Synergism of Accessory Factors in Functional Expression of Mammalian Odorant Receptors*

Hanyi Zhuang1 and Hiroaki Matsunami1,5,2

From the 1Department of Molecular Genetics and Microbiology, 5Department of Neurobiology, Duke University Medical Center, Durham, North Carolina 27710

The discovery of odorant receptors led to endeavors in matching them with their cognate ligands. Although it has been challenging to functionally express odorant receptors in heterologous cells, previous studies have linked efficient odorant receptor expression with N-terminal modifications and accessory proteins, including the receptor-transporting proteins (RTPs) and Ric8b. Here we have shown that a shorter form of RTP1S, supports robust cell-surface and functional expression of representative odorant receptors. Using a combination of accessory proteins, including RTP1S, Ric8b, and G_{olf} a diverse set of untagged odorant receptors were successfully expressed heterologously due to the synergistic effects among the various accessory proteins. Furthermore, the addition of an N-terminal rhodopsin tag to the odorant receptors, along with the same set of accessory proteins, exhibits an additional level of synergism, inducing enhanced odorant receptor responses to odorants and thus defining a more efficient heterologous expression system. We then showed that the presence or absence of different N-terminal tags has little effect on the ligand specificity of odorant receptors, although the amount of receptor expressed can play a role in the ligand response profile. The accuracy of the odorant receptor heterologous expression system involving tagged odorant receptors and various accessory proteins promises success in high throughput de-orphaning of mammalian odorant receptors.

Odorant receptors (ORs), with more than 1000 members in the mouse genome, comprise the largest family among all G protein-coupled receptors. After the initial identification of the OR genes in 1991 (1), one of the foci in the field of olfaction has been the identification of the cognate ligands of ORs (2). The use of recordings from adeno-virally infected rat olfactory sensory neurons first successfully matched an odorant to a cloned OR (3). Although the establishment of a heterologous expression system in cell lines is essential for conducting a large scale analysis of OR ligand specificities, exogenous ORs in cell lines pose a critical problem, namely the inability to functionally express transfected ORs on the plasma membrane, possibly due to endoplasmic reticulum retention, which in turn leads to OR degradation in the proteosome (4, 5).

Krautwurst et al. (6) have pioneered the method of fusing the first 20 amino acids of rhodopsin (Rho tag) to the N termini of ORs to promote their cell-surface expression, although it is not clear how Rho tag enhances expression of the ORs. Other modifications, such as the use of a signal sequence, were used to improve trafficking of OR to the plasma membrane (7–11). However, these modifications increase the functional expression for only a fraction of ORs. It is unclear whether N-terminal modifications have any undesirable effects on OR ligand selectivity, because it is almost impossible to heterologously express untagged ORs to verify their ligand selectivities. Nevertheless, there is no evidence supporting the possibility of N-terminal tags significantly affecting OR ligand specificities. It is known that tagged and untagged OREG (MOR174-9) responded to its cognate ligand, eugenol, suggesting that N-terminal modifications do not affect odorant recognition (10). In addition, Rho-tagged OREG, S6 (MOR42-3), and MOR23 (MOR267-13) expressed in heterologous cells showed a similar ligand response profile as when expressed in isolated olfactory sensory neurons expressing the same receptor (12–17). Furthermore, it has been shown that untagged, N-terminal FLAG-tagged, and C-terminal green fluorescent protein (GFP)-tagged rat I7 receptors expressed in olfactory sensory neurons and N-terminal Rho-tagged I7 expressed in heterologous cells all consistently responded to its ligand, octanal (3, 6, 18–20).

It is likely that specific molecular mechanisms, which are absent in heterologous cell lines, are required for proper OR expression and function. Receptor-transporting protein (RTP) family members were among the first of such accessory proteins that were shown to improve heterologous systems by promoting cell-surface expression of various Rho-tagged ORs (15). RTP1 and RTP2 are specifically expressed in ORs expressed in olfactory sensory neurons in the olfactory epithelium. Co-immunoprecipitation data showed that RTP1 and RTP2 are complexed with various ORs on the HEK293T cells (15). In vivo, they could serve as OR chaperones for folding and/or endoplasmic reticulum export or as co-receptors. In addition, another molecular chaperone, an HSP70 (heat shock protein 70) homolog, Hsc70t, when co-expressed with C-terminal GFP-tagged human ORs, enhanced the num-

* The abbreviations and trivial names used are: OR, odorant receptor; GFP, green fluorescent protein; HEK, human embryonic kidney; Rho, rhodopsin; HA, hemagglutinin; FACS, fluorescence-activated cell sorter; RTP, receptor-transporting protein; Ric8b, resistance to inhibitors of cholinesterase 8 homolog B.

