Olfactory receptors are difficult to functionally express in heterologous cells. They are typically retained in the endoplasmic reticulum of cells commonly used for functional expression studies and are only released to the plasma membrane in mature cells of the olfactory receptor neuron lineage. A recently developed olfactory cell line, odora, traffics olfactory receptors to the plasma membrane when differentiated. We found that undifferentiated odora cells do not traffic olfactory receptors to their surface, even though they release the receptors to the Golgi apparatus and endosomes. This behavior differs from other cell lines tested thus far. Differentiated odora cells also properly traffic vomeronasal receptors of the VN1 type, which lack sequence similarity to olfactory receptors. ODR-4, a protein that is necessary for plasma membrane trafficking of a chemosensory receptor in nematodes, facilitates trafficking of rat olfactory receptor U131 in odora and Chinese hamster ovary cells. Olfactory receptor trafficking from the endoplasmic reticulum to the plasma membrane involves at least two steps whose regulation depends on the maturation state of cells in the olfactory receptor neuron lineage. These results also indicate that some components of the regulatory mechanism are conserved.

The olfactory receptors (ORs) are a large and diverse family of proteins belonging to the superfamily of G protein-coupled receptors (GPCRs). Since the initial isolation of cDNAs encoding rat ORs, ORs have been cloned from several taxonomic groups including mouse, dog, human, fish, chicken (reviewed in Ref. 2), nematodes, and Drosophila (3–5). The GPCR superfamily also includes other chemoreceptors, two divergent types of mammalian vomeronasal receptors and taste receptors in rodents and Drosophila (6–10). Given the large evolutionary distance between these organisms, the varied environments they inhabit, and the evidence that hundreds of OR genes exist in a single species, it is not surprising that chemosensory receptors are diverse sequences that appear to have little in common besides general topology (2).

The capacity to traffic ORs to the plasma membrane efficiently appears to be specific to mature ORNs. ORs do not traffic well to the plasma membrane when expressed in common cultured cell lines, in neuron-like cell lines, and even cell lines derived from the olfactory epithelium (11, 12). This is consistent with the presumption that plasma membrane insertion is necessary for OR function and explains why ORs are difficult to functionally express in heterologous systems. Evidence that mature ORNs traffic ORs to the plasma membrane comes from immunocytochemical detection of ORs in olfactory cilia (13), which appear to lack internal membranes, and from the successful functional expression of an OR in ORNs in vivo (14). In all other cells, we find that ORs are retained in the endoplasmic reticulum (ER) and traffic poorly, if at all, to the Golgi apparatus (12). This appears to be the typical behavior of an OR in a heterologous expression system, although exceptions are known among nonmammalian ORs (15, 16) and other families of chemosensory receptors (17). In addition, plasma membrane trafficking of ORs in heterologous cells can be improved by extending the N terminus of ORs (18, 19). These discoveries serve to underscore our ignorance about the mechanisms regulating trafficking of ORs. Only in Caenorhabditis elegans is the identity of a protein that regulates OR trafficking known. The odr-4 gene, which is expressed specifically in chemosensory neurons, is necessary for trafficking of some ORs to sensory neuron cilia (20). No mammalian homolog of the ODR-4 protein has yet been identified. However, the number of accessory proteins that regulate the trafficking of other types of mammalian GPCRs continues to grow (21–29).

Access to a cell line that mimics the ability of ORNs to traffic ORs would aid the study of OR trafficking. The recently developed odora cells (30) may provide such a model cell line. Differentiated odora cells express neuronal and olfactory markers, including components of the olfactory transduction pathway, and traffic exogenous olfactory receptors to their surface. We found that differentiation is necessary for plasma membrane expression of ORs in odora cells. In undifferentiated odora cells, ORs were not released to the surface but were present in the Golgi apparatus and endosomes. This suggests a two-step model for the regulation of OR trafficking. We also found that differentiated odora cells were able to traffic VN1-type vomeronasal receptors to their plasma membrane and that coexpression of ODR-4 with rat OR U131 facilitated trafficking of U131 to the plasma membrane. These findings are evidence that mechanisms regulating OR trafficking involve conserved functions.

