

# GENETIC VARIATIONS AND POLYMORPHISMS OF G PROTEIN-COUPLED RECEPTORS: Functional and Therapeutic Implications

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■ **Abstract** G protein-coupled receptors (GPCRs) represent a major class of proteins in the genome of many species, including humans. In addition to the mapping of a number of human disorders to regions of the genome containing GPCRs, a growing body of literature has documented frequently occurring variations (i.e. polymorphisms) in GPCR loci. In this article, we use a domain-based approach to systematically examine examples of genetic variation in the coding and noncoding regions of GPCR loci. Data to date indicate that residues in GPCRs are involved in ligand binding and coupling to G proteins and that regulation can be altered by polymorphisms. Studies of GPCR polymorphisms have also uncovered the functional importance of residues not previously implicated from other approaches that are involved in the function of GPCRs. We predict that studies of GPCR polymorphisms will have a significant impact on medicine and pharmacology, in particular, by providing new means to subclassify patients in terms of both diagnosis and treatment.

## INTRODUCTION

The heterotrimeric GTP-binding protein (G protein)-coupled receptors (GPCRs) are members of a large family of proteins found in eukaryotes and certain prokaryotes. The GPCR family is the third most abundant family in *Caenorhabditis elegans*, comprising 5% of its genome with approximately 1100 members (1). The *Drosophila* genome has at least 160 GPCRs whereas it is estimated that there are at least 700 GPCRs in the human genome (2). These plasma membrane-bound receptors have evolved to recognize a diversity of extracellular physical and chemical signals, such as nucleotides, peptides, amines, Ca<sup>2+</sup>, and photons. On recognition of such signals, the GPCRs act via one or more heterotrimeric G proteins to alter the level of intracellular messengers as a proximal event in signaling pathways that influence a wide variety of metabolic and differentiated functions. Because of

their ubiquitous expression and multiple actions, it is not surprising that variation at GPCR loci can be associated with disease states.

In this article, we review recent observations regarding genetic polymorphisms in human GPCRs, in particular therapeutic implications of such polymorphisms. The recent progress in identifying GPCRs, sequencing the genomes of humans and other species, mutational analyses of GPCRs, and discovery of coding region and single nucleotide polymorphisms (SNPs) at human GPCR loci make attempts to review this field a daunting task, at best akin to trying to take a snapshot of a rapidly moving train of information. We have terminated our literature search as of March 2000; information is evolving so quickly as to supercede data available then. We have recently posted on our laboratory web site ([www-insel.ucsd.edu](http://www-insel.ucsd.edu)) the addresses of useful GPCR-related web sites/databases. It is our intention to update this list with links to other sites containing human GPCR polymorphism data, some of which are still under development.

Our emphasis is on naturally occurring GPCR variants and their possible impact on drug therapy. Thus, we have organized the review to focus on structural domains of GPCRs and their role in signal transduction, discussing GPCR mutations that affect cellular response to hormones, neurotransmitters, and other pharmacological agents. We have highlighted data reported for rhodopsin, thyrotropin stimulating hormone (TSH) receptors, and vasopressin 2 ( $V_2$ ) receptors, in part because of the large number of mutations found in their structural domains. Studies on those mutations allow one the opportunity to draw inferences regarding structure-function relationships of GPCRs and, in some cases, on response to agonists and antagonists. Because our principal emphasis is on the therapeutic impact of genetic variation at GPCR loci, we do not discuss structure-function relationships in detail, but interested readers may wish to consult other recent reviews that emphasize such relationships (e.g. 3–9).

## GPCR Classification

GPCRs have been divided into three main classes. These contain the known human GPCRs. Two additional classes encompass fungal pheromone and Dictyostelium GPCRs. Each of these classes has been further divided into subclasses based on a combination of sequence similarity, functional domains, and ligand binding properties (10–11). Though comparison of GPCRs from different classes results in little or no sequence similarity, all GPCRs share a common overall structure (Figure 1*a–c*). Hydropathy analysis predicts that GPCRs have seven hydrophobic transmembrane (TM) helices connected by three intracellular loops (IL) and three extracellular loops (EL). GPCRs also contain an N-terminal extracellular region with N-glycosylation sites and a C-terminal intracellular domain, generally with sites for phosphorylation. Each of these domains has distinct properties depending on the class and subclass of receptors; these properties confer on individual receptors their ligand binding and G protein specificity.

According to their class, GPCRs share additional structural and functional properties, as reviewed by Bockaert & Pin (11), Schöneberg et al (8), and Wess (3), and summarized in Figure 1. Class A contains most known members of the GPCR family and includes three subclasses. Sequence alignment of receptors reveals approximately 20 amino acid residues conserved by most class A GPCRs, which include the following: two Cys residues in EL1 and EL2 that form a disulfide bridge necessary for maintaining proper receptor conformation; the Asp-Arg-Tyr sequence (DRY motif) in the proximal region of IL2; proline residues in the TM regions; an Asn-Pro-X-X-Tyr motif in TM7; and a Cys residue in the C-terminal domain, which can be palmitoylated, thereby forming a fourth IL (Figure 1a). Within class A GPCRs are receptors for photons of light (opsins and rhodopsin), biogenic amines, and other small physiological ligands. The binding site for these molecules is located within the seven TM helices. A second subclass within class A GPCRs includes receptors that respond to chemokines, thrombin, and other small peptides. These ligands bind to the N terminus, the ELs, and regions of the TMs that are close to the ELs. Receptors of the third subclass of class A GPCRs have a relatively large N-terminal domain and bind glycoprotein hormones, such as luteinizing hormone (LH), follicle-stimulating hormone (FSH), and TSH.

The class B GPCRs, the second largest class, contains receptors that bind to higher-molecular-weight hormones, such as glucagon and calcitonin. Class B GPCRs are characterized by long N-terminal regions (>100 residues) involved in ligand binding, six well-conserved Cys residues in the N-terminal region, two well-conserved Cys residues in EL1 and EL2, and approximately 15 other residues that are identical in all members of this class of GPCRs (12) (Figure 1b). Finally, class C, the smallest class of GPCRs, contains the metabotropic glutamate receptors, the GABA<sub>B</sub> receptor, and the calcium sensing receptor (CaR). These receptors possess extremely long extracellular N-terminal domains that are involved in ligand binding and contain several conserved Cys residues in the TM and extracellular regions (Figure 1c).

## Variation at GPCR Loci

As readily accessible, plasma membrane-bound molecular entities that regulate a wide variety of physiological and metabolic processes, GPCRs are commonly used as therapeutic drug targets. To date, numerous studies have reported genetic variation at GPCR loci and their role in human disease and traits (Tables 1–4). A genetic “polymorphism” is defined here as one of at least two commonly occurring (i.e. at least 1% of the population) genetic variants at a locus whereas the term mutation is used for a genetic variant that occurs in an isolated individual or pedigree for germline mutations and for less frequently occurring somatic mutations identified in isolated tissues.

Several hundred mutations have been reported at GPCR loci, most within the coding and 5' untranslated regions. In rhodopsin alone, 63 variants of the receptor have been identified in families with autosomal dominant forms of retinitis

pigmentosa (RP) (13). At least 36 of the 70 reported variants of the  $V_2$  receptor are believed to cause X-linked nephrogenic diabetes insipidus (NDI), a disorder with renal resistance to vasopressin (14). Studies on variation in GPCRs have not been limited to germline mutations. In addition to germline variants of the TSH receptor identified in individuals with, for example, congenital nonautoimmune hyperthyroidism, a large number of somatic variants of the TSH receptor have been identified in toxic adenomas and nodules (15–16). From these examples it is obvious that both somatic and germline mutations at GPCR loci have the potential to contribute to variation in GPCR signaling and function.

In addition to the large number of GPCR variants associated with monogenic diseases, there is growing evidence for GPCR polymorphisms in complex human diseases and traits. For example, two major polymorphisms of the  $\beta_2$ -adrenergic receptor ( $\beta_2$ -AR) have been examined for their association with asthma (17) and hypertension (18). Such GPCR polymorphisms contribute to a complex phenotype in combination with polymorphisms at other genetic loci as well as with environmental factors.

The majority of naturally occurring variants at GPCR loci have been reported only within the past several years. In parallel with these reports, pharmacological studies have primarily involved mutational analyses designed to assess the role of domains in GPCR function. Most of these latter studies have been conducted by using GPCR variants generated through site-directed mutagenesis. Thus, the role of the naturally occurring variants of most GPCR variants in human disease and traits still needs to be elucidated.

