Regulation of G Protein-coupled Receptor Trafficking by Inefficient Plasma Membrane Expression

MOLECULAR BASIS OF AN EVOLVED STRATEGY*

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Despite the prevalence of G protein-coupled receptors as transducers of signals from hormones, neurotransmitters, odorants, and light, little is known about mechanisms that regulate their plasma membrane expression (PME), although misfolded receptors are recognized and retained by a cellular quality control system (QCS). Convergent evolution of the gonadotropin-releasing hormone (GnRH) receptor (GnRHR) progressively decreases inositol phosphate production in response to agonist, validated as a measure of PME of receptor. A pharmacological chaperone that optimizes fold-ability increases PME of human, but not of rat or mouse, GnRHR, consistent with a more rigorous control of ovulation in humans compared with rodents. In contrast, the rodent counterpart receptor is more frequently correctly folded, passed quality control, and was trafficked to the plasma membrane. We confirmed this by identifying the biochemical structures that promote the destabilizing effect of a particular mutant receptor, thereby decreasing PME of this G protein-coupled receptor. The strong and convergent evolutionary pressure for what initially appears to be an increasingly inefficient process suggests a regulatory mechanism with considerable selective advantage.

EXPERIMENTAL PROCEDURES

Materials—pcDNA3.1 (Invitrogen), the GnRH analog, D-tert-butyI-Ser6-des-Gly10-Pro2-ethylamide-GnRH (Buserelin; Hoechst-Roussel Pharmaceuticals, Somerville, NJ), (2S)-2-[5-(2-azabicyclo[2.2.2] oct-2-yl)-1,1-dimethyl-2-oxoethyl]-2-(3,5-dimethylphenyl)-1H-indol-3-yl-N-[2-pyridin-4-yl]thethyl propan-1-amine (IN3; Merck & Co.) (5, 6), myo-[2-3H(N)]inositol (NET-114A; PerkinElmer Life Sciences), DMEM, OPTI-MEM, Lipofectamine, phosphate-buffered saline (Invitrogen), competent cells (Promega, Madison, WI), and Endofree maxi-prep kits (Qiagen, Valencia, CA) were obtained as indicated.

Mutant Receptor—Rodent and human WT and mutant GnHR cDNAs for transfection were prepared as reported (4); the purity and identity of plasmid DNAs were verified by dye terminator cycle sequencing (PerkinElmer).

Transient Transfection—Cells were cultured in growth medium (DMEM, 10% fetal calf serum, 20 μg/ml of gentamicin) at 37 °C in a 5% CO2 humidified atmosphere. For transfection of WT or mutant receptors into COS-7 cells, 5 × 104 cells were plated in 0.25 ml of growth medium in 48-well Costar cell culture plates. 24 h after plating, the cells were washed once with 0.5 ml of OPTI-MEM and then transfectected with 5 ng/well of WT or mutant receptor with 95 ng of pcDNA3.1 (empty vector) to keep the total amount of DNA at 100 ng/well. Lipofectamine was used according to the manufacturer’s instructions. 5 h after transfection, 0.125 ml of DMEM with 20% fetal calf serum and 20 μg/ml of gentamicin were added. 24 h after transfection, the medium was replaced with 0.25 ml of fresh growth medium. Where indicated, 1 μg/ml of IN3 in 1% dimethylsulfoxide (“vehicle”) was added for 4 h in respective media to the cells and then removed 18 h before agonist treatment (7).

