Ric-8B promotes functional expression of odorant receptors

Luiz Eduardo C. Von Dannecker, Adriana F. Mercadante*, and Bettina Malnic†

Departamento de Bioquímica, Instituto de Química, Universidade de São Paulo, Avenida Prof. Lineu Prestes, 748, CEP 05508-000, São Paulo, Brazil

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Odorants are detected by a large family of odorant receptors (ORs) expressed in the nose. The information provided by the ORs is transmitted to specific regions of the brain, leading to odorant perception. The determination of the odorant specificities of the different ORs will contribute to the understanding of how odorants are discriminated by the olfactory system. However, to date only a few ORs have been linked to odorants they recognize, because ORs are poorly expressed on the cell surface of heterologous cells. Here we show that Ric-8B, a putative guanine nucleotide exchange factor for Gαolf, promotes efficient heterologous expression of ORs. Our results also show that Ric-8B enhances accumulation of Gαolf at the cell periphery, indicating that it promotes functional OR expression by improving the efficiency of OR coupling to Gαolf. Expression systems containing Gαolf and Ric-8B should contribute to the functional characterization of ORs.

guanine nucleotide exchange factor | olfactory receptor | synembryn | G protein α olf | heterologous expression

Mammals can discriminate a vast number of odorants with remarkable sensitivity and accuracy. Odorants are first detected by a large family of odorant receptors (ORs) expressed in the cilia of the olfactory sensory neurons (OSNs) of the nose (1). The information provided by the different ORs is then transmitted to the olfactory bulb of the brain, which in turn relays information to the olfactory cortex. A series of experiments indicated that the information provided by the different ORs in the nose is organized into a sensory map in the olfactory bulb (2–4). In the olfactory cortex, which receives input from the bulb, there is another map of OR inputs, different from the map in the bulb (5).

The discrimination of odorants presumably derives from the different ligand specificities of the ORs. Therefore, the determination of the odorant specificities of individual ORs should provide information regarding how odor identities are encoded. However, so far only ≈30 ORs have been linked to odorants they recognized (6–9). The major reason for this fact is that the functional expression of ORs in heterologous cell types has proven difficult, mostly because ORs cannot reach the plasma membrane (10–12). To circumvent this problem, we previously used a combination of Ca^{2+} imaging and single-cell RT-PCR to identify the ORs expressed by olfactory neurons that responded to different aliphatic odorants (6). By using this approach we identified 13 different ORs that responded to various aliphatic alcohols and acids. Our results revealed that the olfactory system uses combinatorial receptor codes to encode odorant identities.

The complete repertoires of mouse and human OR genes have recently been determined from the corresponding genome sequences (13–19). All together, 1,200 intact OR genes were identified in the mouse, and ≈388 intact genes were identified in humans (20). Gene expression studies confirmed that a large fraction of the mouse OR genes is expressed in the olfactory epithelium (21, 22). The information we have on OR family repertoires sets the stage for focused studies on OR ligand specificities that will provide insights into how odorant perception is achieved. However, the method of determining OR ligand specificities we used before (6) is extremely laborious and cannot be easily applied to a large number of ORs and ligands. Therefore, a robust heterologous system for functional OR expression is needed.

Recent advances have improved the expression of ORs in heterologous systems. First, it has been demonstrated that fusion of the 20 N-terminal amino acids of the rhodopsin or serotonin receptor to the N-terminal region of ORs facilitates surface expression (23–25). Tagged ORs can then be cotransfected in heterologous cells with the Gα15/16, which can promiscuously couple receptors to the phospholipase C pathway. Receptor activation by odorants results in an increased intracellular Ca^{2+} concentration, which can be measured at the single-cell level by using Ca^{2+}-sensitive dyes, compensating for the low OR expression efficiency. Similar strategies have been successfully applied by different groups using HEK293 cells (26–28) or different cell lines (9, 29–31).

