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Incorporation of an Additional Glycosylation Site Enhances Expression of Functional Human Gonadotropin-Releasing Hormone Receptor

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Mutation of *N*-glycosylation sites in the mouse gonadotropin-releasing hormone receptor was previously shown to impair its expression in COS-1 cells. We therefore investigated the effects of adding an extra glycosylation site to the human gonadotropin-releasing hormone receptor, as a means for increasing its expression. Covalent labeling of the mutant receptor expressed in COS-1 cells with a gonadotropin-releasing hormone (GnRH) photoreactive analog demonstrated a shift in apparent molecular weight, indicating that the new site was in fact glycosylated. The receptor with extra glycosylation site displayed normal binding affinities for agonists buserelin and [D-Ala^6-Pro^9-NHEt]-GnRH, and the antagonist antide, and a slightly increased affinity for GnRH. Receptor number was increased by 1.7-fold in membrane preparations from cells expressing the mutant receptor, compared with wild-type. Photoaffinity labeling of cell-surface receptors in intact cells demonstrated a 1.8-fold increase in binding sites on the cell surface. The GnRH receptor (GnRHR) with extra glycosylation site conferred a markedly enhanced signaling response to agonist. Dose–response curves for GnRH-stimulated inositol phosphate production were left-shifted by an average of 4.4-fold, and maximal inositol phosphate responses were increased by 1.2 fold, in cells transfected with mutant compared with wild-type receptor, indicating that the increase in binding sites represented functional receptors.These results demonstrate that addition of an extra glycosylation site enhances expression of the human GnRHR, a strategy that may be applicable to other cell-surface receptors.

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Introduction

Strategies for increasing the expression of cell-surface receptors are likely to have important applications in the analysis of receptor structure and function, and in the development of receptor expression systems for high-throughput screening of novel ligands. Gonadotropin-releasing hormone (GnRH) is a decapeptide that acts via receptors on pituitary gonadotrope cells to control the release of gonadotropins. GnRH receptors (GnRHRs) from mouse, rat, human, sheep and cow are members of the G-protein-coupled receptor superfamily (Tsutsumi et al., 1992; Kakar et al., 1992, 1993; Kaiser et al., 1992; Eidne et al., 1992; Reinhart et al., 1992; Brooks et al., 1993; Chi et al., 1993; Illing et al., 1993). The GnRHR is coupled via the G_q/G_{11} group of G-proteins to phospholipase C (Hsieh and Martin, 1992). Its activation results in an oscillatory Ca^{2+} signaling process (Tse and Hille, 1992) and the release of gonadotropins by regulated exocytosis (Davidson et al., 1991).

Peptide GnRH analogs, both agonists and antagonists, have found wide clinical application (Millar et al., 1987); however, nonpeptide analogs for the GnRHR have not yet been described. Recent site-directed mutagenesis studies have begun to provide information on the structure of the ligand-binding site (Flanagan et al., 1994; Zhou et al., 1995).

GnRHRs from five species contain two conserved consensus sequences for *N*-glycosylation (Asn^{18} and Asn^{102}), in the extracellular domains, and the rodent receptors have an additional site at Asn^4. The Asn^4 and Asn^{18} residues of the mouse GnRHR are utilized for glycosylation, whereas the Asn^{102} site is not (Davidson et al., 1995). We previously showed that mutation of either of the glycosylated sites in the mouse receptor led to decreased expression without a change in binding affinity (Davidson et al., 1995).

We previously found that binding studies on the human GnRHR were hampered by the low level of expression in COS-1 cells in transient transfections, compared with the mouse GnRHR (data not shown). Since it was possible that the additional glycosylation of the mouse receptor might explain its higher level of expression, we introduced an extra glycosylation site into the human GnRHR in the same position as in the mouse receptor, as a possible means for increasing its expression. We report here that this mutant receptor (hGnRHR + G) displays increased expression and function in COS-1 cells.

