RELATIONSHIP BETWEEN HOMO-OLIGOMERIZATION OF A MAMMALIAN OLFACTORY RECEPTOR AND ITS ACTIVATION STATE DEMONSTRATED BY BIOLUMINESCENCE RESONANCE ENERGY TRANSFER (BRET)

Fallou Wade1, Agathe Espagne2, Marie-Annick Persuy1, Jasmina Vidic3, Régine Monnerie1, Fabienne Merola2, Edith Pajot-Augy1, and Guenhaël Sanz1

From INRA, UR1197 Neurobiologie de l'Olfaction et Modélisation en Imagerie1, Domaine de Vilvert, F-78350 Jouy-en-Josas, France ; IFR 144 Neuro-Sud Paris France; Laboratoire de Chimie Physique, UMR 80002 Université Paris-Sud 11 and CNRS, Orsay 91405, France ; INRA, UR892 Virologie et Immunologie Moléculaire3, Domaine de Vilvert, F-78350 Jouy en Josas, France.

Address correspondence to: Guenhaël Sanz, INRA, UR1197 Neurobiologie de l'Olfaction et Modélisation en Imagerie, Domaine de Vilvert, F-78350 Jouy-en-Josas, France. Fax: 33 1 34 65 22 41; E-mail: guenhael.sanz@jouy.inra.fr

G-proteins coupled receptors (GPCRs) homo-oligomerization is increasingly reported. However, little is known regarding the relationship between receptor activation and its association/conformational states. The mammalian olfactory receptors (ORs) belong to the GPCRs super-family. In this study, the homo-oligomerization status of the human OR1740 receptor and its involvement in receptor activation upon odorant ligand binding were addressed by co-immunoprecipitation and bioluminescence resonance energy transfer (BRET) approaches using crude membranes or membranes from different cellular compartments. For the first time, our data clearly show that mammalian ORs constitutively self-associate into homo-dimers at the plasma membrane level. This study also demonstrates that ligand binding mediates a conformational change and promotes an inactive state of the OR dimers at high ligand concentration. These findings support and validate our previously proposed model of OR activation / inactivation model based on the tripartite odorant binding protein (OBP)-odorant-OR partnership.

The sense of smell endows mammals with the capacity to recognize and discriminate a large number of odorants. Animals rely on olfactory clues as an essential means for survival through food searching, avoidance of danger and reproduction. The first critical step of odorant detection consists in odorant interaction with olfactory receptors (ORs), which are expressed in olfactory sensory neurons within the olfactory mucosa. Olfactory perception involves a combinatorial code, in which one OR recognizes multiple odorants and different odorants are recognized by different combinations of ORs (1). It is widely accepted that a single olfactory sensory neuron expresses only one OR gene (2). Besides this complex combinatorial coding of odors, involving odorant binding with various affinities, little is known about the molecular mechanisms underlying the initial step in odorant signal transduction. ORs belong to the large super-family of the G-protein coupled receptors (GPCRs). In olfactory sensory neurons, the odorant-activated OR mainly couples to the Golf subunit of a heterotrimeric G-protein, which initiates cellular signaling to generate the olfactory message (3). Odorants are generally volatile hydrophobic molecules and reach ORs through the aqueous olfactory mucus bathing the mucosa. This thin layer contains large amounts of OBPs, members of the lipocalin family (4). In insects, OBPs are thought to interact with ORs and play a scavenger role to maintain ORs in an active conformation (5). In mammals, we have recently demonstrated a tripartite functional interaction between OBPs, odorants and ORs (6).

GPCRs are widely documented to exist as self-associated dimers or higher-order oligomers (7-9). GPCRs oligomerization may play an important role in receptors trafficking to the cell surface and intracellular signaling. Kaupmann et al. have thoroughly demonstrated that heterodimerization is required for the GABABR1 / GABABR2 receptors to ensure both plasma membrane targeting and functionality (10). In contrast, oligomerization only occurs upon ligand interaction at the cell surface for other GPCRs (11). In efforts to identify GPCRs association state, the relationship between activation and oligomerization remains to be understood for many GPCRs including ORs.

To date, no study has provided a clear and undisputable evidence of ORs dimerization, even though heterodimerization of ORs was reported in rodents and insects (12-14). Some
ORs, among which human OR1740, were reported not to heterodimerize with a non-olfactory GPCR, the β2-adrenergic receptor (15). Our previous studies (6,16,17), in line with others (18,19), showed a bell-shaped OR dose-response curve upon odorant stimulation. Several assumptions have been raised to elucidate the decreased ORs response at high ligand concentration (20-22). This observation could result either from a non specific inhibition of the response, or from various association / activation states and conformational changes of ORs. Since protomers association can modulate GPCRs functionality, we suggested that the activity of potential ORs dimers could depend on the number of bound odorant ligands. Indeed, one bound odorant could activate the OR dimer, while two bound odorants, one on each protomer, could hinder signaling due to an inappropriate dimer conformation. We therefore proposed a model for OR-OBP-odorant ligand interactions based on two hypotheses, competitive binding of OBP and odorant to the OR, and association of ORs into homo-dimers. This model allows explaining the bell-shaped dose-response curve observed for receptor interaction with odorants in the absence of OBPs (Fig. 1). On the one hand, in support to this model, we already demonstrated the functional role of OBP for maintaining OR activity at high odorant concentration (6). On the other hand, investigation of the association states of OR is crucial to validate this whole mechanistic model of odorant detection by ORs.

