Published in final edited form as:
Trends Biotechnol. 2009 September ; 27(9): 541–545. doi:10.1016/j.tibtech.2009.06.005.

Enhancement of G Protein-Coupled Receptor Surface Expression

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Abstract

G protein-coupled receptors (GPCRs) mediate physiological responses to a diverse array of stimuli and are the molecular targets for numerous therapeutic drugs. GPCRs primarily signal from the plasma membrane, but when expressed in heterologous cells many GPCRs exhibit poor trafficking to the cell surface. Multiple approaches have been taken to enhance GPCR surface expression in heterologous cells, including addition/deletion of receptor sequences, co-expression with interacting proteins, and treatment with pharmacological chaperones. In addition to allowing for enhanced surface expression of certain GPCRs in heterologous cells, these approaches have also shed light on the control of GPCR trafficking *in vivo* and in some cases have led to new therapeutic approaches for treating human diseases that result from defects in GPCR trafficking.

Introduction

Most vertebrate genomes encode approximately 1000 G-protein coupled receptors (GPCRs), making them the largest class of cell-surface receptors. These receptors are activated by various extracellular ligands (including hormones, neurotransmitters, drugs and sensory stimuli) and couple to intracellular G proteins, which mediate many of the effects on cellular physiology downstream of receptor activation [1]. Due to their location at the cell surface, and the discrete tissue expression patterns that many members of this family exhibit, GPCRs are excellent targets for therapeutics, with nearly half of all current prescription drugs acting through GPCRs [2].

Since GPCRs have proven to be such valuable drug targets, there are numerous ongoing efforts to identify novel compounds that act as agonists, antagonists or allosteric modulators of GPCRs. These studies typically involve expression of a given GPCR in heterologous cells, measurement of a downstream readout of receptor activity, and finally screening of large libraries of compounds for effects on activation of the receptor of interest. Since most GPCR ligands are not membrane-permeable, however, these methods are entirely dependent upon the expression of the receptor at the cell surface, such that the ligand has full access to its binding site. For this reason, it is a major problem for drug discovery efforts that many GPCRs exhibit poor trafficking to the plasma membrane when expressed in heterologous cells.

As with all transmembrane proteins, plasma membrane localization of GPCRs is dependent upon efficient delivery to the plasma membrane [3]. Many factors are involved in receptor delivery to the plasma membrane. The endoplasmic reticulum (ER) is the primary location for

protein folding and maturation, which must proceed properly for a receptor to be released to the Golgi and eventually to the plasma membrane. Treatments that globally disrupt ER function, including modulation of heat shock proteins and inhibition of glycosylation, can lead to altered trafficking of GPCRs as well as many other proteins, but such global approaches have been reviewed elsewhere [4] and will not be covered here. This review will focus on approaches that have been utilized over the past two decades to specifically enhance the trafficking of particular GPCRs to the plasma membrane, including addition of sequences that improve receptor trafficking, removal of sequences that impair receptor trafficking, co-expression with receptor-interacting proteins, and treatment with drugs. Examples of these four distinct approaches will be discussed in the sections below.

Addition of Sequences

One major approach that has been utilized to enhance the plasma membrane expression of GPCRs in heterologous cells is the addition of sequences to the receptors. The first example of this approach was the engineering of an artificial signal sequence onto the N-terminus of the β_2-adrenergic receptor [5], which resulted in a several-fold increase in insertion of the receptor into the plasma membrane. Artificial signal sequences have subsequently been used to enhance the surface expression of other GPCRs, notably the CB1 cannabinoid receptor [6, 7]. It is presumed that signal sequences facilitate receptor interactions with the signal recognition particle (SRP) and SRP receptor, which promote more efficient receptor targeting through the ER and membrane insertion [8].

Other types of sequences, beyond traditional signal sequences, have also been grafted onto the N-termini of certain GPCRs in order to enhance their surface expression. For example, in the case of olfactory receptors (ORs), which are the largest subfamily of GPCRs with more than 300 members in humans and approximately 1000 members in rodents, a variety of N-terminal sequences have been utilized to enhance plasma membrane targeting. Most ORs are inefficiently trafficked to the plasma membrane in heterologous cells [9], but the additions of N-terminal sequences from the serotonin 5-HT3 receptor [10,11] or rhodopsin [12,13] have been shown to markedly enhance heterologous surface expression of many ORs. The use of ORs with modified N-termini has allowed for significant advances over the past few years in defining the pharmacological and signaling properties of this large and diverse family of GPCRs [9].