Materials and Methods

Site-Directed Mutagenesis

The human GnRHR cDNA (Chi et al., 1993) was shortened by removal of 1.3 kb of the 3'-untranslated region with *Ssp*1, and cloned into the *Eco*RV site of the phagemid pcDNA1/Amp (InVitrogen, San Diego, CA). Oligonucleotide-directed mutagenesis was performed using the method of Kunkel et al. (1987). After passage through *Escherichia coli* strain CJ236, uridine-containing single-stranded DNA template was prepared using VCS M13 helper phage (Stratagene). A mutant oligonucleotide ATGGCAAACAA TGCCTCTCTAGAACAGAATC was designed to encode the amino acid substitutions Ser^4 to Asn^4 and Pro^7 to Leu^7, as well as a silent *Xba*I restriction site. The Leu^7 substitution was included, since it has been reported that the consensus sequence N-X-S/T is not glycosylated when followed by proline (Gavel and von Heijne, 1990). The oligonucleotide was phosphorylated with T4 polynucleotide kinase (Promega, Madison, WI), hybridized with the template, and second-strand synthesis was completed using T7 DNA polymerase (Bio-Rad, Hemel Hempstead, UK) and T4 ligase (Bio-Rad). Products of the reaction were used to transform competent *E. coli* strain MV1190. Plasmid DNA from ampicillin-resistant colonies was digested with *Xba*I, and two clones showing the additional restriction site were sequenced, confirming the mutation, at the core facility of the Department of Biochemistry, University of Bristol, UK. Both independent mutant clones were subsequently shown to display the same enhanced response to GnRH (*see* Results and Discussion).

Transfection

Plasmid DNA for transfection was prepared using Qiagen columns (Qiagen, Dorking, UK) according to the manufacturer's instructions. Five different plasmid preparations of mutant cDNA yielded similar results. COS-1 cells were cultured in DMEM (Gibco BRL, Paisley, Scotland) containing 10% fetal calf serum in a 10% CO_2 atmosphere. For inositol phosphate production assays, cells were seeded in 12-well plates 1 d before transfection, at $1.8–8.0 \times 10^5$ cells/well. For binding studies, 10^6 cells were seeded into 75-cm^2 flasks. Cells were transfected by a modification of the DEAE-dextran method (Keown et al., 1990). Cells were washed twice with phosphate-buffered saline (PBS), pH 7.4, and then incubated with 0.5 mL/well or 4 mL/flask of serum-free DMEM containing 2–5 μg/mL plasmid DNA and 0.3 mg/mL DEAE-dextran for 4 h at 37°C. This was replaced with DMEM containing 2% fetal calf serum and 150 μ*M* chloroquine, and incubation was continued for 1 h at 37°C. The cells were washed with PBS, and finally cultured in DMEM with 10% fetal calf serum.

Northern Analysis

Total RNA from COS-1 cells was extracted 48 h after transfection according to the method of Chomczynski and Sacchi (1987). Twenty micrograms of RNA were electrophoresed through a 1% agarose, 16.7% formaldehyde gel and then transferred to a Hybond N membrane (Amersham, Little Chalfont, UK) in 5X SSC. The membrane was baked at 80°C for 10 min and exposed to UV light for 7 s in an Amersham UV crosslinker. A 1.2-kb human GnRHR cDNA fragment and the mouse β-actin fragment were labeled using a Megaprime end-labeling kit (Amersham) for use as probes. Membranes were prehybridized for 2 h, and then hybridized with the probe overnight at 42°C in 0.8*M* NaCl, 20 m*M* PIPES (pH 6.5), 50% formamide, 0.5% sodium dodecyl sulfate (SDS), 100 μg/mL salmon sperm DNA, followed by washing (final wash 0.2X SSC, 0.1% SDS, 60°C for 20 min). The membrane was probed first with the GnRHR probe, followed by quantification of the radioactive signal, and then washed with SDS to remove the first probe, followed by reprobing with the β-actin probe. Probed membranes were analyzed on a Hewlett Packard Instant Imager gel scanner to quantify the amount of receptor RNA.

Inositol Phosphate Production

Twenty-four hours after transfection, cells were labeled for 24 h with 2 μCi/mL myo-[2-^{3}H]inositol (Amersham) in 0.25 mL/well Medium 199 (Gibco) containing 0.3% bovine serum albumin (BSA), and then stimulated with GnRH for 60 min in the presence of 10 m*M* LiCl. Total inositol phosphates were extracted with perchloric acid and chromatographed on Dowex columns as described (Davidson et al., 1990).