**EXPERIMENTAL PROCEDURES**

**Materials**—Suppliers of affinity-purified antibodies were as follows: rabbit anti-KDEL, was from Stressgen, mouse monoclonal antibodies and rabbit polyclonal antisera against GFP were from CLONTECH, mouse monoclonal anti-human transferrin receptor (TIR) was from Zymed Laboratories Inc., mouse monoclonal (16B12) antibodies (mAbs) against the hemagglutinin epitope (HA-1) were from Berkeley Antibody

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differentiated odora cells that were transiently transfected with HA-β2-adrenergic receptor-GFP (a–c) and U131-GFP (d–f), the GFP signal (green) overlaps with the red signal from markers for the ER (KDEL; a and d), the Golgi apparatus (wheat germ agglutinin; b and e), and endosomes (TR2, c and f). The images show transiently transfected cells that were permeabilized prior to immunostaining. Scale bars, 25 μm.

FIG. 2. Olfactory receptor constructs are released from the ER in undifferentiated odora cells. Confocal images show that for both β2-adrenergic receptor-GFP (a–c) and U131-GFP (d–f), the GFP signal (green) overlaps with the red signal from markers for the ER (KDEL; a and d), the Golgi apparatus (wheat germ agglutinin; b and e), and endosomes (TR2, c and f). The images show transiently transfected cells that were permeabilized prior to immunostaining. Scale bars, 25 μm.
Olfactory Receptor Trafficking

Fig. 3. Differentiated odora cells express unrelated chemosensory receptors on the plasma membrane. Differentiated odora cells were transiently transfected with receptors fused to the HA-1 epitope at their N termini. Transfected cells were immunostained prior to fixation so that positive staining would identify only the HA-1 epitope that was expressed on the plasma membrane. The surface staining was apparent in cells transfected with HA-17, HA-VN1, and HA-VN7 but not in cells transfected with HA-DOR64 or HA-TR2. To demonstrate the difference in appearance between surface staining and intracellular staining, differentiated odora cells were transiently transfected with a β2-adrenergic receptor that was tagged with the HA-1 epitope at the N terminus and GFP at the C terminus (HA-β2-GFP). Two images of HA-β2-GFP in the same cell show the surface staining obtained with immunostaining prior to fixation versus the total distribution of the GFP fluorescence, much of which is from receptors in intracellular membranes. The GFP fluorescence of U131-GFP in a differentiated odora cell is shown to demonstrate the network pattern typical of the intracellular distribution of olfactory receptors. Scale bars, 25 μm. wt, wild type.

RESULTS

Undifferentiated odora Cells Do Not Traffic ORs to the Plasma Membrane—The first question we addressed was whether undifferentiated odora cells traffic ORs to their plasma membrane as do the differentiated cells. As a positive control for receptor expression and trafficking, we used HA-β2-adrenergic receptor or β2-adrenergic receptor-GFP. In both differentiated and undifferentiated odora cells, HA-β2-adrenergic receptor was present on the plasma membrane (Fig. 1). As previously reported (30), HA-U131 (rat) was present on the plasma membrane of differentiated odora cells, and the staining pattern was punctate. In undifferentiated odora cells, HA-U131 was not present on the surface. Two other rat ORs, HA-OR5 (not shown) and HA-17 (see Fig. 3), behaved in the same manner as HA-U131. We conclude that whereas differentiated odora cells traffic ORs to the plasma membrane, undifferentiated odora cells do not.

In Undifferentiated odora Cells, ORs Are Present in Endosomes and the Golgi Apparatus—We have previously shown that in cells of several nonneuronal types (CHO, human embryonic kidney 293, Xenopus fibroblasts, and melanophores), inability to express ORs on the plasma membrane is explained by the retention of the receptors in the ER. In contrast, the β2-adrenergic receptor is expressed in the ER, the Golgi apparatus, endosomes, and on the plasma membrane (11, 12). Because undifferentiated odora cells do not traffic ORs to the plasma membrane, we tested whether they also retained ORs in the ER. We used C-terminal fusions of receptors to bGFP-S65T for these colocalization studies; receptors fused to the HA-1 epitope tag or to GFP variants have the same intracellular distribution pattern typical of the intracellular distribution of olfactory receptors.

