Abstract

Despite that recent progress in genomics has elucidated the genomic structure of the olfactory receptors (ORs), most of them are still orphan receptors. The low expression level of ORs in heterologous cells has hampered many attempts to establish cell biological OR assay systems. Recently, we demonstrated that certain G protein-coupled receptors, such as the leukotriene B4 receptor or the dopamine D1 receptor, were efficiently reconstituted on baculovirus budding from infected Sf9 cells. The budded virus (BV) was shown to be mostly free of exogenous proteins other than those related to viral infection, resulting in low-noise assay conditions. Taking advantage of these conditions, we attempted to reconstitute OR complexes on BV. Sf9 cells were coinfected with recombinant baculoviruses harboring the cDNAs encoding adenylyl cyclase, trimeric G-protein, and the receptor: mOR-EG or S6. The coexpression of these proteins was detected by western blot, and the agonist- or antagonist-dependent receptor response was confirmed using ligand-dependent cyclic AMP production. These results demonstrated the successful reconstitution of functional OR complex on BV. Additionally, the expression of OR8B3 on BV, one of human orphan ORs, was also confirmed. This BV expression system is expected to be a highly effective tool for screening unknown ligands for ORs.

Key words: baculovirus, GPCR, olfactory receptor, reconstitution

Introduction

The olfactory receptors (ORs) belong to the G protein-coupled receptors (GPCRs)-forming multigene family and are present on a number of different chromosomes. In humans and mice there are 300 and 1000 intact genes coding for OR proteins, respectively (Godfrey et al. 2004; Malnic et al. 2004). Each individual olfactory neuron expresses only 1 member of the ORs (Chess et al. 1994; Serizawa et al. 2000), but a single group of the ORs can respond to several different odorants (Kajita et al. 2001; Katada et al. 2005). Thus an “odor code,” which is a combinatorial response of ORs to an odorant, is thought to underlie the mechanism of odor discrimination (Malnic et al. 1999; Mori et al. 1999; Hamana et al. 2003).

At present, the odorant specificities of ORs are not well characterized. To search for the ligands for the orphan ORs and analyze their physiological properties, it is necessary that effective assay systems be established. Heterologous cell–based expression systems for ORs, as well as GPCRs in general, have been reported using mammalian and amphibian cell lines (Krautwurst et al. 1998; Wetzel et al. 1999; Kajita et al. 2001; Eglen et al. 2007; Zhuang and Matsunami 2007; Hamana et al. 2010). However, animal cells have a rather high background noise level because of the expression of endogenous GPCRs. Moreover, the OR expression level in heterologous cell systems is quite low, which makes it difficult to obtain an effective reconstruction of the OR signaling complexes (McClintock and Sammetta 2003).

Another cell system is the insect cell line Sf9. The advantage of using Sf9 insect cells, derived from Spodoptera frugiperda, is that they are essentially free of endogenous GPCRs (Butcher et al. 1995), thus providing a low level of background noise in biochemical assays. However, because Sf9 cells are lysed after baculovirus infection to express the exogenous proteins, they cannot be applied for the reconstitution...
system. Meanwhile, there is accumulating evidence that the GPCRs expressed in insect cells are displayed on BV particles (Loisel et al. 1997; Masuda et al. 2003; Saitoh et al. 2006). We previously demonstrated that coinfection of BVs bearing the D1 dopamine receptor, G protein subunits (Gαs, Gβ, Gγ), or adenylyl cyclase (AC) VI gene into Sf9 cells successfully generated BV displaying those 5 exogenous proteins, and formed a receptor complex that produced cyclic AMP (cAMP) on ligand-dependent activation of AC VI (Sakihama et al. 2008).

This evidence encouraged us to examine whether such baculoviral reconstitution would enable the establishment of an odorant-responsive OR assay system. In this study, we demonstrate the functional reconstitution on BV particles of 2 mouse ORs, mOR-EG and S6 (also known as MOR174-9 and MOR42-2, respectively). We also show the expression of other ORs on BV so as to know this assay system is practically applicable for surveying ligands of ORs; I7, one of the well-known mouse ORs (Krautwurst et al. 1998), and OR8B3, one of the human ORs for which the ligand has not yet been identified.

