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Review

Olfactory receptor trafficking to the plasma membrane

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Abstract. Olfactory receptors typically exhibit poor plasma membrane localization and functionality when heterologously expressed in most cell types. It has therefore proven difficult to effectively study olfactory receptor pharmacology and signaling mechanisms using traditional cell culture systems. Over the past few years, a variety of distinct proteins have been reported to interact with olfactory receptors and facilitate olfactory receptor trafficking to the plasma membrane in heterologous cells. Advances in this area have shed significant light on the fundamental factors governing the cell-specific control of olfactory receptor trafficking.

Keywords. Odorant, sensory, GPCR, dimerization, receptor transporting protein, desensitization, surface, expression.

Introduction

With more than 1000 intact genes in rodents and greater than 300 in humans, olfactory receptors (ORs) comprise the largest family of G protein-coupled receptors (GPCRs) in mammalian genomes [1, 2]. Their identification has laid the foundation for decoding vertebrate olfaction, and netted discoverers Linda Buck and Richard Axel a Nobel Prize for their seminal work in this area [3]. Since the groundbreaking discovery of this class of receptors more than a decade ago, ORs have been the subject of intense research interest. To date, however, characterization of OR pharmacology and signaling mechanisms has been limited. Very few members of the vast OR family have identified ligands (reviewed in [4]), and as a result the overwhelming majority of these specialized receptors remain orphans. A key obstacle hindering OR characterization has been difficulty in efficiently expressing these receptors in heterologous cells. When expressed in common cell culture systems, the bulk of OR proteins are detected intracellularly, with very little localization to the plasma membrane [5]. Consequently, reliable results from traditional ligand screening and signaling assays have been difficult to obtain.

Regulation of ORs by kinases and arrestins

In native tissue, ORs localize to the plasma membrane of cilia extending from the dendrites of olfactory sensory neurons (OSNs). Studies in native OSN cilia preparations have demonstrated that olfactory responses undergo rapid termination that is dependent on the actions of protein kinase A and protein kinase C [6]. Additionally, odorant application to cilia preparations has also been found to cause transient phosphorylation of cilia proteins [7]. These data support the postulation that activation of ORs, like many other GPCRs, may be highly regulated through

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desensitization and internalization mechanisms elicited by downstream second messenger-activated proteins.

In addition to regulation by second messenger-dependent kinases, olfactory desensitization also appears to be regulated by receptor-specific kinases. Functional studies have demonstrated a key role for the G protein-coupled receptor kinase 3 (GRK3) in OR signal termination in purified olfactory cilia preparations [8, 9], and GRK3-knockout mice exhibit a loss of odorant-induced desensitization [10]. However, recent GeneChip expression profiling studies revealed only low expression of GRK3 in OSNs [11], and therefore further investigation will be required to conclusively determine the identity of the kinase(s) mediating receptor-specific OR phosphorylation in native cells. In any case, OR desensitization in heterologous cells is due, at least in part, to phosphorylation of sites within the receptor's third intracellular loop followed by internalization *via* β-arrestin 2 association and clathrin-mediated endocytosis [12].

Given the evidence supporting robust desensitization of ORs in native tissue, it is natural to wonder whether the poor cell surface expression of heterologously expressed ORs is due to constitutive activity of the receptors accompanied by persistent internalization. In accordance with this scenario, it was reported that a small portion of heterologously expressed human OR 17–40 properly traffics to the plasma membrane where it then rapidly internalizes *via* clathrin-mediated endocytosis, even in the absence of agonist [13]. Conversely, heterologously expressed human OR 2AG1, a proportion of which properly localizes to the plasma membrane, was found to remain stable at the cell surface until agonist stimulation, whereupon it underwent clathrin-dependent endocytosis [12]. Findings from other groups have demonstrated that heterologously expressed, unstimulated ORs co-localize specifically with endoplasmic reticulum markers [14, 15] rather than endosomal markers, suggesting that in most cases constitutive internalization is an unlikely explanation for the lack of OR protein detected at the plasma membrane of heterologous cells.

