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Targeting of G protein-coupled receptors to the plasma membrane in health and disease

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1. ABSTRACT

G protein-coupled receptors (GPCRs) are cell surface membrane proteins that recognize specific signals (ligands) from an immense number of chemically diverse substances. These receptors act as signal transducers for messages carried by external, systemic, or local stimuli. As complex molecular structures, which must attain specific shapes, newly synthesized GPCRs are subjected to conformational scrutiny at the endoplasmic reticulum level before their passage to the plasma membrane. Such a quality control mechanism guards against aberrant protein structures and checks for proper folding, processing and structural integrity of nascent proteins. Despite this stringent quality control screening mechanism, gain- or loss-of-function mutations that result in GPCR misfolding in the endoplasmic reticulum can manifest themselves as profound effects on health. Understanding the molecular, cellular and energetic mechanisms controlling GPCR intracellular routing is essential for preventing or correcting the conformational abnormalities associated with disease-causing misfolded receptors. This article reviews the mechanisms subserving plasma membrane targeting of GPCRs and describes novel and promising approaches to correct misfolding and misrouting related to various disease states.

2. INTRODUCTION

G protein-coupled receptors (GPCRs) constitute a large and functionally diverse superfamily of membrane proteins whose primary function is to transduce extracellular stimuli into the intracellular environment through the activation of one or more signal transduction pathways. The ligands that recognize and activate these receptors are highly variable in chemical structure and may include photons, odorants, pheromones, hormones and neurotransmitters, and vary in size from small biogenic amines, to peptides, to large proteins. As a result, GPCRs play a key role in regulating an array of biological functions, including cell growth and differentiation, immune responses and cellular metabolism (1-3). Although GPCRs may vary considerably in molecular size, all share a common molecular topology that consists of a single polypeptide chain of variable length that traverses the lipid bilayer seven times, forming characteristic transmembrane (TM) hydrophobic alpha-helices connected by alternating extracellular and intracellular sequences or loops (EL and IL, respectively), with an extracellular NH_2-terminus and an intracellular COOH-terminal tail ("C-tail") (1,4). These receptors characteristically bind large heterotrimeric G-proteins; upon agonist binding, GPCRs undergo

conformational changes that allow exposure of particular sequences to G proteins which, in turn, act as mediators of receptor-evoked effector (enzymes and ion channels) activation (5). Activated GPCRs are rapidly desensitized and internalized via formation of endosomes, where receptor-mediated signaling terminates and the fate of the internalized receptor is determined. Thus, the net amount of a given GPCR at the plasma membrane (PM) will be dictated by: a) its dynamics of intracellular export from their site of synthesis (the endoplasmic reticulum) to their final destination (the PM); b) the fate of the receptor following ligand-stimulated internalization and endocytosis, either to the degradative or recycling pathway; and, c) normal membrane turnover.

Structural alterations provoked by mutations in the gene sequence of GPCRs may lead to abnormal function of the receptor molecule, and eventually to disease. Depending on the location and the nature of the mutation, structural alterations may provoke either gain- or loss-of-function of the affected receptor (1,6). Loss-of-function mutations may alter domains involved in particular functions of the receptor (*e.g.* ligand binding or interaction with coupled effectors) or sequences important for proper folding and intracellular transport of the receptor to the PM. Because of its paramount importance in function, and as a cause of disease, GPCRs currently constitute an important therapeutic target for an array of diseases, including cancer.

The present review focuses on GPCR outward export trafficking from the ER to the PM, and how mutations in GPCR sequence may lead to misfolding and misrouting in certain disease states, ending with the description of promising approaches for overcoming such defects. The gonadotropin-releasing hormone (GnRH) receptor (R; GnRHR), a receptor belonging to family A within the superfamily of GPCRs (Figure 1), is used as an example of the mechanisms that subserve the efficiency of outward trafficking of GPCRs to the PM.

3. THE ENDOPLASMIC RETICULUM QUALITY CONTROL SYSTEM

The synthesis of proteins is tightly regulated at the transcriptional, translational and post-translational levels by multiple signaling pathways. Synthesis and processing of these molecules occurs in association with the endoplasmic reticulum (ER) and Golgi apparatus. The ER has the daunting task of synthesis and assembly of thousand of proteins and provides the specialized environment necessary for folding, glycosylation and oligomeric assembly of proteins prior to their translocation to the Golgi where processing of the protein is completed. As proteins are synthesized in the ER, they fold and adopt distinct conformations that provide a structure recognized by quality control system (QCS) machinery as a structure compatible with ER export (7-13). The ER QCS guards against aberrant protein structures and checks for proper folding, processing and structural integrity of nascent proteins, ensuring proper intracellular trafficking of the newly synthesized protein to the Golgi apparatus, and eventually, to its final destination within the cell (*e.g.* the plasma membrane). By monitoring the structural correctness of newly synthesized proteins, the QCS also prevents accumulation of defective proteins that may potentially accumulate, aggregate and interfere with normal cell function, and provides the means for exporting proteins to other cell compartments (13-17). Since the scrutiny by the ER QCS relies on conformational rather than on functional criteria, even minor alterations in the secondary or tertiary structure of a protein may lead to intracellular retention and degradation. Correct folding and trafficking are delicately balanced, and even minor synonymous polymorphisms in complex transmembrane proteins that affect the timing of co-translational folding may lead to altered function of the protein (18,19).

The ER QCS operates at several levels employing a variety of mechanisms that include a complex sorting system to identify and separate proteins according to their maturation status, as well as the action of specialized folding factors, escort proteins, retention factors, enzymes, and members of major molecular chaperone families (9,20-23). In general, molecular chaperones are ER-resident proteins that bind to and stabilize unstable conformers of nascent polypeptides to facilitate the correct folding, or assembly, of the substrate polypeptide through regulated binding and release cycles. Molecular chaperones also prevent aggregation and/or incorrect interactions between misfolded proteins and other molecules in a crowded and viscous ER environment, thereby preventing their export to other cellular compartments (9,10,14,22,23). Thus, molecular chaperones guard nascent polypeptide chains against potentially unproductive and even toxic interactions that may occur during the different stages of the folding process (*i.e.* while still attached to the ribosome, just after release from the ribosome, as a folding intermediate with exposed hydrophobic surfaces, and even as a misfolded protein). Proteins that do not fulfill the criteria of the ER QCS are retrotranslocated and degraded in proteasomes or lysosomes (24-26).

Similar to other proteins, GPCRs have to be correctly folded in order to pass through the ER QCS. Nevertheless, it has become evident that certain GPCRs are normally exported from the ER in a relatively inefficient manner, leading to restricted PM expression (27-34). For example, it has been found that only a fraction (40-60%) of newly synthesized human GnRH (35) and delta opioid receptors (33) reach a mature conformation compatible with ER export. This natural "inefficiency" in folding and maturation of certain GPCRs in the early secretory pathway may represent a means for controlling their number at the PM level. The possibility that intracellularly retained misfolded or incompletely mature receptors may be rescued from ER trapping and degradation by drugs that act as chaperones (i.e. pharmacological chaperones, or "pharmacoperones") offers a unique opportunity for therapeutic interventions.