Second, it has been demonstrated that ORs can couple to endogenous Gαs or to the olfactory-specific Gαolf, leading to odorant-induced increases in cAMP concentrations (9, 23). The increases in cAMP can be monitored by using the luciferase reporter gene assay (29), which is more sensitive and therefore allows high-throughput analysis of ORs that are expressed at low levels or reach the surface in only a small percentage of cells. Importantly, it was noted by Shirokova *et al*. (9) that coupling of ORs to nonolfactory Gα subunits (such as Gα15 or Gα16) may lead to altered response profiles. Thus, heterologous systems that use endogenous olfactory transduction molecules are more likely to reproduce OR physiological responses.

Third, it was recently demonstrated that coexpression with the olfactory-specific receptor transporting proteins (RTPs) 1 and 2 and receptor expression enhancing protein (REEP) 1 in HEK293T cells promotes OR functional surface expression (8). It was also shown that coexpression with the β_2-adrenergic receptor promotes surface expression of OR M71 in HEK293 cells (32). These results indicate that heterologous expression of ORs can be significantly improved by coexpression with accessory proteins that assist in OR trafficking to the cell surface.

We recently found that Ric-8B, a putative guanine nucleotide exchange factor (GEF) expressed in olfactory sensory neurons, is able to interact with Gαolf (33). GEFs catalyze the exchange of GDP for GTP to generate an activated form of Gα, which is then able to activate a variety of effectors. Consistent with this potential function, we showed that Ric-8B is able to amplify dopamine receptor and β_2-adrenergic receptor signaling through Gαolf (33). Here we investigated whether Ric-8B can also amplify OR signaling through Gαolf. We found that Ric-8B promotes efficient functional expression of ORs in heterologous

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Abbreviations: GEF, guanine nucleotide exchange factor; OR, odorant receptor; RTP, receptor transporting protein; mOR, mouse OR.

*Present address: Departamento do Patologia Básica, Universidade Federal do Paraná, Caixa Postal 19031, Curitiba, Brazil.

†To whom correspondence should be addressed. E-mail: bmalnic@iq.usp.br.

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cells. Our results indicate that Ric-8B can be used to improve heterologous expression systems for ORs, making them more robust and efficient.

Results

To verify whether Ric-8B can amplify signaling through ORs, we checked whether functional expression of the mouse OR (mOR) mOR-EG can be improved by coexpression with Ric-8B. mOR-EG was first cloned by RT-PCR from single OSNs that had responded to the odorant eugenol (23). The receptor specificity was subsequently confirmed by functional expression of the rhodopsin-tagged version of mOR-EG in HEK293 cells (8, 23, 29, 34) or in *Xenopus laevis* oocytes (29).

We first transfected HEK293T cells with the expression vectors for Gαolf, mOR-EG (untagged), and Ric-8B and analyzed whether they responded to eugenol. As shown in Fig. 1*A*, stimulation with eugenol increased cAMP accumulation ≈3-fold, when compared with unstimulated cells. Cells expressing only the rhodopsin-tagged mOR-EG and Gαolf responded to eugenol as previously shown (23), but cells expressing only the untagged mOR-EG and Gαolf did not respond to eugenol, indicating that either a rhodopsin tag or coexpression with Ric-8B is required for functional expression of mOR-EG.

It has been shown before that coexpression of ORs with RTP1 or RTP2 in HEK293 T cells enhances odorant-dependent cAMP production (8). We also tested the effect of RTP1 on the functional expression of untagged mOR-EG. Cells coexpressing Gαolf, mOR-EG, and RTP1 were stimulated with eugenol, and cAMP production was measured. In this case, no cAMP production was induced (Fig. 1*A*). In previous experiments, it had been demonstrated that RTP1 improves mOR-EG function in HEK293 T cells, but in that case, a rhodopsin-tagged mOR-EG was used (8). Whether the RTPs preferentially work as accessory factors for rhodopsin-tagged ORs versus untagged ORs still needs to be determined.