1 Supported by the Ruth L. Kirschstein National Research Service Award from the National Institutes of Health (DC008480).
2 To whom correspondence should be addressed: Dept. of Molecular Genetics and Microbiology, Duke University Medical Ctr., 264 CARL Bldg., Research Drive, Durham, NC 27710. Tel.: 919-684-2777; Fax: 919-681-9193; E-mail: hiroaki.matsunami@duke.edu.
3 The abbreviations and trivial names used are: OR, odorant receptor; GFP, green fluorescent protein; HEK, human embryonic kidney; Rho, rhodopsin; HA, hemagglutinin; FACS, fluorescence-activated cell sorter; RTP, receptor-transporting protein; Ric8b, resistance to inhibitors of cholinesterase 8 homolog B.

© 2007 by The American Society for Biochemistry and Molecular Biology, Inc. Printed in the U.S.A.
ber and level of an OR protein expression, resulting in more cells responding to odors in HEK293T cells, possibly through assistance in folding or trafficking (21). In another case, co-expression with the β2-adrenergic receptor enhanced the cell-surface expression of one OR (22). Amplifying signal transduction is another route to a better heterologous system. Ric8b, an olfactory-specific putative guanine nucleotide exchange factor, has been shown to improve the G_olf signaling cascade with which ORs couple (23, 24).

In this study, we identified a shorter form of RTP1, RTP1S, which promotes the cell-surface expression of ORs even more so than the original RTP1. We then asked whether untagged ORs can be heterologously expressed in the presence of accessory proteins including RTP1S. We investigated possible advantages of co-expressing combinations of different accessory proteins as well as the inclusion of N-terminal tags. We also examined the effect of different epitope tags, namely the Rho, FLAG, and HA tags, on the ligand specificity of representative mouse ORs.

**EXPERIMENTAL PROCEDURES**

**Plasmid Construction**—Rho-(N-MNGTEGPNFYVPSNA-TGVVRC)-, FLAG-(N-MDYKDDDDK-C)-, and HA-(N-MYP-YDVPDYA-C) tags were subcloned into pCI mammalian expression vector (Promega). The tags and pCI were digested with corresponding restriction enzymes. All tags were inserted between the NheI and EcoRI sites in pCI. OR open reading frames were amplified from genomic DNA of C57BL6 (MOR203-1) or DBA2 (Olfr62, S6, and MOR23-1) using KOD DNA polymerase (Toyobo/Novagen) and subcloned in between the MuI and NotI sites in pCI expression vectors containing different tags. RTP1S was amplified from the RTP1L plasmid DNA and subcloned into pCI or HA-pCI. Ric8b, Hsc70t, and G_olf were amplified from cDNA and cloned into pCI. The sequences of the cloned receptors and the accessory proteins were verified by sequencing (3100 Genetic Analyzer, ABI Biosystems).

**Cell Culture and Immunocytochemistry**—HEK293T cells and the HEK293T-derived Hana3A cells (15) were maintained in minimal essential medium (Sigma) containing 10% fetal bovine serum (Sigma) (M10), 500 μg/ml penicillin-streptomycin (Invitrogen), and 6 μg/ml amphotericin B (Sigma). HEK293T cells do not endogenously express RTP1 mRNA (15).

For immunocytochemistry, cells were seeded in a 35-mm dish (Falcon) containing a piece of cover glass coated with poly-d-lysine (Sigma) 24 h prior to transfection in M10. Lipo-fectamine 2000 (Invitrogen) was used for the transfection of plasmid DNA. For all stainings, 0.8 μg of OR DNA and 0.8 μg total of all accessory proteins (RTP1S, RTP1L, Hsc70t, Ric8b, and/or G_olf) or pCI were transfected per dish. 0.27 μg of each accessory protein was transfected, and pCI was co-transfected to make the total amount constant(3.0 μg of blue fluorescent protein was transfected as a control for transfection efficiency. For live cell-surface staining, 24 h post-transfection, cells were incubated in M10 containing 15 mM NaN₃ and 10 mM HEPES (Invitrogen) and the mouse monoclonal anti-rhodopsin antibody, 4D2 (25), at a 1:100 dilution at 4 °C for 45 min. The cells were then washed in Hanks’ balanced buffer solution (Invitrogen) containing 15 mM NaN₃ and 10 mM HEPES followed by incubation with Cy3-conjugated donkey anti-mouse IgG (Jackson Immunologicals) at 4 °C for 30 min and then fixation in 1% paraformaldehyde at 4 °C and mounting in Mowiol.

For permeabilized staining, 24 h post-transfection, cells were fixed in 4% paraformaldehyde for 15 min and permeabilized with methanol at 4 °C. The cells were then washed in phosphate-buffered saline followed by incubation with Cy3-conjugated donkey anti-mouse IgG (Jackson Immunologicals) and Alexa Fluor 488-conjugated donkey anti-rabbit IgG (Invitrogen) at room temperature for 30 min. Slides were mounted and observed under a fluorescent microscope (Axioskop 2; Carl Zeiss).