3.1. Molecular chaperones

As mentioned above, molecular chaperones serve as a control mechanism for recognizing, retaining and

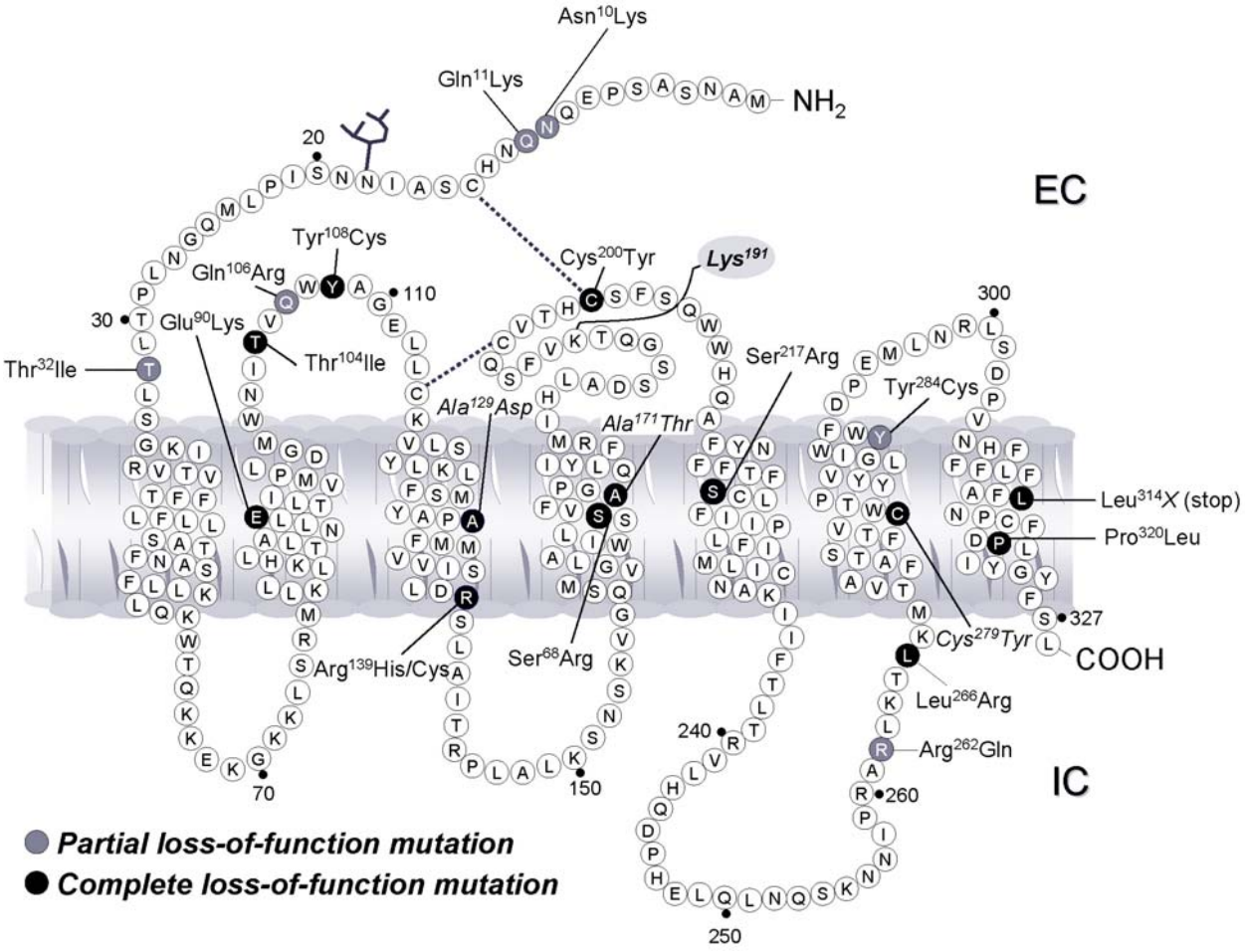

Figure 1. Sequence of the human gonadotropin-releasing hormone receptor and location of the partial and complete inactivating mutations identified to date. The gonadotropin-releasing hormone receptor belongs to the rhodopsin/β-adrenergic-like family of GPCRs (family A), and is coupled to the trimeric $G_{q/11}$ protein localized in the cytoplasm and associated with the intracellular domains of the receptor. Its natural ligand is gonadotropin-releasing hormone, a decapeptide produced by the hypothalamus and released in synchronized pulses to the anterior pituitary to regulate reproductive function. Unlike other members of family A of GPCRs, the human GnRHR exhibits several unique features including the reciprocal exchange of the conserved Asp and Asn residues in the transmembrane helix-2 and -7, the replacement of Tyr with Ser in the Asp-Arg-Tyr motif located in the junction of the helix-3 and the IL-2, and the lack of the carboxyl-terminal extension into the cytosol (187). The dotted lines represent disulfide bridges between Cys^{14} and Cys^{200}, and Cys^{114} and Cys^{196}. The position of Lys^{191} is indicated by the grey oval. EC: Extracellular; IC: Intracellular.

targeting misfolded proteins for their eventual degradation. Although the steric character of the protein backbone restricts the spectrum of protein shapes that are recognized by the stringent quality control mechanisms, some features displayed by proteins, including exposure of hydrophobic shapes, unpaired cysteines, immature glycans, and particular sequence motifs, have been identified as important for chaperone-protein association (16,36). In fact, molecular chaperones possess the ability to recognize misfolded proteins by the exposure of hidden hydrophobic domains or specific sequences (36,37). Through this association, chaperones may stabilize unstable conformers of nascent polypeptides to prevent aggregation and facilitate correct folding or assembly of the substrate via binding and release cycles (23).

Several GPCR interacting proteins that support trafficking to the cell surface have been identified. Nina A (*n*either *i*nactivation *n*or *a*fterpotential A), a photoreceptor-specific integral membrane glycoprotein, is a molecular chaperone that facilitates cell surface membrane expression of the sensory GPCR rhodopsin 1 in *Drosophila melanogaster*; its absence leads to rhodopsin 1 ER accumulation and degradation (38-41). Its mammalian homolog RanBP2 specifically binds red/green opsin molecules and acts as a chaperone aiding proper folding, transport and localization of the mature receptors to the cell membrane (42). ODR4 is a molecular chaperone that assists in folding, ER exit and/or targeting of olfactory GPCRs (*e.g.* ODR10 in the nematode *Caenorhabditis elegans*) to olfactory cilia (43,44). Calnexin and calreticulin

are molecular chaperones that bind a broad range of glycoproteins, including several GPCRs [*e.g.* the GnRHR, vasopressin-2 receptor (V2R), and the glycoprotein hormone receptors, 45-50]. The action of these chaperones predominantly centers on substrate N-glycans present on the newly synthesized proteins, adding hydrophobicity to the folding protein (46,49). When N-linked glycosylation or early glycan processing fails, glycoproteins misfold, aggregate and fail the QCS (48). RAMPs (*r*eceptor *a*ctivity *m*odifying *p*roteins), are proteins that interact with several GPCRs (*e.g.* the calcitonin receptor-like receptor, the vasoactive intestinal polypeptide/pituitary adenylate cyclase-activating peptide receptor, the glucagon receptor and the parathyroid hormone receptor) fostering the transport of the associated receptor to, and regulating its signaling function at, the PM (51), whereas gC1q-R (receptor for globular heads of C1q) interacts with the carboxyl-terminus of the alpha$_{1B}$-adrenergic receptor and regulates the maturation and expression of the receptor (52). Another molecular chaperone is BiP/Grp 78, which is involved in the protective unfolded protein response,, a cell stress program activated when misfolded proteins accumulate and/or aggregate in the ER (53,54). Finally, DriP78 is an ER-membrane-associated protein that binds to the F(x)$_3$F(x)$_3$F motif of the dopamine receptor (and presumably other GPCRs bearing this motif) thereby facilitating its maturation and export to the PM (55). Identification of these particular molecular chaperones is important since they represent a potential target to manipulate ER retention and or export mechanisms, and hence a means for influencing protein trafficking and secretion (56,57).