While most sequences that enhance GPCR trafficking have been added to the N-terminal regions of the receptors, there are also examples of C-terminal additions that enhance receptor surface expression. For example, the rat gonadotropin-releasing hormone receptor (GnRHR) has a very short C-terminal tail and exhibits poor surface expression in most heterologous cells, whereas the catfish GnRHR has a much longer C-terminal region and exhibits robust surface expression in most cell types [14]. Addition of the catfish GnRHR C-terminus onto the rat GnRHR results in a striking improvement in the surface trafficking of the rat version of the receptor [14]. Interestingly, the GnRHR C-terminus is highly variable between species, suggesting that this receptor region may have been subject to intense evolutionary selection pressure as a mechanism for controlling GnRHR expression and functionality [15].

Deletion of sequences

A second major approach that has been taken to enhance the surface expression of certain GPCRs is the deletion of sequences that result in either ER retention or misfolding of the receptors. For example, following the cloning of the gamma-aminobutyric acid receptor $GABA_BR1$ [16], it was widely recognized that the receptor was poorly-trafficked and largely non-functional when expressed in heterologous cells [17]. Truncations of the $GABA_BR1$ C-terminus, or mutation of a specific C-terminal motif (RSRR), were found to relieve ER

retention of $GABA_BR1$ and allowed for robust plasma membrane expression of the receptor [18-20]. Interestingly, the critical RSRR motif on the $GABA_BR1$ C-terminus is similar to ER retention motifs that have been identified on certain ion channels and other transmembrane proteins [21]. However, despite the improved trafficking of the $GABA_BR1$ RSRR mutant, this receptor remained incompetent to couple to G proteins unless it was co-expressed with a related receptor, $GABA_BR2$, which seems to be a required heterodimer partner of $GABA_BR1$ in order to achieve the formation of functional $GABA_B$ receptors [20,22-24].

Analogous to the removal of C-terminal sequences from $GABA_BR1$, removal of N-terminal sequences from GPCRs has in some cases proven to be effective in enhancing receptor surface expression. For example, truncation of 79 amino acids from the N-terminus of the α_{1D}-adrenergic receptor (α_{1D}-AR) was found to dramatically enhance expression of receptor binding sites [25] and plasma membrane localization [26]. In contrast, grafting the α_{1D}-AR N-terminus onto the α_{1A}-AR or α_{1B}-AR was found to markedly impair surface expression of these receptors in heterologous cells [26], suggesting that the α_{1D}-AR N-terminus either possesses an ER retention motif or has difficulty in folding properly. Similar findings have been made for the CB1 cannabinoid receptor, for which it has been shown that truncations to the receptor's N-terminus are capable of greatly enhancing receptor surface expression [7]. For both α_{1D}-AR and CB1, it has been shown that the N-terminal truncations that enhance trafficking do not alter ligand binding [7,26] and that the truncated mutants are therefore useful for achieving enhanced surface expression of functional receptors in heterologous cells.

A key reason why some orphan GPCRs are still orphans at present is because their poor surface expression in heterologous cells has greatly hampered ligand-screening efforts. However, the application of the approaches described above (addition or subtraction of sequences) has in some cases already resulted in orphan receptors that are trafficked more efficiently to the plasma membrane and therefore more suitable for functional experiments. For example, GPRC6A is an orphan receptor that exhibits poor surface expression in heterologous cells [27]. Given its similarity to metabotropic glutamate receptors and $GABA_B$ receptors, it has been assumed that the N-terminus of GPRC6A is most likely the receptor's primary site of ligand binding. GPRC6A is also very similar to a goldfish receptor known as 5.24, which happens to be trafficked well to the plasma membrane in heterologous cells, and thus a chimera was created in which the N-terminus of GPRC6A was fused to the transmembrane and C-terminal regions of the 5.24 receptor [28]. This mutant receptor, lacking the transmembrane and C-terminal regions of wild-type GPRC6A, exhibited efficient trafficking to the plasma membrane and allowed for the identification of positively-charged amino acids, such as arginine, as putative ligands for GPRC6A [28].

