aldrich) for 1 h. CHO cells transfected with GHRH-R HA-tagged cDNA were incubated with monoclonal anti-HA FITC-conjugated antibodies (1:200) (Sigma-Aldrich). Cells were then washed four times with PBS at pH 8 and examined by confocal laser scanning microscopy (LSM 410, Carl Zeiss INC, Oberkochen, Germany) using a 40 x objective lens. All slides were scanned for equivalent times using the same contrast and brightness settings.

Binding assay

To determine whether the missense mutations would alter ligand binding, binding studies were performed using radiolabelled GHRH and HEK 239 membrane extracts. HEK 239 cells were chosen because binding experiments in CHO cells had proven to be technically poorly reproducible. Cells were grown in 100 mm dishes and were transfected at 60–70% confluence with GHRH-R cDNA, using FuGENE 6 transfection reagent. After 48 h, cells were washed, harvested by centrifugation, and homogenized in ice cold homogenization buffer (Hepes 50 mM, NaCl 100 mM, EDTA 10 mM, 0.1 mM EGTA, PMSF 1 mM, protease inhibitor cocktail) and 100 μ M bacitracin (Sigma-Aldrich). Supernatant was removed by centrifugation and membrane pellets were resuspended in binding buffer (TRIS 50 mM, EGTA 2 mM) adding 5 mM of alamethicin (Sigma-Aldrich) for permeabilization. For each sample, one tube was incubated for 1 h at room temperature with approximately 100 000 c.p.m. of Human (1–44)-NH₂ ¹²⁵I-GHRH (Amersham Biosciences), and the other with both human (1–44)-NH₂ ¹²⁵I-GHRH and excess (1 μ M) ‘cold’ [Nle²⁷]human GHRH (1–29)-NH₂. Following 10 min centrifugation at 15 000 g, supernatant with receptor-bound GHRH was assayed using a gamma counter. Counts represent total binding, and the difference between counts in the absence or presence of cold GHRH represents specific binding.

The experiment was repeated three times with three different sets of transfected cells. Results are the means $\pm$ S.D. of the three experiments.

Statistical analysis

Data were analyzed using SPSS statistical package (SPSS Inc., Chicago, IL, USA), and considered statistically significant if $P < 0.05$. ANOVA was used to analyze cAMP data. In the binding experiments, the difference between specific and non-specific binding of each single construct was analyzed by independent sample *t*-test, while ANOVA was performed to compare the specific binding among all different mutants, mock-transfected and WT.

Results

Cyclic AMP assay

Only the cells expressing the WT receptor showed a significant cAMP response to GHRH. The cAMP level in cells expressing the WT receptor was significantly higher compared with all other mutants at 10^{-8} and 10^{-9} M GHRH concentrations (Fig. 1). The lack of response to GHRH by the mutant receptors indicates that all the mutations impair the receptor's ability to transmit GHRH signalling.

Immunoprecipitation

To determine whether the mutated GHRH receptors were translated into mature protein, proteins extracts from CHO cells transiently transfected with either WT or mutant receptors (or with empty plasmid vector) were immunoprecipitated and analysed by Western blotting.

All six mutated receptors were translated into proteins that showed an electrophoretic mobility of about 70–75 kDa (Fig. 2). The shift in the mobility from the displayed size to the expected molecular weight of about 79 kDa (54 kDa for the receptor plus 25 kDa for the GFP-tag), is likely due to the electrophoretic conditions affecting the charge of the glycosylated protein, and no difference in size between the WT and mutants was observed. This experiment was repeated three times, with consistent results. Although immunoprecipitation is not a quantitative assay, the levels of protein expression were similar in all mutants, indicating that the mutations do not negatively affect transcriptional or translational mechanisms and did not cause protein degradation.

Immunofluorescence

To confirm that the receptors are correctly localized into the cell membrane we performed immunofluorescence studies using non-permealized CHO cells transiently

Figure 1 Basal and GHRH-stimulated (10^{-8} M, 10^{-9} M, 10^{-10} M) intracellular cAMP levels in CHO cells expressing wild type (WT) and mutated GHRH-R cDNA's. Mock-transfected cells (Mock) and cell transfected with empty vector (Vector) served as controls. The results are the mean of three separate experiments, each performed in duplicate wells, with each well assayed in duplicate. Results are normalized according to cAMP response to forskolin and are expressed as pmol/well. Bars represent S.E.M. * $P < 0.00001$.

transfected with FLAG-tagged receptor cDNA. Since the FLAG tag is on the extracellular N-terminus of the receptor, only an appropriate localization on the cell membrane would allow interaction with the fluorescent antibody. As shown in Figure 3, using confocal microscopy, no fluorescence was observed either in mock-transfected cells, in cells transfected with vector alone, or in CHO cells transfected with the intracellular C-terminal HA-tagged receptor.