In the present study, we undertook to determine whether mammalian ORs would be present as homo-oligomers in living cells. For this purpose, we heterologously expressed the human OR1740 receptor under optimized experimental conditions in the yeast S. cerevisiae (17,23), and investigated its homo-oligomerization status. Besides a biochemical approach, we used BRET, a well-developed biophysical technology largely employed in the past decade to explore GPCRs oligomerization, to monitor not only ORs homo-dimerization, but also the odorant ligand-mediated conformational changes of the receptors. For the first time, our data show unambiguously that a human OR can form homo-dimers. Interestingly, the BRET approach also allowed establishing that odorant ligand binding induces conformational changes of the ORs dimers, which are compatible with their decreased functional response at high odorant concentration.

**Materials and methods**

**Odorant solutions preparation**- Stock solutions of octanal, vanillin (Sigma-Aldrich), and helional (a gift from Givaudan-Roure, courtesy of B. Schilling (Dübendorf, Switzerland)) were extemporaneously prepared in dimethylsulfoxide (DMSO) at $10^{-4}$ M. $10^{-4}$ M odorant dilutions were performed in water or in BRET buffer (PBS 1X, MgSO$_4$ 0.01% (w/v), glucose 0.1% (w/v)) directly from the stock solution. Further dilutions ($10^{-5}$ to $10^{-8}$ M) were prepared by successive dilutions in water or BRET buffer.

**Plasmids constructs**- The yeast expression vectors pESC-URA and pESC-TRP (Stratagene) were modified as follows. Two multicloning sites (MCS) containing restriction sites and a tag (cmyc for BcMBAN/NABcB; HA for BBSHAN/NAHSBB) were designed and synthesized as single strands (MWG eurofins, France):

- BcMBAN: 5' cccggatccatggaacagaagttgatttccgaagaagacctcctca gatctgcgatcgctagcccc 3'
- NABMcB: 5' ggggcctagcgatcgcagatctgaggaggtcttcttcggaaatcaac ttctgttccatggatccggg 3'
- BBSHAN: 5' cccggatccctgagatctactgcgatcgcatacccatacgatgttcc agattcgcattgactctgcatcgcataccatacgatgttcc agattacgcttaagctagcccc 3'
- NAHSBB: 5' ggggcctagcgatcgcagatcgcataccatacgatgttcc agattacgcttaagctagcccc 3'

BcMBAN and NABMcB ssDNAs or BBSHAN and NAHSBB ssDNAs were heated to 94°C for 5 min, and then cooled to room temperature for annealing. pESC plasmids were digested by Cla I and Nae I, and protruding ends were filled in. Then, both pESC-URA and pESC-TRP plasmids were ligated and amplified in E. coli. The resulting plasmids (pESC del-URA and pESC del-TRP) were double digested by EcoR I and Spe I. The Gal 4 gene was released by EcoR I and Nhe I from the pJH2 vector (24) and inserted into open pESC del plasmids. After ligation, the plasmids were amplified in E. coli. The resulting plasmids pESC del-Gal4-URA and pESC del-Gal4-TRP were linearized by Bam HI and Nhe I. The resulting plasmids were amplified in E. coli.
were constructed and inserted into the pESC-URA or pESC-TRP yeast expression vectors as follows. All primers used are reported in Table 1. OR1740 cDNA without stop codon was PCR amplified using primers 1 introducing the unique BglII restriction site and a linker sequence extension onto which EYFP or Rluc sequences could be added. EYFP gene was site mutated by an A206K replacement as described in Zacharias et al. (25) to avoid self-association of the proteins. EYFP and Rluc sequences to be fused to the OR1740 receptor were generated using primers 2 and 3 respectively harbouring the linker sequence and the unique AspI restriction site. OR1740-Rluc and OR1740-EYFP fusion constructs were generated by PCR amplification using mixtures of DNA fragments to be fused, and primers 4 and 5 respectively. These fusions were digested by BglII and AspI and cloned into the BglII/AspI cloning site of the yeast expression vectors, to obtain pESC-URA-cmyc-OR1740-Rluc and pESC-TRP-cmyc-OR1740-EYFP.

Transformation and yeast growth- The yeast S. cerevisiae strain MC18 (17) was transformed with expression vectors pESC-URA-cmyc-OR1740-Rluc, pESC-TRP-cmyc-OR1740-EYFP and pRGP-Golf (26) for BRET experiments or with pESC-TRP-cmyc-OR1740, pESC-TRP-HA-OR1740, and pRGP-Golf for co-immunoprecipitation (27). The transformed cells were grown and induced at 15°C for 108 h for proteins production as described previously (17).

