A single amino acid substitution in transmembrane helix VI results in overexpression of the human GnRH receptor

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Abstract

Objective: Construction of constitutively active mutants of the GnRH receptor, a member of the G-protein coupled receptor superfamily, would facilitate investigation of the mechanism of receptor activation.

Design: Point mutations were introduced in the human GnRH receptor in positions corresponding to those which caused constitutive activity in other G-protein coupled receptors. The effects of these mutations on ligand binding, receptor intracellular signaling and receptor expression were determined.

Methods: Wild type and mutated receptor cDNAs were expressed in COS-1 cells. Basal and agonist-stimulated inositol phosphate production and ligand binding were determined. In addition, receptor mRNA levels, cell surface receptor stability and rate of internalization were measured.

Results and conclusions: Although none of the mutant receptors exhibited constitutive activity, mutation of Phe-272 in transmembrane helix VI to Leu increased cell surface receptor numbers, with unchanged affinities for radiolabeled agonist, superagonist and antagonist peptides compared with wild type receptor. The cell surface receptor stability and rate of internalization were similar for wild type and F272L GnRH receptors. Thus a single amino acid mutation in transmembrane helix VI causes an increase in cell surface receptor numbers, which appears to result from an increased rate of receptor protein translation, processing or insertion into membranes.

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Introduction

Gonadotropin releasing-hormone (GnRH) is a decapetide which exerts its biological effects via binding to and activating the GnRH receptor (GnRHR) on pituitary gonadotrope cells, to result in the release of the gonadotropins. The GnRHR (1–4), a member of the G-protein coupled receptor (GPCR) superfamily, which is characterized by seven putative transmembrane helices (TMHs), activates phospholipase C via coupling to the Gq/11 class of G-proteins (5).

Some insight into the mechanism of receptor activation has emerged from site-directed mutagenesis of GPCRs (6, 7) and from naturally occurring mutations which cause constitutive activation (8–15). A common mechanism of activation may exist for all GPCRs, regardless of which of the G-proteins they couple to. Several different receptors which couple to different G-proteins have been constitutively activated by mutations in the C-terminal part of intracellular loop III (ICIII), and the middle to intracellular part of TMHs VI and VII (7, 9, 12, 15–21). However, studies on the thyroid-stimulating hormone (TSH) receptor (TSHR) indicate that stimulation of cAMP and phospholipase C dependent cascades may involve different conformational changes in the same receptor, and different intramolecular interactions (14). In addition, a comparison of mutants of the TSHR and luteinizing hormone receptor indicates that some wild type receptors may be less constrained than others in the inactive form, and thus more readily constitutively activated by point mutations (11, 14). The GnRHR has a number of unique structural features such as the absence of a C-terminal tail. This raises the question as to what extent GnRHR conforms to other members of the GPCR family in the role of apparently homologous amino acid residues.

We have systematically incorporated mutations in the GnRHR homologous to mutations which constitutively activate other GPCRs. We report here that none of the GnRHR mutants is constitutively active, although receptor function and/or expression levels are altered. Mutation of Phe at position 272 in TMH VI to Leu gives rise to increased receptor expression relative to the wild type receptor.
Materials and methods

Site-directed mutagenesis

The human GnRHR cDNA (4) was cloned into the EcoRI/Xhol sites of the phagemid pBluecript II SK− (Stratagene, La Jolla, CA, USA). Oligonucleotide-directed mutagenesis was performed using the method of Kunkel et al. (22). Products of the mutagenesis were used to transform competent XL-1 Blue E. coli. Plasmid DNA was extracted from ampicillin-resistant clones and sequenced using a Sequenase kit (United States Biochemical Corporation, Cleveland, OH, USA). Mutant cDNAs were subcloned into the EcoRI/Xhol sites of the mammalian expression vector pCDNAI/Amp (Invitrogen, Carlsbad, CA, USA). Two different F272L mutants were constructed, containing different codons for Leu, i.e. wild type: GCA TTT GCC, F272L-1: GCG CTA GCC and F272L-2: GCG CTT GCC, where TTT is the wild type codon for Phe. All the results shown in the figures and table were obtained with F272L-1.