Comparisons between ORs and other GPCRs

Like the ORs, many other GPCRs also exhibit poor plasma membrane localization upon expression in heterologous cells. This is especially true among families of sensory GPCRs. Members of the bitter taste receptor [16], V2R vomeronasal receptor [17], and trace amine-associated receptor families [18] all fail to localize correctly in heterologous expression systems. Similarly, multiple non-sensory GPCRs also suffer intracellular retention when expressed heterologously (reviewed in [19]). It is not clear why certain GPCRs localize properly at the plasma membrane of native cells but are retained intracellularly when expressed in heterologous cells. It has been widely hypothesized that heterologous cell culture lines may lack one or more critical components present in native cells that are required for proper localization of certain receptors.

To overcome poor heterologous surface expression, several molecular tricks have been developed that can successfully enhance the trafficking of some GPCRs. For example, through addition of the membrane targeting sequence from the serotonin 5-HT_3 receptor [20, 21] or addition of the N-terminal rhodopsin sequence, which may possess a forward targeting signal or simply provide additional glycosylation sites that are important for membrane localization [22–24], a small number of ORs have been successfully studied in heterologous systems. Other studies have foregone the benefits of experimentation in heterologous cells, and instead studied endogenous ORs identified *via* RT-PCR in native OSNs [25] or utilized adenoviral approaches or gene targeting strategies to overexpress defined ORs in OSNs [26–29]. Such techniques have successfully matched OR-ligand pairs and mapped axon convergence of certain receptors. Despite these successes, however, overall there have been limited advances in ligand identification and receptor characterization, especially considering the enormity of the OR repertoire. Thus, over the past several years there has been tremendous interest in understanding how interactions with other proteins might control OR trafficking.

Evidence from Caenorhabditis elegans

The first evidence supporting a role for additional factors in the proper localization of ORs came from observations in *C. elegans.* Early studies on olfaction-deficient worms led to the isolation of the odorant response abnormal 4 (ODR-4) gene, whose protein product was later determined to be expressed specifically in *C. elegans* chemosensory neurons. ODR-4 is required for proper plasma membrane localization of the *C. elegans* olfactory receptor ODR-10 and is thought to aid in receptor folding, sorting, or transport [30]. Subsequent experiments in Chinese hamster ovary cells demonstrated that co-expression with *C. elegans* ODR-4 alleviates the intracellular retention of the rat OR U131, but not that of the rat OR 5 [31], suggesting that unique chaperones might exist for different ORs. Interestingly, a distantly related human

ortholog of ODR-4 (hODR-4) has been identified [32]; however, it is not yet clear if the mammalian version of this protein plays a similar role in regulating OR trafficking as its *C. elegans* counterpart.

OR heterodimerization with other GPCRs

It is well established that components of multi-subunit proteins or members of heterodimer pairs can suffer endoplasmic reticulum retention when missing a partner, perhaps as a result of misfolding [33]. Moreover, several other types of GPCRs that exhibit intracellular retention in heterologous cells have been found to localize properly at the plasma membrane upon co-expression with a specific heterodimerization partner [19]. To ascertain whether heterodimerization might similarly influence mammalian OR localization in heterologous cells, plasma membrane expression of the OR M71 was monitored during co-expression with 42 non-OR GPCRs. In these studies, the β_2-adrenergic receptor and three types of purinergic receptor, the P2Y1, P2Y2, and adenosine A2a receptors, were all found to significantly enhance the plasma membrane localization of M71 in transiently transfected HEK-293 cells. Co-immunoprecipitation and confocal microscopy studies suggested that M71 associates with the GPCRs in a persistent manner at the plasma membrane of transfected heterologous cells [34, 35]. M71 in complex with P2Y1 or P2Y2 was found to activate the mitogen-activated protein kinase pathway in a $G_{i/o}$-dependent manner, while M71 in complex with the β_2-adrenergic receptor coupled to increased cyclic AMP accumulation [34, 35].