Crude membranes preparation- All steps were performed at 4°C unless otherwise indicated. Transformed yeast cells were harvested, washed twice with ice-cold water and resuspended in ice-cold lysis buffer A (50 mM Tris/HCl pH 7.5, 1 mM EDTA, 0.1 mM PMSF, 250 mM sorbitol) and the Complete protease inhibitor cocktail (Roche) prior to membranes preparation as previously described (6,17). The protein concentration of the preparation was determined using the BCA reagent (Pierce) with bovine serum albumin as a standard.

Subcellular fractionation- Crude membranes containing 2 to 5 mg/mL of proteins in 500 µL of 10% sucrose buffer B (10 mM Tris/HCl pH 7.5, 1 mM EDTA, 1 mM DTT and Complete protease inhibitor cocktail) were applied on top of a 11 mL 30% to 70% (w/v) continuous sucrose gradient according to the method of Eraso (28) with slight modifications. After centrifugation for 16 h at 30,000 rpm in a SW41Ti rotor (Beckman) at 4°C, a total of 12 subfractions samples were successively collected from the top (light membrane vesicular fractions) to the bottom of the tube (plasma membrane).

Samples of identical volumes taken from the fractions were immunblotted to check the efficiency of the sucrose gradient to sort the various membranes, notably the endoplasmic reticulum (ER), and plasma membranes. In order to perform BRET assays, sucrose was removed from the subfractions by dilution in buffer B without sucrose and additional centrifugation for 1 h at 30,000 rpm at 4°C. Each pellet was resuspended in buffer B. Protein concentration was measured in each fraction as above.

Co-immunoprecipitation- 500 µg of crude membranes prepared from yeasts expressing cmyc-OR1740 and HA-OR1740, or cmyc-OR1740 alone, were immunoprecipitated using anti-cmyc antibody (Roche Diagnostics) at 10 µg/mL according to the manufacturer’s instructions (Pierce Classic IP Kit). 10 µL of the anti-cmyc immunoprecipitated samples were analyzed by immunoblotting (IB) using anti-HA antibody (Cell Signaling). The same experiment was done using crude membranes prepared from yeasts expressing cmyc-OR1740 and HA-OR1740, or HA-OR1740 alone, and anti-HA antibody for immunoprecipitation and anti-cmyc for immunoblotting.

Immunoblotting- Homogenized crude membranes (5 µg of proteins); subfractionated membranes (5 µg of proteins) from sucrose gradient, or immunoprecipitates were separated by electrophoresis on 10% SDS-PAGE and electrotransferred onto Immobilon-P Transfer Membranes (Millipore). Membranes were blocked with 4.5% no-fat dry milk in buffer C (phosphate buffered saline 1X, 0.1% Tween 20). Membranes were hybridized with either anti-cmyc antibody (for crude and subfractionated membranes), anti-HA antibody (for immunoprecipitates), or antibodies targeting the yeast plasma membrane marker Pma1 (anti-Pma1, abcam) or the yeast ER marker Dpm1p (anti-Dpm1p, Molecular Probes). Anti-mouse or anti-rabbit IgG peroxidase conjugates were used as secondary antibodies (Sigma). The enhanced chemiluminescence reaction was performed using the plus-ECL reagent (PerkinElmer).

Surface plasmon resonance (SPR) measurements- Prior to SPR experiments, membrane fractions were sonicated for 15 min at
2 x 160W, 35 Khz (Sonoclean S2600 sonicator, Labo-modern, Paris, France) on ice-cold water. This yields membrane nanosomes of uniform size of about 50 nm in diameter (29). Real time binding kinetic experiments were conducted on a Biacore 3000 (GE Healthcare Europe). 0.03 mg/mL of yeast nanosomes carrying olfactory receptors were immobilized via their lipidic bilayer on a L1 sensorchip (GE Healthcare). For this, nanosomes diluted at 0.3 µg/ml total proteins in HBS-EP buffer (10 mM HEPES, pH 7.4, containing 150 mM NaCl, 3 mM EDTA, 0.005% P20, 0.005% DMSO) were injected for 20 minutes at 1 µl/min. Then, the obtained immobilized layer was washed with HBS-EP buffer (standby procedure) during several hours to obtain a stable signal. For functional tests, a solution of helional at 5 µM and GTPγs at 10 µM in HBS was injected at 10 µl/min over the captured nanosomes for 4 min and dissociation was registered for 15 min after the end of odorant injection. In control experiments, stimulation was carried out using solutions in which the odorant had been replaced by water. Regeneration of the chip surface was achieved by several 2 min injections of 20 mM CHAPS. The sensorgram observed in the experiment with helional was corrected by subtracting the response observed in the control experiment with water. All measurements were performed at 20°C. Sensorgrams were analyzed using BIAevaluation Software.