In the remainder of this review, we discuss GPCR polymorphisms by taking a “sequential domain approach.” We begin at the amino terminus, citing mutagenesis studies that elucidate roles of this domain on signal transduction, followed by studies identifying naturally occurring genetic variants. Because the number of these variants is very large, we limit our examples to those with functional consequences, associated with disease, or possible therapeutic impact. We continue along the receptor peptide to discuss the TM domains, the intracellular loop domains, and the C-terminal domain. In addition, many pharmacologically altered natural GPCR variants have been reported as a consequence of variation in non-coding regions, expression mechanisms, or dimerization. Thus, we conclude with sections on such variants.

## THE N-TERMINAL DOMAIN

The N terminus of GPCRs is an extracellular domain of variable size, ranging from 154 residues in the calcitonin receptor to 36 residues in the rhodopsin receptor. This domain has several features important for GPCR function, including asparagine residues and motifs for N-glycosylation, which influence intracellular trafficking of receptors to the plasma membrane (19), and cysteine residues that can influence protein folding critical for trafficking of a functional receptor to the cell surface

**TABLE 1** Selected genetic variants of the N-terminal domain<sup>a</sup>

Receptor	Variant	Functional consequence (clinical consequence)	Reference
$\beta_2$ -AR	<i>Arg16Gly</i>	Enhanced agonist-promoted down-regulation	20
	<i>Gln27Glu</i>	Blunted agonist-promoted down-regulation	20
5-HT <sub>1A</sub>	<i>Gly22Ser</i>	Attenuated receptor down-regulation and desensitization	30
5-HT <sub>2C</sub>	<i>Cys23Ser</i>	Decreased agonist binding affinity	32
$\mu$ -Opioid	<i>Asn40Asp</i>	Increased agonist binding affinity	36
Rhodopsin	<i>Thr4Lys</i>	Affects N-glycosylation (RP)	37
	<i>Asn15Ser</i>	Alters N-glycosylation site (RP)	38

<sup>a</sup>RP, Retinitis pigmentosa.

(20). The N terminus of some GPCRs also contains residues involved in ligand binding, activation, and down-regulation. A variety of disease-associated variants have been identified in this domain (Table 1). For example, in rhodopsin, six variants have been identified in patients with a dominant form of RP: *Thr4Lys*, *Asn15Ser*, *Thr17Met*, *Pro23His*, *Pro23Leu*, and *Gln28His*. Variants in this region that have functional consequences but lack clear association with disease are also common.

## Receptor Down-Regulation

$\beta_2$ -ARs are expressed on a wide variety of cell types, including smooth muscle cells, in which the receptors promote relaxation in response to catecholamines and synthetic agonists. Two main polymorphisms have been identified at the  $\beta_2$ -AR loci, and they code for *Arg16Gly* and the *Gln27Glu* variants. Green et al (20) expressed these variants in a heterologous cell system and showed that the variants did not display altered agonist affinity or coupling to G<sub>s</sub> but instead had altered agonist-mediated regulation. The extent of agonist-promoted down-regulation of receptor number, which required several hours of incubation with agonist, was enhanced for the *Arg16Gly* variant but was blunted for the *Gln27Glu* variant. When *16Gly* and *27Glu* were present together, down-regulation resembled that of the *Arg16Gly* variant. Thus, the change at codon16 to Gly dominates over that at codon 27. Green et al (20) suggested that the difference in down-regulation in these variants is due to altered receptor degradation after internalization. Turki et al (21) reported that the *Arg16Gly* variant occurs more commonly in individuals with nocturnal-type asthma. A potentially important pharmacological implication of the *Arg16Gly*

variant is that children homozygous for *Gly16*, compared with those homozygous for *Arg16*, appear less likely to show bronchodilation in response to the  $\beta$ -adrenergic agonist albuterol (22), perhaps because of chronic down-regulation of  $\beta_2$ -AR in response to endogenous catecholamines. Some studies have confirmed the greater extent of *Arg16* homozygotes for bronchodilation in response to  $\beta_2$ -AR agonist (23) whereas others have not observed this (e.g. 24, 25). Although larger numbers of patients need to be examined, consideration of the codon16 polymorphism may prove useful for future therapeutic strategies for asthma that target  $\beta_2$ -ARs.

Because of the role of  $\beta_2$ -AR in vascular smooth muscle relaxation, the *Arg16Gly* and *Gln27Glu* variants of  $\beta_2$ -AR have been examined for association with essential hypertension. Some studies suggest that either the *Gly16* (26–27) or the *Arg16* allele (28) is more frequent in individuals affected with or prone to develop hypertension, but we and our colleagues fail to find such an association (29). As with most complex diseases, the precise role of a variant at an individual locus is difficult to assess, and further studies on different populations will be necessary.

Another naturally occurring N-terminal variant that has been implicated in affecting agonist-induced down-regulation is the *Gly22Ser* variant of the h5-HT<sub>1A</sub> receptor. This variant displays attenuated agonist-promoted down-regulation and functional desensitization with no effect on ligand binding profiles when compared with the wild-type receptor (30). Because the effect of selective serotonin reuptake inhibitors used to treat psychiatric disorders is partially dependent on h5-HT<sub>1A</sub> receptor down-regulation, it is conceivable, but not yet well tested, that individuals carrying such variants may have altered sensitivity to antidepressant treatment.

## Ligand Binding

Residues of the N terminus of certain GPCRs are also involved in ligand binding. Okazaki et al (31) found a *Lys47A<sub>sp</sub>* variant of the calcium-sensing receptor (CaR) in an individual with an autosomal dominant form of hypocalcemia and implicated this codon as important for calcium sensing. Lappalainen et al (32) reported that a *Cys23Ser* variant of the 5-HT<sub>2C</sub> receptor, found at a frequency of 13% in unrelated Caucasian individuals, exhibited lower binding affinity than did wild-type receptor for agonist. This change in binding affinity to 5-HT receptor agonist binding led to studies on association of response to clozapine, which is used for the treatment of schizophrenia. A study of 168 schizophrenic patients found that individuals with Cys at codon 23 responded better to clozapine than did individuals with Ser at this position (33). Further work will be needed to confirm this observation. Interindividual variation in clozapine response has also been associated with variants of another 5-HT receptor (discussed below).

## Receptor Trafficking

Glycosylation and oligosaccharide processing are important for protein trafficking of adrenergic receptors to the plasma membrane (34, 35). The importance of

N-glycosylation has also been shown for other GPCRs. For example, Petäjä-Repo et al (19) studied human  $\delta$ -opioid receptors in a heterologous expression system and found that N-glycosylation of N-terminal asparagine residues within a defined glycosylation consensus motif is initiated cotranslationally in the endoplasmic reticulum (ER) and completed in the *trans*-Golgi network. These fully processed receptors are then transported to the cell surface in 10 min. The authors suggested that the rate-limiting step in this process is the exit of a fully processed GPCR from the ER. Less than 50% of synthesized receptors are fully processed, of which only a fraction attain the proper conformation to exit the ER. Thus, folding and export that rely partially on glycosylation events are potentially key events in the control of GPCR expression and activity.

Several polymorphisms have been identified that alter N-glycosylation in GPCRs. The *Asn40Asp* variant of the  $\mu$ -opioid receptor was found in 10% of a study population of 113 heroin addicts in a methadone maintenance program and 39 controls with no history of drug or alcohol abuse (36). When the population was stratified for ethnicity, this variant was significantly more frequent in Hispanic non-opioid-dependent subjects than in other ethnic groups studied. This variant also displays a higher binding affinity than does wild-type receptor for the endogenous beta-endorphin. A point mutation rhodopsin at codon 15, which changes an Asn residue to Ser, altering a glycosylation site, was described in a five-generation Australian family with RP (37). Another rhodopsin variant, *Thr4Lys*, which affects glycosylation of Asn2 and probably causes rhodopsin not to be incorporated into the membrane, was found in patients with a different form of dominant RP (38).

The examples given here indicate that N-terminal variants affect a variety of functions of GPCRs and have given insight into roles of N-terminal domain residues in GPCR signal transduction. It is worth noting that several of the observed polymorphisms leading to functional consequences, such as agonist-mediated down-regulation, were not expected from prior studies of the N-terminal region. We expect that future studies of polymorphic GPCRs will reveal additional roles for N-terminal amino acid residues.