**Fluorescence-activated Cell Sorter (FACS) Analysis**—HEK293T and Hana3A cells were seeded in 35-mm dishes and then transfected with the same amount of plasmid DNA as in immunocytochemistry. 2 ng of GFP expression vector were transfected per dish as a control for transfection efficiency. 24 h post-transfection, the cells were dissociated in Cellstripper (Mediatech) and transferred to a tube for incubation with the anti-rhodopsin antibody as described in immunocytochemistry and then with phycoerythrin-conjugated donkey anti-mouse IgG (Jackson Immunologicals). 7-Amino-actinomycin D (Calbiochem) was added before flow cytometry to eliminate dead cells from the analysis.

**Generation of Anti-RTP1 Antibody**—The peptide RAPSPT-KPQATGSG located near the C-terminal end of RTP1 was synthesized (AnaSpec, Inc.). Rabbits were immunized according to standard procedures (Duke University Vivarium).

**Western Blot**—HEK293T cells in 35-mm dishes were transfected with RTP1S or RTP1L using Lipofectamine 2000. GFP expression vector was co-transfected as a control. 24 h post-transfection, cells were lysed with sample loading buffer (20 mM Tris, pH 7.5, 2 mM EDTA, 5% SDS, 20% glycerol, 0.002% bromophenol blue, and 0.25 mM dithiothreitol) and sonicated. SDS-PAGE and Western blot analysis were performed according to Mini-Protean 2 Cell (Bio-Rad) protocol. Membranes were incubated with rabbit anti-RTP1 at a 1:1000 dilution and subsequently with donkey anti-rabbit horseradish peroxidase (Jackson Immunologicals). The membrane was then incubated with stripping buffer (25 mM glycine-HCl, pH 2, 1% SDS, 25 mM glycine, and 1% SDS) for 30 min at room temperature and then with rabbit anti-GFP (Invitrogen) at a 1:1000 dilution and subsequently with donkey anti-rabbit horseradish peroxidase. ECL (Amersham Biosciences) was used for detecting proteins on membranes.

**Luciferase Assay and Data Analysis**—The Dual-Glo™ Luciferase Assay System (Promega) was used for luciferase assay essentially as previously described (15). CRE-Luc (cAMP-responsive element firefly luciferase) (Stratagene) was used to measure receptor activation. Renilla luciferase driven by a constitutively active SV40 promoter (pRL-SV40; Promega) was used as an internal control for cell viability and transfection.
efficiency. HEK293T or Hana3A cells were plated on poly-d-lysine-coated 96-well plates (BioCoat; BD Biosciences). Plasmid DNA of ORs and accessory factors was transfected using Lipofectamine 2000. For each 96-well plate, 1 μg of CRE-Luc, 1 μg of pRL-SV40, 5 μg of OR, and 1 μg total of all accessory proteins (RTP1S, RTP1L, Hsc70t, Ric8b, and/or G

were transfected. 0.25 μg of each accessory protein was transfected, and pCI was co-transfected to make the total amount constant. 24 h post-transfection, the medium was replaced with CD293 chemically defined medium (Invitrogen) and then incubated for 30 min at 37 °C. The medium was then replaced with 25 μl of odorant solution diluted in CD293 and incubated for 4 h at 37 °C. We followed the manufacturer’s protocols for measuring luciferase and Renilla luciferase activities. Luminescence was measured using a Wallac Victor 1420 plate reader (PerkinElmer Life Sciences).

Normalized luciferase activity was calculated by the formula [(luc(N) — luc(lowest))/luc(highest) — luc(lowest)], where luc(N) = luminescence of firefly luciferase divided by luminescence of Renilla luciferase of a certain well; luc(lowest) = lowest luminescence of firefly luciferase divided by luminescence of Renilla luciferase of a plate or a set of plates; and luc(highest) = highest luminescence of firefly luciferase divided by luminescence of Renilla luciferase of a plate or a set of plates. Data were analyzed with Microsoft Excel and GraphPad Prism 4 software. The statistical significance of synergism was assessed with a binomial test.

**Calcium Imaging**—Calcium imaging was performed similar to the protocol outlined previously (26). HEK293T cells were seeded on glass-bottom plates with poly-D-lysine-coated cover-slips (MatTek) and plasmid DNA of Olfr62 and either the accessory protein RTP1S, RTP1L, or pCI, and a G protein chimera, G15olf47, was transfected with Lipofectamine 2000 and incubated for 24 h. The same amount of plasmid DNA as used in immunocytochemistry was transfected. G15olf47 is a chimeric G protein in which the last 57 amino acids of G

were replaced by the last 47 amino acids of G

ololf47 was transfected per dish. 24 h post-transfection, cells were incubated with 4 μM Fluo-4 (Invitrogen) and 7 μM Fura-Red (Invitrogen) for 45 min at room temperature. A Leica (Wetzlar, Germany) confocal microscope (excitation at 488 nm; emission at 500–560 nm for Fluo-4 and 605–700 nm for Fura-Red) was used. Cells were exposed to a constant flow of the bath solution, Hanks’ balanced solution supplemented with 10 mM HEPES. Odorant solutions containing various concentrations of 2-coumaranone or 1 μM isoproterenol were applied to the cells for 15 s by changing the bath solution with a peristaltic pump (Rainin Instrument, Oakland, CA). Data were collected at 3-s intervals using the live imaging mode of the Leica confocal software. Ratios of Fluo-4 and Fura-Red were plotted using cells in the entire viewing fields that contained ~300 cells.