3.2. GPCR motifs that promote ER-export or –retention

Properly folded and assembled secretory proteins are segregated from ER-resident proteins into COPII-coated vesicles for exporting to the Golgi to be further processed before being sent to their final destination (58-62). Several mechanisms of bulk-flow, ER-retention and receptor-mediated export have been suggested to operate during this transport step. One mechanism proposed is the association of particular sequences or motifs with COPII-coated vesicles; however, this has only been demonstrated for a few cargoes bearing the diacidic (D*x*E) and dihydrophobic (FF) signals, which are present in the C-tail of the vesicular stomatitis viral glycoprotein (59,63,64), the cystic fibrosis transmembrane conductance regulator protein (65) and the p24 family of proteins (61). Nevertheless, several recently identified motifs have been shown to be involved in GPCR exit from the ER and the Golgi. These include the dileucin motifs E(x)$_3$LL [identified in the human V2R (66-69)], FN(x)$_2$LL(x)3L [in the humanV3R (70)], and F(x)$_6$LL identified in the C-tail of several GPCRs (71), as well as the triple phenylalanine F(x)$_3$F(x)$_3$F motif [(present in the C-tail of the dopamine D1 receptor, the M2-muscarinic receptor and the angiotensin II AT$_{1A}$ receptor (55,72)]. Mutation at these motifs markedly inhibited receptor expression at the PM due to intracellular retention of the altered receptor (36,66,68,73). Studies on export motifs present in the NH$_2$-terminal domain of GPCRs are scarce; nevertheless, recent studies in alpha 2-adrenergic receptors have identified a distinct YS motif within this domain which is important for receptor export from the Golgi (74).

Two highly conserved motifs, the E/DRY motif (at the boundary of the TM segment 3 and the IL2) and the N/DPxxY motif (at the TM segment 7 near the cytoplasmic face of the PM) are important structural determinants in many GPCRs (3,75,76). In some receptors, mutations in these motifs [such as the E/DRY motif in the V2R and the GnRHR, and the N/DPxxY motif in the V2R, GnRHR, endothelin-B receptor, melanocortin-4 receptor and the chemokine receptor 5 (77-81)] may lead to different functional outcomes including defective intracellular trafficking, depending on the particular receptor. On the other hand, the sequence of GPCRs belonging to family A predicts formation of a disulfide bridge between the first and second extracellular loops; this structural feature is associated with the stabilization of the heptahelical structure (1) and mutations near or at either end of this bridge usually result in a complete loss of activity, due to retention in the ER of the mutant receptor (82). In the case of the human (h) GnRHR and rhodopsin, receptors bearing mutations at this location are recalcitrant to or cannot be easily reconstituted with pharmacoperones (77,82).

Post-translational modifications are also important for GPCR export to the cell surface. Two modifications are particularly important: palmitoylation and *N*-linked glycosylation. In some GPCRs, *S*-acylation with palmitic acid of conserved cysteine residues in the C-tail provide an additional site for anchoring of the receptor to the PM, creating a fourth intracellular loop (1,83,84). This palmitic acid-mediated membrane anchoring may potentially decrease agonist-induced receptor internalization thereby extending the residence time of the ligand-bound receptor at the cell surface (84). In some GPCRs, palmitoylation also plays a significant role in receptor export from the ER as abrogation of this modification leads to misfolded structures and intracellular retention of the unpalmitoylated receptor (85-87). Another common post-translational modification is *N*-linked glycosylation at the consensus sequence N*x*S/T (1). This posttranslational modification facilitates folding by increasing protein solubility and stabilizing protein conformation (88-90). For several GPCRs, glycosylation is absolutely required for cell-surface expression of the receptor as mutation of the glycosylation sites lead to intracellular accumulation of mutated receptors (91-96). This is the case, for example, in the gonadotropin receptors in which mutations at the AFNGT sequence of the NH$_2$-terminus alter glycosylation and cause intracellular sequestration of the mutant receptor protein (97,98). In contrast, in other GPCRs, this modification is not absolutely required for receptor transport to the cell surface (99).

4. OLIGOMERIZATION AND RECEPTOR TRAFFICKING

Extensive biochemical and pharmacological studies support the concept of dimerization or oligomerization of GPCRs as a fundamental process of

GPCR activity. It appears that GPCRs approach this issue differently; some receptors are monomeric in the membrane and oligomerize upon ligand binding and activation (100-102), whereas others constitutively form multi-unit complexes as they are synthesized in the ER or processed in the Golgi; an apparent requisite for correct targeting to the cell surface (103-109). Constitutive oligomerization has been demonstrated for a number of GPCRs, including the receptors for $GABA_B$ (110-112), melatonin (113), dopamine D2 (114), vasopressin (115) and serotonin (116) as well as for the delta-opioid receptor (117), the $beta_2$-adrenergic receptor ($beta_2$AR) (118-120) and the follitropin receptor (121). The functions of homo- and hetero-oligomerization at the ER include effective quality control of protein folding prior to export to the PM (103,104,109). For example, inhibiting homodimerization of the $beta_2$AR leads to ER retention and perturbed cell surface targeting (118). In the case of the $GABA_BR$, heterodimerization between $GABA_BR1$ and $GABA_BR2$ is an obligatory prerequisite for cell surface expression of a functional receptor (110,111,122); apparently, formation of a coil-coil domain between the C-tail of the $GABA_B$ receptor subtypes masks an ER retention signal (R*x*R) located in the C-tail of the $GABA_BR1$, thus promoting the ER export of the heterodimer to the PM (111). A similar role in receptor outward trafficking has been shown for the $alpha_{1D}$- and $alpha_{1B}$-adrenergic receptors (123) and the $beta_2$-AR (124).