## TRANSMEMBRANE DOMAINS

The transmembrane (TM) domains of GPCRs are comprised of seven  $\alpha$ -helices imbedded in the lipid bilayer of the plasma membrane. The seven helices are thought to be arranged as a tight, ring-shaped core (5). Similar to most TM proteins, the hydrophobic amino acid residues are presumably arranged to face the lipid bilayer, whereas the more hydrophilic amino acid residues face the core. Furthermore, helix-helix interactions contribute to the functional tertiary structure of the GPCR necessary for receptor folding and stability, ligand binding, and ligand-induced conformational changes for G protein coupling. Thus, mutations in the TM domain can have an array of deleterious effects (Table 2). For example, 11 naturally occurring mutations in the TM domains of rhodopsin of RP patients have been shown to interfere with folding or stability of the receptor (39).

**TABLE 2** Selected genetic variants of the TM domains<sup>a</sup>

Receptor	Variant	Functional consequence (clinical consequence)	Reference
D4	<i>Val194Gly</i>	Decreased agonist binding	44
ET <sub>B</sub>	<i>Cys109Arg</i>	Disruption of putative signal sequence (Hirschsprung's disease)	105
	<i>Trp276Cys</i>	Altered receptor coupling to G <sub>q</sub>	47
FSH	<i>Phe591Ser</i>	Deficient in activating adenylyl cyclase via G <sub>s</sub>	45
V <sub>2</sub>	<i>Lys44Pro</i>	Retention in pre-Golgi compartment (NDI)	14
	<i>Trp164Ser</i>	Defective processing and retention in ER (NDI)	14
	<i>Ser167Lys</i>		
	$\Delta V278/279$	Lacks cell surface expression; retention in Golgi compartment	41
<i>Tyr128Ser</i>	Abolished agonist binding	14	

<sup>a</sup>TM, transmembrane; D4, dopamine 4; ET<sub>B</sub>, endothelin B; FSH, follicle-stimulating hormone; V<sub>2</sub>, vasopressin 2; NDI, nephrogenic diabetes insipidus; ER, endoplasmic reticulum.

Mutations that alter receptor folding can lead to defective posttranslational modification, intracellular transport, or cell surface expression. For example, the TM domains contain several highly conserved Pro residues important for receptor folding and expression. Wess et al (40) demonstrated the significance of such Pro residues in a site-directed mutagenesis study in which three conserved Pro residues (Pro242 of TMV, Pro505 of TMVI, and Pro540 of TMVII) of the m3 muscarinic receptor were changed to Ala. This resulted in a 35- to 100-fold lower expression level than in the wild-type receptor. A change to a Pro residue in the TM domain can also alter receptor expression. The V<sub>2</sub> receptor *Lys44Pro* mutant of TMI, which is found in patients with X-linked NDI, lacks glycosylation, indicating retention in the pre-Golgi compartment. The introduction of Pro is key to receptor folding and subsequent processing, because substitution of another residue, such as the *Lys44Phe* variant, shows correct processing of the receptor, although it also has defective binding.

Other mutants of the TM domain of the V<sub>2</sub> receptor in NDI patients are the *Trp164Ser*, *Ser167Lys*, and *Ser167Thr* in TMIV; all three produce changes in amino acids conserved among GPCRs and show defective processing and retention in the ER, as indicated by their lack of glycosylation (14). In contrast to these mutants, the V<sub>2</sub> receptor mutant with a frame deletion in TMVI,  $\Delta V278/279$ , also found in X-linked NDI patients, shows processing similar to the wild-type receptor but lacks cell surface expression because of retention within the Golgi or post-Golgi compartment (41). These studies on V<sub>2</sub> receptor TM domain mutants have useful therapeutic implications. The defective folding, processing, and cell surface



expression of mutant  $V_2$  receptors that still retain some residual receptor function (ligand binding, AC coupling, and G protein stimulation) probably explains why patients with such mutations fail to respond to standard treatment for X-linked NDI. In these patients, even high doses of the synthetic  $V_2$  receptor agonist desmopressin fail to restore the defective renal tubular concentrating ability observed in NDI (14).

Another naturally occurring variant of the TM domain has been shown to affect translocation of the receptor into the plasma membrane. The *Cys109Arg* mutant of the proximal region of TM1 of the endothelin B receptor was identified in a patient with Hirschsprung's disease (42), a congenital disorder characterized by the absence of ganglion cells in the distal portion of the intestinal tract caused by the premature arrest of neural crest cell migration. This mutation replaces a hydrophobic residue with a charged residue, thereby disrupting the hydrophobic stretch of a putative signal sequence involved in the transport, folding, and proper targeting of the mature receptor from the ER to the plasma membrane. Thus, therapeutic strategies for this disorder (and probably for other disorders) need to consider the possibility of defects in processing, transport, and cell surface expression.

Residues of the TM domain are also involved in ligand binding. Many studies have used site-directed mutagenesis to show altered ligand binding of TM domain mutants. Thus, for example, an extensive mutagenesis study of the TM helices of the melanocortin 1 receptor (MC1R) revealed the importance of several acidic residues in TMII and -III for ligand binding and of aromatic residues in TMIV, -V, and -VI in the formation of a hydrophobic pocket for ligand binding (43). Naturally occurring mutations in the TM domain have also been identified that can alter ligand binding affinity. For example, in the dopamine 4 (D4) receptor, the *Val194Gly* variant of TMIV changes Val residue one position away from the Ser residue critical to dopamine binding. This variant has been identified at a frequency of about 12% in the Afro-Caribbean population and is reported as twofold less sensitive to dopamine, clozapine, and olanzapine than is the wild-type receptor (44). No definitive studies have shown an association of this variant with schizophrenia or other disease. However, TM domain variants of  $V_2$  receptor have been identified as a cause of disease. The *Arg113Trp* mutant of TMIII of the  $V_2$  receptor displays a 20-fold increase in  $K_D$  for the agonist Arg-vasopressin whereas agonist binding in the *Tyr128Ser* mutants of TMIII is completely abolished (14). Both mutations are found in individuals with X-linked NDI. Such results suggest that therapeutic approaches for the treatment of NDI, and perhaps other diseases, will need to consider the binding properties of each affected individual's mutant receptor.

Analysis of naturally occurring variants has also implicated the TM domain in G protein coupling. Variants of TMVI in two members of the glycoprotein hormone receptor subfamily of GPCRs, FSH receptor and LH receptor, result in receptors deficient in the ability to activate adenylyl cyclase via  $G_s$ . The FSH receptor mutant, *Phe591Ser*, eliminates cAMP production when expressed in COS cells. A similar

effect is seen when this mutation is introduced in a homologous position in the LH receptor (45); this mutant receptor is able to bind human chorionic gonadotropin with an affinity similar to that of the wild-type receptor but is defective in its ability to activate adenylyl cyclase (46). A variant of the endothelin B receptor, in which a highly conserved Trp residue at position 276 in TMV is substituted with a Cys, impairs ligand-mediated increases in  $\text{Ca}^{2+}$  levels in transfected cells, most likely by altering receptor coupling to  $\text{G}_q$  (47). This variant was identified in a Mennonite pedigree and was found to associate with Hirschsprung's disease such that homozygotes and heterozygotes for the variant had, respectively, a 74% and 21% risk for the disease.

## Dimerization

Several studies provide evidence that GPCRs can exist as homodimers or heterodimers, and receptors in this state have altered receptor function (e.g. 48, 49). Given the potentially important role of homodimerization in the function of GPCRs, identifying key domains for dimerization is important for assessing the role of polymorphisms and for developing new therapeutic agents. Hebert et al (50) showed that agonists favored  $\beta_2$ -AR homodimerization. Furthermore, using evidence from a sequence in the glycophorin A protein that is essential for homodimerization, [LIXXGVXXGVXXT], Hebert et al proposed that a sequence in TM6 of  $\beta_2$ -AR, [LKTGLIIMGTFTL], is responsible for  $\beta_2$ -AR dimerization. Consistent with this proposal, they found that addition of this peptide competed for intact GPCRs and reduced homodimerization. The functional consequences of the reduction in homodimerization was a significant reduction in agonist-stimulated adenylyl cyclase activity.