The percent overlap values for the three compartments are not expected to sum to 100%, because each compartment was tested in different cells, and other compartments that we did not test (e.g., proteolytic compartments and transport vesicles) presumably contain receptors. OR trafficking in undifferentiated odora cells shows a pattern that differs from nonneuronal cells and from mature ORNs. The ORs are released from the ER and are present in the Golgi apparatus and in TIR-positive endosomes but are not detected on the plasma membrane. Our previous results (12) suggest that exit from the ER is a regulated process that depends on structural determinants in the C-terminal part of an OR. These new data suggest a second regulatory process, occurring in a post-Golgi compartment and sensitive to the differentiation state of the ORN.

Differentiated odora Cells Traffic to the Plasma Membrane Chemosensory Receptors from Unrelated Families—We further
investigated whether differentiated odora cells are capable of plasma membrane expression of other families of chemosensory receptors. We made HA-1-tagged versions of rat OR I7, rat vomeronasal receptors VN1 and VN7, Drosophila OR dor64, and rat taste receptor TR2. These groups of chemosensory receptors do not share significant sequence homology but are thought to share the general GPCR topology. As shown in Fig. 3, HA-I7, HA-VN1, and HA-VN7 were detected at the cell surface as punctate or concentrated regions of immunostaining done on living cells. The appearance of this surface staining differed greatly from the intracellular distribution of receptors in these cells. The intracellular distribution was a network that extended throughout the cell, often with high-intensity regions near the nucleus. This is shown in Fig. 3 as the GFP fluorescence of HA-\textit{b}2-GFP and U131-GFP expressed in differentiated odora cells. Receptors fused at the C termini to GFP show trafficking behavior identical to the same receptors containing the N-terminal HA-1 epitope tag (12). These data argue that HA-I7, HA-VN1, and HA-VN7 were present on the plasma membrane of the differentiated odora cells. The efficiency of expression we observed (0.5–2% of the cells) was similar to that for HA-U131 and HA-OR5. As observed with HA-U131 and HA-OR5, none of these chemosensory receptors were detected on the plasma membrane of undifferentiated odora cells (not shown). We did not detect surface staining in undifferentiated (not shown) or differentiated odora cells transfected with HA-TR2 and Drosophila HA-DOR64; the intensity of nonpermeabilized staining in these cells was similar to very weak background staining occurring in control cells. We conclude that differentiated odora cells are capable of properly trafficking at least two unrelated types of chemosensory receptors. The underlying mechanisms must therefore recognize both OR and VN1 receptor families, suggesting that these receptors have some common features.

\textit{Odr-4 Facilitates Surface Expression of U131—}If diverse chemosensory receptors share trafficking behavior in heterologous expression systems, as our data indicate, they should also be expected to share the ability to interact with proteins that regulate their trafficking. Thus far, the only protein known to regulate trafficking of an OR is ODR-4, a unique protein expressed in chemosensory neurons of \textit{C. elegans}, where it is necessary for the surface expression of chemosensory receptor ODR-10. In odr-4 mutants, chemosensory receptor ODR-10 is confined to an intracellular network in the cell body of the receptor neurons (20). When coexpressed with HA-U131 in undifferentiated odora cells, ODR-4 caused an increase in surface expression of HA-U131 in a small (0.3–0.5%) population of cells (Fig. 4A). Coexpression with ODR-4 did not have any effect on the surface expression of HA-OR5 (not shown). The staining intensity of the cells expressing HA-U131 on their surface varied. To estimate the effect of