BRET assays- 3.10^7 yeast cells expressing cmyc-OR1740-Rluc (BRET donors) alone or with cmyc-OR1740-EYFP (BRET acceptors) were pelleted and disrupted with 3 min incubation successively in isopropanol dry-ice bath (-25°C) and 25°C water bath. Disrupted cells were then resuspended in BRET buffer (PBS 1X, MgSO4 0.01% (w/v), glucose 0.1% (w/v)). Crude membranes from the same yeast cells or subfractionated membranes were also used for BRET assays. Disrupted yeast cells or membranes carrying 10 µg of proteins were then distributed in a white 96-well microplate (Nunc). Coelenterazine h (Promega) substrate was added at a final concentration of 5 µM in the BRET buffer. Emitted luminescence and fluorescence were measured simultaneously using a LB 941 Multimode Reader TriStar (Berthold), with emission filters respectively at 485 nm and 530 nm. Luminescence emission was checked to be similar for the various samples tested (membranes from yeast expressing OR1740-Rluc alone or co-expressing OR1740-Rluc and various amounts of OR1740-EYFP). The BRET ratio is expressed as emission at 530 nm over emission at 485 nm (30) and the normalized BRET ratio is deduced as the BRET ratio for OR1740-Rluc and OR1740-EYFP minus the BRET ratio for OR1740-Rluc alone, (mBret = (530/485 [OR-Rluc+OR-YFP]- 530/485 [OR1740-Rluc]))x1000). Immunoblot analysis using an anti-cmyc antibody was performed with membranes carrying cmyc-OR1740-Rluc and cmyc-OR1740-EYFP to evaluate the (OR1740-EYFP/OR1740-Rluc) ratio for the BRET saturation assay.

RESULTS

**ORs homo-dimerization assessment by co-immunoprecipitation.** Immunoblots of membranes from yeast cells expressing cmyc-OR1740 show two bands with apparent molecular masses of 27 kDa and 49 kDa (Fig. 2). These bands have been suggested to correspond respectively to the monomer and dimer forms of the OR1740 receptor (31). To investigate ORs homo-dimerization, we then co-immunoprecipitated potential OR1740 oligomers using crude membranes prepared from yeast cells co-expressing cmyc-OR1740 and HA-OR1740. As shown in Fig.3, non specific bands appear common to both the co-immunoprecipitated proteins (lane 1) and the control sample (lane 2), but additional bands corresponding to the monomer, dimer and oligomer forms of the OR1740 receptor are specifically present in the co-immunoprecipitated proteins. Thus, the present results demonstrate that HA-OR1740 co-immunoprecipitated with cmyc-OR1740, supporting the hypothesis that ORs exist as homo-dimers in the cells. We performed the cross-study using anti-HA antibody for immunoprecipitation, and anti-cmyc antibody to reveal the immunoblots. Again, we found that cmyc-OR1740 co-immunoprecipitated with HA-OR1740, but we could not prevent the presence of non specific bands either (data not shown).

**ORs homo-oligomerization assessment by BRET.** Our initial results based on immunoblotting and co-immunoprecipitation analyses support the hypothesis that ORs self associate. However, these qualitative techniques suffer from a lack of precision as to the nature of this association, either promiscuity-driven or specific dimerization. We have therefore taken advantage of the BRET technique, a tool based
on energy transfer, to elucidate the specificity of the association of ORs heterologously expressed in yeast cells. BRET assays were conducted either with disrupted yeast cells co-expressing OR1740-Rluc (donors) and OR1740-EYFP (acceptors) or with crude membranes prepared from these cells. We checked by SPR measurement upon ligand stimulation (6,17) that the OR1740 receptor fused to Rluc and the OR1740 receptor fused to EYFP are functional as OR1740 (data not shown). BRET signals are reported in Fig. 4A. Significant BRET signals were measured, providing evidence that ORs exist in homo-association form. Furthermore, the BRET level was higher for experiments carried out with membranes compared to experiments using whole disrupted yeast cells.

Random clustering of the ORs arising from an excessive expression level could result in non specific bystander energy transfer interaction and thus in a non specific BRET signal (32,33). To address this question BRET measurements were performed as a function of the amount of acceptor (OR1740-EYFP) relative to the donor (OR1740-Rluc), starting from many different yeast clonal populations. Immunoblots were performed to evaluate the relative amount of OR1740-Rluc and OR1740-EYFP proteins within crude membranes from these clones. The BRET signal increases as a function of the OR1740-EYFP/OR1740-Rluc ratio, with a trend towards saturation at high ratios (Fig. 4B), suggesting a specific interaction of the receptors.

Homo-oligomerization of OR1740 receptor at the subcellular level. OR oligomerization may occur at different levels of OR expression. It may be required for OR maturation, folding and plasma membrane targeting, or to allow or modulate OR response to its odorant ligand. We thus investigated whether OR oligomerization takes place as early as the endoplasmic reticulum level or later, at the plasma membrane level. For this purpose, BRET was measured in subcellular membrane fractions, obtained from crude membranes of yeast cells co-expressing OR1740-Rluc and OR1740-EYFP and fractionated following the sucrose gradient procedure described in the Materials and Methods section. The subcellular fractions were analysed by immunoblotting with antibodies targeting the yeast ER and plasma membrane markers Dpm1p and Pma1 (Fig. 5B) to qualify their cellular origin. A BRET signal was measurable in all fractions from the endoplasmic reticulum to the plasma membrane (Fig. 5A). Interestingly, BRET levels significantly increased from inner fractions to plasma membrane fractions.