Based on the model of Hebert et al (50), Bai et al (51) suggested that a similar domain plays a role in the high level of dimerization observed in the CaR and identified a putative consensus dimerization motif, [LMALGFLIGYTCL], in TMV of CaR. Although no further studies have been done to provide evidence that this domain is in fact playing a role in the dimerization of CaR, a naturally occurring frameshift mutation at codon 747, identified in an individual with familial hypocalciuric hypercalcemia syndrome, terminates the receptor in TMV (52). Pearce et al (53) showed that this truncated receptor, which is lacking TMV, cannot form dimers under the same conditions as do wild-type CaRs; it is likely that domains lacking in the truncated CaR are responsible for the loss of dimerization properties. It is interesting that several other mutations in CaR have been isolated in this region. Watanabe et al (54) isolated a *Phe788Cys* substitution just downstream of the putative consensus dimerization domain in a Japanese family with severe familial hypoparathyroidism. De Luca et al (55) described a patient with sporadic hypoparathyroidism who was heterozygous for a *Leu77Arg* mutation. This mutation lies within the putative consensus dimerization domain: [LMAL<sup>773</sup>GFLIGYTCL]. Functional studies of this mutant have not been reported, but it would be interesting to see whether there is an observed difference in dimerization and

signaling. In receptors for which dimerization is critical for function, as with the CaRs, a mutant receptor may act as a dominant negative, greatly reducing the function of the expressed normal receptor. For example, the *Arg185Gln* N-terminal mutant of the CaR, which was identified in a female infant with hyperparathyroidism, is thought to exert a strong negative effect on the function of the wild-type CaR, resulting in an unusually severe form of hypercalcemia (56).

## INTRACELLULAR LOOP DOMAINS

Whereas amino acid sequences in the TM domain contribute primarily to ligand binding and structural stability, the IL domains are particularly important for receptor interactions with signaling and regulatory proteins. The N- and C-terminal regions of IL3 have been implicated in G protein coupling and specificity for class A and B GPCRs (57–59); IL1 and IL2 may also be important in G protein interactions (3). The IL domains of GPCRs are also involved in interactions with other proteins, such as  $\beta$ -arrestins (60), and contain consensus sites for phosphorylation by G protein receptor kinase (GRK) (61) and second messenger kinases, such as protein kinase A (62) and protein kinase C (63), which mediate receptor phosphorylation and desensitization. Further, a Pro-rich motif has been identified in IL3 of some GPCRs (e.g.  $\beta$ -AR and D4) as a site for interaction with the Src homology domains of such proteins as Nck and Grb2, which may be involved in receptor internalization (64–66). Variants of the IL domains have been identified that can result in constitutive activity of GPCRs (Table 3), which suggests that

**TABLE 3** Selected genetic variants of the IL, EL, and C-terminal domains<sup>a</sup>

Receptor	Variant (domain)	Functional consequence	Reference
$\beta_1$ -AR	<i>Gly389Arg</i> (C)	Enhanced agonist-stimulated coupling to G <sub>s</sub>	108
D2	<i>Ser311Cys</i> (IL)	Decreased agonist binding affinity	74
	<i>Pro310Ser</i> (IL)	Altered receptor coupling to G <sub>i</sub>	74
ET <sub>B</sub>	<i>Ser390Arg</i> (C)	Altered G protein coupling (Hirschsprung's disease)	105
P2Y <sub>2</sub>	<i>Arg334Cys</i> (C)	Putative additional palmitoylation site	103
Rhodopsin	<i>Arg135Gly</i> (IL)	Unable to mediate G protein release of GDP	85
TSH	<i>Ala623Iso</i> (IL)	Constitutive activity	15
	<i>Ala623Gly</i> (IL)		
	<i>Ala623Ser</i> (IL)		
V <sub>2</sub>	<i>Arg137His</i> (IL)	Unable to couple G <sub>s</sub>	84

<sup>a</sup>IL, Intracellular loop; EL, extracellular loop; AR, adrenergic receptor; D2, dopamine 2; ET<sub>B</sub>, endothelin B; TSH, thyroid stimulating hormone; V<sub>2</sub>, vasopressin 2.

the wild-type receptor sequence of IL2 and IL3 helps maintain the inactive conformation of GPCRs whereas constitutively active substitutions stabilize, as do agonists, the active conformation of the receptors.

Dopamine receptors are the most well-documented GPCRs for which polymorphisms have been identified in IL domains. Variants of dopamine 2 (D2) receptors have been associated with a number of disorders, including substance abuse and schizophrenia (67–70). The *Pro310Ser* and *Ser311Cys* of the D2 receptors are located in IL3, with similar distribution patterns in different human populations. The *Cys311* polymorphism occurs at a frequency of 3% in the Taiwanese Chinese population (71), about 2% in the Japanese population (72), and 3% in the Caucasian population (73). The *Pro310Ser* variant is rarer, occurring at a frequency of 0.4% in Caucasians (73). Cravchik et al (74) showed that the ligand binding affinity of the *Ser311Cys* variant for dopamine was approximately twofold lower than the affinities of the *Pro310Ser* variant and D2 wild-type. Despite the small difference in binding affinity, there was a marked difference in the ability of variants to couple to  $G_i$  and inhibit adenylyl cyclase activity. Although wild-type receptor inhibited forskolin-stimulated levels of cAMP synthesis by >90%, the *Pro310Ser* and *Ser311Cys* variants were able to inhibit forskolin response by only 24% and 58%, respectively. Such results suggest that these IL3 variants have a decreased ability to allow for the agonist-induced conformational changes required to activate  $G_i$ .

Another intriguing polymorphism of dopamine receptors is in D4 receptors, which, like D2 receptors, inhibit adenylyl cyclase activity. The antipsychotic agent clozapine has increased affinity for D4 compared with D2 receptors, perhaps in part explaining its antipsychotic activity (75). Among the numerous polymorphisms obtained in the amino-terminal and TM regions is a highly polymorphic, hyper-variable 48-bp tandem repeat, termed variable number tandem repeat, in IL3 [as reviewed by Van Tol (76)]. Individuals possess two to ten tandem repeat units; at least 19 different sequence variants have been identified in the individual units, and in excess of 20 different polymorphic forms of the D4 receptor have been identified in IL3. In spite of this considerable variation in IL3, few definitive data have documented that these polymorphisms are associated with disease, although some evidence suggests an association of the repeat polymorphism with novelty seeking and attention deficit hyperactivity disorder. In addition, pharmacological studies have not revealed major differences between the different repeat polymorphic variants of IL3 in D4.

A highly conserved sequence found in members of class A GPCRs is the Glu/Asp-Arg-Tyr/His (E/DRY/H) motif located at the TM3-IL2 junction. Site-directed mutagenesis of the Asp residue of the DRY motif results in constitutive activity of GPCRs, including  $\beta_2$ -AR (77), the cannabinoid receptor (78), and the histamine  $H_2$  receptor (79). Mhaouty-Kodja et al (80) provide evidence that the Asp142 residue of the DRY motif in the  $\alpha_{1b}$ -AR plays a role in receptor interaction with regulatory proteins such as GRK2 and  $\beta$ -arrestin. The authors suggest that the negative charge of this residue may be directly involved in interacting with GRKs and  $\beta$ -arrestins or that mutations of Asp142 may change the conformation of IL2 and IL3 such that these loops can no longer bind to activate GRK2, thereby

reducing desensitization. The second residue of the DRY motif, Arg, is the most conserved amino acid of this motif, as revealed through sequence alignment of 620 receptors (81). Mutations of the Arg residue impair receptor-mediated signal transduction in M<sub>1</sub> muscarinic receptors (82). Scheer et al (83) recently demonstrated that substitution of the Arg143 of the DRY motif in  $\alpha_{1b}$ -AR by several different amino acid residues results in an array of functional effects. A change to Ala or Ile resulted in a complete loss of receptor-mediated response, whereas a change to Lys conferred constitutive activity to the receptor. Using computer-simulated mutagenesis and results from site-directed mutagenesis experiments, the authors conclude that Arg143 of the DRY motif in  $\alpha_{1b}$ -AR mediates receptor activation by enabling the intracellular loops to achieve the proper conformation for interacting with the G protein.