Cells coexpressing mOR-EG and Gαolf with both Ric-8B and RTP1 also showed eugenol-induced cAMP accumulation, but to lower amounts than cells coexpressing mOR-EG and Gαolf with Ric-8B alone (Fig. 1*A*).

We next analyzed whether coexpression with Ric-8B affects mOR-EG odorant specificities. Cells were transfected with mOR-EG (untagged), Gαolf, and Ric-8B and exposed to nine odorants with unrelated structures, including eugenol. Even though high concentrations (300 μM) of odorants were used, only eugenol was able to elicit significant responses (Fig. 1*B*). Unexpectedly, no responses were detected when cells were stimulated with vanillin, an odorant that is able to activate the rhodopsin-tagged version of mOR-EG (Fig. 1*B*; refs. 8 and 23). These results show that Ric-8B is able to amplify OR signal transduction through Gαolf only when the receptor is activated by a specific ligand. The fact that untagged mOR-EG does not respond to vanillin, although rhodopsin-tagged mOR-EG does, suggests that modified ORs (rhodopsin-tagged) may show different odorant specificities when compared with their unmodified (untagged) versions.

In previous studies we used single-cell RT-PCR to identify OR sequences from mouse olfactory neurons that had responded to specific aliphatic odorants (6). One of these ORs, mOR-S6, responded to nonanedioic acid, but not to the other aliphatic odorants. Functional expression in heterologous cells of some of these ORs, including rhodopsin-tagged mOR-S6, has been difficult to achieve and has only recently been accomplished, by two different groups (8, 9). We next tested whether mOR-S6 expression can also be improved by coexpression with Ric-8B. Cells coexpressing rhodopsin-tagged mOR-S6 and Gαolf alone, or together with Ric-8B, did not show significant increases in cAMP production when stimulated with nonanedioic acid (Fig. 2*A*). Cells coexpressing rhodopsin-tagged mOR-S6, Gαolf, and RTP1