**Chemicals**—All odorants were purchased from Sigma, except octanoic acid, which was purchased from Calbiochem.

**RESULTS**

**Characterization of a Shorter and More Potent Form of RTP1**—During the course of analyzing the function of a deletion series of RTP1 in OR expression, we found that one of the deletions, which started from the second methionine of the open reading frame, induced stronger expression of ORs when co-expressed in HEK293T cells. We named this shorter form, lacking the N-terminal 36 amino acids, RTP1S, whereas the RTP1 previously described in Saito et al. (15) was renamed RTP1L. The putative translation start site of RTP1S is in agreement with those of the other RTP members (Fig. 1A).

Western blot analysis using lysates from HEK293T cells expressing RTP1S or RTP1L as well as from the olfactory epithelium revealed that the RTP1 proteins expressed in the olfactory epithelium appeared to migrate to the same position as that of RTP1S, whereas no band corresponding to RTP1L was seen, suggesting that RTP1S is a major form of RTP1 produced in vivo (Fig. 1B). Expressed sequence tag mining revealed three expressed sequence tags corresponding to RTP1 in which one of them contained the first methionine of RTP1L, and the 5’-ends of the other two cDNAs start between the first methionines of RTP1L and RTP1S.

To compare the function of RTP1S and RTP1L, we first examined the cell-surface expression of a set of four diverse mouse ORs, Olfr62 (MOR258-5), S6, OREG, and MOR203-1, all modified with an N-terminal Rho tag, which corresponds to the first 20 amino acids of human rhodopsin. The ORs were co-expressed with RTP1S, RTP1L, or a control vector in HEK293T cells. In all four cases, co-expression of RTP1S resulted in more robust cell-surface expression compared with co-expression of RTP1L, although the effect of RTP1L was clear when compared with negative controls (Fig. 1C). To quantify the cell-surface OR proteins, we performed FACS analysis for Olfr62 and S6. Fig. 1D demonstrates that RTP1S is more potent than RTP1L in inducing cell-surface expression of ORs, although RTP1L was sufficient to promote cell-surface expression of the ORs (15). In the Western blot analysis shown in Fig. 1B, the intensity of the bands corresponding to RTP1S and RTP1L were comparable. A control blot of co-transfected GFP shows similar GFP expression in cells expressing RTP1S and RTP1L, indicating that similar amounts of RTP1S and RTP1L are produced in cells. This suggests that the stronger activity of RTP1S in inducing OR cell-surface expression is not attributable to a higher level of protein expression.

Next, we compared the effect of RTP1S and RTP1L on the functional activation of ORs. A luciferase reporter gene assay was used to assess OR response upon ligand stimulation. Because ORs couple with stimulatory G proteins, leading to intracellular cAMP increases, luciferase gene expression mediated by a cAMP-responsive element can be used to measure activation of ORs by their ligands in HEK293T cells (15, 27). For three ORs, Olfr62, S6, and MOR23-1, co-expression of RTP1S induced more cAMP-mediated luciferase reporter gene product, indicating that RTP1S is more potent for the induction of functional cell-surface expression (Fig. 2A). Similarly, calcium imaging analysis showed that Olfr62 responded to its cognate ligand, 2-coumaranone, more robustly and at lower concentrations when co-expressed with RTP1S than when co-expressed with RTP1L (Fig. 2B).

H. Zhuang and H. Matsunami, manuscript in preparation.
To explore the mechanism by which RTP1S enhances OR functional expression, we examined the subcellular localization of RTP1S and Olfr62 in HEK293T by permeabilized staining (Fig. 2C). The majority of the signals for both proteins were seen in intracellular compartments. The expression of RTP1S co-localized with that of Olfr62, suggesting possible interaction between the two proteins.

Synergism of Accessory Proteins in the Functional Expression of Untagged ORs—Untagged ORs typically produced little or no ligand-induced responses, presumably because of poor cell-surface expression. To address this, we used accessory proteins recently implicated in the promotion of functional expression of mammalian ORs, including RTP1S, RTP1L, Ric8b, and Hsc70t, and asked whether co-expression of the accessory proteins is sufficient to confer functional expression of untagged ORs in heterologous cells.