Although naturally occurring mutations of the Asp residue have not been reported, disease-associated mutations of the conserved Arg residue of the DRY sequence have been identified in the V<sub>2</sub> receptor and rhodopsin. Rosenthal et al (84) identified an *Arg137His* variant of V<sub>2</sub> receptor found segregating in individuals of a family with X-linked NDI. The *Arg137His* variant was able to bind Arg vasopressin with normal affinity but was unable to couple to G<sub>s</sub>. Several mutations of the Arg residue have also been identified in rhodopsin (*Arg135Gly*, *Arg135Leu*, *Arg135Pro*, *Arg135Trp*) in individuals suffering from dominant forms of RP. Functional studies on the *Arg135Gly* variant showed that this mutant receptor was able to bind the G protein transducin but was unable to mediate the release of GDP on light stimulation (85). A mutation of the Arg of the DRY motif has also been reported in the melanocortin-1 receptor, which is thought to contribute to skin and hair pigmentation in humans. The *Arg142His* variant has been identified in an individual with red hair (86). The contribution of this allele to the red hair phenotype in humans has not been determined; functional studies on this variant are lacking.

Three variants of the TSH receptor have been found in individuals with thyroid disease at the amino acid position homologous to Ala293 of  $\alpha_{1b}$ -AR, a site at which mutagenesis yields constitutive activation (87). The two somatic mutations, *Ala623Ile* and *Ala623Ser*, and one germline mutation, *Ala623Val*, result in constitutively active receptors (15), which decrease the response to endogenous or exogenous TSH. Although we are not aware of studies documenting expression in humans, it is intriguing that site-directed mutagenesis studies on amino acid residues homologous to the Ala293 of  $\alpha_{1b}$ -AR in  $\alpha_2$ -AR and  $\beta_2$ -AR also result in constitutively active receptors (88, 89). Studies on  $\alpha_{1b}$ -AR,  $\alpha_2$ -AR, and  $\beta_2$ -AR showed increased constitutive activity of these mutants combined with increased agonist-independent GRK2-mediated phosphorylation. (See page 624 for Note.)

## THE EXTRACELLULAR LOOP DOMAINS

Of the GPCR domains discussed in this review, a smaller number of polymorphisms have been reported in the EL domains compared with other GPCR regions (Table 3). The most prominent structural feature of these domains is the disulfide

bridge found in most GPCRs that is formed by two conserved Cys residues of EL1 and EL2. An additional disulfide bridge between a Cys in EL3 and the N-terminal domain can also occur and has, for example, been found in the angiotensin receptor. These disulfide bridges are thought to be important for proper receptor folding, with consequences for intracellular transport (as discussed in the N-Terminal Domain section), and the formation of a ligand binding pocket (90–92). Mutations that contribute to additional Cys residues in EL domains can compete for disulfide bond formation with the conserved Cys and perturb receptor function. For example, the *Gly185Cys* NDI-causing mutation of EL2 of the  $V_2$  receptor has impaired binding, possibly because of competition with the conserved EL2 cysteine for the formation of a disulfide bridge with the conserved EL1 Cys (93). Site-directed mutagenesis studies have shown that the EL domains play a key role in ligand binding and ligand specificity in other GPCRs (e.g. 94–96). Tsukaguchi et al (41) reported that the *Arg202Cys* variant of the  $V_2$  receptor, found in a patient with NDI, has impaired binding and decreased activation of adenylyl cyclase by vasopressin. The binding impairment of the mutant receptor suggests that this residue participates in ligand recognition. Cotte et al (97) showed that a Leu residue at the same position in the rat  $V_2$  receptor facilitates ligand binding, whereas the Arg at position 202 in the human receptor is not favorable. A change to Cys at this position may constitute an even more negative determinant restricting receptor interaction with ligand. Such effects would imply differences in the ability of the variant receptors to be activated by endogenous and exogenous agonists.

## THE C-TERMINAL DOMAIN

The intracellular carboxy-terminal (C-terminal) domain is involved in several aspects of GPCR signaling. All GPCRs contain Ser and/or Thr residues in this domain, which can serve as sites for G protein receptor kinase (GRK)-mediated phosphorylation and receptor desensitization (98, 99). Some GPCRs contain a cysteine residue in the C-terminal domain, which can serve as a site for palmitoylation. This can create a fourth IL because of the ability of the palmitoylated cysteine to insert in the plasma membrane. Also contained in the C-terminal domain upstream of the palmitoylated cysteine residue and downstream of TMVII is a G protein-binding domain in which variants have been identified (Table 3). More recent evidence suggests that the C terminus may be involved in interactions with other proteins that mediate GPCR signaling, such as the recently identified calcyon (100), PDZ domain-containing proteins (101), and Homer/Ves1 proteins (102), although GPCR variants that alter interactions with these proteins have not yet been established.

Class A GPCRs contain the palmitoylated cysteine in the C terminus and this can be a site for polymorphism. Janssens et al (103) identified a polymorphism with a frequency of 0.2 in the P2Y<sub>2</sub> receptor, which produces an *Arg334Cys* substitution at a potential site for palmitoylation. Although there were no major pharmacological differences in response to natural agonists tested between the two polymorphic

receptors, the authors observed slight variation in the time course of second messenger generation:  $\text{InsP}_3$  accumulation was slower in the *Cys334* variant than in the *Arg334* variant. This site may have pharmacological relevance.  $\text{P2Y}_2$  receptors are expressed in airway epithelial cells and respond to the nucleotides UTP and ATP by increasing intracellular calcium and thereby regulating chloride conductance in a manner alternative to the activity of the cystic fibrosis transmembrane regulator (CFTR). As such,  $\text{P2Y}_2$  receptors are under study as therapeutic targets with nucleotide analogs for the treatment of cystic fibrosis and chronic bronchitis. Thus, expression of this polymorphism might impact therapeutic utility of nucleotides in the treatment of such diseases.

The *Arg492Cys* variant of the  $\alpha_{1a}$ -adrenergic receptor was identified in a study of patients with benign prostatic hypertrophy (104). The substitution of a Cys at residue 492 may confer a palmitoylation site in the C terminus next to the postulated palmitoylation site at 490 for this receptor. This polymorphism showed no marked pharmacological differences in binding or receptor-mediated intracellular  $\text{Ca}^{2+}$  levels, nor was it differently distributed between patients with benign prostatic hypertrophy and normal control subjects. However, the distribution between the Japanese and US populations was substantially different: *Arg492* is the major allele in the Japanese population (90%), whereas *Cys492* is the major allele in the US population (66%).

Prior to the palmitoylated cysteine in the proximal region of the C-terminal domain is a region sometimes referred to as the fourth IL domain, which contains a G protein binding site. Variants in this region that alter G protein coupling have been identified in at least two different GPCRs. Tanaka et al (105) identified a *Ser390Arg* variant of the endothelin B ( $\text{ET}_B$ ) receptor in a Japanese patient with Hirschsprung's disease. This variant has binding affinities similar to the wild-type  $\text{ET}_B$  receptor but is characterized by a decreased ligand-induced intracellular calcium level and decreased inhibition of adenylyl cyclase activity. A similar region of the related  $\text{ET}_A$  receptor contains the putative palmitoylation site and forms a fourth cytoplasmic loop required in G protein coupling (106). The data suggest that the replacement of a Ser at position 390 with the positively charged Arg residue might cause a decrease in G protein coupling through conformational changes.

The same region in two other class A GPCRs, the  $\beta_2$ -AR and  $\alpha_2$ -AR, has been implicated in G protein binding (107); a naturally occurring polymorphism of this region has been identified in the  $\beta_1$ -AR. Mason et al (108) observed that the initially described "wild-type" sequence of  $\beta_1$ -AR, which codes for a Gly at residue 389 in the putative G protein binding domain, actually occurs at a frequency of 0.26, whereas a "gain-of-function" *Arg389* polymorphism is at a frequency of 0.74 and thus more likely to be the true wild-type. Several types of experiments, such as receptor-promoted binding of  $\text{GTP}\gamma\text{S}$  to  $\text{G}_s$  and adenylyl cyclase assays, showed that agonist-stimulated coupling to  $\text{G}_s$  was enhanced in the *Arg389* allele in comparison to the *Gly389* allele. Given the key role of  $\beta_1$ -AR as the predominant  $\beta$ -AR subtype in the heart involved in responses to  $\beta$ -adrenergic agonists, genetic variation at  $\beta_1$ -AR might contribute to interindividual differences in the response to  $\beta$ -blockers in the treatment of cardiovascular diseases. The study by Mason et al

(108) emphasizes the importance of population studies to identify the expression and frequency of the genetic variants.