Transfection

Plasmid DNA for transfection was prepared using Qiagen columns (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. COS-1 cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) (Gibco, Paisley, UK) containing 10% fetal calf serum (Highveld Biological Pty Ltd, Lyndhurst, Gauteng, Republic of South Africa) in a 10% CO₂ atmosphere. Cells were seeded at 2 × 10⁵ per well in poly-D-lysine coated 12-well plates 1 day before transfection by a modified DEAE-dextran method (23). Cells were washed once with Hepes DMEM and then incubated with 0.6 ml/well serum-free DMEM containing 3.3 μg/ml plasmid DNA and 0.3 mg/ml DEAE-dextran for 4 h at 37 °C. The cells were incubated for a further 1 h at 37 °C with DMEM containing 2% fetal calf serum and 200 μmol/l chloroquine, after which they were incubated in DMEM containing 10% DMEM for 2 min. The cells were then cultured overnight in DMEM with 10% fetal calf serum. The cells were washed with Hepes DMEM after each step.

Inositol phosphate (IP) production

Twenty-four hours after transfection, cells were incubated overnight with 2 μCi/ml myo-[2-³H]inositol (Amersham, Bucks, UK) in 0.5 ml/well Medium 199 (Gibco) containing 2% fetal calf serum. Total IPs were then measured as previously described (24).

Radioligand binding

GnRH (pyroGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH₂) and GnRHA ([α-Ala³-N-Me-Leu⁵,Pro⁹-NH₂]GnRH), a potent GnRH agonist, were synthesized by conventional solid phase synthesis. Ant26 ([Ac-α-p-ClPhe³,β-p-ClPhe⁶, δ-Trp⁹,δ-Lys⁴,δ-Ala¹⁰NH₂]GnRH), a competitive GnRH antagonist, was a gift from D Coy (Department of Medicine, Tulane University School of Medicine, New Orleans, LA 70112, USA). Whole cell receptor ligand binding was performed to determine ligand affinities and receptor numbers. For agonist binding studies, transfected COS-1 cells were washed with cold Buffer I (140 mmol/l NaCl, 4 mmol/l KCl, 20 mmol/l Hepes, 8 mmol/l glucose, 0.1% fatty acid-free BSA, 1 mmol/l CaCl₂, 1 mmol/l MgCl₂, pH 7.4) and then incubated for 3–4 h on ice in Buffer I containing 100 000 c.p.m. [¹²⁵I-GnRHA (50 pmol/l) and varying concentrations of unlabeled GnRHA. Antagonist binding studies were performed at 37 °C, and cells were incubated for 1 h with 100 000 c.p.m. [¹²⁵I-Ant26 (50 pmol/l). In both agonist and antagonist assays, incubation was terminated by washing the cells in cold Buffer I containing 0.5% BSA. Cells were removed from the dishes with 0.1 mol/l NaOH at room temperature and their radioactivity counted in a scintillation counter. Non-specific binding was estimated by incubating untransfected cells with 100 000 c.p.m. of the labeled ligand, in the absence of unlabeled ligand.