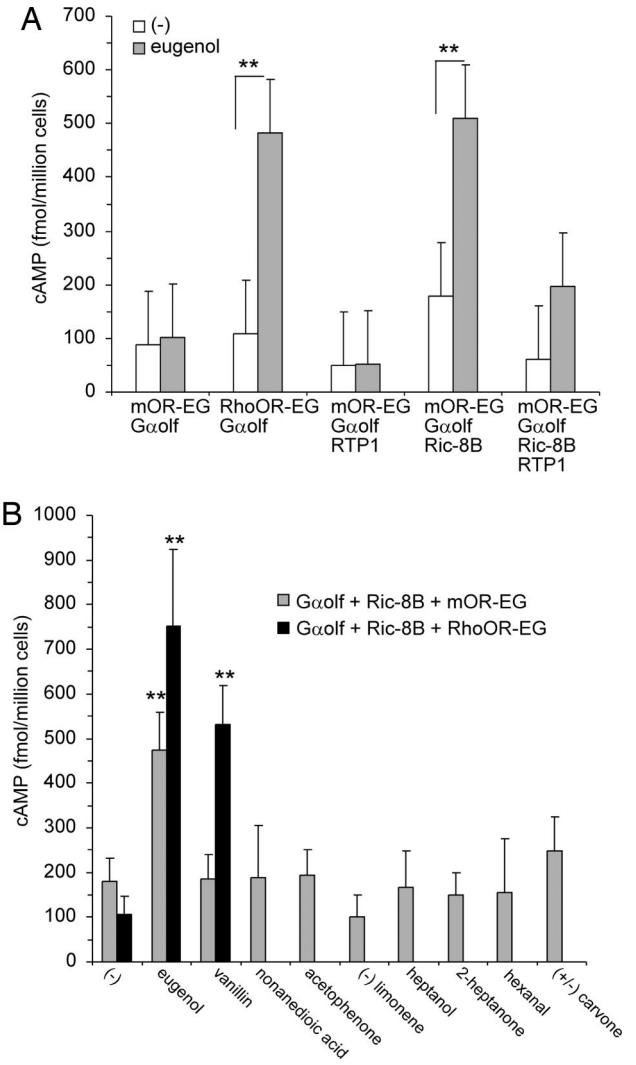

Fig. 1. Ric-8B promotes functional expression of mOR-EG. (*A*) Production of cAMP was measured in HEK293 T cells transfected as indicated with Gαolf, Ric-8B, RTP1, mOR-EG, or rhodopsin-tagged mOR-EG (RhoOR-EG) expression vectors. Activity was recorded in the absence (white bars) or presence (gray bars) of 300 μM eugenol. The data are expressed as means ± SD from three different experiments. cAMP accumulation is presented as femtomole per million cells. (**, $P < 0.007$.) (*B*) Production of cAMP was measured as described above in cells transfected with Gαolf, Ric-8B, and mOR-EG expression vectors (gray bars) or with Gαolf, Ric-8B, and rhodopsin-tagged mOR-EG (RhoOR-EG) expression vectors (black bars). Activity was recorded in the absence (−) or presence (300 μM) of the indicated odorants. [**, $P < 0.009$ compared with transfection of the same expression vectors but no addition of odorant (−)].

showed a slight, but not significant, increase in cAMP production when stimulated with the odorant ($P = 0.1753$; Fig. 2*A*). Significant cAMP production was observed only when cells coexpressed rhodopsin-tagged mOR-S6 and Gαolf with Ric-8B plus RTP1. In this case, activation with increasing concentrations of nonanedioic acid resulted in increasing amounts of cAMP accumulation, as shown in Fig. 2*B*. The same experiments were also performed by using untagged mOR-S6, but no increase in cAMP production was observed (data not shown).

Altogether, these results indicate that Ric-8B can amplify OR signal transduction through Gαolf and can be used alone or in combination with RTP1 to improve functional expression of ORs in heterologous cells, making it more robust and more sensitive. The fact that coexpression with Ric-8B allows func-

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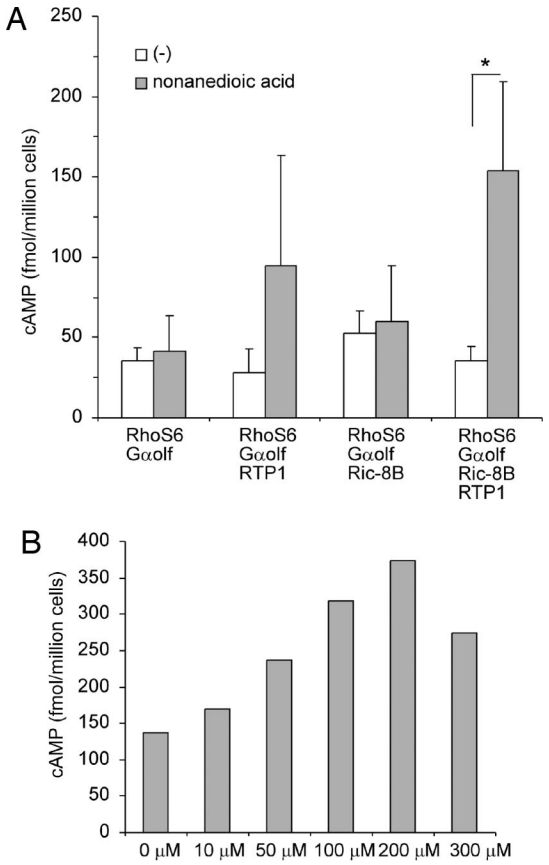

Fig. 2. Ric-8B promotes functional expression of mOR-S6. (*A*) Production of cAMP was measured in HEK293 T cells transfected as indicated with Gαolf, Ric-8B, RTP1, and rhodopsin-tagged mOR-S6 (RhoS6) expression vectors. Activity was recorded in the absence (white bars) or presence (gray bars) of 300 μM nonanedioic acid. The data are expressed as means ± SD from three different experiments. cAMP accumulation is presented as femtomole per million cells. (*, $P = 0.0219$.) (*B*) Production of cAMP was measured in cells expressing Gαolf, Ric-8B, RTP1, and rhodopsin-tagged mOR-S6 after stimulation with the indicated concentrations of nonanedioic acid. The data are expressed as the means from two different experiments.