Materials and methods

Antibodies and reagents

Monoclonal mouse anti-hemagglutinin (HA) antibody (clone HA7), monoclonal mouse anti-FLAG (clone M2) antibody, and horse radial peroxidase–conjugated goat anti-mouse or anti-rabbit IgG were from Sigma. Monoclonal mouse anti-V5 antibody and monoclonal mouse anti-histidine (His) was from Invitrogen. The polyclonal rabbit anti-rhodopsin antibody and monoclonal mouse anti-histidine (His) was from Acris. The monoclonal mouse anti-histidine (His) was from Santa Cruz Biotechnology. Eugenol, bovine G-protein oligodendrocyte (clone C20) were from Millipore. The monoclonal mouse anti-histidine (His) was from Invitrogen. The monoclonal mouse anti-rhodopsin antibody (clone 4D2) was from Millipore. The polyclonal rabbit anti-rhodopsin antibody and monoclonal mouse anti-histidine (His) was from Invitrogen. The monoclonal mouse anti-rhodopsin antibody (clone 4D2) was from Millipore. The polyclonal rabbit anti-rhodopsin antibody and monoclonal mouse anti-histidine (His) was from Invitrogen. The monoclonal mouse anti-rhodopsin antibody (clone 4D2) was from Millipore. The polyclonal rabbit anti-rhodopsin antibody and monoclonal mouse anti-histidine (His) was from Invitrogen.

Recombinant baculovirus construction and Sf9 cell culture

The cDNA for mOR-EG, S6, and I7 were obtained from plasmids reported previously (Hamana et al. 2010). For convenience, the plasmid construction is briefly described in this study. The coding sequences of mOR-EG, S6, and I7 were amplified from mouse genomic DNA by polymerase chain reaction (PCR) and cloned into the EcoRI/BamHI sites of Rho-tag/phCMV1. The cDNA for OR8B3, which was cloned into the Nhel/BamHI sites of phCMV1, was a kind gift from Dr Hitoshi Nomura of the Chugai Pharmaceutical Company. The chemically synthesized coding sequence with the FLAG tag sequence at the N-terminal end of the receptor transport protein-1s (RTP1S) containing an EcoRI/KpnI site that was cloned into pUC57 was purchased from GenScript. The cDNA fragments for the ORs and RTP1S were subcloned into a baculovirus transfer vector (either pBlueBacHis2 or pFastBac-1 from Invitrogen). For the N-terminal or C-terminal expression of tag-fused OR, 5′ or 3′ PCR primers containing the following sequences were designed so as to link with approximately 3-mer of the 5′ or 3′ site of the OR, with an appropriate restriction site at the 5′ or 3′ end for insertion into the baculovirus transfer vectors: ATGTACCATCAGTGTTCCAGATTAGCCT for the HA-tag, ATGGACTACAAGAGTACGATGCAAG for the FLAG tag, ATGAACGGCCAGGAGGCCCACAC TTCTAG TGCCCTTCTCCAAGG CCACCGGGCG TTGGT for the human rhodopsin tag (Rho-tag), or TCAC GTAGAATCGAG CCGAGAGAGGTTA GGGAG TAGGCGTACC for the V5-tag. The chemically synthesized cDNA for mouse AC III was purchased from GenScript. The AC III gene was subcloned into pFastBac-1 as described above, without an expression tag. Recombinant baculoviruses were generated using the Bac-to-Bac system (Invitrogen). A recombinant virus harboring the cDNA of the rat Gαolf or bovine G-protein β1γ2 subunit dimer (G121 γ2), in which the Gβ1 and Gγ2 genes are tandemly aligned (Nakamura et al. 1995), was prepared as described (Sakihama et al. 2008). A recombinant virus containing the G-protein αi subunit (Gαi) cDNA was prepared as described (Masuda et al. 2003). Sf9 cells were cultured in Grace’s supplemented media (Invitrogen) containing 10% fetal calf serum, 0.1% Fluoronic F-68 (Invitrogen), 100 U/mL penicillin and 100 μg/mL streptomycin in a 1-L spinner flask at 27 °C. All of the experiments using the recombinant cDNA, Sf9 cells, and baculovirus in this study were performed under the guidelines for use of living modified organisms in research and development provided by The University of Tokyo.