Co-expression with partners

Most, if not all, GPCRs act primarily at the plasma membrane in the native cell types in which they are endogenously expressed. If a given GPCR exhibits poor surface trafficking in heterologous cells, a common explanation is that the heterologous cells must lack one or more receptor-interacting partners that normally promote the proper folding and trafficking of the receptor in native cells. Thus, over the past two decades, there has been intense interest in identifying GPCR-associated proteins [29-31], and many of the identified interactions have indeed helped to explain cell-specific differences in GPCR trafficking.

One major class of interactions that can in some cases greatly promote GPCR surface expression is interactions with other GPCRs (also known as heterodimerization or hetero-oligomerization) [32]. For example, as noted above, co-expression of $GABA_BR1$ with $GABA_BR2$ results in a massive enhancement in $GABA_BR1$ surface expression [33]. One consequence of this heterodimerization is believed to be the masking of the aforementioned

ER retention motif present on the $GABA_BR1$ C-terminus [19], providing an example of how two distinct approaches (deletion of a sequence and co-expression with an appropriate partner to mask a sequence) can in some cases enhance the surface targeting of a given GPCR via related mechanisms. There is strong evidence that the interaction between $GABA_BR1$ and $GABA_BR2$ is also essential for $GABA_BR1$ trafficking *in vivo*, since the brains of $GABA_BR2$ knockout mice exhibit a striking redistribution of $GABA_BR1$ and substantial loss in $GABA_B$ receptor functional activity [34].

In addition to $GABA_B$ receptors, there are a number of other GPCRs that have been found to exhibit enhanced surface expression in heterologous cells upon co-expression and association with other GPCRs [32]. For example, the aforementioned α_{1D}-adrenergic receptor has been shown to assemble with the α_{1B}-adrenergic receptor [35,36] and β_2-adrenergic receptor [37] in a manner that strongly promotes α_{1D}-AR surface trafficking in heterologous cells. Similarly, co-assembly of some ORs with certain members of the adrenergic and purinergic receptor families can enhance OR surface expression in heterologous cells [38,39]. Along these same lines, co-expression of the taste receptor T1R1 with its related receptors T1R2 and T1R3 has been shown to result in greatly enhanced functionality that is believed to correlate with improved surface expression, although the work undertaken in this area so far has focused more on assessing changes in receptor activity and pharmacology following heterodimerization than on addressing any changes in receptor trafficking [40].

There are a variety of other interactions, beyond receptor-receptor associations, that have been found to strongly regulate GPCR trafficking and surface expression. Some of these GPCR-interacting partners have been identified in genetic screens. For example, in screens for mutations that affect visual transduction in *Drosophila*, the cyclophilin-related protein *nina A* was identified as a rhodopsin partner required for rhodopsin folding and transport [41,42]. Subsequent work has revealed that the vertebrate homolog of *nina A*, RanBP2, associates with vertebrate opsins to regulate their folding, trafficking and surface expression [43]. Similarly, in screens for mutations that affect chemosensory signaling in *C. elegans*, the protein odorant response abnormal 4 (ODR-4) was identified and shown to associate with certain olfactory receptors [44,45]. It is not known, however, if the vertebrate ortholog of ODR-4 plays a comparable role for any vertebrate receptors [46]. Other GPCR-interacting partners that promote receptor trafficking include GEC1, which promotes surface expression of mammalian κ-opioid [47] and prostaglandin EP3 receptors [48], RACK1, which enhances trafficking of thromboxane A_2 receptors [49], Usp4, which increases plasma membrane expression of adenosine A_{2A} receptors [50], ATBP50, which regulates the transport of angiotensin AT_2receptor to the cell surface [51], and DRIP78, which enhances surface expression of angiotensin II AT_1 receptors [52].

A variety of transmembrane proteins, including receptor activity modifying proteins (RAMPs), receptor transporting proteins (RTPs), receptor expression enhancing proteins (REEPs), melanocortin receptor accessory proteins (MRAPs), and the M10 family of major histocompatibility (MHC) proteins have been identified during the past decade as GPCR-interacting proteins that promote the surface expression of specific subsets of GPCRs. The RAMPs were first identified as key regulators of the trafficking and functionality of the calcitonin receptor-like receptor (CRLR), an orphan receptor that had proven difficult to study until the realization that associations with RAMPs were required for its efficient plasma membrane localization [53]. The three members of the RAMP family are now known to interact with several Class B GPCRs, as well as a Class C GPCR, the calcium-sensing receptor [54], to influence receptor trafficking and pharmacology [55]. The RTP and REEP proteins were first identified in screens for proteins that enhance olfactory receptor functionality [56]. RTP1 and RTP2 are selectively expressed in the olfactory epithelium [56], and their roles in controlling OR trafficking have shed light on the underlying reasons why ORs are efficiently