Pharmacological chaperones rescue misfolded mutants of the gonadotropin-releasing hormone (GnRH) receptor (GnRHR) isolated from patients with hypergonadotropic hypogonadism (1, 2) and mutant proteins associated with other diseases (3). For the GnRHR, such “pharmacopers” are cell-permeant antagonists that promote correct folding of mutants, thereby allowing them to escape retention and degradation by the quality control system (QCS). Accordingly, these drugs are valuable for assessing whether loss of plasma membrane expression (PME) of particular mutants is due to misfolding in contrast to diminished mRNA expression, loss of ligand binding, or effector coupling. An unexpected finding was that pharmacopers elevate plasma membrane expression of human, but not rat or mouse, WT GnRHRs (4). This led to the consideration that the folding and structural requirements for hGnRHR are significant, more significant than those for the rodent GnRHR, consistent with a more rigorous control of ovulation in humans compared with rodents. In contrast, the rodent counterpart receptor was more frequently correctly folded, passed quality control, and was trafficked to the plasma membrane. We confirmed this by identifying the biochemical structures that promote the destabilizing effect of a particular mutant receptor, thereby decreasing PME of this G protein-coupled receptor. The strong and convergent evolutionary pressure for what initially appears to be an increasingly inefficient process suggests a regulatory mechanism with considerable selective advantage.

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2 The abbreviations used are: GnRH, gonadotropin-releasing hormone; GnRHR, GnRH receptor; PME, plasma membrane expression; QCS, quality control system; WT, wild type; ECL, extracellular loop; DMEM, Dulbecco’s modified Eagle’s medium; IP, inositol phosphate.
Inositol Phosphate (IP) Assays—27 h after transfection, cells were washed twice with 0.50 ml of DMEM/0.1% bovine serum albumin/20 μg/ml of gentamicin and “pre-loaded” for 18 h with 0.25 ml of 4 μCi/ml myo-[2-3H(N)]-inositol in inositol-free DMEM. The cells were washed twice with 0.30 ml of DMEM (inositol free) containing 5 mM LiCl and treated for 2 h with 0.25 ml of a saturating concentration of Buserelin (10⁻⁷ M) in the same medium. Total IP was determined (8). This assay has been validated as a sensitive measure of PME for functional receptors when expressed at low amounts of DNA and stimulated by excess agonist (1–5, 7, 9–13).

Binding Assays—Cells were cultured and plated in growth medium as described except that 10⁵ cells in 0.5 ml of growth medium were added to 24-well Costar cell culture plates (cell transfection and medium volumes were doubled accordingly). 23 h after transfection, the medium was removed and replaced with 0.5 ml of fresh growth medium. 27 h after transfection, cells were washed twice with 0.5 ml of DMEM containing 0.1% bovine serum albumin and 20 μg/ml of gentamicin, and 0.5 ml of DMEM was added. After 18 h, cells were washed twice with 0.5 ml of DMEM/0.1% bovine serum albumin/10 mM HEPES, and a range of concentrations of ¹²⁵I-Buserelin (1.25 × 10⁵ to 4 × 10⁶ CPM/ml) (4) in 0.5 ml of the same medium were added to the cells. Cells incubated at room temperature for 90 min (14). After 90 min, the media were removed and radioactivity was measured as previously described (15).

To determine nonspecific binding, the same concentrations of radioligand were added to similarly transfected cells in the presence of 10 μM unlabeled GnRH. Saturation binding curve fits and calculations were computed using SigmaPlot 8.02 (Jandel Scientific Software, Chicago, IL); a non-linear one-site binding model was used to calculate Bₘₕ values (16), which are displayed as percentages of respective WT levels.

Statistics—Data (n ≥3) were analyzed with one-way analysis of variance and then paired Student’s t test (SigmaStat 3.1; Jandel Scientific Software); p <0.05 was considered significant.

RESULTS

Rat and Human GnRHR (hGnRHR) are >88% Homologous yet Have Unique Features—Fig. 1 shows the hGnRHR, indicating residues that differ in the rat (ovals; the rat substitution is shown first in each pair of letters). Lys¹⁹¹ (absent in rat and mouse GnRHR) is associated with diminished PME of the human sequence and is shown by an enlarged circle in ECL2. The thermodynamic “favorability” of the interspecific differences is indicated by numbers in parentheses adjacent to the changes. Zero is neutral; positive numbers are favored; negative numbers are disfavored.