We first co-transfected the ORs with RTP1S, RTP1L, Ric8b/G_{o,olf} and Hsc70t in various combinations in HEK293T cells (Fig. 3). Co-expression of RTP1S was found to be sufficient for the detection of ligand-induced OR activation for all four ORs. Consistent with the results shown in Fig. 2A, RTP1S was found to be more potent than RTP1L using untagged ORs. Co-expression of Ric8b was effective with one OR, OREG, consistent with a previous report (23), although we did not observe luciferase induction for the other three ORs in this condition. Hsc70t alone did not significantly enhance OR activation in our experiments. Importantly, in all four cases and at all three concentrations, co-expression of RTP1S and Ric8b resulted in more robust OR activation compared with a simple sum of both ($p = 0.00024$). These results suggest that RTP1S and Ric8b synergistically enhanced the function of the ORs. We then examined the same combinations of accessory factors in Hana3A cells, which is a HEK293T-derived cell line stably expressing RTP1L, RTP2, REEP1, and G_{o,olf}. We have previously shown that Hana3A cells can induce the functional expression of various Rho-tagged ORs and are thus an ideal foundation for building a more powerful OR heterologous expression system (15). We found more robust OR functions in all cases, consistent with the previous report. The synergism between RTP1S and Ric8b was, however, not as pronounced in these cells, possibly because of saturation of OR activation at higher ligand concentrations. Yet, it is clear that the addition of both RTP1S and Ric8b/G_{o,olf} in both HEK293T and Hana3A cells constitutes the most robust conditions for untagged OR expression among the conditions tested.

Synergism among Accessory Proteins and the Rho Tag in an OR Heterologous Expression System—Next, we examined the effect of the widely used Rho tag of ORs in conjunction with
Accessory Factors of Odorant Receptors

**FIGURE 2.** The effect of RTP1S and RTP1L on the functional activation of various ORs in heterologous cells. A, luciferase activity for Rho-tagged ORs (Olfr62, S6, and MOR23-1) co-transfected with RTP1S, RTP1L, or pCI in HEK293T cells. ORs were stimulated with their corresponding ligands (nonanediolic acid for S6, 2-coumaranone for Olfr62, and octanoic acid for MOR23-1) at various concentrations (1, 10, and 100 μM). The y-axis denotes normalized luciferase activity ± S.E. (n = 3). B, calcium imaging of HEK293T cells transfected with Rho-Olfr62 and RTP1S, RTP1L, or pCI. Cells were stimulated with buffer solution, 1 μM isoproterenol and increasing concentrations of 2-coumaranone (10, 100, and 300 μM) and 1, 10, and 100 μM). Isoproterenol stimulated endogenously expressed β2-adrenergic receptors. C, permeabilized staining of HEK293T cells co-transfected with C-terminal HA-tagged RTP1S and N-terminal FLAG-tagged Olfr62. Cells were stained with mouse anti-HA and rabbit anti-FLAG (scale bar = 10 μM).

The combination of accessory proteins in enhancing OR functions. We used the same set of four ORs and cloned each OR with a Rho tag at the N terminus. An untagged version of each OR was included as a control. We assessed the luciferase activity of the ORs in the presence of the combination of the accessory proteins that induced robust luciferase assay response in the untagged condition, that is, RTP1S and Ric8b/Golf. We found that the addition of Rho tag alone was not sufficient to induce functional expression of Olfr62 and MOR203-1 when expressed in HEK293T cells. Rho tag induced weak functional expression of S6 and OREG, as previously reported (6, 17, 28) (Fig. 4A). Moreover, when expressed with RTP1S and Ric8b/Golf, Rho-tagged ORs responded far better than untagged ORs in four cases, indicating synergistic effects of the Rho tag and the accessory proteins (p = 0.00293). This combination with the Rho tag and one or two structurally similar control ligands in Hana3A cells co-expressing RTP1S. ORs with either Rho or FLAG tag generally responded to lower concentrations of agonists compared with HA-tagged or untagged ORs. In the cases of S6, OREG, and MOR203-1, the relative ligand specificities of the ORs with different tags were similar to each other, although different N-terminal modifications had differential effects in the level of functional OR expression (Fig. 5, B–D). In contrast, we observed that, in the case of Olfr62, Flag- or HA-tagged, or the untagged version of the OR responded the best to 2-coumaranone, whereas Rho-tagged Olfr62 showed similar responses to 2-coumaranone, coumarin, piperonal, and benzaldehyde (Fig. 5A). These apparent differences of relative ligand specificities could have been caused by different N-terminal tag sequences. Alternatively, the differences of the amount of cell-surface OR molecules could cause differences in relative ligand
specificities. To test this, we expressed less Rho-tagged Olfr62 by transfecting smaller amounts of vectors. As a result, we observed gradually decreasing amounts of cell-surface expression under these conditions (Fig. 6, A and B). When we expressed a lesser amount of the Rho-tagged OR, the dose-response curves shifted toward the higher concentrations, indicating a reduction in receptor potency. At the same time, with the decreasing amount of OR transfected, the relative ligand specificities resembled more the FLAG-, HA-tagged, or untagged Olfr62, in that 2-coumaranone was clearly the most potent ligand, suggesting that the quantity of cell-surface OR molecules, not the N-terminal Rho tag, has a more significant contribution in relative ligand specificities of Olfr62 (Fig. 6C).