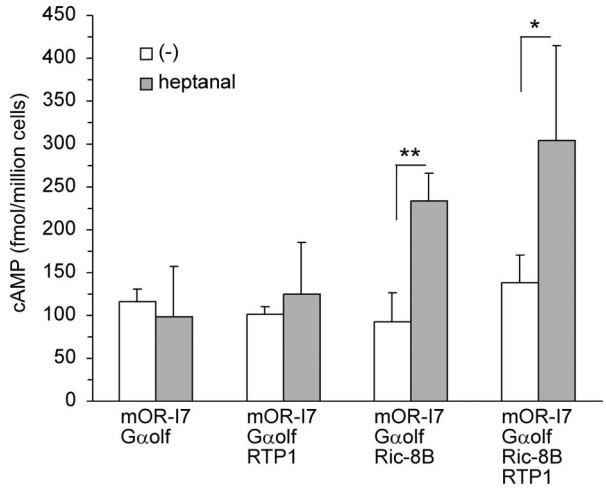

Fig. 3. Ric-8B promotes functional expression of mOR-I7. Production of cAMP was measured in HEK293 T cells transfected as indicated with Gαolf, Ric-8B, RTP1, and mOR-I7 expression vectors. Activity was recorded in the absence (white bars) or presence (gray bars) of 300 μM heptanal. The data are expressed as means ± SD from three different experiments. cAMP accumulation is presented as femtomole per million cells. (*, $P = 0.0288$; **, $P = 0.0035$.)

tional expression of the untagged mOR-EG also indicates that, at least for some ORs, no rhodopsin tags would be required.

To determine whether the effects observed for mOR-EG and mOR-S6 can be extended to other ORs as well, we analyzed whether another OR with a known ligand, mOR-I7, can also be functionally expressed with the addition of Ric-8B. Rat OR-I7 was first shown to recognize octanal by using recombinant adenovirus to drive expression of the receptor in OSNs in the olfactory epithelium (35). mOR-I7 was shown to preferentially recognize the odorant heptanal instead of octanal when coexpressed with Gα15/16 in HEK293 cells (24). Subsequent experiments with GFP-tagged mouse olfactory neurons that endogenously express mOR-I7 (36) and with HeLa cells that coexpress rhodopsin-tagged mOR-I7, Gαolf, and CNGA2 (9) both confirmed the receptor specificity for heptanal.

We repeated the same transfection experiments by using expression vectors for mOR-I7 (untagged), Gαolf, Ric-8B, and RTP1. Cells expressing mOR-I7, Gαolf, and Ric-8B responded to heptanal, as shown in Fig. 3. Conversely, cells transfected with mOR-I7, Gαolf, and RTP1 (but not Ric-8B) did not significantly respond to heptanal. Cells coexpressing mOR-I7, Gαolf, Ric-8B, and RTP1 also responded to heptanal. These results show that Ric-8B can help functional expression of untagged mOR-I7 as well as mOR-EG and thus suggest that it may promote functional expression of other untagged ORs as well.

We next analyzed whether Ric-8B affects the cellular localization of Gαolf in HEK293T cells, because we previously demonstrated that Ric-8B directly interacts with Gαolf (33). Immunofluorescence experiments show that Ric-8B and Gαolf are colocalized at the cell periphery (Fig. 4 *E–G*). In addition, we observed that coexpression with Ric-8B increases 2-fold the number of cells that show a strong peripheral localization of Gαolf, versus the more diffuse and weaker staining pattern observed when Gαolf is expressed alone (Fig. 4 *A–D* and *H–J*). Accordingly, coexpression with the alternatively spliced version of Ric-8B, Ric-8BΔ9, which does not interact with Gαolf (33), does not result in an increase in the number of cells showing a strong peripheral staining for Gαolf (Fig. 4*J*). These results are in agreement with the recent demonstrations that *Drosophila* Ric-8 is involved in membrane localization of heterotrimeric G proteins (37–39) and indicate that Ric-8B enhances accumulation of Gαolf at the periphery of HEK293T cells.