![Diagram of the human WT GnRHR, emphasizing those residues that differ compared with rat WT GnRHR (shown Rat/Human in black circles). An enlarged circle shows the position of Lys¹⁹¹ in ECL2. The “favorability” of substitutions (www.russell.embl-heidelberg.de/aas/aas.html, for membrane proteins) of the interspecific differences is indicated by numbers in parentheses adjacent to the changes. Zero is neutral; positive numbers are favored; negative numbers are disfavored.](image)

| SPECIES/AMINO ACID | # AA | AA7 | AA168 | AA189 | AA191 | AA202/203 |
|-------------------|------|-----|-------|-------|-------|------------|
| mouse Mus musculus | 327  | L   | I     | P     | -     | P          |
| rat Rattus norvegicus | 327  | L   | I     | P     | -     | P          |
| cow Bos taurus     | 328  | P   | -1    | S     | Q     | 0          |
| sheep Ovis aries   | 328  | P   | -1    | S     | Q     | 0          |
| pig Sus scrofa     | 328  | P   | -1    | S     | Q     | 0          |
| horse Equus caballus | 328  | P   | -1    | S     | Q     | 0          |
| dog Canis familiaris | 328 | P   | -1    | G     | Q     | 0          |
| human Homo sapiens | 328  | P   | -1    | S     | Q     | 0          |
| bonnet macaque m radiata | 328 | P   | -1    | S     | Q     | 0          |
| guinea pig C porcellus | 328 | S   | -2    | S     | Q     | 0          |
| possum T vulpecula | 328  | L   | 0     | I     | Q     | 0          |
terminal and ECL2 (Cys\textsuperscript{14}-Cys\textsuperscript{199/200}) and the ECL1 and 2 (Cys\textsuperscript{114}-
Cys\textsuperscript{195/196}) as indicated.

The Cys\textsuperscript{114}-Cys\textsuperscript{195/196}, but Not Cys\textsuperscript{14}-Cys\textsuperscript{199/200}, Bridge Is Required for
Optimal Routing of the Rat and Mouse GnRHR, whereas Both Are
Needed for hGnRHR—We assessed the potential bridge requirements
by converting each of the four Cys to Ala (Fig. 2) in the mouse, rat, or
human sequence. For all sequences, the bridge between ECL1 and ECL2
(Cys\textsuperscript{114}-Cys\textsuperscript{195/196}) appears essential because replacement of either Cys
residue by Ala resulted in loss of IP in response to 10\textsuperscript{-7} M Buserelin (a
saturating concentration of a metabolically stable agonist of the
GnRHR) (10). Under the conditions described under "Experimental
Procedures," the IP response of a functional receptor to a saturating
concentration of agonist is a sensitive measure of PME (1–5, 7, 9–13).
Pharmacoperone IN3 did not effect rescue (shown only for human, Fig.
2, inset). Replacement of either Cys in the bridge between the NH\textsubscript{2}-
terminal and the ECL2 resulted in loss of IP production in the human
but had a more modest effect in the mouse sequence and virtually no
effect in the rat sequence. The human mutants (C14A or C200A) were
rescued by IN3, suggesting that the absence of this bridge resulted in a
rescuable misfolded protein.

The Requirement for the Potential Cys\textsuperscript{14}-Cys\textsuperscript{200} Bridge Is Lost in the
Absence of Lys\textsuperscript{191} or Other Insertions at Position 191—Deletion of Lys\textsuperscript{191} from the human WT sequence
also rescued mutants (C14A and C200A), indicating a relation between these
two sites and that the presence of Lys\textsuperscript{191} diminishes the probability of
this association. Interestingly, the effect of the Lys\textsuperscript{191} is not wholly an
effect of charge because converting it to uncharged Ala\textsuperscript{191}, negatively
charged Glu\textsuperscript{191}, or modestly positively charged Gln\textsuperscript{191} also produces
inefficient PME of the human sequence compared with hGnRHR lacking
Lys\textsuperscript{191}, although to somewhat different levels (Fig. 3C).