For membrane binding assays, transfected COS-1 cells were homogenized in binding buffer (1 mmol/l Hepes, 0.1 mmol/l EDTA, pH 7.4) and centrifuged at 13 000 g for 30 min at 4 °C. For agonist binding, the crude membrane pellet was resuspended in binding buffer (7.5 × 10⁵ cell equivalents/tube) and incubated with 100 000 c.p.m. [¹²⁵I-GnRHA (50 pmol/l), and varying concentrations of unlabeled GnRHA for 2 h on ice. For antagonist binding, homogenized COS-1 cells were centrifuged for 10 min at 1000 g at 4 °C to remove cell debris and nuclei, to reduce non-specific binding. The resulting supernatant was then centrifuged at 13 000 g for 30 min at 4 °C as for the agonist binding assay. The resuspended membrane pellet was incubated with 100 000 c.p.m. [¹²⁵I-Ant26 (50 pmol/l), and varying concentrations of unlabeled Ant26 for 30 min at 37 °C. Maximum binding of [¹²⁵I-Ant26 was achieved after 15 min under these conditions and was stable for at least 1 h. In both agonist and antagonist assays, the incubation was terminated by the addition of 3 ml ice-cold 0.01% (w/v) aqueous polyethylenimine (Sigma, St Louis, MO, USA) and immediate filtration through GF/C filters (Whatman, Maidstone, Kent, UK) which were presoaked in 1% (w/v) polyethylenimine, followed by three washes with 3 ml 0.01% polyethylenimine. Non-specific binding was estimated in the presence of 10⁻⁶ mol/l unlabeled Ant26 for both agonist and antagonist binding assays. Non-specific binding of [¹²⁵I-Ant26 ranged from 4000 to 7000 c.p.m. while maximum specific binding was 12 000 to 16 000 c.p.m. in the wild type GnRHR.

Northern blot analysis

Total RNA from COS-1 cells was extracted 48 h after transfection according to the method of Chomczynski &
Saccos (26). Twenty micrograms RNA were electrophoresed through a 1% agarose, 16.7% formaldehyde gel and then transferred to a Hybond N+ membrane (Amersham) in 5×SSC. The membrane was baked at 80°C for 10 min and exposed to UV light for 7 s in an Amersham UV crosslinker. The 1.2 kb human GnRHR cDNA fragment and a β-actin cDNA fragment were labeled using a Megaprime end-labeling kit (Amersham), for use as probes. Membranes were pre-hybridized for 2 h and then hybridized with the probe overnight at 42°C in 20 mmol/l Pipes pH 6.5, 0.8 mol/l NaCl, 50% formamide, 0.5% SDS, 100 μg/ml salmon sperm DNA, followed by washing (final wash 0.2× SSC, 0.1% SDS, 60°C for 20 min). Probed membranes were then analyzed on a Hewlett-Packard Instant Imager gel scanner (Packard Instruments, MD, CT, USA) to quantitate the amount of receptor and β-actin RNA.

**Receptor internalization**

Forty-eight hours after transfection, the cells were washed in ice-cold Buffer I and then incubated with 500,000 c.p.m. 125I-GnRHA for 3 h on ice. The cells were then moved into a 37°C water bath and incubated for the indicated times to allow internalization, without removing the radiolabeled peptide from the medium. After the incubation, the cells were transferred to an ice bath and washed twice with cold PBS. Externally bound ligand was collected by a 10 min acid wash (50 mmol/l acetic acid, 150 mmol/l NaCl), while the internalized ligand was measured by solubilizing with 0.1 mol/l NaOH.

**Cell surface receptor stability**

Forty-eight hours after transfection, cells were incubated with or without cycloheximide in the absence of ligand, at 37°C. At the indicated times, the cells were transferred to 4°C and incubated with 100,000 c.p.m. 125I-GnRHA for 3 h. The cells were washed twice with cold PBS and the externally bound ligand was removed by a 10 min acid wash (50 mmol/l acetic acid, 150 mmol/l NaCl).

**Data reduction**

Graphpad Prism (Graphpad Software, San Diego, CA, USA) was used for four-parameter non-linear curve fitting to estimate peptide concentrations required to stimulate half-maximal (EC50) IP production and to half-maximally inhibit binding of radioligand (IC50). LIGAND (26) was used to estimate the dissociation constant (Kd) and receptor numbers in the competition binding studies. Figures show representative experiments (unless stated otherwise), in which data points are the mean ± S.D. of triplicate or duplicate determinations. The mean values were used to calculate the percentage wild type for each experiment. The average of the percentage wild type values obtained is shown ± S.D. in Table 1.