**DISCUSSION**

In this study, we first identified a shorter version of RTP1, RTP1S, as more potent and likely to be a major form of RTP1 that is produced by the olfactory sensory neurons. Using RTP1S and other previously identified accessory molecules, we showed that a variety of untagged ORs can be functionally expressed in heterologous cells as a result of synergistic effects among the accessory factors. Although the addition of N-terminal tags generally enhanced the expression of ORs compared with untagged ORs, the tags themselves appeared to have minimal effects on the ligand specificities of the ORs.

We found that the shorter RTP1S is more robust in enhancing cell-surface expression and ligand-dependent activation of ORs. This indicates that the first 36-amino-acid stretch of RTP1L may have inhibitory effects on OR functional expression. In a previous paper, we deduced the position of the first methionine of RTP1, because it was the first methionine in the open reading frame (15). In addition, this methionine is conserved in RTP1 in various mammalian species including human, macaque, cow, mouse, and rat. However, here we have shown by Western blot analysis that the molecular weight of RTP1 expressed by olfactory sensory neurons is the same or very similar to that of RTP1S, indicating that the second AUG is used as a start codon. We cannot exclude the possibility that the first AUG is actually used and RTP1L is processed to a smaller form that has similar molecular weight to RTP1S in the olfactory sensory neurons. Another possibility is that the first methionine is not included in the major transcript. Cell-surface expression and luciferase assay activity for the ORs tested were more robust with RTP1S than RTP1L, indicating that RTP1S is generally more effec-
FIGURE 4. **Heterologous expression of ORs is enhanced by the synergy between the N-terminal rhodopsin tag and the accessory proteins Ric8b/Golf and RTP1S.**

A, functional activation of ORs (Olfr62, S6, OREG, and MOR203-1) through synergy among Rho tag, Ric8b/Golf, and RTP1S. HEK293T cells were transfected with Rho-tagged OR with RTP1S and Ric8b/Golf (lanes 1), untagged OR with RTP1S and Ric8b/Golf (lanes 2), Rho-tagged OR with pCI (lanes 3), and untagged OR with pCI (lanes 4). Luciferase activities were measured after stimulation with their corresponding ligands at various concentrations (the same as described in the legend to Fig. 3). The y-axis denotes normalized luciferase activities ± S.E. (n = 3). B, cell-surface staining of the various Rho-ORs co-transfected with various combinations of accessory proteins (RTP1S, Hsc70t, Ric8b/Golf, RTP1S + Ric8b/Golf, and pCI). Cell-surface expression is seen in the case of RTP1S and RTP1S + Ric8b/Golf (scale bar = 50 μm). C, FACS analysis of cell-surface expression of Rho-tagged ORs (Olfr62 and S6) transfected with various combinations of accessory proteins (the same as described for B). Transfection with no OR was used as a control.
tive than RTP1L, although further investigation is needed to test whether RTP1S is always more effective in the functional expression of ORs.

We have shown here a subcellular co-localization of RTP1S and Olf62 in HEK293T cells. We have previously shown interaction between RTP1L and MOR203-1 by co-immunoprecipitation (15). We infer that RTP1S could interact with ORs in a similar manner as RTP1L. Both pieces of evidence point to the possibility that RTP1S promotes the trafficking of ORs through direct interaction.

By expressing the accessory proteins RTP1S and Ric8b/Golf in combination, we succeeded in the heterologous functional expression of a diverse set of unmodified ORs, which were known to be notoriously difficult to express. For a yet higher level of functional activation, combining the use of the Rho tag with the accessory proteins proved to be useful due to a synergistic effect. How do the different RTP1Ss and Ric8bs work synergistically? One possibility is that RTP1S functions in the chaperoning and trafficking of OR proteins to increase cell-surface expression. On the other hand, Ric8b alone does not have significant effects on OR cell-surface expression; it is likely to enhance G_{olf}/G_{s}-mediated signal transduction. The fact that a higher basal level of activation was observed when the Ric8b/Golf pair was co-transfected with ORs supports this idea (23).