Particular Amino Acids Co-evolved with the Appearance of Lys\textsuperscript{191} or
Other Insertions at Position 191—The table in Fig. 1 compares the most
strongly, thermodynamically disfavored amino acid substitutions
among GnRHR sequences for various species (amino acids 7, 168, and
203 (202 in rat and mouse)). Amino acid 189 was included because of its
physical proximity to Lys\textsuperscript{191}. Of the 40 amino acid differences between
rodent and human GnRHR, it was striking that three of the four sites
identified in the Fig. 1 table (amino acids 7, 168, and 189) also co-evolved
with the insertion of an amino acid at position 191. The fourth (amino
acid 202 in the rodent/203 in other species) changed in the human
sequence.

FIGURE 2. Mutation of Cys residues involved in
two disulfide bridges in the rat, mouse, and
human GnRHR receptors. IP production in
response to 10\textsuperscript{-7} M Buserelin was assessed as
described under “Experimental Procedures.” The
inset shows IN3 rescue of human WT GnRHR and
the four human Cys-Cys bridge-breaking mutants.
In this and subsequent figures, data are presented
as % WT with S.E. of at least three independent
experiments performed in replicates of six.
GPCR Folding and Inefficient Plasma Membrane Expression

FIGURE 3. Effect of the human WT GnRHR Lys191 residue and its relation to the disulfide bridge between amino acids 14 and 200 (199 in rat) compared with rat GnRHR. A, effect of adding or deleting Lys191 in mouse, rat, and human WT receptor. IN3 rescue data for human WT (black bar) and human GnRHR (–Lys191, hatched bar) are in the right panel. B, effect of breaking the Cys14–Cys199/200 bridge and adding or deleting Lys191. C, effect of replacing Lys191 in human WT receptor with Ala, Glu, and Gln residues.

Exchange of Human and Rat ECL2 Appears to Influence the Proximity between Cys14 and Cys200—Fig. 4 shows the effect of mutating amino acids in the human second extracellular loop (hECL2) to the rat sequence (rendering the human ECL2 sequence more rat-like from amino acids 186 to 203), as well as several different or multiple changes selected based on proximity to this region (Fig. 4A). The difference between the rat (Ala) and human (Thr) at position 190 was ignored because of the close conservation of these amino acids and the observation that mouse, guinea pig, canines, and ungulates are identical to humans at this site. The data show that replacement of the rat sequence by hECL2 enhances PME whether or not Lys191 is present (Fig. 4B, left). The reverse combination in which hECL2 is replaced by the rat ECL2 significantly blunts the effect of removal of Lys191 and diminishes the overall level of PME (Fig. 4B, right). These results were unexpected because the rat WT normally expresses at a higher level than the human WT. Such mutants (including the human GnRHR with mutation to the rat sequence at ECL2 alone (Fig. 4B), 168/ECL2, 161/168/ECL2, 182/ECL2, 189, 186, 203, or 189/203, Fig. 4D) are rescued with phosphorone IN3 (not shown) to levels above that observed for WT receptor. Moreover, deletion of Lys191 (Fig. 4, B and D), a modification associated with increased routing to the plasma membrane, also rescues these mutants. These observations confirm that the effects of mutating ECL2 are due to misfolding and misrouting rather than altered interactions with effectors or altered levels of mRNA synthesis. Also surprising, replacement of the rodent ECL2 with the human sequence did not restore the ability of Lys191 to interfere with folding or routing and actually increased the level of PME.