Preparation of the Sf9 membrane and BV fraction

Sf9 cells (2 × 10⁶ cells/mL) were coinfected in combination with recombinant baculoviruses, as follows: (multiplicity of infection [M.O.I.] 5, 2, 2, 0.5, or 5 for the receptor, FLAG-RTP1S, Gαolf, G121 γ2, or AC III, respectively). For the control experiment, which is designated as “non-OR” in this article, Sf9 cells were coinfected with the recombinant baculoviruses for the same set of components, but the baculovirus for the OR was replaced with the wild-type form of the virus not carrying any OR genes. The Sf9 membrane or BV fraction was collected at 48 h or 72-h postinfection, respectively. BV was isolated from the culture medium of infected Sf9 cells as described (Loisel et al. 1997; Masuda et al. 2003). The pellets of the BV fraction were resuspended in Tris-buffered saline containing 1 mM EDTA, 50 μM E-64, 2 μg/mL aprotinin, and 10 μg/mL leupeptin, and stored at 4 °C. The membrane fractions of the Sf9 cells
were prepared as described (Windh and Manning 2002), resuspended in HE/Pt buffer (20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid [HEPES], pH 7.4, 2 mM EDTA, 10 μg/mL leupeptin, 0.1 mM phenylmethylsulfonyl fluoride) containing 2 mM DTT and stored at −70 °C.

**Western blotting**

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) was carried out as described (Laemmli 1970), except that the GPCR-containing samples were not heat-treated so as to avoid aggregation. The gel consisted of either a 10% (10% gel) or 7.5–15% gradient concentration (7.5–15% gel) of polyacrylamide. Immunoblotting was carried out as previously described (Masuda et al. 2003).

**AC assay**

The AC assay was carried out as described (Dessauer 2002), with certain modifications. The BV or membrane fractions were added to the AC buffer (50 mM HEPES [pH 8.0], 0.6 mM EDTA, 5 mM MgCl₂, 0.01% bovine serum albumin, 1 mM ATP, 0.1 mM 1-methyl-3-(2-methylpropyl)-7H-purine-2,6-dione, 3 mM trisodium phosphoenolpyruvate, 5 U/mL pyruvate kinase, 5 U/mL myokinase, 10 μM GDP, 1 nM GTP). The final volume was 50 μL. For the assay of the BV fraction, saponin was added to the buffer at a final concentration of 0.02%. The assay was started by the addition of agonists or forskolin, proceeded for 60 min at room temperature, and was then stopped with 12 μL of 50 mM HEPES (pH 7.4) containing 3% Tween-20. cAMP was determined using an AlphaScreen cAMP assay kit according to the manufacturer’s instructions. Values were read on a Fusion-α microplate analyzer (PerkinElmer). The assays were performed in duplicate for the 3 independent BV preparations unless otherwise specified. The measured values were normalized to the amount of cAMP produced. The resulting 6 normalized data points were subjected to statistical analysis by nonlinear regression with SigmaPlot software (Systat Software), and the EC50, IC50, minimum enzyme activity (Vmin), and maximum enzyme activity (Vmax) were obtained, along with the standard errors (SE).

Significant differences between the dose-response curve of the non-OR control and OR, or 2 dose-response curves of 2 ORs, were analyzed by 2-way analysis of variance followed by a Holm–Sidak post hoc test. A P value <0.05 was considered statistically significant.

**Results**

**Expression of olfactory receptor on BV and in the Sf9 membrane**

To determine whether ORs were expressed on BV in the Sf9 membrane, we infected Sf9 cells with recombinant baculoviruses containing HA-mOR-EG or HA-S6 cDNA and incubated them for different times until recovery of the Sf9 membrane and BV fractions. A non-OR control was prepared as well. Western blot analysis revealed the expression of HA-mOR-EG and HA-S6 at 48 h postinfection in the Sf9 membrane fraction, whereas the expression took place at 72 h postinfection on BV (Figure 1). The apparent molecular masses of approximately 26 kDa for HA-mOR-EG and 28 kDa for HA-S6 were detected on the BV fractions, and they were approximately 10 kDa smaller than the predicted sizes deduced from the cDNA sequences (35 kDa for HA-mOR-EG and 37 kDa for HA-S6). In addition to the lower band, a 48-kDa band for HA-mOR-EG and a 50-kDa band for HA-S6 were also detected. These lower and higher bands probably correspond to a monomer and dimer of the OR molecule, respectively. To examine whether the full-sized receptor molecule was expressed, we expressed a dual-tagged mOR-EG or S6 harboring an HA-tag on the N-terminal and a V5-tag on the C-terminal (i.e., HA-mOR-EG-V5 or

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**Figure 1** Expression of ORs on BV. Sf9 cells were infected with recombinant baculovirus harboring the cDNA of HA-mOR-EG or HA-S6 and incubated for the indicated times. Membrane preparations of Sf9 and the BV fraction were subjected to SDS-PAGE (20 μg protein/lane from each preparation, 10% gel) followed by western blot analysis using a mouse anti-HA monoclonal antibody (clone HA7). The arrow and arrow head indicate the monomer and dimer of the OR, respectively.
HA-S6-V5) in Sf9 cells and on BV. The western blot analysis of HA-mOR-EG-V5 and HA-S6-V5 demonstrated that the band detected with the specific antibody against either the N- or C-terminal tag was of the same molecular size on SDS-PAGE, as indicated in Figure 2.