Ligand-mediated conformational change of OR1740 dimers. Since the self-association of the OR1740 receptor was demonstrated to take place without ligand, it is constitutive and not ligand-induced. The BRET technique has already been employed to investigate a possible conformational change induced by ligand binding on receptor dimers (34). We thus undertook to explore whether, helional, the preferential agonist of OR1740, induced a conformational change of the OR1740 dimer, resulting in a change of the BRET level. For this purpose, we performed BRET experiments with crude membrane preparations in the presence or absence of helional. Helional 10^{-5} M was used since this concentration was previously shown to induce a large response of the OR1740 receptor (35). As shown in Fig. 6A, helional stimulation of the OR1740 receptor significantly increased the BRET level as compared to the pre-existing level. In contrast, octanal and vanillin, which do not activate the OR1740 receptor (36), did not promote such a change in the BRET signal (Fig. 6A). The helional-induced increase in resonance energy transfer may result from an OR conformational change bringing the BRET donors and acceptors closer to each other or in a favorable orientation. To further investigate the ligand-mediated conformational changes at the subcellular level, BRET was measured in membrane subfractions with or without helional stimulation. In the presence of helional, the BRET level exhibited a significant increase only from plasma membrane fractions, whereas it remained unchanged at a basal level in inner and ER fractions (Fig. 6B). So, it seems that only the mature receptors having reached the plasma membrane exhibit a ligand-induced BRET signal modification due to their interaction with a ligand. This correlates with the receptors functional activity at this location (34).

Ligand-induced BRET modulation correlates with ligand-induced OR activity level. Our group previously demonstrated a bell-shaped dose-response of OR1740 to its odorant ligand (17), using measurements of the functional interaction of the ligand with the receptor by SPR experiments. We proposed a model in which the decrease of OR1740 response at high helional concentration was ascribed to a ligand-induced conformational change of the OR1740 dimer.
resulting in receptor inactivation, whereas the response reached a maximum at intermediate helional concentration (6). Here, we monitored the variation of BRET level upon odorant stimulation with various concentrations of helional. As was observed in our previous Surface Plasmon Resonance experiments (6), the variation of BRET level upon odorant stimulation, plotted as a function of helional concentration, exhibits a bell-shaped curve (Fig. 7). It therefore appears that the OR1740 dimer conformational changes induced by various helional concentrations elicit an evolution in the BRET level which correlates with that of the functional response. The most functionally active dimer states, i.e. at intermediate helional concentrations (10^-6M - 10^-5M), result in the largest BRET increase, whereas the inactive dimer states, at the highest helional concentrations, induce no change in basal BRET level. These observations tend to confirm our model according to which dimer activity or inactivity depends on ligand concentration, through the level of ligand binding site occupancy, which mediates conformational change towards active or inactive states.

**DISCUSSION**

A review by Gurevich et al. (37) elegantly titled "How and why do GPCRs dimerize?" has tackled a question of great importance. In the past decade, a corpus of studies (http://data.gpcr-okb.org/gpcr-okb/oligomer/list) has documented GPCRs oligomerization states, which are thought to play a crucial role in the maturation pathway and plasma membrane targeting of receptors. Some GPCRs oligomerize upon ligand binding while others, such as GABA_B and CCR5 receptors, associate earlier during the maturation pathway. Controversial statements have been issued over the proper expression, localization, and function of GPCRs oligomers (38,39). Many assumptions have been made but few evidences thoroughly demonstrate that GPCRs oligomerization takes place early in the biosynthetic process, and that GPCRs are targeted to the plasma membrane as constitutive oligomers (40-43). Up to now, no consensus has emerged from the literature about the relationship between GPCRs oligomerization status and receptors activation (20,43,44). In particular, little is presently known about olfactory receptors self-association, although they constitute the largest GPCR subfamily. However, some authors have pointed out the implication of the association of some ORs with other non-olfactory GPCRs, to reach the cell surface (12,14,45). Some evidences support a strong relationship between ligand binding and GPCR oligomers signaling (46,47). We have proposed a model based on two hypotheses: (i) a tripartite interaction between OBPs, odorants and ORs (6) and (ii) an activation state of ORs depending on their dimerization status and the number of bound ligands. Indeed, one odorant ligand binding to the dimers would favor an active form of the OR. In contrast, OR dimers with two odorant molecules, each binding to a receptor protomer, would blunt signaling due to a dimer conformation inappropriate for signaling. The first assumption was investigated in the framework of this model, and validated (6). It was concluded that OBP plays a crucial role in maintaining OR activity at high odorant concentration.