Mutations in Amino Acids within ECL2 or in Structures Flanking It Also Regulate the Relation between the NH2-terminal and ECL2—The effects of mutating individual amino acids within and flanking ECL2 were examined (Fig. 4C, rat; Fig. 4D, human) because these might impact the relation between Cys14 and Cys199/200 and regulate the probability of bridge formation. Making the hGnRHR rat-like at position 161, 168, 182, 186, or 203 did not have a marked effect on the (otherwise) WT receptor. Changes at 161 or 186 increased PME of the WT sequence. When Lys191 was deleted from the hGnRHR sequence a second mutation at position 161, 168, 182, 186 or 203 exacerbated the effect of the deletion, but mutation at 189 alone or the combination of ECL2 with 168 diminished the increased PME observed due to deletion of Lys191 to near WT levels. Likewise, the combined mutation of 189 and 189/203 resembled the effect of mutating ECL2 only. Finally, the human sequence (Fig. 4D) tolerated changes from amino acids 155–161, further from Cys199/200, including the effect of Lys191 removal, unless these were combined with the rat ECL2. Combining changes at positions 161 and 168 (in effect, moving the rotational influence of Ser on the protein backbone further from Cys199/200 with ECL2 resulted in loss of activity of WT and the Lys-deleted sequence.

In the rat, mutations at 161, 168, 182, 186, and 189 increased WT PME (Fig. 4C). Less dramatic increases were seen with mutation in position 202 of rat GnRHR. When combined mutants (189/202, 168/ECL2, or 182/ECL2) were made, the effect of the mutant that exacerbated PME appeared to predominate. Also in the rat sequence, several mutants into which Lys191 was inserted showed higher PME (161, 168, 189, 202); 182 and 186 showed more modest effects. The proximity required for Cys bonds to form (1–2 Å) explains why exceedingly precise alignment is needed for Cys14–Cys200 and why twisting of the support sequences (transmembrane segments 4, 5) or those that abut on that area (i.e. ECL1, 3) also impacts bridge formation.

Other Sites Impact on the Net Level of Receptor PME and Allow the Effect of Lys191—Modifications of the hGnRHR at positions 4, 7, and 10 resulted in PME above the level of WT and amplification of the effect of deletion of Lys191 (Fig. 5A). Mutation at these same sites in the rat GnRHR did not have a measurable effect on WT PME, although modifications at 4, 7, or 10 in the presence of hECL2 enhanced PME.

Mutations in human positions 24 and 27 (Fig. 5A) decreased PME of WT GnRHR and blunted the effect of deletion of Lys191. In the rat, homologous mutations at residues 24 and 27 increased WT and WT(+Lys191) PME, perhaps due to the replacement of a Thr (C9 branched, presenting bulkiness near the backbone) with an amphipathic Met24 that allows the side chain to be buried and increases the chance of bridging the NH2-terminal with ECL2.

We created an additional series of interspecific chimeras. Fig. 5, B (rat) and C (human), shows the dramatic effect of modifications at amino acids 112, 207/208, 224/225, 299/300, 301/302, as well as selected combinations of these sites. Unlike the majority of mutations associated with ECL2, these are all steric in nature, because hydrophobicity and charge are largely conserved.
In the rat sequence, mutations were made at 299 alone or in combinations. For all mutants PME was blunted. Insertion of Lys$^{191}$ with 299 mutants also had diminished PME. Mutations at 207, 224, 301 were also associated with elevated PME, with or without Lys$^{191}$.

Of note, substitutions at positions 161, 207/224, 229/301 (Figs. 4 and 5) each allowed destabilization by Lys$^{191}$ in the rat sequence, presenting the possibility that contributions from multiple sites were important. Individual homologous mutations in the human sequence did not totally ablate the ability of the deletion of Lys$^{191}$ to decrease PME; some actually increased the effect, again consistent with the view that several different sites contribute to the final effect of Lys$^{191}$. In addition, mutations at 300 and 302 dramatically increased overall PME in the human sequence, with or without Lys$^{191}$, and decreased the same in the rat sequence.