Reconstitution of mOR-EG or S6 receptor complex on BV

To reconstitute the OR complex, we coinfected Sf9 cells with at least 4 different recombinant baculovirus, each of which contained the cDNA for AC III, \(G_{olf} \), \(G_\beta_1\gamma_2\), or the receptor (mOR-EG or S6), respectively. At 72 h postinfection, the BV fraction was recovered and subjected to SDS-PAGE followed by western blot analysis. It is reported that RTP1 and RTP2 support and increase the localization of mOR-EG and S6 on the plasma membrane of mammalian cells (Saito et al. 2004; Zhuang and Matsunami 2007), and the N-terminally truncated short form of RTP1, termed RTP1S, was demonstrated to be more effective than full size RTP1 (Zhuang and Matsunami 2007). To investigate the effect of RTP1S, RTP1S cDNA-carrying recombinant baculovirus was additionally coinfected. As shown in Figure 3, all the components of the receptor complexes were clearly detected in both of the reconstituted receptor complexes. The apparent molecular mass of the recombinant proteins matched the calculated sizes, except for the receptors. The panel labeled “OR” shows that the mOR-EG and S6 levels were unchanged either with (lanes 2 and 4) or without (lanes 1 and 3) the coexpression of RTP1S (also see the panel on FLAG-RTP1S expression). Small changes in the expression level of \(G_{olf}\) and \(G_\gamma\), were seen along with the coexpression of RTP1S. For comparison, western blot of the components of the mOR-EG complex and non-OR control on BV was performed, and no difference in the expression levels of the components other than the receptor was observed (Figure 3, the panels marked “mOR-EG complex” and “non-OR”).

Ligand-dependent activation of AC III in the reconstituted OR complex on BV

Ligand binding to an OR induces the detachment of \(G_{olf}\) from the \(G_{olf}G_{\beta_1\gamma_2}\) complex and induces \(G_{olf}-AC\) III coupling, which enhances the enzymatic activity for converting ATP to cAMP (Hepler and Gilman 1992). To evaluate the function of the reconstituted OR complex, we measured ligand-dependent cAMP production using the AlphaScreen method. First, to examine whether the N-terminally fused expression tag would exert an effect on receptor function, we compared the ligand-dependent response of HA-, FLAG-, or Rho-fused mOR-EG on BV. The expression of each of the differently tagged mOR-EG in the reconstituted complex on BV was confirmed by western blot (Figure 4a). In Figure 4b, it was shown that the 3 dose-response curves for these differently tagged mOR-EG receptors were closely overlapped and there was no significant difference among them in terms of the EC50 value or maximum enzyme activity. Next, we tested whether the coinfection of Sf9 cells with recombinant baculoviruses containing HA-mOR-EG or HA-S6 with FLAG-RTP1S cDNA would enhance the ligand-dependent response of the reconstituted OR complex on BV. As shown in Figure 5a,5c, the dose-response curves for mOR-EG and S6 with or without the presence of RTP1S did not exhibit any evident change in the EC50 values or maximum enzyme activities.

Dependency of \(G_{olf}\) on ligand-stimulated activation of AC III in the reconstituted OR complex on BV

To confirm that the AC activation caused by ligand-OR binding was specifically dependent on the \(G_{olf}-AC\) III interaction, we performed an anomalous reconstitution of mOR-EG or S6 on BV, in which \(G_{olf}\) was replaced with \(G_{ar}\), and tested whether this system would respond to the stimulation with either of the specific ligands, that is, eugenol for mOR-EG or cc9 for S6. As shown in Figure 5b,5d, \(G_{ar}\) containing mOR-EG or HA-S6 complex did not respond to
eugenol or cc9 stimulation, respectively, indicating that the AC III in the mOR-EG or HA-S6 reconstituted on BV was activated specifically through $G^{\alpha}_{\text{olf}}$.