Photoaffinity Labeling

[D-Lys^6-*N*-azidobenzoyl]GnRH was synthesized according to Hazum (1981). Five micrograms of the peptide were iodinated using chloramine T and purified using reverse-phase HPLC. Transfected COS-1 cells or αT3 cells (a gonadotrope-derived mouse pituitary tumor cell line; Windle et al., 1990) in 100 mm dishes were incubated with 1.5 mL of buffer A containing 5×10^6 cpm [^{125}I-D-Lys^6-*N*-azidobenzoyl]GnRH in the presence or absence of 2 μ*M* unlabeled GnRH agonist, at 24°C for 30 min. The concentration of radioligand used (10–40 n*M*) was several-fold higher than its K_d (approx 1 n*M*). The dishes were irradiated for 30 s using a Spectroline TR-312A UV light box. The cells were detached, homogenized in binding buffer

(10 m*M* HEPES, 1 m*M* EDTA, pH 7.4), and centrifuged for 10 min at 400*g* to remove nuclei. The supernatant was centrifuged at 10,000*g* for 30 min, and the membrane pellet resuspended in 0.25 mL binding buffer/dish of cells. Labeled receptors were denatured by addition of 0.5% SDS and heating at 60°C for 15 min. The SDS concentration was then decreased to 0.2% by dilution with buffer, and a fivefold molar excess of Triton X-100 was added. Deglycosylation was performed with 5 U/mL peptide *N*-glycosidase-F (Boehringer-Mannheim, Randburg, South Africa) for 18 h at 37°C. Samples were dissolved in SDS sample buffer (Garfin, 1990), and electrophoresed on 10% SDS-polyacrylamide gels, which were stained with Coomassie blue and autoradiographed. Radioactivity on gels was quantitated using a Packard Instantimager, or radioactivity was counted in bands cut out of gels.

Radioligand Binding

Protocol A (Membrane Preparations)

Transfected COS-1 cells were homogenized in binding buffer and centrifuged at 15,000*g* for 30 min at 4°C. The membrane pellet was resuspended in binding buffer and incubated (7.5×10^5 cell Eq/tube) with 60,000 cpm [^{125}I-D-Ala^6,*N*-Me-Leu^7,des-Gly^{10}-NHEt]-GnRH (GnRH-A) and varying concentrations of unlabeled peptide for 90 min on ice. The incubation was terminated by the addition of 3 mL PBS containing 0.1% BSA and immediate filtration through GF/C filters (Whatman). Nonspecific binding was estimated in the presence of 10^{-7}*M* unlabeled [D-Ala^6,*N*-Me-Leu^7,des-Gly^{10}-NHEt]-GnRH.

Protocol B (Whole-Cell Assay)

Cells were scraped from dishes and 10^5 cells/tube were incubated with 100,000 cpm [^{125}I]-buserelin plus unlabeled peptides for 30 min at 24°C, after which cells were centrifuged through oil as described (McArdle et al., 1992).

Data Reduction

Peptide concentrations required to stimulate half-maximal IP production (EC_{50}) and to inhibit binding of radioligand (IC50) half-maximally were estimated by nonlinear regression using Sigmaplot (Jandel Scientific). Figures 3 and 4 show representative experiments, in which data points are the mean ± SEM of triplicate or duplicate determinations. Means were compared using paired or unpaired Student's *t*-tests, as appropriate.

Results and Discussion

After covalent photoaffinity labeling with [^{125}I-D-Lys^6-*N*-azidobenzoyl]GnRH, membranes from αT3 cells and from COS-1 cells transfected with GnRHR cDNAs showed broad labeled bands (Fig. 1), as previously described (Hazum, 1981; Janovick et al., 1993; Perrin et al., 1993; Davidson et al., 1995). These bands represented GnRHRs, since (1) labeling was abolished in the presence of 10^{-6}*M* unlabeled GnRH agonist, and (2) no such labeled bands