Curiously, it appears that co-expression of Ric8b/Golf and RTP1S induces an additional level of OR cell-surface expression than when RTP1S is expressed by itself, raising the possibility that Ric8b has roles in OR trafficking in concert with RTP1S. It is probable that the synergistic effect between Ric8b/Golf and RTP1S on the functional activation of ORs might be, in part, due to their synergistic enhancement of OR trafficking to the cell-surface, despite the fact that Ric8b has only been implicated in the OR signal transduction. It would be interesting to ask in the future whether association of RTP1S with the ORs somehow enhances the effects of Ric8b on OR coupling with the G proteins or vice versa. How the tags actually work remains elusive. They may promote trafficking of the ORs to the cell surface, stabilize the OR proteins, or enhance transcription/translation of the ORs (10). The issue is currently difficult to resolve due to a lack of OR-directed antibodies.

We showed that N-terminal tags do not have large effects on OR ligand selectivities for four different receptors. This is cons-

---

**FIGURE 5.** Effect of different N-terminal tags on the relative ligand specificities of various ORs. Various differentially tagged ORs (Olf62 (A), S6 (B), OREG (C), and MOR203-1 (D)) were transfected into Hana3A cells and stimulated with their corresponding set of four or five ligands and one or two controls. Ligand specificity is largely preserved in the case of S6, OREG, and MOR203-1. The y-axis denotes normalized luciferase activity ± S.E. (n = 4).
consistent with previous reports showing that various Rho-tagged ORs, OREG, S6, MOR23, and rat I7, have a similar ligand response profile in heterologous cells and isolated olfactory sensory neurons (3, 6, 12–20, 28). Our results are also consistent with the notion that odorants are recognized by a combination of the transmembrane domains but not by the extracellular N-terminal domain (6, 10, 29).

We found that the amount of OR expression may affect its relative ligand specificities. One possible explanation is that a relatively small amount of cell-surface receptors are needed to achieve a response for their best ligands, whereas more surface ORs are required to cause activation of OR signaling for weaker ligands. As a result, when we express a relatively large amount of functional ORs, the differences between strong and weak ligands appeared to be lost. Our results are consistent with previous studies on receptor activities of other G protein-coupled receptors. It has been shown that increasing the amount receptors expressed can increase receptor activity (30–32), and in some cases, increasing G protein-coupled receptor signaling especially enhances receptor activity to weak agonists (33). Because OR proteins are likely to be abundantly expressed in the olfactory sensory neurons (34, 35), this condition where more OR is expressed is likely to mimic the ligand specificity of the ORs in the olfactory sensory neurons. Our results raise the possibility that the amount of OR expressed may modify the ligand-induced OR activation in olfactory sensory neurons, because the amount of OR proteins may vary in individual cells.

In fact, it is reported that olfactory sensory neurons expressing the same OR exhibited a wide dynamic range of response kinetics (36).

Our current work paves the way for conducting screenings in a robust heterologous expression system for large scale identification of odorant-OR pairs. Because Rho- or FLAG-tagged ORs, especially Rho-ORs in most cases, show increased functional expression while retaining their ligand specificity compared with the untagged version, the inclusion of a Rho tag, along with the accessory factors RTP1S and Ric8b/Golf, comprises an optimal heterologous OR expression system.

Acknowledgments—We thank Charles Luetje for pCI-S6, Kazushinge Touhara for mOREG cDNA, Dr. Molday for mouse anti-Rho antibody, Douglas Marchuk, Raphael Valdivia, and Joseph Heitman for generous sharing of equipment, Qiuyi Chi and Momoka Kubota for expert technical assistance, Joel Mainland for expert advice on statistics, Mike Cook for flow cytometry, and Hubert Amrein, Marc Caron, Dan Tracey, Charles Luetje, and members of the Matsunami and Amrein laboratories for critical review of the manuscript.

REFERENCES
1. Buck, L., and Axel, R. (1991) Cell 65, 175–187
2. Mombaerts, P. (2004) Nat. Rev. Neurosci. 5, 263–278
3. Zhao, H., Ivic, L., Otaki, J. M., Hashimoto, M., Mikoshiba, K., and Firestein, S. (1998) Science 279, 237–242