Introduction of ER retention motifs (e.g. R*x*R motif) into GPCRs may lead to retention of the modified receptor and also to hindering of the cell surface delivery of an homologous unmodified receptor (118), a dominant-negative effect. The dominant-negative effect of mutant receptors on wild-type (WT) receptor trafficking has been demonstrated for a number of GPCRs (125-128), including the hGnRHR (129,130). Of the 21 mutations of the hGnRHR reported in patients with hypogonadotropic hypogonadism (HH) (Figure 1), 7 mutant receptors were partially functional when expressed in heterologous cell systems; the remaining mutant receptors were non-functional (77,78,131). When several of the non-functional receptors were co-expressed with the WT receptor in heterologous cell systems, it was discovered that these non-functional receptors also inhibited WT GnRH receptor function, a dominant-negative effect (129,130). Creation of a protein chimera in which the green fluorescent protein sequence was added to the carboxyl terminus of the wild-type hGnRHR sequence allowed the use of confocal microscopy to localize wild-type receptors that were co-expressed with the dominant-negative mutant receptors. The dominant-negative action that the mutant GnRH receptors have on the wild-type receptor appears to be due to ER retention of an aggregate of wild-type and mutant proteins (35,132) (Figure 2). The wild-type and mutant receptors appear to form oligomers in the ER and those oligomers were retained and presumably degraded.

It is important to mention that oligomerization in the ER, as part of the intracellular transport of GPCRs, seems to be a more general mechanism applicable to other membrane proteins. Although the intrinsic mechanism(s) that subserve these protein-protein associations as well as the mechanistic basis for the general need for oligomerization of membrane proteins is not completely understood, recent studies suggest that oligomerization of certain membrane proteins facilitates their recognition by the dimeric molecular chaperones 14-3-3 epsilon- and zeta-, which in turn probe for the valency and spatial arrangement of recognition domains (i.e. the carboxyl tails), functioning as a checkpoint for forward trafficking of maturing multimeric proteins (133).

5. MISFOLDED GPCRs AS DISEASE ETIOLOGY

It is well recognized that point mutations of receptors, enzymes and ion channels frequently result in protein misfolding and subsequent retention by the cellular QCS (77,131,134-143). Other factors may also trigger protein misfolding, including temperature, oxidative stress and activation of signaling pathways linked to protein folding and quality control (35,144). One well-studied example of this abnormality is cystic fibrosis, a disease caused by mutations of the cystic fibrosis transmembrane conductance regulator (CFTR). In this disease the Phe^{508}del mutation, which is found in ~70% of patients with this condition, leads to chaperone-mediated ER retention and rapid degradation of the incompletely processed cAMP-regulated chloride transmembrane channel (144-146). It is important to note, that misfolding can result in protein molecules that *retain* intrinsic function (*e.g.* in their intrinsic ability to participate in particular functions such as substrate or ligand recognition, receptor activation or coupling to effectors) and, that for reasons of mislocation only, cease to function normally and result in disease.

Misfolding and deficient outward trafficking of GPCRs frequently lead to disease (Table 1). This is the case, for example, with the autosomal dominant form of retinitis pigmentosa, X-linked nephrogenic diabetes insipidus and HH. In retinitis pigmentosa, ER trapping of misfolded mutant rhodopsin eventually leads to rod photoreceptor degeneration (82,147-150) followed by cone degeneration. Mutations in the V2R gene cause X-linked nephrogenic diabetes insipidus, a disease characterized by an inability to concentrate urine despite normal or elevated plasma concentrations of the antidiuretic hormone arginine vasopressin (80,151-157). Nearly 70% of V2R mutants causing X-linked diabetes insipidus are unable to reach the cell surface membrane and respond to agonist stimulation (80). Mutations leading to receptor misfolding and resultant misrouting of the hGnRHR cause congenital HH, a disease characterized by reproductive failure due to partial or complete inability of the pituitary gonadotropes to respond to agonist (158,159). The majority (~90%) of the hGnRHR mutants whose function has been examined to date are trafficking-defective receptors as disclosed by mutational studies and/or response to pharmacological chaperones (77,130,131,160) (see below). Because reproductive failure is not life-threatening, it is likely that many cases (particularly partial HH forms) go undiagnosed and individual mutants, if severe in function, are not transmitted to progeny.

Table 1. Loss-of-function diseases or abnormalities caused by GPCR misfolding

Disease or abnormality	GPCR	Pharmacoperones	Refs
Retinitis pigmentosa	Rhodopsin	9-cis-retinal, 11-cis-retinal, 11-cis-7-ring retinal	82, 147-49, 233, 234
Nephrogenic diabetes insipidus	V2R[§]	SR121463 (satavaptan), SR49059 (relcovaptan), VPA-985, YM087, OPC41061 (tolvaptan), OPC31260	80, 152-157, 232, 233
Hypogonadotropic hypogonadism	GnRHR	Indoles, quinolones, erythromycin-derived macrolides	77, 158, 228-231
Familial hypocalciuric hypercalcemia	CaR	NPS R-568	217
Male pseudohermaphroditism Hypergonadotropic hypogonadism	LHR		97, 161, 162
Ovarian dysgenesis	FSHR		97, 163-165
Congenital hypothyroidism	TSHR		166-169
Hirschsprung´s disease	E-BR		176, 177
Red head color and fair skin (RHC) phenotype and propensity to skin cancer	MC1R	NBI-A	170, 171, 237
Obesity	MC3R		174
Obesity	MC4R		172, 173
Resistance to HIV-1 infection	CCR5		178

Abbreviations: V2R: Vasopressin Type-2 Receptor; GnRHR: Gonadotropin-releasing hormone receptor; CaR: Calcium-sensing receptor; LHR: Lutropin (luteinizing hormone) receptor; FSHR: Follitropin (follicle-stimulating hormone) receptor; TSHR: Thyrotropin (thyroid stimulating hormone) receptor; E-BR: Endothelin-B receptor; MC1R: Melanocortin-1 receptor; MC2R: Melanocortin-2 receptor [or adrenocorticotropin (ACTH) receptor]; MC3R: Melanocortin-3 receptor; MC4R: Melanocortin-4 receptor; CCR5: Chemokine receptor-5.