Association with non-olfactory GPCRs does not appear to be a general phenomenon by which the trafficking of all ORs to the plasma membrane of heterologous cells can be enhanced. Instead, the β_2-adrenergic receptor and the purinergic receptors seem to selectively interact with only certain ORs, as evidenced by facilitation of the cell surface expression of the M71 subfamily member mOR171-4, but not two distantly related ORs, rat I7 and human OR 17–40 [34, 35]. Likewise, another study also demonstrated that β_2-adrenergic receptor co-expression had no effect on the heterologous surface localization of several ORs distantly related to M71, including rat I7 [36]. It is important to point out that the above-described studies on OR interactions with other receptors were performed in heterologous cells and it is not clear whether β_2-adrenergic, P2Y1, P2Y2, and A2a receptors are expressed at physiological levels in OSNs. *In situ* hybridization and immunohistochemical studies have provided evidence for the expression of these receptor subtypes in the olfactory epithelium [34, 35, 37, 38], but, in the absence of electron microscopy analyses, it is difficult to determine conclusively whether the receptors are truly expressed in OSNs or rather in surrounding cells. Relevant to this issue, a recent GeneChip expression profiling study of OSN-expressed genes did not reveal significant expression of β_2-adrenergic, P2Y1, P2Y2, and A2a receptors [11]. However, even if these receptor subtypes are not highly expressed in OSNs, the observations that these receptors associate with ORs and drive OR surface expression in transfected heterologous cells still demonstrate that ORs have the capacity to form dimers (or higher order oligomers) and that such oligomerization can influence OR trafficking.

In parallel with the aforementioned studies on mammalian ORs, advances in the field of *Drosophila* olfaction have also indicated that insect ORs require receptor heterodimerization for proper localization and function. In the case of *Drosophila*, evidence suggests that all typical ORs must heterodimerize with a divergent OR called OR83b to correctly localize and function in olfactory sensory neurons of the fly [39, 40]. In heterologous cells, functional expression of typical *Drosophila* ORs is also significantly enhanced upon expression with OR83b [40]. This observation of OR heterodimerization in the fly bears similarity to the capacity for dimerization of some mammalian ORs, but whereas obligate heterodimerization with OR83b appears to be requisite for all fly ORs, obligate heterodimerization with one partner is not likely to be a general phenomenon among mammalian ORs [34]. Furthermore, it is not clear how closely analogies can be drawn between *Drosophila* and mammalian ORs, as recent evidence suggests that the transmembrane topology of *Drosophila* ORs may be quite distinct from their mammalian counterparts [41].

Receptor transporting and expression enhancing proteins as chaperones

Three novel proteins that promote the functional plasma membrane expression of heterologously expressed ORs were identified using gene expression analysis techniques from single olfactory sensory neurons. Two of these proteins were termed receptor transporting protein 1 and 2 (RTP1 and RTP2), and the other was named receptor expression enhancing protein 1 (REEP1) [36]. Immunocytochemistry and fluorescence-activated cell sorting analysis indicated that RTP1 and RTP2 significantly enhanced the cell surface expression of the ORs mOR203-1, mOREG, mORS46, mOR62, and rat I7, all containing N-

terminal rhodopsin tags. REEP1 exerted similar effects, although to a lesser extent, while related RTP and REEP family members exhibited no enhancement of mOR203-1 surface expression. Co-immunoprecipitation studies in HEK-293T cells demonstrated physical interactions between ORs and RTP1 and REEP1. Moreover, physical associations between RTP1 and ORs may influence the cell surface localization of both proteins, as RTP1 expressed alone exhibited poor surface localization that was greatly increased upon co-expression with mOREG [36].

In an effort to capitalize on the enhanced surface expression of ORs in heterologous cells, RTP1, RTP2, and REEP1 were stably expressed in HEK-293T cells together with a cyclic AMP response element-luciferase reporter gene, thereby creating a recombinant system in which OR-ligand pairs could be screened. These cells were used to successfully generate rough odorant response profiles for 11 ORs, including 7 orphans [36]. Such findings demonstrate that ORs reaching the plasma membrane *via* interactions with RTP and REEP proteins are functionally active.