5 H. Saito, Q. Chi, H. Zhuang, O. Awonuga, and H. Matsunami, manuscript in preparation.
4. Lu, M., Echeverri, F., and Moyer, B. D. (2003) *Traffic* **4**, 416–433
5. McClintock, T. S., Landers, T. M., Gimelbrant, A. A., Fuller, L. Z., Jackson, B. A., Jayawickreme, C. K., and Lerner, M. R. (1997) *Brain Res. Mol. Brain Res.* **48**, 270–278
6. Krautwurst, D., Yau, K. W., and Reed, R. R. (1998) *Cell* **95**, 917–926
7. Wetzel, C. H., Oles, M., Wellerdieck, C., Kuczkowiak, M., Gisselmann, G., and Hatt, H. (1997) *Brain Res. Mol. Brain Res.* **48**, 270–278
8. Wellerdieck, C., Oles, M., Pott, L., Korsching, S., Gisselmann, G., and Hatt, H. (1997) *Chem. Senses* **22**, 467–476
9. Gaillard, I., Rouquier, S., Pin, J. P., Mollard, P., Richard, S., Barnabe, C., Demaille, J., and Giorgi, D. (2002) *Eur. J. Neurosci.* **15**, 409–418
10. Katada, S., Tanaka, M., and Touhara, K. (2004) *J. Neurochem.* **90**, 1453–1463
11. Spehr, M., Gisselmann, G., Poplawski, A., Riffell, J. A., Wetzel, C. H., Zimmer, R. K., and Hatt, H. (2003) *Science* **299**, 2054–2058
12. Malnic, B., Hirono, J., Sato, T., and Buck, L. B. (1999) *Cell* **96**, 713–723
13. Oka, Y., Katada, S., Omura, M., Suwa, M., Yoshihara, Y., and Touhara, K. (2006) *Neuron* **52**, 857–869
14. Abaffy, T., Matsunami, H., and Luetje, C. W. (2006) *J. Neurochem.* **97**, 1506–1518
15. Saito, H., Kubota, M., Roberts, R. W., Chi, Q., and Matsunami, H. (2004) *Cell* **119**, 679–691
16. Touhara, K., Sengoku, S., Inaki, K., Tsuboi, A., Hirono, I., Sato, T., Sakano, H., and Haga, T. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96**, 4040–4045
17. Kajiya, K., Inaki, K., Tanaka, M., Haga, T., Kataoka, H., and Touhara, K. (2001) *J. Neurosci.* **21**, 6018–6025
18. Ivic, L., Zhang, C., Zhang, X., Yoon, S. O., and Firestein, S. (2002) *J. Neurobiol.* **50**, 56–68
19. Belluscio, L., Lodovichi, C., Feinstein, P., Mombaerts, P., and Katz, L. C. (2002) *Nature* **419**, 296–300
20. Imai, T., Suzuki, M., and Sakano, H. (2006) *Science* **314**, 657–661
21. Neuhaus, E. M., Mashukova, A., Zhang, W., Barbour, J., and Hatt, H. (2006) *Chem. Senses* **31**, 445–452
22. Hague, C., Uberti, M. A., Chen, Z., Bush, C. F., Jones, S. V., Ressler, K. J., Hall, R. A., and Minneman, K. P. (2004) *Proc. Natl. Acad. Sci. U. S. A.* **101**, 13672–13676
23. Von Dannecker, L. E., Mercadante, A. F., and Malnic, B. (2006) *Proc. Natl. Acad. Sci. U. S. A.* **103**, 9310–9314
24. Von Dannecker, L. E., Mercadante, A. F., and Malnic, B. (2005) *J. Neurosci.* **25**, 3793–3800
25. Laird, D. W., and Molday, R. S. (1988) *Investig. Ophthalmol. Vis. Sci.* **29**, 419–428
26. Ishimaru, Y., Inada, H., Kubota, M., Zhuang, H., Tominaga, M., and Matsunami, H. (2006) *Proc. Natl. Acad. Sci. U. S. A.* **103**, 12569–12574
27. Katada, S., Nakagawa, T., Kataoka, H., and Touhara, K. (2003) *Biochem. Biophys. Res. Commun.* **305**, 964–969
28. Shirokova, E., Schmiedeberg, K., Bedner, P., Niessen, H., Willecke, K., Raguse, J. D., Meyerhof, W., and Krautwurst, D. (2005) *J. Biol. Chem.* **280**, 11807–11815
29. Abaffy, T., Malhotra, A., and Luetje, C. W. (2007) *J. Biol. Chem.* **282**, 1216–1224
30. Kenakin, T. (1996) *Pharmacol. Rev.* **48**, 413–463
31. Chen, G., Way, J., Armour, S., Watson, C., Queen, K., Jayawickreme, C. K., Chen, W. J., and Kenakin, T. (2000) *Mol. Pharmacol.* **57**, 125–134
32. Ford, D. J., Essex, A., Spalding, T. A., Burstein, E. S., and Ellis, J. (2002) *J. Pharmacol. Exp. Ther.* **300**, 810–817
33. Burstein, E. S., Spalding, T. A., and Brann, M. R. (1997) *Mol. Pharmacol.* **51**, 312–319
34. Barnea, G., O’Donnell, S., Mancia, F., Sun, X., Nemes, A., Mendelsohn, M., and Axel, R. (2004) *Science* **304**, 1468
35. Strotmann, J., Leval, O., Fleischer, J., Schwarzenbacher, K., and Breer, H. (2004) *J. Neurosci.* **24**, 7754–7761
36. Grosmaître, X., Vassalli, A., Mombaerts, P., Shepherd, G. M., and Ma, M. (2006) *Proc. Natl. Acad. Sci. U. S. A.* **103**, 1970–1975