**Results**

**Effects of GnRHR mutations on IP production**

Point mutations were introduced separately at six positions of the GnRHR by site-directed mutagenesis: A261I and L266I in ICIII, F272L and F276Y in TMH VI and F309E and C317Y in TMH VII. Wild type and mutant receptors were screened for increased basal IP production in the absence of ligand, a feature of constitutively active receptors. The basal IP production in the absence of transfected DNA, as well as for wild type and mutant receptors was in the range 75–130 c.p.m., showing that neither the wild type nor any of the mutant GnRHRs was constitutively active.

Although the F272L GnRHR did not display an increase in basal IP production, the dose–response curve for agonist-induced IP production was left-shifted with respect to the wild type, showing a 3-fold increase in potency (Fig. 1).

**Ligand binding characteristics of the F272L GnRHR**

In the TSHR, mutation of Phe at position 631 to Leu caused the receptor to be constitutively active (21). Alignment of the TMHs revealed that Phe-272 of the human GnRHR is the equivalent of Phe-631 of the TSHR. The mutation F272L did not increase basal IP production above that of the wild type receptor. However, the binding data showed that this mutant had two to four times more specific binding than the wild type receptor (Figs. 2 and 3).

Homologous competition binding studies with GnRHA in whole cell binding assays show that the F272L GnRHR has the same affinity (Kd = 5.6 ± 0.9 nmol/l) as the wild type (Kd = 4.5 ± 1.1 nmol/l) (Table 1). Heterologous competition binding in whole cells was performed using 125I-GnRHA and unlabeled GnRH. The affinity of the F272L GnRHR for GnRH (Kd = 3.9 ± 0.6 nmol/l) was unchanged from that of the wild type receptor (Kd = 4.4 ± 1.9 nmol/l).

In homologous agonist competition binding in whole cells, the same affinities for the F272L (Kd = 4.0 ± 1.5 nmol/l) and wild type GnRHRs (Kd = 3.6 ± 1.1 nmol/l) were obtained. In all three competition assays, the F272L GnRHR showed several-fold more receptor expressed on the cell surface than the wild type receptor (Table 1).

Binding experiments were also performed on total cell membranes with displacement by GnRH, GnRHA and Ant26, for both wild type and the F272L mutant GnRHR. These results showed a similar increase in receptor numbers for the mutant receptor relative to wild type, with unchanged affinity for the ligands.
The F272L mutant was back-mutated to the wild type sequence and the back-mutated construct was shown to have wild type binding (data not shown), showing that the mutation at the single Phe-272 amino acid residue in the construct was responsible for the increase in F272L GnRHR expression.

Two F272L GnRHR constructs with different codons for Leu resulted in similar increases in specific binding compared with the wild type receptor (F272L-1 = 341%, and F272L-2 = 376%).

Radioligand binding of mutant GnRHRs with different substitutions at Phe-272 and Phe-276

To examine the chemical basis of the increased receptor numbers, Phe-272 was mutated to Ala, Glu, Lys and Tyr. Binding assays showed that only the F272L GnRHR had an increased receptor number (Fig. 4). When Phe-272 was mutated to a positively or negatively charged amino acid, no binding was detected (Fig. 4). These mutants also did not produce IPs in response to agonist stimulation (data not shown). When Ala or Tyr was substituted at position 272, the resulting mutant receptors had decreased specific binding, but had similar affinities for agonist ligand, compared with the wild type (Fig. 4, Table 1 and not shown).

Phe-276, one turn of a putative alpha helix after Phe-272 in TMH VI, was mutated to Leu to see if the effect observed for the F272L GnRHR was dependent on the Phe position within the TMH. The F276L mutant had no binding which could be detected (Fig. 4) or agonist-stimulated IP production (data not shown). The F276Y GnRHR mutant had the same binding (Fig. 4) and IP production characteristics (data not shown) as the F272Y mutant.