A short form of RTP1, called RTP1S, has been identified and appears to exhibit even greater facilitation of OR cell surface localization in heterologous cells than the long form of RTP1. Furthermore, compared to co-expressing RTP1S plus unmodified ORs, expressing RTP1S together with N-terminal rhodopsin-tagged constructs, plus several other accessory factors including the OR G protein, $G\alpha_{olf}$, and an olfactory-specific putative guanine nucleotide exchange factor, Ric8b, yielded the greatest enhancement of cell surface localization and functionality for a number of different ORs. Additionally, Western blots of olfactory epithelium suggested that this more potent RTP1S may be the dominantly expressed form of the protein *in vivo* [42]. Overall, these studies demonstrated that proper heterologous plasma membrane localization of a number of different ORs is significantly enhanced by co-expression with specific members from the RTP and REEP families of proteins.

In addition to their expression in olfactory sensory neurons, recent evidence suggests RTP1, RTP2, and REEP1 also exhibit testicular expression [43]. Interestingly, several ORs are expressed in the testis as well [44–46]. However, the extent to which RTP and REEP proteins are potentially able to influence the surface localization of testis-expressed ORs is presently not known, as it has not yet been demonstrated that these proteins are specifically localized to spermatids, where the ORs are found. Also unknown are the mechanisms by which RTP and REEP proteins exert their effects on OR surface targeting. Nonetheless, utilizing RTP1, RTP2, REEP1, and particularly RTP1S, in conjunction with other accessory factors appears to be a useful way to study various types of ORs in recombinant systems. Moreover, these proteins may also be critical for endogenous OR trafficking in olfactory sensory neurons, although this idea must be tested using transgenic approaches.

Heat shock proteins as OR chaperones

HSP70 heat shock proteins are molecular chaperones that interact with and assist in the correct folding of improperly folded proteins [47]. Hsc70t is a constitutively expressed member of this family, previously found to be expressed in spermatids [48], and more recently identified in the olfactory epithelium of both humans and mice [49]. Since ORs are specifically expressed in these same tissues, Hsc70t involvement in OR folding and trafficking was examined. Co-transfection of numerous GFP-tagged ORs (human 1F12, 17-4, 3A1, 52E2, 51I2, 2AG1, 52B4, 4C15, mouse I7) with Hsc70t in HEK-293 cells significantly increased the overall cellular expression of OR protein as assessed by immunofluorescence assays and Western blotting. This enhanced expression appeared to be particularly robust for ORs known to be expressed in spermatids, such as human OR 17-4 [49]. Moreover, co-expression with Hsc70t increased the number of human OR 17-4 transfected cells that responded to the ligand bourgeonal, from 1% to 5–10%, suggesting that more functional ORs were present on the cell surface upon Hsc70t co-expression [49].

To further investigate the effects of Hsc70t on OR expression, the human OR orphan 2AG1 was co-expressed with Hsc70t and cells were exposed to sequential subdivisions of a mixture of 100 potential odorants until a single compound, amylbutyrate, was identified as the best ligand, activating 5–10% of all cells tested. HEK-293 cells transfected with 2AG1, but not Hsc70t, also responded to amylbutyrate (although not as frequently), suggesting that Hsc70t co-expression does not alter functional response properties [49]. Subsequent studies co-expressing Hsc70t with various other ORs and in combination with some of the aforementioned RTP proteins have yielded insignificant effects [42]. These studies, however, mainly examined ORs whose expression in the testis is unknown. Thus, it is possible that Hsc70t plays a more important chaperone role for testis localized ORs *versus* nasal epithelium localized ORs.