**Breaking the Cys$^{14}$-Cys$^{200}$ Bridge Allows Assessment of the Stability of the Association of the NH$_2$-terminal and ECL2 in Human and Rat Mutants**—To determine the stability of the NH$_2$-terminal-ECL2 relation and the persistence of destabilization by Lys$^{191}$ created by the transfer of human substitutions to the rat sequence, we prepared "broken bridge" mutants (i.e. C14A, Fig. 5D) for rat and human sequences indicated by brackets (Fig. 4, B–D, and Fig. 5, B and C).

The human sequence in which position 189 was converted to rat (Q189P) results in modestly more PME than hWT and a considerable blunting of the effect of removal of Lys$^{191}$ (Fig. 4D). When the bridge is broken in this sequence, the ability to create a functional receptor is lost and not rescuable by deletion of Lys$^{191}$. This observation means that formation of the bridge occurs at a time when the association of these regions is intimate.
The observation that all rat sequences in which we successfully transferred the destabilizing effect of Lys191 with human substitutions lose this effect when the bridge is unable to form (Fig. 5D, C14A) suggests that failure to create the bridge during synthesis results in a lost opportunity for Lys191 to modulate this effect. The impact of Lys191 can only occur at the time when the bridge is able to form (likely at the time of synthesis) and not once the molecule is complete.

The human with rat substitutions at positions 112, 208, 300, and 302 is remarkable, as these amino acids flank and appear to stabilize the association of positions 14 and 200 even in the absence of the Cys bridge (i.e. when C14A is present). In this curious molecule, which is primarily human, we observe rat-like PME levels (rat WT GnRHR typically reaches the plasma membrane with about twice the efficiency of the human WT) and lack of requirement for the Cys14-Cys200 bridge yet maintenance of sensitivity to the effect of destabilization by Lys191.

Quantification of Plasma Membrane GnRHRs by Radioligand Binding—To confirm that the results measured in the functional assay (IP production) reflected changes in actual receptor numbers (in contrast to changes in the efficiency of coupling to G proteins, for example), we used radioligand binding to quantify receptor numbers of selected WT and mutants (Fig. 6). We included rat and human WT GnRHR, as well as human WT (which normally contains Lys191) with this residue deleted, and rat WT (which normally does not contain Lys191) with this residue added. We also included representative protein-encoding plasmids with the exchange of the human and rat ECL2 for both species, as well as representatives of human sequences with C14A. This modifica-
FIGURE 6. PME of GnRHR and mutants from protein-encoding plasmids was assessed in a radioligand binding assay using [125I]-Buserelin, a GnRHR agonist. Plasmids expressing rat and human sequences are shown as percent of WT for the respective template species. Plasmids for rat and human WT GnRHR, as well as human WT (which normally contains Lys191) with this residue deleted and rat WT (which normally does not contain Lys191) with this residue added, were expressed as described under “Results.” Representative protein-encoding plasmids with exchanged human and rat ECL2 for both species were included, as well as representatives of human sequences containing C14A. This modification results in breaking the apparent bridge between positions 14 and 200, a modification that is without effect in the rat. We also included human mutants in which the rat characteristics (less requirement for the Cys bridge and higher expression levels) were conveyed (112/208/300/302).

**DISCUSSION**

Lys191 in the hGnRHR sequence (328 amino acids) appears to raise the folding criteria and structural requirements for the hGnRHR compared with its rat/mouse counterparts that lack this residue (327 amino acids) or hGnRHR from which Lys192 was deleted (1–4). The view that Lys191 increases the percentage of misfolded/misrouted receptors is supported by the observation that a pharmacorone, which corrects folding errors, enabling mutants to pass through the cellular QC, also increases the PME of the human WT, but not rat GnRHR. This observation also allowed us to exclude ligand binding, effector coupling, or level of mRNA expression as a cause of reduced mutant PME (10–11). Radioligand binding data and studies in which pharmacorones are used to correct folding and sequential routing indicate that, at the low cDNA levels transfected, IP production is a good measure of functional receptors at the plasma membrane.