Northern blot analysis

To determine whether the increased expression of the F272L GnRHR relative to the wild type receptor was caused by an increase in GnRHR mRNA levels, RNA was extracted from COS-1 cells transfected with the F272L GnRHR or wild type receptor cDNA. Similar amounts of RNA were analyzed by Northern blotting, using the full-length GnRHR cDNA as a radiolabeled probe (Fig. 5). The membrane was stripped and re-probed with a radiolabeled β-actin probe. Relative levels of GnRHR mRNA were quantified by measuring the radioactive signal in the GnRHR band as a ratio of the signal in the β-actin band. For two independent experiments, the ratio of wild type GnRHR mRNA relative to F272L GnRHR mRNA, normalized for β-actin, was 1.25 ± 0.23.

Receptor internalization

The increased cell surface receptor numbers of the F272L GnRHR relative to wild type could be due to a decrease in the rate of receptor internalization from the

### Table 1

Ligand affinity and relative receptor number data for wild type and mutant receptors from whole cell and membrane binding assays. Data are the mean ± S.D. with the number of experiments in brackets. For whole cell binding, wild type Ro values in pmol/l for GnRHA, GnRH and Ant26 were 77.48 ± 17.22, 55.06 ± 10.91 and 240.04 ± 66.02 respectively. For membrane binding assays, wild type Ro values in pmol/l for GnRHA, GnRH and Ant26 were 48.23 ± 21.41, 40.12 ± 27.51 and 60.67 ± 21.13 respectively.

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Whole cell</th>
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<td>Kᵦ (nmol/l) (%) wild type</td>
<td>Ro (pmol/l)</td>
<td>Kᵦ (nmol/l) (%) wild type</td>
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<td>Ro (pmol/l)</td>
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<tr>
<td>Wild type</td>
<td>4.5 ± 1.1</td>
<td>100</td>
<td>0.7 ± 0.4</td>
<td>100</td>
<td>0.7 ± 0.4</td>
<td>100</td>
<td>0.7 ± 0.4</td>
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<tr>
<td>F272L</td>
<td>5.6 ± 0.9</td>
<td>392.0 ± 67.2</td>
<td>0.7 ± 0.4</td>
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<td>0.7 ± 0.4</td>
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<td>0.7 ± 0.4</td>
<td>100</td>
</tr>
<tr>
<td>F272Y</td>
<td>2.0 ± 0.9</td>
<td>ND</td>
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(ND, not determined. Ro, receptor number in pmol/l.)
cell surface. To investigate this possibility, cells transfected with wild type or F272L GnRHR were incubated with $^{125}$I-GnRHA on ice and then transferred to 37°C to allow internalization. Internalization was stopped by placing the cells on ice and washing with cold PBS. An acid wash was used to remove cell surface ligand, and internalized ligand was measured after solubilizing the cells in NaOH. Agonist-induced internalization showed that the F272L mutant internalized at the same rate as the wild type (Fig. 6). More receptors were measured both externally and internally for the mutant than for the wild type. This is consistent with the presence of more total receptors for the mutant than the wild type, in the absence of a decreased rate of internalization.

**Cell surface receptor stability**

The increase in receptor numbers of the F272L GnRHR relative to wild type could be due to an increased stability of the unliganded mutant receptor on the cell surface. Receptor binding as a function of time was measured in the absence or presence of the protein synthesis inhibitor cycloheximide. Cycloheximide caused a similar fractional decrease in cell surface receptor numbers in both

![Graph](image1.png)

**Figure 1** IP production in response to GnRH for the wild type (●) and the F272L mutant (○). $^{[3]}$H]Inositol-labeled transfected COS-1 cells were incubated for 1 h with the indicated concentrations of GnRH. EC$_{50}$ values for wild type and F272L GnRHRs were 60.0 ± 5.6 and 16.3 ± 4.1 pmol/l respectively. The absolute values for wild type basal and agonist-stimulated maximal IP production were 112 ± 12 and 7210 ± 1861 c.p.m. respectively. Data are means ± s.d. of triplicate determinations, and are normalized to percentage of wild type data.