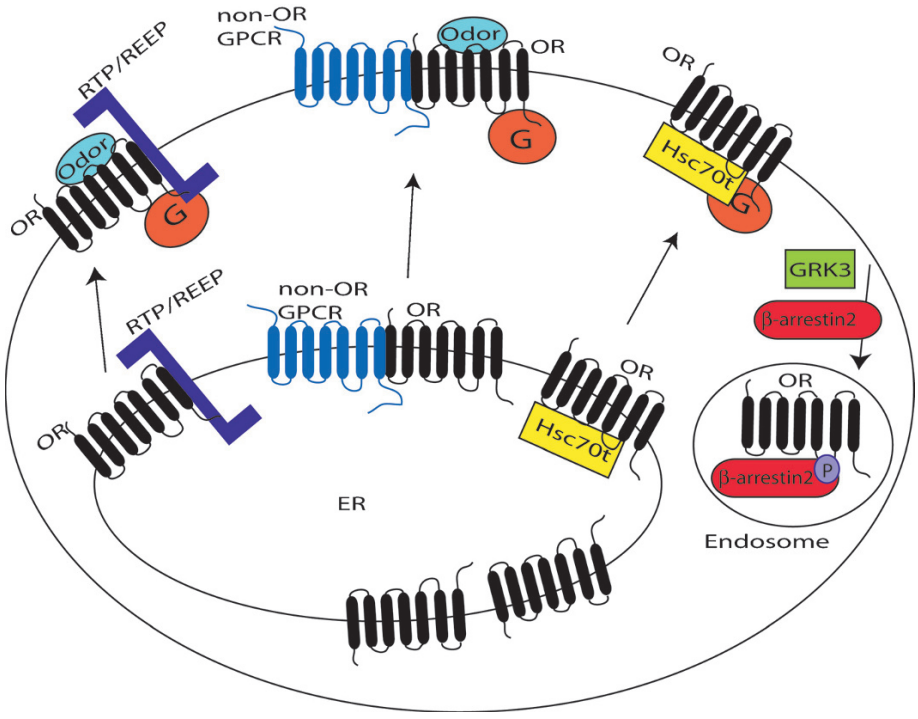

Figure 1. Olfactory receptor (OR) localization at the plasma membrane can be facilitated by co-expression with multiple accessory proteins. ORs expressed alone in heterologous cells typically exhibit endoplasmic reticulum (ER) retention. However, enhanced cell surface localization and functionality of ORs has been observed upon co-expression with specific receptor transporting proteins (RTPs), receptor expression enhancing proteins (REEPs), non-OR G protein-coupled receptors (GPCRs), and the heat shock protein Hsc70t. Plasma membrane-bound ORs are activated by odorants to elicit coupling to G proteins. Activated ORs are phosphorylated at specific residues by G protein-coupled receptor kinase 3 (GRK3) and other protein kinases, causing desensitization. Desensitized ORs may then undergo clathrin-mediated internalization into endosomes *via* association with β-arrestin 2.

Summary of OR accessory proteins

This review has summarized what is currently known about receptor-associated proteins that regulate OR surface expression in heterologous cells. As discussed, ODR-4 in *C. elegans* and OR83b in *Drosophila* have both been demonstrated to enhance the trafficking and functional activity of ORs from these species *in vitro* as well as *in vivo.* In mammals, certain members of four distinct protein families, non-OR GPCRs, RTP, REEP, and HSP70, all exhibit the capacity to facilitate appropriate localization and functionality of particular ORs in HEK-293 cells (Fig. 1). However, it is not clear at present if any of these proteins play a comparable role in influencing the trafficking of endogenous ORs in OSNs. Moreover, the specific mechanisms by which these accessory proteins enhance cell surface expression are presently not understood. In the case of non-OR GPCRs, RTP1, and REEP1, co-immunoprecipitation studies suggest that these proteins may directly interact with the ORs, potentially to facilitate correct folding or to mask an endoplasmic reticulum retention signal. Whether Hsc70t also associates directly with ORs remains unclear.

Non-OR GPCRs, RTPs, REEPs, and Hsc70t do not share significant amino acid sequence similarity with each other, and thus it is possible that each unique accessory protein exerts its effects by different means. These accessory proteins may potentially carry out complementary functions, each acting at a particular step in the maturation and trafficking of ORs. Indeed, multiple mechanisms may be involved in regulating the targeting of a receptor family as large and diverse as that of the ORs.

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