targeted to the plasma membrane in olfactory sensory neurons but not in heterologous cells. Other members of the RTP and REEP families have wider tissue distribution patterns and have been shown to promote the surface expression in heterologous cells of T2R bitter taste receptors [57] and μ/δ opioid receptor heterodimers [58]. MRAP and MRAP2 have been shown to associate with the melanocortin 2 receptor (MC2R) and dramatically enhance surface expression of this receptor in a variety of cells [59-62]. Naturally-occurring mutations to MRAP cause defects in the trafficking and functionality of MC2R, resulting in an inherited disorder known as familial glucocorticoid deficiency type 2 [59]. MRAPs also have been shown to associate with MC3R and MC4R to reduce the signaling activity and/or surface expression of these receptors [63], so MRAP effects on receptor functionality appear to be receptor-specific. Finally, V2R vomeronasal receptors proved difficult to study in heterologous cells until the finding that co-expression and interactions with M10 MHC molecules and β_2-microglobulin were capable of dramatically enhancing V2R surface expression in heterologous cells [64].

Treatment with pharmacological chaperones

A number of naturally-occurring GPCR mutations have been identified that cause human disease by impairing normal receptor trafficking. The approaches described above for enhancing the surface targeting of GPCRs - addition of sequences, deletion of sequences and co-expression with partners - can be useful for enhancing the surface expression of GPCRs in heterologous cells, but these approaches are not likely to be useful in a clinical setting without significant further advances in gene therapy technology. Thus, there has been tremendous interest over the past few years in identifying small molecules that can bind to poorly-trafficked, disease-causing GPCRs with the goal of enhancing the surface expression and functionality of these receptors. Such molecules are often referred to as “pharmacological chaperones,” “pharmacochaperones” or “pharmacoperones” [65,66].

An example of the potential utility of pharmacological chaperones comes from work on nephrogenic diabetes insipidus (NDI), which is a rare X-linked disease characterized by loss of anti-diuretic response to the hormone arginine-vasopressin resulting in an inability to concentrate urine. NDI has been linked to a variety of mutations in vasopressin V2 receptors (V_2Rs), with the majority of these mutations causing V_2R to be retained in the ER and degraded [67]. Treatment of cells with certain membrane-permeant V_2R antagonists has been shown to restore cell surface expression of ER-retained V_2R mutants [65,68-70]. These findings are believed to be due to binding of the antagonists to the already-misfolded V_2R in the ER, resulting in the stabilization of receptor structure and trafficking of V_2R to the plasma membrane. Clinical studies have indeed provided proof-of-concept evidence that vasopressin receptor-targeted pharmacological chaperones can have beneficial effects in patients suffering from NDI [71].

Retinitis pigmentosa (RP) is another disease caused by mutations to a GPCR. Characterized by progressive photoreceptor degeneration and eventual retinal dysfunction, RP has been linked to a number of mutations in various gene products encoding nearly all of the components of the visual signaling pathway, including rhodopsin [72]. Mutations within the gene encoding rhodopsin are observed in one particular form of RP, autosomal dominant RP, and result in a mutated receptor that is retained intracellularly with no 11-*cis*-retinal binding. The most common mutation of this type is P23H [73-75]. P23H rhodopsin mutants can be rescued by treatment of cells with an 11-*cis*-retinal analog, 11-*cis*-ring-retinal, which results in restoration of receptor surface expression [76] in a manner that is analogous to the above-described rescue of V_2R surface expression by V_2R antagonists.

A third example of disease-causing mutations that affect GPCR trafficking comes from studies on mutations to GnRHR, which can result in hypogonadotropic hypogonadism (HH), a

condition characterized by disrupted sexual maturation. The GnRHR mutations that cause HH result in misfolded receptors that are not properly targeted to the plasma membrane. As in the above-described cases of pharmacological chaperones for V_2R and rhodopsin, certain GnRHR antagonists have been found to be capable of rescuing mutant GnRH receptors by restoring the surface expression and signaling activity of the mutant receptors [66,77,78].