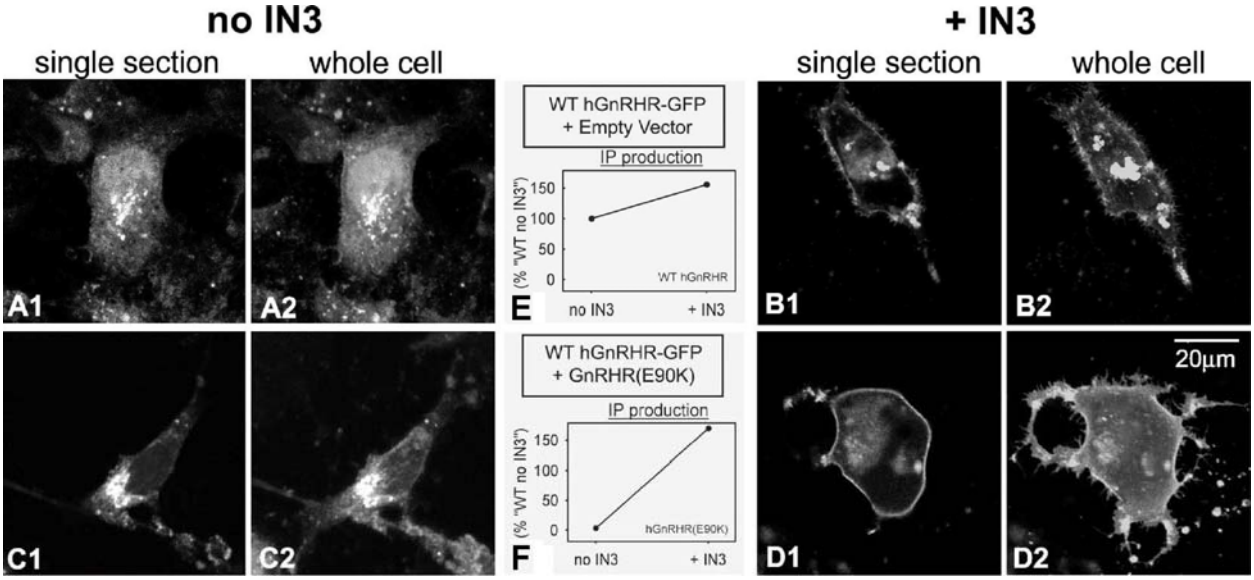

Figure 2. The dominant-negative effect of a GnRH mutant on plasma membrane expression of wild type (WT) human GnRHR. In these images the human GnRHR is labeled with green fluorescent protein (GFP) and the $Glu^{90}Lys$ ($E^{90}K$) mutant is unlabeled. A-D, Confocal micrographs of cells coexpressing the GFP-tagged WT human GnRHR and empty vector (A-B) or human GnRHR($E^{90}K$) (C-D), and stained with ER-Tracker dye. Micrographs are either single-confocal sections or overlay projections of all sections through the cell, as noted. In the presence of a pharmacoperone (IN3), the GFP-tagged WT receptor showed greater plasma membrane localization when expressed alone (B) or with the human GnRHR mutant $Glu^{90}Lys$ ($E^{90}K$) (D). The misfolded mutant, itself, is recognized by the cell's QCS as defective and retained in the ER for degradation. Reprocessing can also cause the WT receptor to be retained, presumably due to oligomerization. Pharmacoperones which correct folding, reduce this event. E-F, Inositol phosphate production in cells individually transfected with the GFP-tagged WT human GnRHR *plus* empty vector (E) or GFP-tagged WT human GnRHR plus the untagged mutant (F), in the absence or presence of the pharmacoperone. Reprinted from reference 132 with permission of the Endocrine Society. Copyright 2004, The Endocrine Society.

Misfolding of GPCRs as a cause of disease may be substantially more common than previously recognized. This is suggested by the recognition, in other GPCRs, of mutations that provoke intracellular retention of the abnormal (and presumably misfolded and/or incompletely processed) receptor in the ER or ER/Golgi intermediate compartment leading to decreased or absent cell surface membrane expression as a consequence. Trafficking-defective mutants of the glycoprotein hormone receptors (lutropin, follitropin, and thyrotropin receptors) have been described in patients with Leydig cell hypoplasia, a rare autosomal recessive form of male pseudohermaphroditism (161,162), in women with ovarian dysgenesis (163-165), and in congenital hypothyroidism (166-169). The melanocortin-1 receptor, which is a major determinant for variations in skin and hair pigmentation, has been found to

be mutated at different locations in patients with skin and hair abnormalities, and increased susceptibility to skin cancers (170). Among the 60 or so mutants described, at least four display decreased cell surface expression (171). Intracellular retention of mutants from two other melanocortin receptors, the melanocortin-3 and melanocortin-4 receptors associated with regulation of fat deposition and energy homeostasis, have been detected in patients with morbid obesity (73,172-174). Mutations in the calcium-sensing receptor leading to intracellular retention of the abnormal receptor have been found in patients with familial hypocalciuric hypercalcemia (175), whereas mutations that lead to intracellular trapping of the endothelin-B receptor have been detected in patients with Hirschsprung's disease or aganglionic megacolon (176,177). Intracellular trapping of the chemokine receptor 5 has also been observed in a subset of subjects with resistance to HIV infection (178).

The fact that mutations causing misfolding and intracellular retention of the receptor protein do not always modify domains involved in agonist binding, receptor activation or effector coupling, offers a unique opportunity to correct misrouting and rescue mutants by pharmacologic means, thereby restoring function and, potentially, curing disease. Further, it has also become clear that variable amounts of some WT GPCRs are normally misrouted, presumably as a result of misfolding (27,29,30,33,179-182), suggesting that this level of post-translational control may itself be amenable to pharmacological intervention and provide another level of potential therapeutic intervention (141) (see below).

6. INEFFICIENT ROUTING OF WT GPCRS

It is currently recognized that inefficient folding and maturation of GPCRs is not only limited to mutants bearing alterations in their primary sequence, but also occurs with wild-type proteins. Normally inefficient maturation and degradation have been demonstrated for a number of GPCRs, including the dopamine D_2-receptor (34), the lutropin receptor (182,183), the delta-opioid receptor (33,179), the V2R (28), olfactory receptors (29,30), cannabinoid receptors (27) and the hGnRHR (35). In these receptors, inefficient maturation leads to retrotranslocation and proteosomal degradation of a substantial amount of misfolded/incompletely folded newly synthesized receptors. In the case of olfactory receptors, which are poorly expressed at the cell surface membrane, ER retention via interaction with the chaperone calnexin impedes trafficking of the receptor to the Golgi apparatus to complete carbohydrate processing (30). A similar mechanism probably operates for other incompletely folded/processed receptors, such as the delta-opioid receptor and the lutropin receptor (179,182). Although the mechanisms subserving the ER retention of WT receptors remain to be elucidated, potential mechanisms may include exposure of retention motifs and sequestration of the receptors from the COPII machinery and/or poor coupling to the export machinery due to misfolding. In any case, it has been speculated that this inefficiency of WT receptors to acquire a mature conformation compatible with ER export might represent a fine-tuning mechanism through which the cell efficiently regulates the number of functional receptors at the PM. In the case of the hGnRHR, which also is normally inefficiently trafficked to the cell surface membrane, recent studies strongly suggest that this natural inefficiency has resulted from strong and convergent evolutionary pressure, producing receptor molecules that are sensitive to single changes in chemical charge and are delicately balanced between expression at the PM and retention/degradation in the ER (31,32,141,184,185).