Removal of Lys191 or use of pharmacorone obviates the requirement for an apparent Cys14-Cys200 bridge in the hGnRHR required for correct folding. Because inserting Lys191 alone into the rat or mouse sequence (>88% homologous with the human) did not impart the requirement for this bridge between the NH2-terminal and ECL2, we sought to identify the other components of the requisite motif.

We examined thermodynamically unfavorable amino acid substitutions after recognizing that these frequently co-evolved with the appearance of the “extra” amino acid at position 191 and were proximal to it and to the bridge. An extra amino acid at position 191, not necessarily Lys, is present in all non-rat/mouse mammalian species that have been sequenced to date.

Insertion of Lys191 or Glu191 is associated with substitution of Leu7 for Pro7, except in the guinea pig in which case a less favorable change is made (Ser7). Furthermore, Lys191 is associated with a change from Ile168 to Ser168 in primates, whereas Glu191 is associated with Ser168 in the guinea pig and ungulates but Gly168 in the case of canines. Such substitutions are always unfavorable. Ile is Cβ branched with two non-H substituents attached to the β carbon, presenting bulkiness near the protein backbone and restricting conformations that the main chain can adopt.

Insertions at position 191 also correlate with a change of the Pro169 to Gin189, neutral for membrane proteins but unfavorable (−1) in the extracellular space. Only primates show the change from Pro202 to Ser203.

Glu191 is found in the sequence of many pre-primate mammalian GnRHs (Fig. 1, table). Although it too can destabilize bridge formation, it is markedly less effective than Lys191 found in primates. This further supports the progressive trend of limiting GnRHR expression at the plasma membrane in higher evolved species.

The effect of mutations associated with human disease emphasizes the importance of the Cys14-Cys200 bridge. Mutants S168R and S217R are in a previously reported “zone of death” (1) and cannot be rescued by any of several different chemical classes (indoles, quinolines, and erythromycin macrolides) of pharmacorones tried, which is a rare circumstance because the vast majority of mutants are resuable by all classes (5). This observation and the physical relation between transmembrane segments 4 and 5 and ECL2 make it attractive to consider that (charge-altering) mutations in these two residues exert their influence by regulating the position of ECL2 and the intimacy of Cys14 and Cys200.

Because of charge considerations, the unfavorable exchange of Ser and Arg likely moves the ECL2 into a position from which the formation of a Cys bridge is improbable and the mutant never passes the cellular QCS even in the presence of pharmacorones. The dramatic effect of the mutation at position 217 supports the view that the Cys14-Cys200 bridge is not created until the transmembrane segment 5 is formed.

The importance of position 217 is emphasized by recognizing that the homologous residue in rodents (amino acid 216) is associated with substantial differences in both trafficking and dominant negative action between the rat (Ser217) and mouse (Gly217) GnRHR (10), despite >96% homology. The substitution Ser217 with a highly flexible Gly217 results in loss of PME of the mutant, an effect that is resuable by deletion of Lys191 (10).