Discussion

The determination of OR ligand specificities has been hampered by the fact that ORs cannot be easily expressed in heterologous cells. The olfactory cilia of olfactory neurons, where ORs are expressed *in vivo*, are highly specialized sensory organelles and contain all of the molecules necessary for olfactory signal transduction (40). The highly organized arrangement of these components within the cilia seems to be essential for efficient activation of the downstream signaling events. The understanding of the detailed molecular mechanisms involved in odorant signal transduction *in vivo* should contribute to the establishment of an efficient heterologous system for OR expression. Here we show that Ric-8B, a putative GEF which is normally expressed in olfactory sensory neurons and interacts with Gαolf (33), promotes efficient OR expression in heterologous cells. It is important to note that Ric-8B not only improves heterologous expression of ORs but may also be important for the endogenous odorant signaling pathway.

To date, mostly tagged ORs have been successfully expressed in HEK293 cells (7). Here we show that functional expression of untagged mOR-EG and mOR-I7 can be achieved by coexpres-

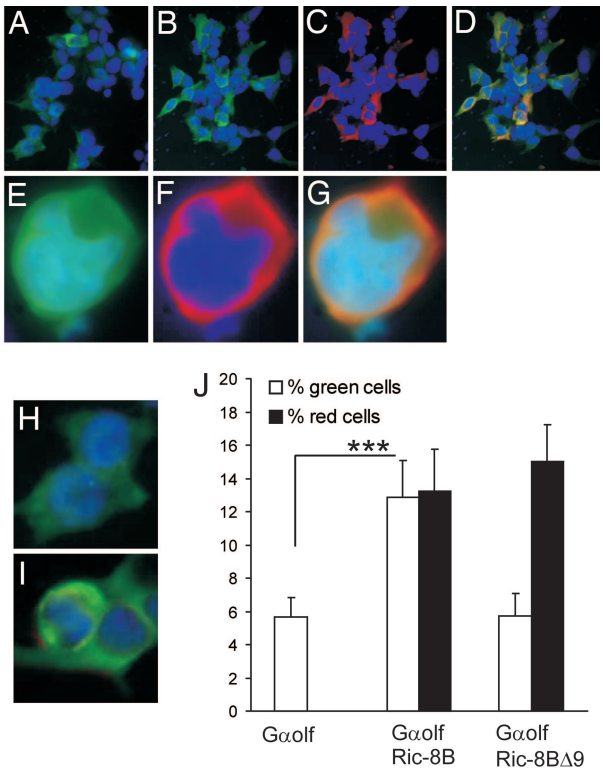

Fig. 4. Cellular localization of Gαolf and Ric-8B detected by immunofluorescence. (*A–D*) HEK293T cells transfected with Gαolf (*A*) or Gαolf and Ric-8B (*B–D*) expression vectors were permeabilized and immunostained by using anti-Gαolf (*A* and *B*) or anti-FLAG (*C*) and visualized on a fluorescence microscope. The merge between the images shown in *B* and *C* is shown in *D*. (*E–G*) Cells coexpressing Gαolf and Ric-8B and double-labeled with anti-Gαolf (*E*) and anti-FLAG (*F*) antibodies show that Gαolf and Ric-8B colocalize at the cell periphery (*G*, a merge of *E* and *F*). (*H–J*) Representative cells transfected with Gαolf alone (*H*) or with Gαolf and Ric-8B (*I*) stained with anti-Gαolf are shown. The percentages of cells with strong Gαolf fluorescent signal, like the ones shown in *I* (green cells), and with Ric-8B or Ric-8BΔ9 signal (red cells) in cells transfected with Gαolf alone, with Gαolf and Ric-8B, or with Gαolf and Ric-8BΔ9 were counted (700 cells from three independent experiments) and are shown in *J*. (***, $P < 0.0001$.) Nuclei were visualized with Hoechst dye.