Studies with pharmacoperones have shown that PM expression of the WT hGnRHR *but not* the WT rat and mouse GnRHRs increase substantially upon exposure to these agents (132,141,180,184,185), indicating that a large portion of the hGnRHR is normally inefficiently trafficked to the cell surface membrane, retained by the QCS, and likely degraded. On the other hand, the observation that the human receptor is so sensitive to alterations of single charges in the receptor structure, suggests that the hGnRHR is precariously balanced between retention in the ER and routing to the PM, which is not seen in rats or mice, animals that route their GnRHRs to the PM with higher efficiency (132,141,180,184,185). Several studies (31,32,184,185) indicate that this natural inefficiency of the human receptor represents an evolved mechanism designed to control expression and function of this receptor: *i)* mutations leading to misfolded hGnRHRs have less impact on trafficking in rat and mouse wild-type GnRHRs; *ii)* a particular feature of primate GnRHRs is the presence of a lysine residue at position 191, located in the EL2 (Figure 3), that restricts the GnRHR PM expression (186). Non-primate mammals utilize a less-effective Glu^{191} in this position (or Gly^{191} in the opossum, all 328 amino acids) while rats and mice do not have this insertion at all [327 amino acids, (187, 188)] and a higher proportion of translation product of both rodent receptors is expressed at the PM (180, 184, 185); *iii)* the presence of Lys^{191} limits the number of hGnRHR molecules that may be potentially exported from the ER to the PM through a mechanism involving formation of the Cys^{14}-Cys^{200} bridge, which apparently stabilizes the human receptor in a conformation compatible with ER export (141,185). In the rat GnRHR, formation of this bridge (Cys^{14}-Cys^{199}; 199 is the orthologous position in the rat to human 200) is not an essential requirement for correct folding, as mutations in any of these positions do not affect agonist-stimulated intracellular signaling (131); and, *iv)* construction of human receptors that were more "rat-like" led to receptors that expressed at the higher levels associated with the rat receptor and lacked the requirement for the Cys^{14}-Cys^{200} bridge (141,185). Mutagenesis experiments (based on the identification of the thermodynamically unfavored changes among GnRHR from various animal species, the amino acid residues that frequently coevolved with the appearance of the "extra" amino acid in position 191 in primates or that were proximal to it and to the Cys^{14}-Cys^{200} bridge, and speculation based on the physical relationship between amino acids in the three dimensional structure of the receptor molecule) were performed. These revealed that residues located in the NH_2-terminus and in the EL2 as well

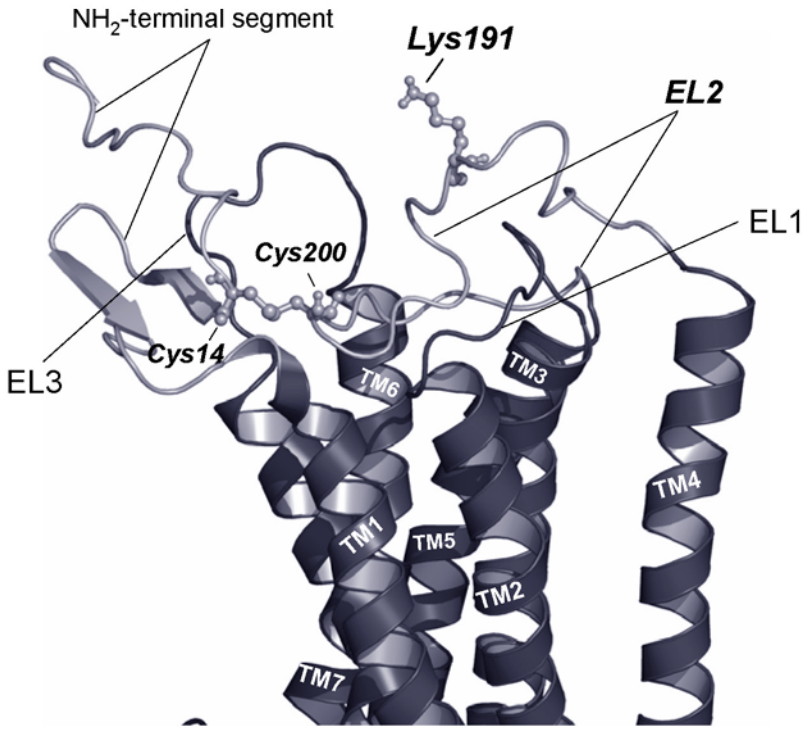

Figure 3. Close-up of the predicted structure of the upper two thirds of the human GnRHR. The antiparallel transmembrane (TM) helices are represented by coiled structures, the extracellular loops (EL) 1 and 3 by dark gray cords, and the EL2 and NH_2-terminus by light gray cords. Cys^{14} and Cys^{200}, which form a disulfide bridge between the EL2 and the NH_2-terminus, and Lys^{191} in the EL2, are rendered as spheres and sticks. The optimized model shown was generated by molecular dynamics simulation and visualized using the program PyMol (DeLano Scientific, San Francisco, CA), and was kindly provided by Eduardo Jardón-Valadez and Angel Piñeiro from the Faculty of Chemistry of the National University of Mexico (UNAM), Mexico City, Mexico.

as sequences flanking this loop (i.e. within TM segments 4 and 5) and those that abut on that area (ELs 1 and 3), presumably control the destabilizing role of Lys^{191} on the formation of the Cys^{14}-Cys^{200} bridge (185). To our knowledge, there are no similar studies in other GPCRs that are normally inefficiently routed attempting to identify whether or not such a limited ER export of the receptor to the PM follows the evolutionary pattern found in the GnRHR.

7. STRATEGIES FOR STABILIZING MISFOLDED PROTEINS

Several approaches have been applied to manipulate the ER QCS and salvage defective proteins *in vitro*. Among these are the use of physical methods (144,189-192), non-specific protein stabilizing agents (such as polyols and sugars) (193), genetic modification of mutant proteins ("genetic rescue") (194-196), and the use of template molecules or pharmacoperones that correct errors in folding and restore activity by correct routing ("pharmacological rescue") (21,192,197,198)..

As mentioned above, studies on the biosynthesis and localization of the CFTR Phe^{508}del mutant showed that the mutation leads to subtle misfolding that does not grossly interfere with proper function, but that leads to intracellular retention of the mutant protein (145,189). Expression of this mutant in Xenopus oocytes and in Sf9 insect cells (which are maintained at lower temperatures than mammalian cells) led to detection of chloride channel activity owing to the processing sensitivity of nascent proteins to temperature (189). Incubation at reduced temperatures (20-30^o C) reverted processing of the CFTR mutant toward the WT channel, allowing the cAMP-regulated Cl^- channel to be expressed at the cell surface membrane (189,190). Similarly, increased expression of several conformationally defective GnRHRs, bearing different point mutations, resulted from incubating transfected cells at lower (32^o C) temperatures (35). Thus, it appears that for certain temperature-sensitive, misfolded proteins, lower temperatures prevent aggregation in the ER and allow the defective proteins to escape the QCS facilitating their trafficking to their site of action. Another effective strategy to rescue the function of misfolded proteins is by incubating cells expressing the mutant protein with stabilizing agents. Incubation of stable CFTR Phe^{508}del transfectants with the cellular osmolytes glycerol or trimethylamine N-oxide led to accumulation of functional Phe^{508}del protein and an increase in whole cell Cl^- conductance (146,193). Low molecular weight compounds such as 4-phenylbutyric acid and deuterated water, can stabilize proteins against thermally induced denaturation (199-201), whereas sarco(endo)plasmic reticulum Ca^{2+} ATPase pump inhibitors act by promoting the release of Ca^{2+} to the cytosol, thereby modifying the

level of activity of molecular chaperones (202). Although chemical chaperones can rescue some misfolded proteins, they are nonspecific and might potentially increase secretion of many different proteins in various cellular compartments leading to inappropriate changes in the levels and/or secretion of other proteins, which may be highly undesirable (134). It has been observed, however, that glycerol, 4-phenylbutyric acid and trimethylamine *N*-oxide selectively increase the secretion efficiency of alpha1-antitrypsin without influencing that of other proteins or decreasing proteasomal degradation (136,203,204). Another strategy to rescue misfolded proteins is by introducing specific sequences into the conformationally abnormal protein. This approach either over-expresses or stabilizes molecules rendered unstable by genetic defects and, in theory, does not provoke global changes in the ER secretory activity unless a particular agent capable of enhancing the transcription of several genes is used to obtain such an effect (194). For example, in the case of WT or mutant hGnRHRs, addition of an intracellular carboxyl-terminal extension from other species (205), or GPCRs (206), [the carboxyl-terminal extension is important in cell surface membrane expression of the GnRHR through a dual effect: its presence decreases internalization rates resulting in increased net membrane expression (205) and also functions by increasing the stability of the receptor, promoting its transport to the cell surface (180,207)] or deletion of Lys^{191} dramatically increased PM expression in both cases (129,196,208,209). Nonetheless, genetic approaches are probably redundant as therapeutic intervention because, if it were possible to access the gene sequence, the primary error could be directly corrected.