Our present results using co-immunoprecipitation and BRET approaches demonstrate that the human OR1740 receptor exists as a non ligand-induced homo-dimer in yeast cells. Indeed, both disrupted yeast cells heterologously expressing OR1740 fused to Rluc and OR1740 fused to EYFP, and the membrane fraction prepared therefrom, exhibited BRET signals demonstrating the OR1740 receptor self-association. Interestingly, the highest BRET level (by a factor of 2.5) was reached with the membrane preparations relative to disrupted yeast cells (Fig. 4A). Rather than ascribing this phenomenon to some receptor enrichment in the membrane fraction, we infer that clearing disrupted cells from debris and cytoplasm could result in a decrease in the occurrence of phenomena that quench the bioluminescence emission and lower the BRET signal. Reaching a sufficient expression level in heterologous systems is crucial to allow monitoring GPCRs oligomerization, but overexpression can elicit random clustering of the receptors and their non-specific self-association, at densities high enough to elicit non specific, bystander energy transfer and thus to non specific BRET signals (11,32-34). It is possible to distinguish between these bystander effects and those due to specific protomer association by undertaking systematic studies of BRET or FRET levels against donor and acceptor densities (32,48). Indeed, specific BRET (or
FRET) depends on the acceptor/donor ratio, but not on donor and acceptor surface densities, whereas non-specific BRET arising from random clustering varies with acceptor density and is insensitive to the acceptor/donor ratio. In addition, the increase in specific BRET with acceptor/donor ratio should saturate at a level determined by the intrinsic stoichiometry of the receptor complex. We therefore performed BRET measurements as a function of the amount of acceptor (OR1740-EYFP) relative to the donor (OR1740-Rluc). For this purpose, we faced a major difficulty: since yeasts self-regulate gene expression, they could not be made to express increasing amounts of acceptors while the donor amount remained fixed. Thus, the saturation plateau investigation was limited by the natural ratio observed in the various clones studied. This limitation is often by-passed in other studies through the use of mammalian cells, which allows modulating expression levels by varying the amount of transfected cDNA (39,43). However, in spite of these limitations, the BRET assay showed a saturation curve tendency as a function of the OR1740-EYFP/OR1740-Rluc ratio (Fig. 4B). This indicates the presence of a specific component in the interaction of receptors (33,43).

In our previous work (6), we demonstrated a tripartite interaction between OBPs, odorants and ORs, in which OBPs play a crucial role in preserving OR activity at high odorant concentration. However, the OBP-odorant-OR partnership is not sufficient to fully explain the complexity related to the molecular mechanisms of OR-odorant interactions and signaling. One of the major events in GPCRs activation is the receptor conformational change occurring upon ligand binding. This conformational change inducing signaling downstream of the receptors has been recently reviewed (49). BRET studies have been used to prove that ligands can mediate conformational changes in constitutive receptor oligomers such MT2R, CCR5, DORs (34,39,50). In an additional approach to prove the specificity of the receptors interaction into dimers, we thus undertook to monitor the BRET signals upon odorant ligand stimulation (Fig. 6). Stimulation with helional significantly increased the BRET level as compared to the pre-existing BRET (Fig. 6A). In contrast, vanillin and octanal which do not activate the OR1740 receptor (36), did not significantly change the BRET signal. This result further confirms the specificity of the selfassociation of the OR1740 receptor in the yeast cells, through the conformational change upon ligand binding. Interestingly, BRET levels measured increased from ER membrane fractions to the plasma membrane fractions. Our observation differs from what is reported by Issafras et al. who have detected comparable BRET signals from CCR5 receptor expressed in ER and plasma membrane fractions resolved on a sucrose gradient (39). The effect we observe may be interpreted in terms of maturation and/or localization. The BRET increase suggests that the ratio of OR dimers relative to monomeric species could be larger at the plasma membrane level, or that the conformation of the dimers could evolve from the ER to the plasma membrane, being more suitable for BRET at the plasma membrane level. Receptor maturation along the expression pathway may participate in this evolution. However, it must be stressed that, as the receptor density is probably higher in the ER fractions than at the plasma membrane, the lower BRET signals in the ER may well correspond, in whole or in part, to a non-specific component. In all cases, it is difficult to conclude on the oligomerization state of the receptor at the early stages of its biosynthesis. We observed that only the BRET measured from the plasma membrane fractions increased in the presence of helional, while that measured from the ER fractions remained unchanged (Fig. 6B). The increase in BRET level in the plasma membrane fraction upon ligand exposure may be ascribed to the ORs being mature at the cell surface, a compulsory condition for adequate signaling. Both the oligomerization and ligand-induced conformational changes of GPCRs are known to significantly modulate (increase or decrease) the FRET and BRET signals (9,11,43,44,51). Here, ligand binding clearly induces a conformational change of OR1740 receptors, thus eliciting changes in distance or relative orientation between donor and acceptor, which enhance energy transfer efficiency.

The bell-shaped curve of BRET level variation upon stimulation with increasing helional concentrations is compatible with the bell-shaped curve exhibited by ORs functional response in our previous experiments (6,16,17). Ligand-mediated conformational rearrangement of the ORs within the dimers is a plausible explanation that fits into the two-state model (active and inactive states) of OR dimers depending on ligand concentration. Indeed, the decrease of functional response at high helional
concentration can be ascribed to ligand induced conformational change of the protomers within the OR1740 dimer resulting in dimer inactivation, and is corroborated by ligand-induced BRET variation.