Contrarily, cells expressing the WT or mutant receptors showed a comparable pattern of surface fluorescence, indicating that proper translocation towards the cell membrane and insertion of the mutated receptors had occurred.

Binding studies

Since immunoprecipitation and immunofluorescence studies indicated that the protein is translated in its entire sequence and expressed on the cell surface, we wanted to determine whether the inability of the GHRH to trigger

the physiological response to GHRH-R was due to an impaired capacity to bind to its receptor.

Crude membrane pellets were obtained by homogenization and permeabilization from cells expressing either the WT or the mutant GHRH-Rs. Permeabilization with alamethicin was performed in order to enhance specific binding, as previous studies have demonstrated that functional receptors are present also at internal sites of vesicles (Gaylinn *et al.* 1994, 1999). GHRH binding was measured by competing assay using 125 I-human GHRH (Fig. 4). Analysis of specific binding by ANOVA showed that membranes from cells expressing the WT GHRH-R had a significantly higher binding compared with membranes from mock-transfected cells and from cells expressing the mutant receptors ($P < 0.05$). Interestingly, when we used independent *t*-test to analyse the response to competitive binding between 'cold' and 125 I-GHRH in the WT GHRH-R and in each mutated GHRH-R, we found that some specific binding had also occurred in the mutant K329E ($P < 0.002$ vs non-specific binding).

Figure 2 Immunoprecipitation of the mutated receptors transiently transfected into CHO cells. Protein extract from mock-transfected cells (Mock) and cell transfected with empty vector (Vec) served as negative control, while WT receptor was used as a positive control. The upper band, present in all lanes, is non-specific and likely represents immunoglobulins.

Figure 3 Immunofluorescence of WT and mutated receptors expressing extracellular FLAG-epitope in non-permeabilized CHO cells. Mock-transfected CHO cells (Mock), CHO cells transfected with empty vector (Vec) and with WT receptor expressing the intracellular HA-tag (WT-HA) served as negative controls. All images were scanned with a confocal microscope for equivalent times.

Discussion

The importance of the presence of a functional GHRH-R for the proliferation of somatotroph cells, GH synthesis and secretion, and ultimately for linear growth is demonstrated by the murine model *little* (*lit*) mouse, and by several human *GHRH-R* mutations. Indeed, *GHRH-R* defects are now recognized as the cause of approximately 10% of

autosomal recessive IGHD (Salvatori *et al.* 2001a). Studies on the *lit* GHRH-R (Gaylinn *et al.* 1999) have shown that the mutant receptor is unable to bind to GHRH.

The GHRH-R belongs to class family B-III of the GPCR superfamily, together with receptors for glucagon, glucagon like peptide-1, secretin, vasoactive intestinal peptide (VIP), gastric inhibitory peptide, and pituitary adenylate cyclase-activating peptide. Using chimeras

Figure 4 Binding of human ¹²⁵I-GHRH to membranes from mock-transfected HEK 293 cells (Mock) or cells transfected with WT or mutant GHRH-R cDNA. Black bars represent total binding of ¹²⁵I-GHRH; grey bars describe binding of ¹²⁵I-GHRH in presence of 1 μM 'cold' competitor GHRH (non-specific binding). Each data point is the mean of three different experiments, each performed in duplicate. Bars represent S.E.M. Analysis by ANOVA indicates that specific binding of WT receptor is statistically significantly higher compared with all the mutated receptors (**P*<0.02).