Manipulation of ER retention mechanisms may also be a potentially useful strategy to influence receptor trafficking. Several studies have shown that manipulation of components involved in the ER export machinery may also selectively influence receptor PM expression and function. This is the case for the small GTPase Rab1 protein, a member of the Rab GTPases family of proteins (210); Rab1 is specifically localized in the ER and Golgi apparatus and regulates anterograde transport of several proteins from the ER to and through the Golgi (211-214). Attenuation of Rab1 function by expressing dominant-negative Rab1 mutants or siRNA-mediated depletion of endogenous Rab1 inhibited cell surface expression of a number of endogenous GPCRs (*e.g.* the angiotensin-1 receptor and the beta2-AR) and promoted their accumulation in the ER and the Golgi (212,214).

In the case of the hGnRHR and the V2R, interaction with the chaperone calnexin has been documented (48,50). Since a proportion of the WT hGnRHR is retained in the ER (131,132), we recently examined the possibility that calnexin may mediate ER retention of the WT GnRHR (50) and compared the effect of this molecular chaperone on the rat GnRHR, which is more efficiently trafficked to the PM. Expression of the WT hGnRHR with calnexin decreased receptor expression by about half, thereby diminishing the receptor mediated second messenger production. The rat receptors were also retained by calnexin but, since a larger proportion of the rat GnRHR normally reaches the PM, there was no effect on maximal receptor signaling. Calnexin appears to retain a proportion of both human and rat GnRHRs in the ER, likely by means of a physical interaction between the proteins. In the presence of a pharmacoperone, there was a calnexin-mediated increase in hGnRHR signaling, probably reflecting an increase in ER export to the PM. The pharmacoperone-stabilized receptors seemed to be more efficiently routed to the PM. Thus, calnexin appears to act as a quality control protein for the GnRHR by retaining misfolded receptors and steering properly folded or conformationally stabilized receptors to the PM. Most of the rat GnRHR is properly folded and expressed at the PM (186,205,208); such very high expression is consistent with the observations that the rat receptor is not rescued by pharmacoperone exposure (180). Only when the cDNA of rat receptor was decreased 12.5-fold, did the additional calnexin decrease inositol phosphate production. Further, when siRNA was used to knockdown the transfected calnexin, the hGnRHR signaling was restored. Calnexin siRNA had little effect on the already robust rat GnRHR signaling (50).

Calnexin co-expression with hGnRHR chimeras bearing the intracellular carboxyl-terminal extension, or without Lys^{191}, no longer affected signaling. Thus, either calnexin does not interact with these modified (and more conformationally stable) receptor molecules (particularly the receptor from which the Lys^{191} has been deleted), or alternatively, any reduction in membrane expression did not diminish second messenger production, as is seen with the rat GnRHR. In the case of the V2R, it has been shown that calnexin interacts with both the WT and misfolded mutant V2Rs (Arg^{337}X(stop) and Ser^{315}Arg mutants) (48). However the half-lives of receptor-calnexin interaction varied depending on the particular receptor; retention of misfolded V2Rs was associated with longer interaction times between the mutant receptors and calnexin, suggesting that this chaperone could play a role in the intracellular retention of misfolded GPCRs and that this retention can be minimized by stabilizing the conformation of the misfolded receptor molecule.

More recently, new classes of substances that penetrate cells and selectively promote cell surface delivery of misfolded mutant V2Rs retained either in the ER or the post-ER compartment have been described (202,215). These compounds promote the release of Ca^{2+} into the cytosol and thereby affect chaperone (*e.g.* calnexin) function. Peptide compounds that penetrate into the ER-Golgi intermediate compartment via the retrograde transport pathway, but fail to reach the ER, act by selectively promoting Ca^{2+} release from the ER-Golgi compartment, promoting PM rescue of misfolded receptors retained in this particular location (155). It is important to emphasize that these particular compounds do not correct folding but rather affect chaperone-protein interaction by changing the intracellular Ca^{2+} environment (*i.e.* are not *true* pharmacoperones); consequently, PM rescuing might not always be accompanied by functional rescue of the misfolded receptor. These compounds are good examples of the feasibility to control the functional level of molecular

chaperones and/or GPCR-interacting proteins, which still represent an underappreciated therapeutic target.

Pharmacoperones appear to be among the most promising therapeutic approaches to treat conformational disorders (80,134,137,198,216,217). Pharmacoperones are small, membrane-permeable peptide or non-peptide ligands that increase the conformational stability of misfolded proteins by interacting directly with the target protein, thereby enabling their exit from the ER (134,197,198,218). The advantage of this approach is that the binding selectivity of the pharmacoperone is such that it will not affect the beneficial degradation of other misfolded proteins that need to be eliminated.

In transthyretin amyloidogenesis, for example, several small molecules may bind with high affinity to the unoccupied binding sites within the transthyretin molecule leading to stabilization of the native state of the protein, decreasing the concentration of the intermediate species, and thereby, amyloid formation (204,219). Short beta-sheet breaker peptides have been designed for blocking the conformational changes and aggregation undergone by beta-amyloid (220). These synthetic mini-chaperones, which have a structure homologous to the central hydrophobic region of the fibril aggregate, inhibit and dissolve beta-amyloid aggregates *in vitro* and *in vivo* (221-223). The competitive alpha-galactosidase A inhibitor, 1-deoxy-galactonojirimycin, increases the activity of the Arg^{301}Gln mutant form of this enzyme (whose retention in the ER leads to the lysosomal storage disease, Fabry´s disease in humans) and facilitates its ER export and transportation to lysosomes in fibroblasts expressing the mutant enzyme (138,224,225). Similar results have been obtained by treating fibroblasts from patients with Gaucher disease (which results from mutations in lysosomal beta-glucosidase leading to the accumulation of glycosylceramide in macrophages) with the enzyme inhibitor N-(*n*-nonyl) deoxynojirimycin (226).