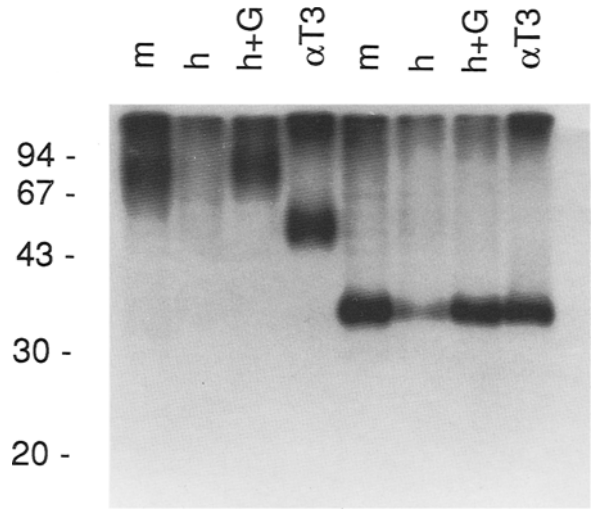

Fig. 1. Autoradiograph of photoaffinity-labeled GnRHRs. m, Mouse GnRHR (COS-1 cells); h, wild-type human GnRHR (COS-1 cells); h + G, human GnRHR with extra glycosylation site (COS-1 cells); αT3, αT3 cells. The left-hand four lanes show native receptors, and the right-hand four lanes show deglycosylated receptors. Molecular-weight markers (kDa) migrated at positions shown in the left margin.

were present in untransfected cells. GnRHRs expressed in COS-1 cells showed apparent molecular weights in a range between 55 and 85 kDa, whereas the GnRHR from half-maximallyT3 cells migrated at 45 kDa (Fig. 1). The broadness of the bands was the result of heterogeneity of glycosylation, since all the GnRHR types migrated as sharp bands of apparent mol wt 32 kDa after enzymatic deglycosylation (Fig. 1). For comparison, the apparent molecular weight of the major form of the GnRHR expressed in primary pituitary cells has been reported as 60 kDa (rat; Hazum, 1981), 60 kDa (mouse; Iwashita and Catt, 1985), 57–59 kDa (rat; Nikolics et al., 1988), and 46–60 kDa (rat; Janovick et al., 1993). Thus, the extent of glycosylation is a labile parameter that is very cell-type-dependent, and even in the gonadotrope-derived αT3 cell, the glycosylation of the GnRHR is not the same as in the pituitary gland.

The wild-type human GnRHR was labeled to a lower intensity than the mouse receptor (Fig.1), consistent with previous findings indicating a lower level of expression of the human receptor in COS-1 cells (data not shown). In optimally exposed autoradiographs, the human GnRHR exhibited a lower apparent mol-wt range than the mouse receptor (Fig. 2, lanes 1 and 2), a finding that is consistent with two glycosylation sites in the mouse compared with one in the human receptor. The mutant human GnRHR with extra glycosylation site (hGnRHR + G) was more intensely labeled than the wild-type human GnRHR, and also displayed a higher apparent mol-wt range (Fig. 2, lane 3), indicating that the extra site is in fact utilized for glycosylation. Interestingly, the hGnRHR + G showed a slightly higher apparent molecular weight than the mouse receptor, indicat-

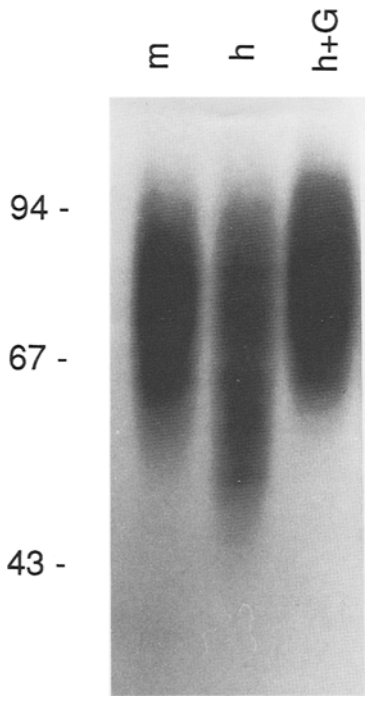

Fig. 2. Autoradiograph of photoaffinity-labeled GnRHRs. m, Mouse GnRHR (COS-1 cells); h, wild-type human GnRHR (COS-1 cells); h + G, human GnRHR with extra glycosylation site (COS-1 cells). Molecular-weight markers (kDa) migrated at positions shown in the left margin.

ing that sequence differences other than at the glycosylation sites also influence the degree of glycosylation.