Table 1 Summary of the location of the six missense mutations and their relevant changes in amino acid sequence and influence on polarity

Amino acid change	Change in polarity	Location in GHRH-R domains
H137L (histidine → leucine)	Polar → non polar	1st transmembrane domain
L144H (leucine → histidine)	Non polar → polar	1st transmembrane domain
A176V (alanine → valine)	Non polar → non polar	2nd transmembrane domain
A222E (alanine → glutamic acid)	Non polar → polar	3rd transmembrane domain
F242C (phenylalanine → cysteine)	Non polar → polar	4th transmembrane domain
K329E (lysine → glutamic acid)	Polar → polar	6th transmembrane domain

of the GHRH-R and VIP and secretin receptors, DeAlemeida and Mayo (1998) have elegantly shown that both N- and C-termini of the GHRH-R are necessary for ligand binding and triggering of signalling. The N-terminal extracellular domain plays a fundamental role in initial interaction with the ligand, while residues in the transmembrane domains, in conjunction with the extracellular loops, could have a role in secondary interaction and in determining binding specificity.

All the small deletions, splice mutations or nonsense GHRH mutations described to date either insert a premature stop codon or cause the messenger RNA to be out of frame. Resulting receptors, if translated at all, would lack the C-terminal domain and/or most of the transmembrane domains, possibly preventing the localization of the truncated receptor into the cell membrane or interaction with G proteins. Contrarily, the GHRH-Rs bearing missense mutations are more likely to be translated into mature protein allowing more extensive studies on how the mutation may interfere with the GHRH signalling pathway. Receptor malfunction could be due to instability and early degradation of the mature protein, improper folding, alterations in intracellular transport or insertion into cell membrane, or in binding of the ligand. Although there is evidence that GPCR's can exist as dimers, to date no mutation has been found that alters dimerization (Milligan 2004).

Our group has reported six naturally occurring missense mutations that cause familial autosomal recessive IGHD. The six amino acids that are mutated are conserved in all mammalian GHRH-Rs (human, rat, pig, bovine, and ovine) cloned to date (Horikawa *et al.* 2001), pointing to their importance in receptor function and confirming that these changes are not polymorphisms. Indeed, when expressed in eukaryotic cells, the mutated receptors are not able to activate the intracellular messenger cascade.

To investigate the expression of the mutated proteins, we transiently transfected CHO cells either with WT or mutant GHRH-R cDNA tagged with an intracellular GFP-tag. Receptors were immunoprecipitated and detected by Western blot. All mutant receptors are expressed and are of identical size as the WT. The degree of expression seems not to be altered, suggesting that the

mutations do not interfere with posttranscriptional events. The size of band displayed on the Western blot is not in perfect accordance with the expected size of 79 kDa, which could be due to electrophoretic conditions affecting the charge of the proteins and therefore their mobility. Another possible explanation is that the glycosylation of human GHRH-R expressed in HEK 293 is altered (Gaylinn *et al.* 1994). Nevertheless, the electrophoretic migration of the mutant receptors is identical to the one of the WT protein.

The expression of the mutated receptors is also confirmed by the results of the immunofluorescence studies. All mutant receptors containing an extracellular FLAG-tag display immunofluorescence in non-permeabilized cells, indicating that the receptor is properly transferred through the Golgi apparatus to the cell surface.

Once we determined that the mutated receptors were properly expressed on the cell surface, we investigated their ability to bind to labelled GHRH. Binding studies revealed that all the mutated receptors had reduced ligand binding compared with WT receptor. The K329E mutation seems to retain some binding capacity, as shown by the difference between specific and non-specific binding, but its binding ability remained significantly lower than the wild type. The K329E mutant does not cause any rise in intracellular cAMP. We can only speculate on the reason of this finding. As shown in Table 1 all of the six mutations are located within the transmembrane domains and four (H137L, L144H, A222E, and F242C) produce modifications in the degree of solubility of the amino acid within the lipid layer of the cell membrane. It is conceivable that a change of polarity causes alterations in proper protein folding and receptor function. Since the mutation K329E causes the change from one polar amino acid to another (lysine to glutamic acid) it is possible that the conformation of the protein is conserved to a point that would still allow some degree of ligand binding but not further signalling.

Unfortunately, to date little is known regarding the role of specific residues in the transmembrane domains of class B GPCR's and limited data are available on the mechanism through which mutations in these regions may affect receptor function.

To determine how the amino acid changes cause the inability of the receptor to bind the ligand would require the creation of three-dimensional models analysing how the single amino acid changes affect the tertiary structure of the protein.

In conclusion, we have shown that the six naturally occurring and disease-causing missense *GHRH-R* mutations identified to date allow proper surface expression of the receptor. They cause the receptor to be unable to bind to GHRH, impairing its ability to transmit intracellular signalling and ultimately to stimulate GH secretion.

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