In the case of GPCRs, it has been shown that agonists and antagonists of the receptor may promote cell surface delivery of the misfolded receptor from the ER (21,28,31,77,80,134,137,158,197,215,218,227). Although the molecular mechanism(s) involved in pharmacoperone-mediated rescue are poorly understood, it has been suggested that they could act on misfolded proteins by increasing protein stability, thereby preventing misfolding and aggregation of the nascent proteins or inhibiting their rapid degradation (28). In the case of mutant membrane receptors, pharmacoperones may restore not only cell surface delivery, but also functional activity whenever the mutations in the protein do not include domains involved in binding to agonist or interaction with signaling proteins or effectors. The efficiency of this rescuing approach will depend on the particular structure of the pharmacoperone (that determines selectivity toward the target protein) and the severity of the folding defect present in the target protein (28,35). For example, in the case of the Ser^{168}Arg and Ser^{217}Arg hGnRHR mutants, which are completely recalcitrant to pharmacological rescue, replacement of any of these serine residues (which in the three-dimensional structure of the receptor are located in the lipid membrane-contact phase of the helix) by the highly hydrophilic arginine would conceivably disrupt not only the orientation of the corresponding helix but also the net hydrophobicity of its external phase and hence its interaction with the lipid membrane. In the case of the Pro^{320}Leu hGnRHR mutant, we have found that the abnormal protein is also unrescuable by genetic approaches that rescue other misfolded hGnRHRs (e.g. the Glu^{90}Lys hGnRHR mutant (196)); because the peptide backbone of proline is constrained in a ring structure, occurrence of this amino acid is associated with a forced turn in the protein sequence and its replacement may severely disturb the structure of the helix. Similarly, mutant human V2Rs displaying amino acid exchanges at the interface of the TM-2 and TM-4 (His^{80}Arg, Trp^{164}Arg, and Ser^{167}Leu mutants), are resistant to pharmacoperone-mediated cell surface delivery, likely because the replacing residues leads to a severe folding defect (28).

As shown in Figure 1, several inactivating mutations (including two leading to deletion of large sequences) in the hGnRHR gene have been described, to date, as a cause of HH (141,158,159). The ability of different GnRHR peptidomimetics to rescue defective GnRHR mutants has been extensively analyzed (77,131,137,228). The peptidomimetics assessed as potential rescuers came from three different chemical classes (indoles, quinolones, erythromycin-derived macrolides) which were originally developed as GnRH peptidomimetic antagonists (228). These particular pharmacoperones were selected for study as potential pharmacoperones considering their predicted ability to permeate the cell membrane and specifically interact with the hGnRHR with a known affinity (229-231). All but three [Ser^{168}Arg, Ser^{217}Arg, and L^{314}X(stop)] of the 17 mutants tested were completely or partially rescued with pharmacoperones (77,78,198,228). As mentioned above, the Ser^{168}Arg and Ser^{217}Arg GnRHRs are mutants in which the thermodynamic changes leading to receptor distortion are too great to effect rescue (141). Accordingly, even though these two mutants are not rescued by pharmacoperones, their failure to route correctly is attributable to misfolding, and probably not to an intrinsic inability to potentially participate in particular receptor functions such as ligand binding, receptor activation or G-protein coupling. In the case of misfolded V2Rs, it has been shown that distinct hydrophobic, cell membrane permeable antagonists effectively rescue function of several misfolded, traffic-defective V2R mutants that cause diabetes insipidus in humans (79,80,227,232,233). These observations are important considering that ~70% of V2R mutations leading to disease are due to receptor misfolding (157). The fact that the effect of these antagonists on mutant V2R expression could not be mimicked by a V2R impermeant antagonist, and that the antagonist pharmacoperones did not rescue the function of the mutants that are normally expressed at the cell surface membrane, is an indication that the cell membrane permeable antagonists acted intracellularly to promote maturation and targeting of misfolded mutants to the PM (227). More recently, the effect of the peptidomimetic $V1_AR$/V2R antagonist

SR49059 (79,232) to rescue function of V2R misfolded receptors (Arg^{137}His, Trp^{164}Ser, and 185-193del) in patients with nephrogenic diabetes insipidus was examined. This pilot trial revealed a drop in urine production and water intake as well as a significant increase in urine osmolarity in response to this compound (80).

In addition to the misfolded GnRHR and the V2R mutants described above, for which non-peptide antagonists have proved to be useful as pharmacoperones, there are other conformationally defective GPCRs in which these drugs have been demonstrated to be efficacious in rescuing function or preventing abnormal accumulation of the defective molecule. In retinitis pigmentosa, folding and rescue of the Pro^{23}His mutant rhodopsin associated with this retinal degenerative disease have been achieved by exposure of the cells to 11-*cis*-7-ring-retinal, a seven-membered ring variant of 11-*cis*-retinal, the chromophore of rhodopsin that plays a central role in the photoactivation process (234,235). In the case of PM expression-deficient mu-opioid receptors and melanin concentrating hormone receptor-1 mutants, different cell permeable agonists and antagonists have been shown to effectively enhance cell surface expression of the mutant receptors (236,237).

The overall data indicate that pharmacoperones represent a novel approach for the potential development of defined therapeutic strategies for an array of diseases caused by incorrectly routed cell surface or secreted proteins.

8. PERSPECTIVE

From the above discussion, it is evident that a large number of biological systems appear to rely on assessment of fidelity of protein structure and folding by the QCS. For many GPCR point mutations or polymorphisms that lead to disease, the underlying mechanism resides in misfolding of the protein and its resultant inability to reach the PM and exert function. The growing number of examples of misfolded GPCRs as a cause of disease indicates that misfolding, retention, and aggregation of conformationally-defective membrane receptors may be more common than previously recognized. In this scenario, the traditional view that mutational inactivation always reflects loss of intrinsic function needs to be reassessed, considering misrouting as the potential mechanism subserving the loss of function of the abnormal receptor. Recognition of this alternate concept immediately presents the therapeutic opportunity to rescue mutants either by manipulating the ER QCS or through rescuing by pharmacological chaperones. The recent observations showing that previously synthesized, misfolded proteins retained by the QCS are still rescued by pharmacoperones (28,160), suggest that determining the pattern of pharmacoperone administration *in vivo* need not consider whether the target protein is being synthesized at the time of drug administration. Nevertheless, in the case of conformational diseases caused by formation of intracellular protein aggregates as the central disease pathogenesis (150,238), it is possible that the aggregates may prove thermodynamically very stable and thus rescue may not be possible by this means; this may result in the development of "lifestyle" drugs that may be taken regularly in order to prevent the onset or progression of the disease in susceptible individuals. In fact, in an experimental model of cerebral amyloidosis, the main effect of pharmacoperones is on prevention of fibril formation by acting on fibril intermediates (221,223). Another consideration is that the half-life of ER-retained mutants may be short (239), forcing pharmacoperones to be present for as protracted a period as possible if optimal rescue is the goal. Nonetheless, in the case of certain GPCR mutants that are retained in the ER (150,238), this appears to be possible and certainly will facilitate therapeutic intervention.

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Abbreviations: GPCRs: G protein-coupled receptors; TM: transmembrane; EL: extracellular loops; IL: intracellular loops; PM: plasma membrane; GnRH: gonadotropin-releasing hormone; GnRHR: gonadotropin-releasing hormone receptor; ER: endoplasmic reticulum; QCS: quality control system; V2R: vasopressin-2 receptor; WT: wild-type; HH: hypogonadotropic hypogonadism; CFTR: cystic fibrosis transmembrane conductance regulator

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