sion with Gαolf and Ric-8B. The use of untagged ORs instead of rhodopsin-tagged ORs for heterologous expression is greatly advantageous, because it is not clear whether receptor protein modifications interfere with the odorant responses. It has been demonstrated, for example, that even one single amino acid change in the primary structure of an OR may lead to altered odorant specificity (24). The addition of 20 aa from rhodopsin to the N terminus of an OR may also alter its specificity. Accordingly, we show that rhodopsin-tagged mOR-EG responds to vanillin, although untagged mOR-EG does not (Fig. 1*B*). Therefore, the use of Ric-8B should contribute to the better determination of the authentic odorant specificities of the ORs.

The untagged mOR-S6 could not, however, be functionally expressed in any of the conditions we tested [when Ric-8B, RTP1, or both were coexpressed (data not shown)]. In our experiments, mOR-S6 could be functionally expressed only with a rhodopsin tag and in the presence of RTP1. Coexpression with Ric-8B and RTP1 enhanced the activation of rhodopsin-tagged mOR-S6, indicating that Ric-8B can potentiate the effect of RTP1. It is important, however, to note, that coexpression with Ric-8B and RTP1 does not necessarily lead to increased sensitivity. As shown in Fig. 1*A*, coexpression of mOR-EG with Ric-8B and RTP1 results in decreased eugenol-induced cAMP accumulation, when compared with the same experiment performed in the absence of RTP1. Therefore, coexpression with both RTP1 and Ric-8B should be used with caution, and the best conditions for each OR in this case should be determined.

In summary, some ORs can be functionally expressed in their untagged version with the help of Ric-8B, whereas other ORs are more difficult to express: they require a rhodopsin tag, Ric-8B, and RTP1. In any case, it is clear from our results that the addition of Ric-8B increases both the intensity of the responses and the number of members of the OR family that can be functionally expressed in heterologous cells without an N-terminal tag.

It is believed that the major reason for the inefficient expression of ORs in HEK293 cells is the fact that they are retained in the endoplasmic reticulum and cannot reach the cell surface (10, 11). Therefore, accessory proteins that help ORs to overcome this problem would be necessary to enable functional OR expression in heterologous cells. The RTPs seem to fulfill this accessory function, because it was demonstrated that they promote surface expression of ORs (8). The mechanisms through which Ric-8B enhances functional expression of ORs remain unknown. Here we show that Ric-8B enhances cellular peripheral localization of Gαolf. It is possible that the presence of higher amounts of Gαolf at the plasma membrane results in increased OR signal transduction. Another possibility, which still needs to be investigated, is that Ric-8B would enhance OR targeting to the cell surface.

Finally, we cannot exclude the possibility that Ric-8B acts as a GEF to amplify Gαolf signaling, although it may be only one of the mechanisms responsible for our findings. Considering that only a small number of ORs can reach the cell surface, only a few Gαolf molecules would be stimulated by the activated receptors. Ric-8B would then work as a GEF to amplify these low levels of Gαolf stimulation. In the absence of Ric-8B, no amplification would occur, and no detectable cAMP signal would be generated. This possibility is consistent with the recent observation that odorant–receptor interaction is extremely short in duration and that an individual odorant-bound receptor has a very low probability of activating one downstream G protein molecule (41). If this condition is the case, Ric-8B could be used to increase the probability of G protein activation, even if only a small number of receptors can reach the cell surface.

In conclusion, our results demonstrate that Ric-8B significantly improves the ability to identify OR ligand specificities in heterologous expression systems. The employment of Ric-8B in a high-throughput system will allow the functional screening of the OR family members and thereby provide further insights into the mechanisms of odor perception.