Conclusion and Perspectives

The various approaches described in this review - additions and deletions of receptor sequences, co-expression with partners, and treatment with pharmacological chaperones - have proven useful in enhancing the plasma membrane expression of various GPCRs in heterologous cells (Figure 1). Such enhanced surface expression of receptors can greatly facilitate drug discovery efforts focused on GPCRs as therapeutic targets. In addition to providing insights into how to improve receptor surface trafficking in heterologous cells, studies in this area have also shed tremendous light on the regulation of the receptors *in vivo*, with many of the examples of receptor-associated proteins described above being shown in knockout studies to be critical for receptor regulation in native tissues. Moreover, the recent advances in the characterization of pharmacological chaperones that enhance receptor trafficking in heterologous cells can have direct translation to the clinic and might have utility in the future treatment of a number of human diseases.

Acknowledgments

The authors' research is supported by grants from the National Institutes of Health.

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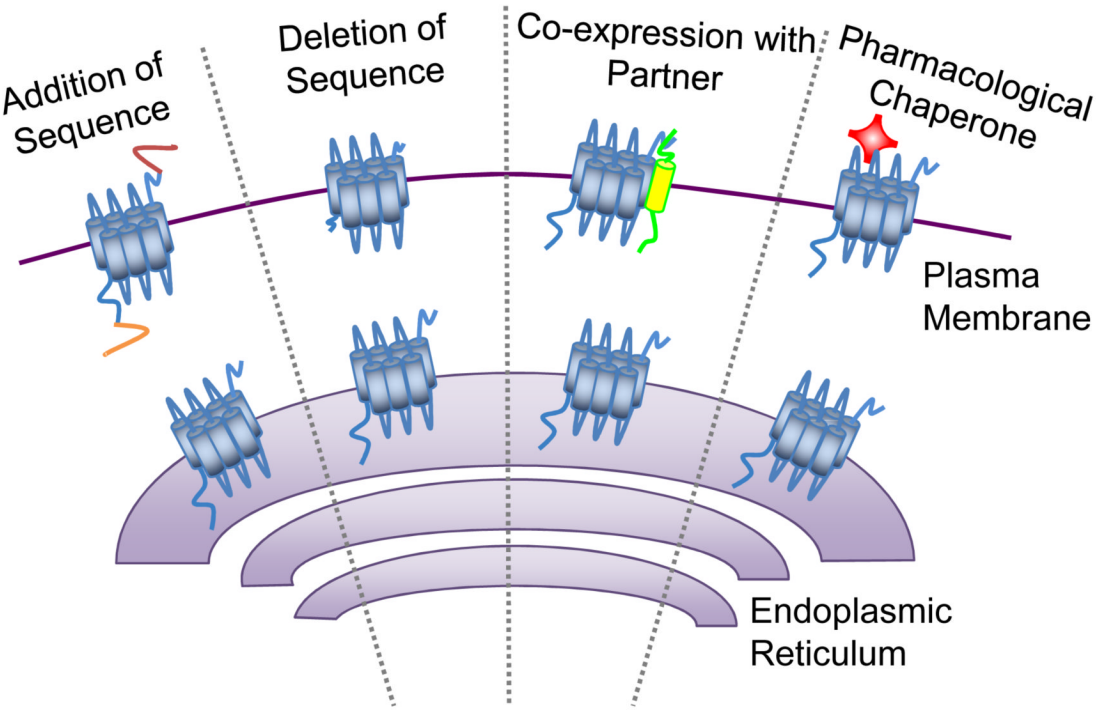

Figure 1.

Approaches for enhancement of G protein-coupled receptor surface expression.

When expressed in heterologous cells, many GPCRs exhibit poor plasma membrane trafficking, which can be enhanced using a variety of methods. For instance, addition or deletion of receptor sequences can in some cases greatly improve receptor surface expression. In other situations, co-expression with specific receptor-interacting partners can strongly promote proper surface trafficking. These receptor-interacting partners can either be transmembrane proteins, as illustrated in this schematic figure, or cytoplasmic protein that associate with intracellular domains of the receptor. Finally, pharmacological chaperones can release certain misfolded receptors from the endoplasmic reticulum and allow their enhanced trafficking to the plasma membrane.