To determine whether the response of the reconstituted OR was ligand-specific, we checked the interaction of cc9 with the mOR-EG complex and eugenol with the S6 complex. Neither exhibited cAMP production at any dose of the ligands tested (Figure 5b,5d), indicating that the reactions of the reconstituted ORs on BV were indeed ligand-specific.

Dose-dependent response of reconstituted mOR-EG on BV to vanillin

We further tested whether vanillin, a compound with close structural similarity to eugenol, would mimic the eugenol response of the reconstituted mOR-EG. As shown in Figure 6a, the mOR-EG complex on BV displayed a ligand-dependent response to vanillin with an EC50 value and maximum enzyme activity similar to eugenol.

The EC50 values and the minimum and maximum enzyme activities (Vmin and Vmax, respectively) for all of the OR complexes tested are summarized in Table 1.

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**Figure 3** Coexpression of the components of the OR complexes. Sf9 cells in suspension culture were coinfected with the recombinant baculoviruses harboring the cDNAs of AC III, $G^{\alpha}_{\text{olf}}$, $G^{\beta}_{1}$, $G^{\gamma}_{2}$, and HA-mOR-EG or HA-S6, with or without FLAG-RTP1S. The M.O.I. for each virus was 5, 2, 2, 0.5, and 5 for OR, FLAG-RTP1S, $G^{\alpha}_{\text{olf}}$, $G^{\beta}_{1}$, $G^{\gamma}_{2}$, and AC III, respectively. After 72 h of incubation, the BV fractions were collected and the lysates were subjected to SDS-PAGE (20 µg protein/lane from each preparation, 7.5–15% gel) followed by western blot analysis using an anti-HA monoclonal antibody for the ORs, an anti-FLAG monoclonal antibody for RTP1S, and polyclonal antibodies against $G^{\alpha}_{\text{olf}}$, $G^{\beta}_{1}$, $G^{\gamma}_{2}$, and AC III. The arrowhead in each panel indicates the corresponding component. The panels with the heading “non-OR control” and “mOR-EG complex” show the components expressed without OR and with mOR-EG, respectively.
Figure 4  Expression and ligand-dependent activation of BV reconstituted ORs fused with different expression tags. The BV fraction expressing the recombinant OR components was prepared from an Sf9 suspension cell culture, as indicated in the Figure 3 legend, and collected at 72-h postinfection. (a) The western blot analysis of the mOR-EG fused with the different tags expressed in the receptor complexes on BV. About 20 µg protein/lane from each preparation was applied to a 10% gel. (b) A 10 µg amount of each of the BV fractions was subjected to cAMP production assay at the various concentrations on a 10⁻¹⁰ x dilution line of eugenol, starting from the highest concentration of 1 mM (n = 6). (●, ○, ▼), or (△) represents the curve for the HA-, FLAG-, Rho-mOR-EG reconstitution complex or non-OR control, respectively. The *, †, or ‡ symbol indicates a significant difference in cAMP production between the non-OR control and HA-, FLAG-, or Rho-mOR-EG, respectively (P < 0.05).

Table 1  EC50 value, and minimum and maximum enzyme activities of AC assay for each reconstituted OR complex on BV

<table>
<thead>
<tr>
<th>Reconstituted OR complex</th>
<th>Ligand</th>
<th>EC50 (µM)</th>
<th>IC50 (µM)</th>
<th>Vmin (pmol cAMP/min/mg protein)</th>
<th>Vmax (pmol cAMP/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA-mOR-EG</td>
<td>Eugenol</td>
<td>44.7 ± 15.3</td>
<td>109.1 ± 1.96</td>
<td>141.7 ± 3.45</td>
<td></td>
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<tr>
<td>FLAG-mOR-E</td>
<td>Eugenol</td>
<td>48.7 ± 16.9</td>
<td>107.2 ± 0.49</td>
<td>139.4 ± 1.74</td>
<td></td>
</tr>
<tr>
<td>Rho-mOR-EG</td>
<td>Eugenol</td>
<td>31.1 ± 6.98</td>
<td>108.2 ± 1.24</td>
<td>134.8 ± 1.69</td>
<td></td>
</tr>
<tr>
<td>HA-mOR-EG</td>
<td>Eugenol</td>
<td>43.2 ± 16.9</td>
<td>107.4 ± 1.85</td>
<td>144.0 ± 3.72</td>
<td></td>
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plus FLAG-RTP1s

| HA-mOR-EG               | Vanillin| 43.0 ± 5.17| 107.8 ± 1.17| 142.3 ± 1.59                  |                               |
| HA-S6                   | cc9     | 43.0 ± 5.85| 104.7 ± 1.31| 148.6 ± 1.94                  |                               |
| HA-S6 plus FLAG-RTP1s   | cc9     | 31.5 ± 6.99| 104.4 ± 2.17| 151.4 ± 2.95                  |                               |