A substitution 20 residues away from the end of the C-terminal domain of the 5-HT<sub>2A</sub> receptor, creating the *His452Tyr* variant, has been identified in two independent cohorts of unrelated patients treated with clozapine (109). Functional data on this variant are lacking; however, the study did find a statistically significant association of the *Tyr452* allele with nonresponders to clozapine. Although the precise target of clozapine's therapeutic action is not known, studies such as these, which associate variants of a neurotransmitter receptor to which the drug binds, strengthen the candidacy of these receptors as important therapeutic targets.

## NONCODING-REGION POLYMORPHISMS AND OTHER TRANSCRIPTIONAL AND TRANSLATIONAL MECHANISMS ALTERING EXPRESSION

A large number of polymorphisms have been identified within the promoter region and 5' untranslated region (UTR) of GPCRs; fewer polymorphisms have been reported thus far for other noncoding regions, such as in the 3' UTR and introns (Table 4). Because levels of receptor expression are important in determining the magnitude and sensitivity of response to agonists (110), it is not surprising that GPCR polymorphisms in noncoding regions have been associated with disease and altered drug responses. In this section, we give examples of such polymorphisms and suggest mechanisms by which expression of GPCRs may be altered.

Aside from SNPs that have been identified in the noncoding region of human GPCR loci, several other mechanisms that alter receptor expression have been identified but are rare or have only been identified in the GPCR loci of nonhumans. Such disease-related polymorphisms can show altered response to endogenous

**TABLE 4** Selected genetic variants the noncoding region<sup>a</sup>

Receptor	Variant (region)	Functional consequence (clinical consequence)	Reference
B <sub>2</sub>	-412C/G (5'UTR)	Destroys transcription factor binding site	122
β <sub>2</sub> -AR	-47T/C (5'LC)	Decreased receptor expression	131
CCR5	-1454G/A (5' UTR)	Reduced promoter activity	123
D4	-521C/T (promoter)	Decreased transcriptional activity	114
D2	-141C Ins/Del (promoter)	Lower promoter activity	115
ET <sub>B</sub>	Splice variant	Defective in G protein coupling	136

<sup>a</sup>5'UTR, 5' untranslated region; AR, adrenergic receptor; 5'LC, 5' leader cistron; CC, chemokine receptors; D4, dopamine 4; D2, dopamine 2; Ins, insertion; Del, deletion; ET<sub>B</sub>, endothelin B.



hormones or neurotransmitters, and presumably to exogenous agents as well, although few studies have directly tested this inference. An example is the insertion of a 226-bp, short, interspersed nucleotide element within the 5' flanking intronic region needed for pre-mRNA lariat formation and proper splicing in the hypocretin receptor 2 gene (*HCRTR2*), which binds orexin peptides derived from the precursor peptide hypocretin. Deficiency of hypocretin can produce sleep pathologies resembling narcolepsy (111); faulty splicing of *HCRTR2* yields a receptor incapable of proper signal transduction and results in narcolepsy in dogs (112). Such studies provide a hypothesis for assessing the pathogenesis of human narcolepsy and a potential drug target for the disease.

RNA editing, a mechanism whereby the primary nucleotide sequence of an RNA transcript is posttranscriptionally modified, provides another means to generate expression of functionally different GPCRs. Niswender et al (113) showed that the brain can express two different edited forms of the human 5-HT<sub>2C</sub> receptor, each with different constitutive activity from the nonedited form. A more common mechanism for altering expression of GPCRs is alternate splicing. This is discussed in more detail below.

## 5' UTR

As indicated above, dopamine (D) receptors are targets of antipsychotic drugs, and polymorphisms affecting the expression of dopamine receptor genes (*DRD*) can contribute to individual variation in response to drug therapy. Okuyama et al (114) identified a  $-521C/T$  polymorphism in the 5' promoter region of *DRD4*. Reporter assays were used to assess the effect of this polymorphism on receptor expression and revealed that the  $-521T$  allele had 40% less transcriptional activity than did  $-521C$ . The authors observed a weak association of the more transcriptionally active polymorphism with schizophrenia. However, because an elevation of *DRD4* mRNA is found in the frontal cortex of postmortem brains from schizophrenics, the authors proposed that this polymorphism may be a candidate for a genetic factor that influences both schizophrenia and the response to clozapine therapy, which targets D4 receptors, an idea that requires further testing.

As with the D4 receptor, the D2 receptor may be involved in schizophrenia and is a major site of action of neuroleptic agents used in its treatment. Arinami et al (115) identified a  $-141C\text{Ins/Del}$  polymorphism in D2 receptors and an association of the insertion polymorphism with schizophrenia in a case-control study in a Japanese population. In reporter assays, the  $-141C\text{Del}$  polymorphism had lower promoter activity. Breen et al (116) reported the reverse association in a case-control study of schizophrenia in a Caucasian population, but they attributed the results to linkage disequilibrium with a nearby polymorphism. This *DRD2* polymorphism serves as an example that genetic background in different populations can contribute to a role of specific polymorphisms in disease and drug response.

A number of 5' UTR and promoter polymorphisms have been identified in other candidate genes for schizophrenia, although further investigation is required to

establish their role in this or other diseases. Because a decreased density of 5-HT<sub>2A</sub> receptors has been reported in the frontal cortex of schizophrenic patients (117), many studies have been conducted to identify polymorphism in the promoter region of its gene. A  $-1438G/A$  polymorphism was found, but the results showed no clear association with schizophrenia (118). However, Collier et al (119) have reported that this polymorphism is associated with anorexia nervosa. Polymorphisms have been identified in two other candidate genes for schizophrenia, but functional analysis and studies associating these polymorphisms with this or other diseases are lacking:  $-19G/A$  and  $-18C/T$  in the 5' UTR of the 5-HT<sub>5A</sub> receptor gene (120), and  $-707C/G$  and  $-343A/G$  of the DRD3 promoter (121).

Considerable effort has been directed at defining noncoding changes in GPCRs that may be associated with cardiovascular diseases. One example is the gene for the bradykinin B<sub>2</sub> receptor. Erdmann et al (122) assessed the role of three novel, but rare, promoter variants isolated in individuals with cardiac disease: A  $-412C/G$  variant, identified in a patient with dilated cardiomyopathy, destroys a binding site for the transcription factor Sp1, which affects basal gene transcription; a  $-704C/T$  mutation, identified in an individual with hypertrophic cardiomyopathy, destroys the binding site of a nuclear binding protein; and a  $-78C/T$  mutation, also isolated from an individual with dilated cardiomyopathy, reduces protein binding of an unidentified protein. It will be of interest to determine whether these polymorphisms influence efficacy or toxicity of agents such as angiotensin converting enzyme (ACE) inhibitors, which blunt the degradation of bradykinin.

Perhaps the best example of promoter polymorphism affecting a noninherited disease is that of the CC chemokine receptor-5 gene (*CCR5*) promoter polymorphism in HIV-1 pathogenesis. McDermott et al (123) identified an A/G polymorphism at bp 59029 of the *CCR5*, 1454 bp upstream of the initiation site in the *CCR5* promoter. Both promoter alleles were common (43%–68% allelic frequency for  $-1454A$ , depending on race). In vitro promoter activity of the  $-1454G$  polymorphism was 45% lower in activity than was  $-1454A$ . In a cohort of HIV-1 seroconvertors lacking both *CCR5* $\Delta$ 32 and *CCR2-641* alleles, the  $-1454G/G$  individuals progressed to AIDS on average 3.8 years more slowly than did the  $-1454A/A$  individuals. *CCR5*  $-1454G/G$  thus appears to be protective relative to *CCR5*  $-1454A/A$ , and about twice as protective relative to *CCR5* $\Delta$ 32 or *CCR2-641*. This effect may result from reduced *CCR5* mRNA production. These results identify a site in the *CCR5* promoter that may be a useful target for treatment of HIV-1 infection.