Materials and Methods

Odorants. Odorants were purchased from Sigma (1-heptanol), Aldrich (hexanal), or Fluka (remaining odorants). Stock solutions (250 mM) were made up in DMSO and diluted into the serum-free media to give the indicated concentrations before the experiments.

cDNAs and Expression Vectors. The full-length cDNA coding region for RTP1 was cloned by PCR using cDNAs prepared from mouse olfactory epithelium and subcloned into the XhoI and KpnI restriction sites of the pcDNA3.1(−) expression vector (Invitrogen). The cDNAs corresponding to the full-length sequence of Gαolf, Ric-8B, and Ric-8BΔ9 were cloned by PCR using cDNAs prepared from mouse olfactory epithelium and subcloned into the XhoI site of the pcDNA3.1(−) expression vector (Gαolf) or into the BamHI site of the pcDNA3 vector containing a FLAG epitope (pcDNA3/5′F, Ric-8B, and Ric-8BΔ9) (42). mOR-EG, mOR-I7, and mOR-S6 coding regions were amplified by PCR from C57BL/6 mouse genomic DNA and subcloned into the XhoI and KpnI restriction sites of the

pcDNA3.1(−) plasmid. The OR plasmids rhodopsin-tagged mOR-EG and rhodopsin-tagged mOR-S6 were provided by K. Touhara (University of Tokyo, Tokyo) and H. Matsunami (Duke University Medical Center, Durham, NC), respectively. The plasmids containing untagged mOR-EG and rhodopsin-tagged mOR-EG were sequenced again, to make sure that the only difference between the two was the presence of the sequence coding for the rhodopsin tag in rhodopsin-tagged mOR-EG.

Tissue Culture cAMP Detection. HEK293 T cells were maintained in DMEM supplemented with 10% (vol/vol) FCS, 1 mM glutamine, 50 μg/ml streptomycin, and 50 units per milliliter penicillin at 37°C with 5% CO_2. Cells (0.5×10^5) were plated into each well of a 96-well plate for 16–20 h and transfected by using the Lipofectamine reagent (Invitrogen) with constructs expressing the different cDNAs (100 ng per well each). After 3 h of transfection, medium was replaced by serum-free media and incubated for 40 h. Serum-free media containing the agonist (300 μM) were added, and cells were incubated for 10 min. cAMP was measured by using the cAMP Enzymeimmunoassay (Amersham Pharmacia Biosciences) and following the manufacturer's protocol.

Immunofluorescence Microscopy. Cells were plated on Lab Tek chamber slides, transfected with Lipofectamine reagent as described above, and fixed in 3.7% paraformaldehyde in PBS for 15 min at room temperature. The fixed cells were permeabilized with 0.01% Triton X-100 in blocking buffer (5% normal horse serum and 2% BSA in PBS) for 1 h at room temperature. Cells were then incubated in 2-fold diluted blocking buffer containing 1:500 anti-FLAG (Sigma, for Ric-8B and Ric-8BΔ9 stainings) or 1:80 anti-Gαolf (K19, sc-385, Santa Cruz Biotechnology, for Gαolf staining). Alexa-Fluor 568 anti-mouse or Alexa-Fluor 488 anti-rabbit secondary antibodies (Molecular Probes) were used to visualize the FLAG-tagged Ric-8B and Ric-8BΔ9 or Gαolf, respectively. Cells were counterstained with 0.1 mg/ml Hoechst dye to visualize the nuclei. Images of fluorescent cells were obtained on a Nikon TE300 fluorescence microscope.

Statistical Analysis. Statistical analysis was performed by using GRAPHPAD PRISM version 4.0 (GraphPad, San Diego). Data are presented as means ± SD and were analyzed by using a two-tailed unpaired Student's *t* test. Significance was set as $P < 0.05$. The *P* values for the different comparisons are indicated by asterisks in Figs. 1–4.

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