HA-S6b

| fcc9                   | 83.4 ± 17.1| 104.8 ± 3.97| 127.8 ± 1.12                  |                               |

Values represent average ± SE (n = 6).

Values were calculated from fcc9 dose-response curve of reconstituted HA-S6 containing 40 µM cc9 at all assay points.
Effect of an S6 antagonist on the reconstituted receptor on BV

Because fcc9 was observed to be an antagonist against the S6 expressed in HEK293 cells (T. Sato, personal communication), we examined whether fcc9 would also exhibit an antagonistic effect on S6 reconstituted on BV. We tested the dose-dependent inhibitory effect of fcc9 on the cAMP production induced by 40 μM of cc9, a concentration near the EC50 value. Fcc9 treatment resulted in a clearly reversed sigmoidal curve (Figure 6b), indicating that fcc9 worked as an antagonist against reconstituted S6 on BV. On the other hand, fcc9 did not exert any effect on the mOR-EG complex on BV in the presence of 40 μM eugenol (Figure 6c). The IC50, Vmin, and Vmax values are shown in Table 1.

Expression of other olfactory receptors on BV

We tested whether other ORs could be expressed on BV. The baculovirus harboring the cDNA of His-fused-I7 and...
His-fused-OR8B3 were generated using the Bac-N-Blue system (Invitrogen) according to the manufacturer’s instruction, and they were infected into Sf9 cells. The BV fractions were subjected to western blot analysis and clear monomer and dimer bands of each reconstituted OR were observed (Figure 7).

**Discussion**

The aim of this study was to reconstitute OR complexes on BV using the 2 ORs, mOR-EG and S6 as an example, and to determine whether they were functional in terms of firing the initial stage of the signal transduction machinery in a ligand-dependent fashion.

Figure 6  Dose-dependent activation of reconstituted mOR-EG on BV with vanillin, and the inhibitory response of reconstituted S6 on BV against an S6 antagonist. (a) The BV fraction expressing the mOR-EG complex or S6 complex was prepared and subjected to cAMP production assay, as indicated in the Figure 4 legend, using vanillin as the ligand (n = 6). The curves for the mOR-EG complex (●), S6 complex (○), and non-OR control (▼) are displayed. The * symbol indicates a significant difference in cAMP production between the non-OR control and HA-mOR-EG (P < 0.05). (b) The BV fraction expressing the S6 complex was prepared and subjected to cAMP production assay, as described above, using various concentrations on a 10^{-15} \times dilution line of fcc9 in the presence of 40 \mu M of cc9 (n = 6). (c) Reconstituted mOR-EG on BV was subjected to the same assay using fcc9 as described in (b), in the presence of 40 \mu M of eugenol instead of cc9 (n = 6).
Despite the reported difficulty in expressing ORs in heterologous cell systems (McClintock and Sammeta 2003), both mOR-EG and S6 were clearly detected in Sf9 cells and on BV under these experimental conditions (Figure 1). An immunopositive band with a molecular mass of approximately 28 kDa on SDS-PAGE analysis was appropriate for the OR monomer as reported using mammalian cells (Gat et al. 1994; Katada et al. 2003). In addition, the appearance of the 28-kDa and 48-kDa bands suggests that the OR molecules dimerize on BV as in mammalian cells. The size discrepancy between the predicted and apparent molecular masses suggested an unexpected degradation of the ORs, but an examination performed with double-tagged ORs on both the N- and C-termini using different expression tags confirmed the expression of the full size ORs on Sf9 cells and BV (Figure 2).

The coexpression of all of the components of the receptor complexes on BV was also confirmed (Figure 3). This indicates that BV can display the OR proteins at almost the same level as other GPCR proteins, such as BLT1 and DR1 (Masuda et al. 2003; Sakihama et al. 2008).