### 3' UTR

The angiotensin II type 1 receptor gene (*AT1R*), which is expressed in vascular smooth muscle cells and regulates growth and vasoconstriction in response to angiotensin II, has been particularly well studied for a 3'UTR polymorphism, an A/C transversion found at position 1166. Bonnardeaux et al (124) and Wang et al (125) observed an increase in allelic frequency of *1166C* in populations of Caucasian individuals with essential hypertension and inferred that this polymorphism

imparted a small effect on blood pressure. Szombathy et al (126) evaluated fewer than 100 subjects but reported that the *1166C* allele, although not more common in hypertension, was associated with higher values for systolic and diastolic blood pressure in overweight, hypertensive patients. These results suggest that the *1166C* polymorphism is potentially involved in the regulation of blood pressure, with the effects of the genotype being more pronounced in overweight, and perhaps older, subjects. Miller et al (126a) evaluated 66 healthy Caucasian subjects and demonstrated the association of the C allele with lower baseline renal function, as assessed by several indices, and with greater hemodynamic and renal responses to the AT receptor antagonist losartan. The effect of this polymorphism may contribute to other cardiovascular diseases as well. Szombathy et al (127) reported an association of the *1166C* polymorphism with mitral valve prolapse syndrome in Caucasians. Although the A/C alleles at nucleotide 1166 of *AT1R* failed to show an association with early coronary disease in a study by Alvarez et al (128), a significant interaction between a deletion polymorphism of ACE was found. Individuals homozygous for the deletion polymorphism of ACE and the *1166C* polymorphism of *AT1R* had an increased risk for coronary artery disease. This suggests the importance of genetic background in the involvement of polymorphisms with disease and perhaps in drug response as well.

## 5' Leader Cistron

The 5' UTRs of eukaryotic mRNA can contain sequences that regulate the efficiency of translation of the mRNA. One example is upstream AUG sequences that code for initiation of short open reading frames (uORFs), which influence the translation of the main ORF (129). Recent data suggest that polymorphism can occur in such uORFs and that such polymorphisms can influence GPCR expression. For example, the  $\beta_2$ -AR contains a short ORF termed the 5' leader cistron (5'LC). This sequence begins 102 bp upstream of the  $\beta_2$ -AR coding sequence and encodes a putative 19-amino acid peptide. This 5'LC inhibits  $\beta_2$ -AR translation; mutational inactivation of the 5'LC increases receptor expression (130). McGraw et al (131) have identified a T to C transversion polymorphism at -47, leading to a putative peptide product containing an Arg instead of a Cys at residue 19 (*Arg19Cys*) in the C terminus of this ORF. Of particular importance, this 5'LC-*Arg19* variant acted to lower the basal level of receptor expression. Although this result will require confirmation from other studies because others have obtained somewhat different results (132), it could provide a potential explanation for at least part of the substantial intersubject variability in  $\beta_2$ -AR expression. Expression of the 5'LC polymorphism for this receptor (and perhaps other GPCRs) could be a key determinant of both "basal" and agonist-regulated levels of receptor expression in light of evidence that the 5'LC-*Arg19* variant is in linkage disequilibrium with the coding sequence polymorphisms at codons 16 and 27, which influence susceptibility of receptors to undergo agonist-promoted down-regulation, as discussed above (131). Yamada et al (133) found an increased frequency of the 5'LC-*Arg19* variant in obese individuals compared with nonobese individuals. In addition, they found

a polymorphism at  $-20$  in which the  $-20C$  allele was in linkage disequilibrium with the  $-47C$  polymorphism.

Another example of a 5'LC is found in the promoter of the D3 receptor gene, which encodes a putative peptide of 36 amino acids. Sivagnanasundaram et al (121) found that a polymorphism in the 5'LC, the *Lys9Glu*, and two SNPs in the promoter region,  $-707C/G$  and  $-343 A/G$ , and a coding polymorphism *Ser9Gly* were in tight linkage disequilibrium and associated with schizophrenia. Further studies will be needed to confirm and extend these findings. Conceivably, this or other 5'LCs could provide therapeutic targets to regulate GPCR expression.

## Alternate Splicing

Molecular diversity in the GPCRs is also achieved through alternate splicing. For example, *DRD2* generates two alternative RNA isoforms, the  $D_{2L}$  (long) and the  $D_{2S}$  (short), that differ by a 29-amino acid sequence in the third cytoplasmic loop of the receptor protein (134). Because such isoforms of GPCRs may differ in pharmacological properties and can be expressed differentially in tissues and in development, it is important to consider alternate splicing events in therapeutic drug design targeting GPCR isoforms. For example, the full-length CaR is expressed in undifferentiated keratinocytes, whereas its expression is decreased as keratinocytes differentiate, at which time there is an increase in an alternatively spliced "loss of function form" of CaR, which lacks exon 5 (135). A splice variant has also been identified in the  $ET_B$  receptor. The binding properties of this  $ET_B$  splice variant were similar to those of wild-type  $ET_B$  receptors, but the splice variant is defective in G protein coupling, as implied by a lack of increase in inositol phosphate accumulation in response to agonists (136). To date, no studies have directly assessed the role of splice variants in differences in drug response, but evidence from the receptors discussed above suggest that this may be a key area for further studies.

A splice variant of the 5-HT<sub>4</sub> receptors has also been identified in humans (137). This variant has a shorter C-terminal domain that contains a unique sequence with two Pro-Val repeats, which cause the receptor to have constitutive activity. This splice variant has been found only in brain, whereas 5-HT<sub>4</sub> receptors are expressed in a wide variety of tissues, including brain, colon, urinary bladder, esophagus, and heart, and 5-HT<sub>4</sub> receptor agonists, such as metoclopramide and cisapride, are used therapeutically as prokinetic drugs. Such drugs have been shown to be superagonists in colliculi neurons but only partial agonists or antagonists in other cells (138). Differentially expressed splice variants probably contribute to such tissue-specific responses.

## TRUNCATED RECEPTORS

Genetic variations altering multiple domains through frameshift mutations have also been identified; these frameshift mutations result in truncated receptors that can influence the pathogenesis and treatment of disease. Here, we give two examples, one that results in disease and another that confers a selective advantage

to the carrier for protection against disease. We make mention of the possibility of truncated receptors because the latter has given insight into new therapeutic approaches to disease.

In a study of families with morbid obesity, Vaisse et al (139) identified the presence of a heterozygous 4-bp insertion in the coding sequence of the melanocortin 4 receptor (MC4R). This insertion results in a truncated receptor that lacks TMVI and TMVII. MC4R is expressed in the hypothalamus, and studies of mice have implicated this receptor in the regulation of body weight. Mice that do not express MC4R display morbid obesity with hyperinsulinemia. Another study identified a 4-bp deletion in one individual from a cohort of severely obese children (140). This deletion at codon 211 results in a frameshift that introduces a stop codon in the region encoding TM5. Although functional studies of these truncated receptors were not done, these variant receptors are likely to be nonfunctional and probably contribute to the obese phenotype in individuals carrying these variants.

A deletion in the coding region of the *CCR5* can also be beneficial. The 32-bp deletion of nucleotides 794 to 825 of the coding region of the *CCR5* results in a frameshift that truncates the receptor after codon 206 (141) and is common to many populations, including those of European descent, Indians, and Middle Easterners (142). Although the truncation does not seem to produce an aberrant phenotype in individuals carrying this variant, this polymorphism is a factor in HIV-1 resistance in Caucasians with complete penetrance; infected Caucasian individuals heterozygous for the polymorphism show a slower rate of disease progression. Because the individuals homozygous for the truncated receptor seem to be normal, the existence of this polymorphism suggests that *CCR5* may be a good candidate for antiretroviral therapy. Hall et al (143) reported that individuals carrying the deletion polymorphism are at reduced risk of developing asthma, and this may help explain the high prevalence of this mutation in the general population. Thus, *CCR5* may also be a potential therapeutic target for asthma therapy, in addition to therapy related to HIV infection, as noted above.

## SUMMARY AND CONCLUSION

It is apparent that numerous naturally occurring variants exist in virtually all domains of GPCRs, but thus far, definitive understanding of their impact on disease and drug therapy is incomplete, with the exception of only a small number of receptors and subsets of variants. We believe that there are three main reasons for this current gap in knowledge. (a) A comprehensive compilation of all common and important variants is not yet available for most GPCRs. Completion of the human genome project and more extensive analyses of SNPs of GPCR genes should be forthcoming (from within months to a few years) and should define the "suspects" that will need to be interrogated. (b) Detailed and precise functional data have not been available for the vast majority of GPCR variants. (c) Careful statistical genetic analyses have not generally been undertaken to define relationships between GPCR variants, disease, and response to drugs largely because of the lack of sufficient data to achieve statistical significance.