![Figure 4](https://example.com/figure4.png)
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ODR-4 in odora cells, we acquired for each transfection condition the first 12 to 20 images of the cells that were visually perceived as being above the background in both transfected and control cells. We quantified staining in unmodified images of a standard size by digitally integrating pixel values between grayscale levels 40 and 254 using the integration function of NIH Image. Representative images from an odora cell experiment are shown in Fig. 4A, and the intensity values are presented on Fig. 4B. For this experiment, the mean intensity values were as follows: 1782 ± 1981 for ODR-4 coexpression with HA-U131, and 135 ± 177 for HA-U131 alone (mean ± S.D.; arbitrary units; the data represent 4 independent transfections). In another experiment we transfected, stained, and collected the intensity data for HA-U131 + ODR-4 (329 ± 320; mean ± S.D.; four independent transfections) and HA-U131 (49 ± 55), HA-OR5 + ODR-4 (81 ± 50), and HA-β2-adrenergic receptor (1396 ± 1557). To test whether coexpression with any protein was sufficient to promote surface expression of HA-U131, other proteins were coexpressed with HA-U131. We tested hAIP1 (fused to the FLAG epitope), which has no sequence similarity to ODR-4, because it was detected in a yeast two-hybrid screen as a possible binding partner of a fragment of U131. Coexpression with hAIP1 did not increase plasma membrane expression of HA-U131 (Fig. 4B, inset). Coexpression with hGFP-S65T gave identical negative results. ODR-4 had a significant effect (p < 0.05, with averaged intensity values from two separate sets of four transfections each, normalized to the mean value of U131 + ODR-4 in each case) on the plasma membrane expression of U131. The action of ODR-4 did not appear to be correlated with an increased amount of receptor protein. Coexpression of U131-GFP and ODR-4 in undifferentiated odora cells was compared with cotransfection of U131-GFP plus the empty vector pJG3.6. The resulting distributions of GFP fluorescence intensity values were similar, but the peak intensity values from the cells transfected with U131-GFP plus pJG3.6 was 30% higher. Although ODR-4 worked in two cell lines, including a nonolfactory line, it is probably unsuitable for use in functional expression screening of the odorant selectivity of ORs. Its effects are relatively weak and appear to be limited to a subset of mammalian ORs. Nevertheless, our data do suggest parallels between the mechanisms of regulation of the plasma membrane expression of chemosensory receptors in C. elegans and in mammals.

DISCUSSION

The ability to traffic ORs to the plasma membrane appears to be limited to ORNs. When expressed in nonolfactory cell lines, ORs are retained in the ER (12). Odora cells are an exception to these generalities, perhaps because they resemble ORNs (30). First, they traffic ORs to the plasma membrane when in the undifferentiated state. Second, in the undifferentiated state they show a unique pattern of OR trafficking; ORs traffic into the Golgi apparatus and endosomes but not to the plasma membrane. These data predict that as progenitor cells progress through differentiation to become mature ORNs they may acquire stepwise changes in their ability to move ORs toward the plasma membrane. Our results further indicate that the mechanisms regulating OR trafficking have conserved features. First, trafficking of the VN1 type of vomeronasal receptors depends on the differentiation of odora cells just as do ORs. Second, the ability of a C. elegans protein, ODR-4, to improve plasma membrane trafficking of a mammalian OR is similar to its ability to facilitate trafficking of a C. elegans OR.

Our results support a model of OR trafficking that has two regulatory checkpoints (Fig. 5). The first checkpoint is exit from the ER. Because undifferentiated odora cells, unlike other cell lines, can traffic ORs past the ER, the mechanism allowing ORs to cross the ER checkpoint may be expressed relatively early in the differentiation of ORNs. Because ORs are not present in the plasma membrane of undifferentiated odora cells, but are present there after differentiation, a second checkpoint may occur at a post-Golgi compartment. We do not yet have evidence of whether ORs traffic directly to endosomes from the Golgi apparatus or arrive there by rapid internalization from the plasma membrane. The proteins that regulate these checkpoints remain obscure.