![Graph](image2.png)

**Figure 2** Agonist binding displacement curves for the wild type (●) and F272L mutant (○) GnRHR. Transfected COS-1 cells were incubated with $^{125}$I-GnRHA and indicated concentrations of unlabeled GnRHA at 4°C for 3 h. Data are means ± s.d. of triplicate determinations, and are normalized to percentage of wild type data. A representative experiment is shown.
receptors (Fig. 7), suggesting that the F272L GnRHR has a half-life on the cell surface similar to that of the wild type receptor.

**Discussion**

Six sites were mutated in the human GnRHR, corresponding to positions of amino acids leading to constitutive activity when mutated in other G\textsubscript{i}, G\textsubscript{s} or G\textsubscript{q}/G\textsubscript{11}-coupled GPCRs. The failure of the GnRHR to be constitutively activated by any of the mutations in this study indicates that a common mechanism of activation does not exist for all GPCRs. This, together with the low intrinsic basal activity of the GnRHR relative to that of the TSHR, indicates that the equilibrium between the active and inactive GnRHR strongly favors the inactive form when compared with certain other GPCRs like the TSHR.

Mutation of a Phe at position 631 in TMH VI to a Leu in the TSHR causes the receptor to be constitutively active (21). The F631L mutant showed an increase in basal cAMP production, but binding of \textsuperscript{125}I-labeled TSH was unchanged compared with the wild type. When the TMHs of the TSHR are aligned with the human GnRHR, Phe-272 is the equivalent of Phe-631. The F272L mutant exhibited a similar maximal IP response to GnRH stimulation and unchanged affinities for GnRH, GnRHA and Ant26 (Figs 1, 2 and 3, Table 1) compared...
with the wild type GnRHR. An unexpected finding was the marked overexpression of the F272L GnRHR mutant. Results from both whole cell and a membrane binding assay (Table 1) show that the receptor number is increased both extracellularly (whole cell assay) and intracellularly (total membrane binding). The affinities for GnRH, GnRH agonist and GnRH antagonist were indistinguishable in the wild type and F272L mutant receptors. The consistently higher affinity of both the mutant and wild type receptor for all the ligands in the total membrane assay compared with the whole cell assay most likely reflects the low salt concentration in the membrane binding assay compared with the physiological medium used with whole cell binding (27). The lower EC50 for IP production in response to GnRH obtained for the F272L mutant compared with wild type GnRHR is consistent with an increase in receptor numbers on the cell surface, in the presence of a receptor reserve (Fig. 1).

We have used binding data to calculate receptor numbers on the cell surface and in cell membranes, as a measure of GnRHR protein expression, since we have not to date been able to raise antibodies to the GnRHR which recognize the expressed receptor on the cell

**Figure 5** Northern blot of wild type and F272L mutant GnRHR RNA (shown by the arrows) in transfected COS-1 cells. Total RNA was isolated from COS-1 cells transfected with wild type and F272L mutant GnRHRs, electrophoresed on an agarose/formaldehyde gel, transferred to nitrocellulose and hybridized with radiolabeled GnRHR or β-actin cDNA. An autoradiogram of the membrane is shown. The ratio of GnRHR to β-actin signal for wild type was 0.54 and for F272L, 0.39.

**Figure 6** Agonist-induced internalization of wild type and F272L receptors. Cell surface receptors were measured for the wild type (●) and F272L GnRHR (□) by removal of [125I]-GnRHA with an acid wash (upper panel). The amount of internalized receptors was determined by solubilizing the cells with 0.1 mol/l NaOH (lower panel). Untransfected COS-1 cells (○) were used as a control. Data are means ± s.d. of duplicate determinations from four independent experiments.
surface, and FLAG and HA epitope tagged receptor does not display wild type characteristics (A Katz & RP Millar, unpublished observations).