A starting point for the analysis of GPCR variants is a comparison of detected sequence variations with information available in public databases, such as Genbank. It is thus intriguing that as new data become available for GPCR loci, one learns that the database version of the wild-type form of the receptor is actually a rarer variant than certain other “polymorphic” forms (e.g. 108, 131). This is particularly the case when one begins to look at different populations, implying a need to consider that there may be no single wild-type form of a given receptor and that, instead, one must qualify such information as relevant for only certain (sub)populations. Such an idea has potentially important implications in terms of disease associations, disease progression, and drug therapy, especially if different receptor alleles show important functional differences.

We propose that the following set of studies be conducted to establish the role of a GPCR polymorphism in a complex disease (or trait) and in order to assist in designing appropriate pharmaceutical strategies for the treatment of the disease: (a) population studies to ascertain the distribution of the polymorphism in affected and unaffected individuals in different ethnic populations, (b) *in vitro* studies to assess the impact of the polymorphism on GPCR structure-function, and (c) *in vivo* studies to determine the physiological and pharmacological consequences of the polymorphism both in normal subjects and those with a given disease.

As suggested above, efforts to decipher the role of GPCR polymorphisms in pathogenesis and therapy of complex diseases must consider the varying expression of GPCR variants among different populations. Several studies suggest that there are ethnic differences in the distribution of GPCR polymorphisms (144–146). For some GPCRs, this variation may manifest in ethnic differences in clinical phenotypes. For example, a number of studies have observed ethnic variation in the efficacy of  $\beta$ -AR antagonists as antihypertensive drugs: Chinese men are the most sensitive, African-American men are the least sensitive, and Caucasian-American men show intermediate sensitivity (147, 148). A thorough population study of the  $\beta$ -ARs, the targets of these drugs, is necessary to understand this observed difference in response to medication. Hence, to design appropriate therapeutic strategies for a complex disease, a study of the population distribution of the polymorphism must be conducted to determine the ethnic distribution of the polymorphism in affected and unaffected individuals, and to determine whether the genetic background of the individual will affect response.

Second, *in vitro* studies are required to assess whether the polymorphism has an impact on GPCR function. Studies on polymorphic receptors commonly employ heterologous expression systems to assay for binding properties, changes in the level of second messengers, G protein coupling, and desensitization and internalization. Drawbacks of such systems include the possible loss of functional information due to receptor expression outside the genetic background of the individual carrying the polymorphism, or due to expression outside the genetic background of its endogenous cell. More informative strategies might employ isolated cells or cell lines that endogenously carry the polymorphic receptor, preferably isolated from individuals who carry the polymorphism. For example, McGraw et al (131)

used human airway smooth muscle cells endogenously expressing various forms of the  $\beta_2$ -AR to study the impact of receptor polymorphisms on receptor binding and cyclic AMP formation. We believe that the use of cells endogenously expressing GPCR polymorphisms may provide particularly relevant systems for in vitro functional studies designed to enhance understanding of the role of GPCR polymorphisms in complex diseases and their impact on drug therapy.

Ultimately, the role of a GPCR polymorphism must be related to the in vivo setting. Pinpointing the effect of a single polymorphism to a complex trait can be difficult, as the contribution of a polymorphism at an individual locus is generally small and large numbers of subjects may need to be studied to provide strong statistical power for association and linkage. The possibility that linkage disequilibrium may account for observed patterns of statistically significant patterns of linkage to a particular locus must also be carefully considered.

Pharmacologists must be alert to the usefulness of information gleaned from both in vitro and in vivo studies of endogenous agonists (e.g. hormones and neurotransmitters) acting on polymorphic receptors. Such results can yield unexpected, but potentially important, hints regarding structurally important residues involved in the regulation of receptor expression and function. In addition, information derived from studies of polymorphic receptors has the potential to reveal regions of receptors—or the receptors themselves, as in the case of HIV infection and the CC5 receptor—to which new types of therapeutic agents might be targeted. Overall, the impact of studies on GPCR polymorphisms in therapeutics is likely to be considerable as we move into an era in which we are able to subclassify groups of patients with what was previously considered a disease/syndrome, and as we recognize that pharmacodynamics depends as much, or more, on underlying genetic differences between individuals than on environmental factors. Given the key role of GPCRs in signal transduction and functional regulation in virtually every organ system, studies of polymorphisms of GPCRs seem destined to play a major role in therapeutics in this decade and beyond.

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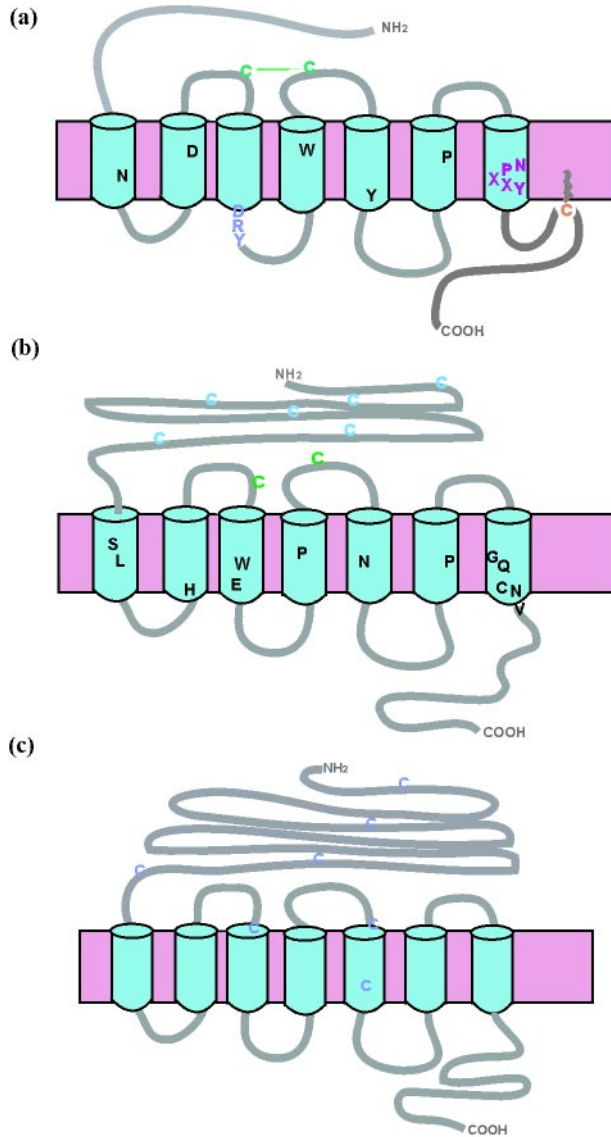
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#### NOTE ADDED IN PROOF

A recent study by Morello et al (89a) introduces a new therapeutic pathway for disease caused by mutations in the IL domains. The  $\Delta 62-64$  mutant of the V<sub>2</sub> receptor, found in NDI patients, is retained intracellularly in the ER due to improper protein folding and maturation. Treatment with cell permeable, nonpeptide antagonists partially rescued cell surface expression of this mutant receptor, and these receptors coupled to adenylyl cyclase with the same efficiency as the wild type receptors. The authors reported that they were also able to partially rescue mutants of the TM domains that exhibit defective binding due to improper cell surface expression. This work suggests a novel therapeutic approach in which cell permeable antagonists would act as pharmacological chaperones to stabilize improperly folded mutant receptors and enable their release from the ER, thus allowing the mutant receptors to resume proper function.





**Figure 1** Diagram of main structural features of the three classes of GPCRs. (a) Class A GPCRs share approximately 20 amino acid residues in common, including two Cys residues (in green) which form a disulfide bridge between EL1 and EL2, a Asp-Arg-tyr (DRY) motif in IL2, a Asn-Pro-X-X-Tyr (NPXXY) motif (in magenta) in TMVII, and a Cys residue (in orange) that serves as a site for palmitoylation in the C-terminal domain. (b) Class B GPCRs share six conserved Cys residues (in blue) in their N-terminal domain, two highly conserved Cys residues (in green) in the EL1 and EL2 domains, and several conserved amino acid residues (indicated by their one letter code). (c) Class C GPCRs are characterized by their long N-terminal domain and conserved Cys residues in the extracellular and TM regions.