In conclusion, the present work thoroughly demonstrates that the human OR1740 receptor exists as a constitutive homo-oligomer, which expands the growing list of GPCRs oligomers. As for other receptors (GABA<sub>B</sub>, hLHR and CCR5), OR1740 receptor dimers seem to be formed early in the endoplasmic reticulum, which may play a role in quality control of the receptor biosynthesis, trafficking to the cell surface and functional signaling. Our results also show that a ligand-mediated conformational rearrangement occurs at the level of the receptor dimers, modulating the pre-existing BRET signals. Although we associate the decrease of the response at high odorant concentration with the homo-oligomerization status of the OR1740 receptor, the physiological relevance of such an association remains to be fully elucidated.

REFERENCES

1. Malnic, B., Hirono, J., Sato, T., and Buck, L. B. (1999) Cell 96(5), 713-723
2. Mombaerts, P. (2004) Curr Opin Neurobiol 14(1), 31-36
3. Firestein, S. (2001) Nature 413(6852), 211-218
4. Steinbrecht, R. A. (1998) Ann NY Acad Sci 855, 323-332
5. Xu, P. (2005) Science 310(5749), 798-799
6. Vidic, J., Grosclaude, J., Monnerie, R., Persuy, M. A., Badonnel, K., Baly, C., Caillol, M., Briand, L., Salesse, R., and Pajot-Augy, E. (2008) Lab Chip 8(5), 678-688
7. Park, P. S., Filipek, S., Wells, J. W., and Palczewski, K. (2004) Biochemistry 43(50), 15643-15656
8. Duncan, R. R., Bergmann, A., Cousin, M. A., Apps, D. K., and Shipston, M. J. (2004) J Microsc 215(Pt 1), 1-12
9. Pfleger, K. D., and Eidne, K. A. (2005) Biochem J 385(Pt 3), 625-637
10. Kaufmann, K., Malitschek, B., Schuler, V., Heid, J., Froestl, W., Beck, P., Mosbacher, J., Bischoff, S., Kulik, A., Shigemoto, R., Karschin, A., and Bettlert, B. (1998) Nature 396(6712), 683-687
11. Milligan, G., Wilson, S., and Lopez-Gimenez, J. F. (2005) J Mol Neurosci 26(2-3), 161-168
12. Hague, C., Hall, R. A., and Minneman, K. P. (2004) Mol Interv 4(6), 321-322
13. Neuhaus, E. M., Gisselmann, G., Zhang, W., Dooley, R., Stortkuhl, K., and Hatt, H. (2005) Nat Neurosci 8(1), 15-17
14. Bush, C. F., Jones, S. V., Lyle, A. N., Minneman, K. P., Ressler, K. J., and Hall, R. A. (2007) J Biol Chem 282(26), 19042-19051
15. 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(37), 13672-13676
16. Sanz, G., Schlegel, C., Pernollet, J. C., and Briand, L. (2005) Chem Senses 30(1), 69-80
17. Minic, J., Persuy, M. A., Godel, E., Aioun, J., Connerton, I., Salesse, R., and Pajot-Augy, E. (2005) Febjs 272(2), 524-537
18. Araneda, R. C., Peterlin, Z., Zhang, X., Chesler, A., and Firestein, S. (2004) J Physiol 555(Pt 3), 743-756
19. Ko, H. J., and Park, T. H. (2006) Biol Chem 387(1), 59-68
20. Springael, J. Y., Urizar, E., Costagliola, S., Vassart, G., and Parmentier, M. (2007) Pharmacol Ther 115(3), 410-418
21. Han, Y., Moreira, I. S., Urizar, E., Weinstein, H., and Javitch, J. A. (2009) Nat Chem Biol 5(9), 688-695
22. Chabre, M., Deterre, P., and Antonny, B. (2009) *Trends Pharmacol Sci* **30**(4), 182-187
23. Pajot-Augy, E., Crowe, M., Levasseur, G., Salesse, R., and Connerton, I. (2003) *J Recept Signal Transduct Res* **23**(2-3), 155-171
24. Price, L. A., Kajkowski, E. M., Hadcock, J. R., Ozenberger, B. A., and Pausch, M. H. (1995) *Mol Cell Biol* **15**(11), 6188-6195
25. Zacharias, D. A., Violin, J. D., Newton, A. C., and Tsien, R. Y. (2002) *Science* **296**(5569), 913-916
26. Crowe, M. L., Perry, B. N., and Connerton, I. F. (2000) *J Recept Signal Transduct Res* **20**(1), 61-73
27. Schiestl, R. H., Dominska, M., and Petes, T. D. (1993) *Mol Cell Biol* **13**(5), 2697-2705
28. Eraso, P., Mazon, M. J., and Portillo, F. (2006) *Biochim Biophys Acta* **1758**(2), 164-170
29. Vidic, J., Pla-Roca, M., Grosclaude, J., Persuy, M. A., Monnerie, R., Caballero, D., Errachid, A., Hou, Y., Jaffrezic-Renault, N., Salesse, R., Pajot-Augy, E., and Samitier, J. (2006) *Anal Chem* **79**(9), 3280-3290
30. Angers, S., Salahpour, A., Joly, E., Hilairet, S., Chelsky, D., Dennis, M., and Bouvier, M. (2000) *Proc Natl Acad Sci U S A* **97**(7), 3684-3689
31. Cook, B. L., Ernberg, K. E., Chung, H., and Zhang, S. (2008) *PLoS One* **3**(8), e2920
32. Milligan, G., and Bouvier, M. (2005) *Febs J* **272**(12), 2914-2925
33. Bacart, J., Corbel, C., Jockers, R., Bach, S., and Couturier, C. (2008) *Biotechnol J* **3**(3), 311-324
34. Ayoub, M. A., Couturier, C., Lucas-Meunier, E., Angers, S., Fossier, P., Bouvier, M., and Jockers, R. (2002) *J Biol Chem* **277**(24), 21522-21528
35. Vidic, J. M., Grosclaude, J., Persuy, M. A., Aioun, J., Salesse, R., and Pajot-Augy, E. (2006) *Lab Chip* **6**(8), 1026-1032
36. Jacquier, V., Pick, H., and Vogel, H. (2006) *J Neurochem* **97**(2), 537-544
37. Gurevich, V. V., and Gurevich, E. V. (2008) *Trends Pharmacol Sci* **29**(5), 234-240
38. Margeta-Mitrovic, M., Jan, Y. N., and Jan, L. Y. (2000) *Neuron* **27**(1), 97-106
39. Issafras, H., Angers, S., Bulenger, S., Blanpain, C., Parmentier, M., Labbe-Jullic, C., Bouvier, M., and Marullo, S. (2002) *J Biol Chem* **277**(38), 34666-34673
40. Terrillon, S., Durroux, T., Mouillac, B., Breit, A., Ayoub, M. A., Taulan, M., Jockers, R., Barberis, C., and Bouvier, M. (2003) *Mol Endocrinol* **17**(4), 677-691
41. Salahpour, A., Angers, S., Mercier, J. F., Lagace, M., Marullo, S., and Bouvier, M. (2004) *J Biol Chem* **279**(32), 33390-33397
42. Herrick-Davis, K., Weaver, B. A., Grinde, E., and Mazurkiewicz, J. E. (2006) *J Biol Chem* **281**(37), 27109-27116
43. Guan, R., Feng, X., Wu, X., Zhang, M., Zhang, X., Hebert, T. E., and Segaloff, D. L. (2009) *J Biol Chem* **284**(12), 7483-7494
44. Hebert, T. E., Gales, C., and Rebois, R. V. (2006) *Cell Biochem Biophys* **45**(1), 85-109
45. Larsson, M. C., Domingos, A. I., Jones, W. D., Chiappe, M. E., Amrein, H., and Vosshall, L. B. (2004) *Neuron* **43**(5), 703-714
46. Armstrong, D., and Strange, P. G. (2001) *J Biol Chem* **276**(25), 22621-22629
47. Baker, J. G., and Hill, S. J. (2007) *Trends Pharmacol Sci* **28**(8), 374-381
48. Kenworthy, A. (2002) *Trends Biochem Sci* **27**(9), 435-437
49. Kobila, B. K., and Deupi, X. (2007) *Trends Pharmacol Sci* **28**(8), 397-406
50. Audet, N., Gales, C., Archer-Lahlou, E., Vallieres, M., Schiller, P. W., Bouvier, M., and Pineyro, G. (2008) *J Biol Chem* **283**(22), 15078-15088
51. Cheng, Z. J., and Miller, L. J. (2001) J Biol Chem 276(51), 48040-48047