The mutant receptor showed normal binding affinities for three GnRH analogs (2 agonists, 1 antagonist) and a slightly increased affinity for GnRH (Fig. 3 and Table 1). To assess receptor number, as well as the proportion of receptors localized on the cell surface relative to the total number of receptors in all cellular compartments, binding studies were performed using both homogenized membrane preparations and whole cells. Maximal binding, reflecting the number of binding sites, was increased by an average of 1.7-fold (1.7 ± 0.08, mean ± SEM, n = 5 experiments) in homogenized membrane preparations (protocol A), and by 2.2-fold (2.2 ± 0.15, mean ± SEM, n = 4 experiments) in whole cells (protocol B). The number of cell-surface binding sites measured by photoaffinity labeling in intact cells, followed by quantitation of bands on SDS-PAGE gels, was increased by 1.8-fold (1.8 ± 0.17, mean ± SEM, n = 5 experiments). The intensity of labeling in the photoaffinity crosslinking experiments is a reflection of receptor number, since photolabeling was performed under near-saturating conditions, where binding is insensitive to small changes in binding affinity. Levels of mutant receptor mRNA quantitated by Northern blotting were not increased compared with wild-type (data not shown), indicating that the additional glycosylation mediates increased receptor number at a posttranscriptional step.

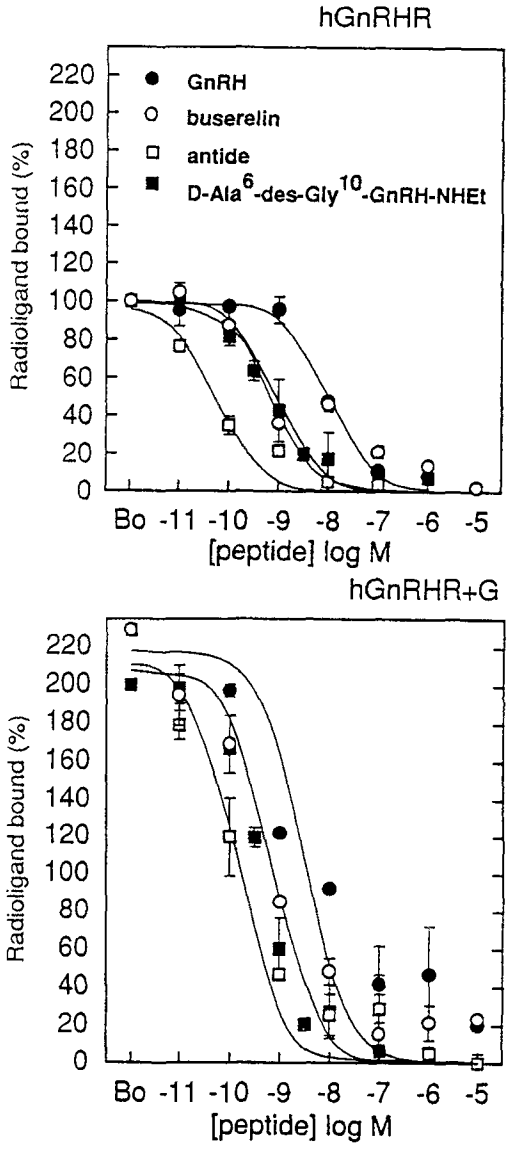

Fig. 3. Radioligand displacement curves for GnRH and analogs. Data are from representative experiments, normalized to a percentage of maximal wild-type receptor binding. Data for GnRH, buserelin, and antide were obtained in whole cells (protocol B) and for D-Ala^6-des-Gly^{10}-GnRH-NHEt using membranes (protocol A).

Incorporation of the additional glycosylation site markedly enhanced GnRH-stimulated inositol phosphate production in transfected COS-1 cells (Fig. 4). In six independent experiments, the EC50 for GnRH-stimulated inositol phosphate production was left-shifted by an average of 4.4-fold compared with the wild-type receptor, whereas the maximal response was increased by an average of 1.2-fold (Table 1). The expected enhancement of the inositol phosphate response to GnRH resulting from the combination of increased affinity and receptor number may be calculated from a simple "operational model" (Kenakin, 1993). It can be shown that if receptor number (Rt) and binding affinity (K_d) change without a change in coupling efficiency, then:

$$IP_{max_1}/IP_{max_2} \times (EC_{50_2}/EC_{50_1}) = (K_{d_2}/K_{d_1} \times (Rt_1/Rt_2) \quad (1)$$