The model does make predictions, however, about the nature of these regulatory proteins. It predicts that the critical proteins have positive effects, either directly on the OR by activating other proteins to promote OR trafficking or by disinhibiting processes that suppress OR trafficking. There are examples of other GPCRs whose membrane trafficking in heterologous cells improves in the presence of specific accessory proteins (23–25). We have now shown that at least one OR, U131, behaves similarly when coexpressed with the C. elegans protein, ODR-4. Our model predicts that mammalian ORNs ex-

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2 T. McClintock, C. Josefowicz, S. C. Bose, and A. Gimelbrant, unpublished results.
press proteins with functions analogous to ODR-4. Because ODR-4 does not traffic to the cilia of sensory neurons with its receptor in *C. elegans*, and because ODR-4 promotes plasma membrane trafficking of U131 in a cell line where U131 is normally retained in the ER, we suspect that ODR-4 and its predicted functional homologs in vertebrates are involved in the exit of ORs from the first checkpoint at the ER. Why ORs are retained in the ER in the first place is not yet clear, but misfolding or the lack of a partner for heteromeric proteins are common causes of active ER retention of plasma membrane proteins by ER quality control mechanisms (32, 33). The second, post-Golgi checkpoint is more mysterious. It may be specific to cells of the ORN lineage, but this has not been tested directly. The proteins regulating this checkpoint may interact with ORs either at the Golgi apparatus or endosomes to target the ORs onward to the plasma membrane. It is possible that they release ORs from the proteins that promoted exit of the ORs from the first checkpoint. Alternatively, the second checkpoint may consist of proteins that encounter ORs at the plasma membrane, trapping them there and slowing OR recycling to endosomes. The latter possibility is analogous to other GPCRs whose accumulation in certain subdomains of the plasma membrane is regulated by specific accessory proteins (for review see Refs. 34 and 35). A third alternative is that processing at the ER can generate receptor proteins with differing trafficking capacities, some capable of reaching endosomes and others capable of reaching the plasma membrane. This would be consistent with the ability of ODR-4 to promote plasma membrane trafficking in both CHO cells, where ORs are retained in the ER, and in undifferentiated *odora* cells, where ORs reach the Golgi apparatus and endosomes. Whatever the mechanism, the reason for such apparently complex regulation of OR trafficking is unknown. Perhaps it is related to the hypothesis that the olfactory cilium is a privileged plasma membrane domain containing a unique subset of proteins.

An alternative to our model is the possibility that mature olfactory receptor neurons lack an otherwise widely expressed factor that suppresses plasma membrane trafficking of ORs. We cannot exclude this possibility, but it is not consistent with the ability of ODR-4 to act as a positive factor or with analogies to positive control mechanisms that regulate the trafficking of other GPCRs (23–25). A more complex mechanism, such as depicted in our model, therefore seems justified. The model is not meant to suggest that negative factors do not play significant roles in regulating OR trafficking, however. For example, ER quality control mechanisms may play a significant inhibitory role if nascent ORs are misfolded or lacking a binding partner, something that is consistent with the effects of truncation on OR trafficking (12). An interdependence of positive and negative factors would be consistent with the apparent complexity of OR trafficking.

Given the paucity of conserved residues among ORs in particular, and among chemoreceptor proteins in general, wide variability in trafficking behavior in heterologous cells might be expected. Indeed, there are examples of ORs that traffic well to the plasma membrane in at least some heterologous cells (15, 16, 36). However, these are the exceptions rather than the norm. Instead, our results emphasize the conservation of the mechanisms regulating trafficking of chemoreceptor proteins. First, the trafficking behavior of the VNI type of vomeronasal receptor mirrors that of ORs. They traffic to the plasma membrane of *odora* cells only when these cells are differentiated. The nature of ODR-4 to increase the plasma membrane trafficking of at least one vertebrate OR suggests conservation of at least part of the mechanism regulating trafficking. The absence of sequence similarity among the receptor proteins we tested argues that the conserved functions we identified depend on protein signal features more complex than the primary structure. This is consistent with the view that a small number of accessory proteins are responsible for regulating the trafficking of the entire OR repertoire.

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