C200Y is another disease-associated mutation (12–13, 17–19) that results in loss of the Cys14-Cys200 bridge and decreased PME; rescue by a pharmacorone (10–13) supports the significance of the bridge for creating a correctly folded moiety. Consistent with that view, the homologous mutation in the rat or mouse GnRHR is a fully functional receptor. Indeed, the observation that many human disease-associated mutations recreated in rodent templates are functional (10) emphasizes the increased significance of the QCS in the human. It appears that a balance is created (in the human) between retention by the QCS and routing to the membrane. Accordingly, the human, but not rat or mouse, GnRHR is extremely sensitive to naturally occurring point mutations (10) that perturb this balance.
Of rodents, animals with large litters, only the guinea pig is known to have added an amino acid (Glu) at position 191 of the GnRHR. Interestingly, the guinea pig, a hystricomorph that diverged very early in rodent evolution, has a long luteal phase (a primate characteristic). Most non-rodent mammals such as cows, sheep, pigs, dogs, and horses also contain Glu\(^{191}\), suggesting that the loss of an amino acid in the homologous position is a specialization associated with very short reproductive cycles in rats and mice. Unlike all other reported mammalian sequences, the opossum (a non-placental mammal that places fetuses in a marsupium) has an uncharged, racemic Glu\(^{191}\) that may reflect the early divergence of this group. In hGnRHR, substitution of uncharged (Ala\(^{191}\)) or negatively charged (Glu\(^{191}\)) amino acids appears to have early divergence of this group. In hGnRHR, substitution of uncharged amino acid but supports the view that the extra amino acid provokes crowding and a change in the relative positioning of amino acids that normally leads to Cys bridge formation. Noting that various species have inserted Lys\(^{191}\), Gly\(^{191}\), or Glu\(^{191}\) suggests convergent evolutionary pressure for a means to use this position as a regulatory mechanism.

The observation that residues that regulate the efficiency of trafficking continue to evolve, even among the upper primates, suggests that selective pressure remains for this regulatory approach. Ser\(^{203}\) and Leu\(^{300}\) are uniquely human as other mammals have Pro\(^{203}\) or Val\(^{300}\). Several conservative modifications were present in regions that might impact the putative Cys\(^{14}\)-Cys\(^{199/200}\) bridge. These modifications appear to participate in recreating the bridge-stabilizing effect of Lys\(^{191}\) in the rodent, suggesting that the effect in human is the impact of multiple evolutionary changes. Among the conserved modifications, Leu\(^{224/225}\) was changed to Phe\(^{225}\) only in canines, whereas only in humans was Val\(^{300}\) converted to Leu. There also appeared to be a relation between Asp\(^{301/302}\) because this residue is Glu\(^{302}\) in rodents lacking an insertion at position 191. The ability of particular residues to have an impact at such an apparent distance is both a theme to the interactive nature of this G protein-coupled receptor and the failure of two-dimen- sional representations to portray accurately the proper spatial relations.

The progressive decrease of PME within mammals began in pre-mammalian classes. The GnRHR in fish and birds (species that produce large numbers of offspring at low metabolic energy per unit and low survival and have a single gonadotropin hormone) contains a cytoplasmic carboxy-terminal tail that increases net expression and effector coupling efficiency of the receptor at the plasma membrane (11, 22). Truncation of the tail in these species results in diminished PME, whereas production of a chimeric structure of a rodent GnRHR containing the catfish tail increases PME (11, 22). Piscine and avian GnRHRs also lack the cysteines needed to bridge the NH\(_2\)-terminal region and the ECL2; such residues are absolutely conserved among mammalian GnRHRs. Because the number of receptors per cell expressed on the plasma membrane (23, 24) cycles and thereby controls gonadotropin levels and in turn ovulation, it may be that the advent of complex primate ovarian cycling requires the ability to regulate promptly the GnRHR without the requisite time for transcription and translation.

The observation that hGnRHR is inefficiently expressed at the plasma membrane is intriguing, because this is the result of a progressive and convergent evolutionary trend toward diminished PME of the GnRHR, adds metabolic cost to create unused receptors, and creates an increased sensitivity to mutation. Potentially, this mechanism provides a source of GnRHR needed for rapid availability without transcription or translation. A similar mechanism appears to regulate the human \(\delta\) opioid receptor because permeable agonists and antagonists also facilitate post-translational processing and increased export of the ligand-stabilized receptor from the endoplasmic reticulum to the cell surface (25). Other reports indicate that other receptors (Glur1, \(\alpha_1\)D adrenoceptor, odorant, and Luteinizing hormone receptors) are likewise inefficiently expressed at the plasma membrane (26–30); this suggests that restricted trafficking may be a more commonly occurring means of regulating protein availability than presently appreciated.

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