Table 1
Ligand Binding and Inositol Phosphate Stimulation Parameters for Wild-Type hGnRHR and Receptor with Extra Glycosylation Site (hGnRHR + G)

		hGnRHR	hGnRHR + G	
Maximal binding (Bo) (% of wild type)				
Protocol A		100%	171% ± 8% (5)	
Protocol B		100%	218% ± 15% (4)	
Photoaffinity labeling		100%	181% ± 17% (5)	
Binding affinity (IC_{50}, n*M*)				
Radioligand	*Displacing Ligand*			
^{125}I-buserelin	Buserelin	1.00 ± 0.10 (2)	0.79 ± 0.07 (2)	NS
^{125}I-buserelin	Antide	0.28 ± 0.11 (3)	0.24 ± 0.08 (3)	NS
^{125}I-GnRH-A	GnRH-A	0.59 (1)	0.46 (1)	
^{125}I-buserelin	GnRH	8.37 ± 1.66 (4)	2.48 ± 0.30 (3)	$p < 0.05$
^{125}I-His^5-D-Tyr^6-GnRH	GnRH	19.1 (1)	3.9 (1)	
Inositol phosphates				
EC_{50} (GnRH), pM		75.1 ± 13.6 (6)	16.9 ± 6.7 (6)	$p < 0.01$
IP_{max} (%)		100	118 ± 9.1 (6)	NS

[a] Binding using ^{125}I-GnRH-A and ^{125}I-His^5-D-Tyr^6-GnRH was performed according to protocol A, and using ^{125}I-buserelin according to protocol B. Maximal binding data are expressed as % of the wild-type value in the same experiment. Data are the mean ± SEM of *n* experiments, where *n* is given in parentheses.

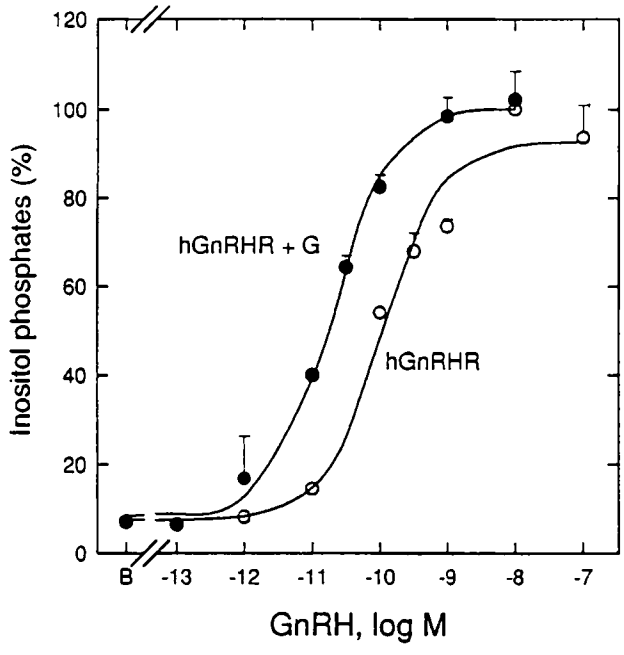

Fig. 4. GnRH-stimulated inositol phosphate responses of wild-type hGnRHR (open circles) and hGnRHR with additional glycosylation site (filled circles). Data show a representative experiment.

where IP_{max} is the maximal inositol phosphate response and EC_{50} is the GnRH concentration giving half-maximal inositol phosphate response. Applying Eq. 1 to measured values for K_d for GnRH (Table 1) and Rt (1.8-fold increase) yielded a predicted 4.9-fold increase in the value of $IP_{max_1}/IP_{max_2} \times (EC_{50_2}/EC_{50_1})$, in good agreement with the value of 5.2 ± 2.0 (mean ± SEM) obtained from measured values of IP_{max} and EC_{50} in six independent inositol phosphate stimulation experiments.

In conclusion, we have shown that introducing an extra glycosylation site enhances expression of functional human GnRHR in COS-1 cells. This will facilitate future mutagenesis and structural analyses of this receptor, as well as the establishment of cell lines expressing the human GnRHR for use in drug screening programs. This strategy for increasing expression may also be applicable to other receptors and plasma membrane proteins. The findings strengthen the concept that glycosylation is an important determinant of cell-surface receptor expression.

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