The functionality of the reconstituted OR complex was assessed by assaying ligand-dependent cAMP production. The reconstituted complex of either mOR-EG or S6 displayed a typical ligand-dependent cAMP production response curve (Figure 5a, 5c). The EC50 value of approximately 10⁻⁵ M obtained in this assay is compatible with that reported in other mammalian cell expression systems (Zhuang and Matsunami 2007; Yoshikawa and Touhara 2009; Hamana et al. 2010). The cAMP production of the S6 complex induced by the S6-specific agonist cc9 was inhibited by the putative antagonist fcc9 (Figure 6b), as in the case of the HEK293 expression system (T. Sato, personal communication). This clearly demonstrates that the OR response on BV solely takes place in a ligand-dependent fashion. Moreover, because the ligand-dependent cAMP production did not occur when G₍olf was replaced with Gᵢ, the OR response observed here was effected through the G₄olf-dependent signaling complex (Figure 5b, 5d). Together, these findings indicate that the OR complexes were functionally reconstituted on BV.

The similar responsiveness of mOR-EG to vanillin as to eugenol (Figure 6a) and the specific interactions of eugenol and cc9 with mOR-EG and S6, respectively, in the complex on BV (Figure 5), indicate that the reconstituted ORs on BV retain the ligand specificity of their function, as they do in other OR expression systems.

We further examined the effect of N-terminal fusion tags and an accessory protein on the expression level of ORs, as well as on their receptor function on BV. In mammalian cell expression systems, N-terminal fusion of a HA-, FLAG- or Rho-tag on ORs has been reported to enhance the translocation of the OR molecules to the plasma membrane (Gat et al. 1994; Ivic et al. 2002; Saito et al. 2004; Zhuang and Matsunami 2007). Such an enhancement of membrane translocation would be expected to improve receptor function in Sf9 cells. However, as shown in Figure 4b, the receptor function of the reconstituted mOR-EG fused with such N-terminal tags on BV did not result in a significant change in either the EC50 or maximum activity of the ligand-dependent cAMP production, in contrast to the conspicuous changes seen in mammalian cell systems (Zhuang and Matsunami 2007).

Furthermore, the effects of RTP1S on mOR-EG or S6 expression in Sf9 cells and the receptor function of the reconstituted BV were also tested. In our experiments, the receptor function of the reconstituted OR complex on BV did not display any conspicuous improvement as a result of the presence of RTP1S (Figure 5a, 5c). A small reduction of the expression level of G₄olf and Gᵢ was observed in the reconstituted complex when RTP1S was coexpressed. The expression level of each component of the complex is quite sensitive to the ratio of the coinfecting viruses harboring each component, and the ratio of the viruses chosen for this study is the one currently reported to be optimum for obtaining clear ligand-receptor dose responses. It is highly unlikely that the ability of RTP1S was significantly modulated by the small changes in the components, because while tuning the assay with different ratios of viral infections there appeared no sign of improved receptor function due to the copresence of RTP1S (data not shown). In this regard, we speculate that

**Figure 7** Detection of the expression of OR8B3, a human orphan OR, on BV. Sf9 cells were infected with recombinant baculovirus harboring the cDNA of His-I7 or His-OR8B3, and incubated for 72 h. The BV fraction of each of them was subjected to SDS-PAGE (20 µg protein/lane from each preparation, 7.5–15% gel) followed by western blot analysis using a mouse anti-His monoclonal antibody. The arrow and arrow head indicate the monomer and dimer of the OR, respectively.
the baculovirus reconstitution system may have different features in reconstructing heterologous receptor complexes compared with the mammalian cell system.

As the expression level of each component of the OR complex depends on the number of the recombinant viruses used, it is important to set up a good M.O.I. ratio for each virus harboring the cDNA of each component. Once this condition is optimized, it is easy to reproduce the same BV preparation in a large amount of homogeneous quality. It is thus an advantageous feature of the OR reconstitution on BV that it provides a reliable assay system more easily than other heterologous mammalian cell–based systems. Because we have been able to detect other recombinant orphan ORs on BV in our preliminary trial, this technique would be expected to be generally applicable to OR complexes. An example of detecting the expression of OR8B3 on BV, one of the orphan ORs (Figure 7), is sufficiently encouraging to suggest that this will be of practical utility. Together with the recent report that an application of a heterologous cell system (Saito et al. 2009) and dissociated olfactory sensory neurons (Nara et al. 2011) led to successful identification of orphan ORs, it is shown that BV reconstitution can serve as a high throughput combinatorial assay for orphan ORs and to search for new OR functions.

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