In order to examine the role of the amino acid side chain in the increased expression, we investigated the effects of other substitutions at position 272 of the GnRHR by constructing the mutants F272Y, F272A, F272K and F272E. The non-conservative mutations to Lys and Glu resulted in non-functional receptors (Fig. 4 and data not shown). The conservative F272Y GnRHR had characteristics similar to those of the wild type receptor. The characteristics of the F272A GnRHR (20% total specific binding compared with wild type) most likely reflect a pronounced decrease in receptor expression. Introduction of hydrogen bonding residues or a small hydrophobic residue all decreased receptor numbers. Thus a large hydrophobic residue is required in position 272 for high receptor expression.

Other underlying mechanisms which could account for overexpression of the F272L GnRHR were investigated. Increased protein expression could be due to increased mRNA synthesis or stability. Northern blot analysis of the F272L and wild type GnRHR RNA expressed in COS-1 cells revealed no differences in steady-state mRNA levels, indicating that the difference in the receptor expression is due to post-transcriptional events (Fig. 5).

Since two F272L GnRHR constructs with different codons for Leu resulted in similar increases in specific binding compared with the wild type receptor, secondary structure differences during translation are unlikely to be the cause of overexpression. However, it is possible that translation of the F272L GnRHR mRNA is more efficient than that for the wild type receptor, for reasons other than secondary structure of the nucleic acids. However, we did not detect any difference in the amount of translated receptor protein for the F272L GnRHR compared with wild type receptor in an in vitro translation assay (data not shown).

A decreased rate of receptor internalization from the cell surface could also account for increased cell surface receptor numbers. However, we showed that a decreased rate of agonist-induced internalization is not responsible for the increased receptor numbers as the F272L GnRHR does not internalize at a slower rate than the wild type (Fig. 6). There was more radiolabeled ligand both externally and internally for the F272L GnRHR compared with the wild type receptor. This indicates that the F272L mutant has an increased receptor number throughout the cell, compared with the wild type. This is substantiated by the membrane binding assays, which also showed the increased receptor numbers for the mutant receptor. The data suggest that the Leu-272 mutation increases the rate of translation, membrane insertion or folding of the receptor protein, such that the rate of delivery to the cell surface is increased.

The majority of point mutations in the TMHs of the GnRHR and other GPCRs decrease receptor expression on the cell surface, possibly due to folding defects (JS Davidson, CA Flanagan, JP Hapgood, RP Millar & DB Myburgh, unpublished observations and reference 28). Thus, it is unusual to find a mutation in the GnRHR which results in an increased receptor expression. The characteristics of the F272Y GnRHR are similar to wild type receptor and indicate a requirement for an aromatic or large lipophilic residue at this position for wild type expression, while mutations to residues with acidic, basic, hydrophilic or small hydrophobic side chains decrease expression, possibly by disruption of TMH
VI, or of important interhelical interactions. One turn above the Phe-272 in the putative alpha helix of TMH VI is another Phe (Phe-276). We considered it might be instructive to determine whether mutation of this Phe would have a similar effect on expression. The F276L mutant, in contrast to the F272L mutant, was non-functional. However, mutation to Tyr (F276Y) resulted in wild type characteristics as shown for F272Y. These results indicate that while the aromatic character of Phe residues in TMH VI is important for wild type receptor expression and function, the effects of mutation of Phe to Leu on overexpression are position specific.

Since natural mutation of Phe-272 to the higher-expressing and fully functional F272L would require only one nucleotide change, the question might be posed as to why this mutation has not occurred naturally. Since the GnRH gene is highly regulated by GnRH itself and by gonadal steroids and peptides (29), selective forces may have favored low basal expression and selected against more highly expressing sequences.

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