FOOTNOTES

We are grateful to Ralf Jockers and Jean-Louis Banères for their fruitful advises. This work was funded by INRA and Agence Nationale de la Recherche (NOSE, ANR-07-PCVI-0027-01)

TABLE

Table 1: Primers used for PCR amplifications. The restriction sites are underlined, and the linker sequence is in bold letters.

| Primers | Sequences 5′- 3′ | Restriction site |
|---------|------------------|------------------|
| 1 (sense) | GGACCAAGATCTCAGCCAGAATCTGGGGCCAATGGA | BglII |
| 1 (antisense) | GCACCCTCACACAGCCAGTGCCTCCTCCCTGTGA | |
| 2 (sense) | ACTGGCTGGTGAGCGTGCTGGTTTATTTGGTGAGCAAGGGCGAGGA | AsiSI |
| 2 (antisense) | ACCTCGGCGATCGCTTACTTGTACAGCTCGTCCATGCC | |
| 3 (sense) | ACTGGCTGGTGAGCGTGCTGGTTTATTTGGTGAGCAAGGGCGAGGA | AsiSI |
| 3 (antisense) | ACCTCGGCGATCGCTTACTTGTACAGCTCGTCCATGCC | |
| 4 (sense) | GGACCAAGATCTCAGCCAGAATCTGGGGCCAATGGA | BglII |
| 4 (antisense) | ACCTCGGCGATCGCTTACTTGTACAGCTCGTCCATGCC | AsiSI |
| 5 (sense) | GGACCAAGATCTCAGCCAGAATCTGGGGCCAATGGA | BglII |
| 5 (antisense) | ACCTCGGCGATCGCTTACTTGTACAGCTCGTCCATGCC | AsiSI |

FIGURE LEGENDS

Fig. 1. OR1740 functional response to helional or vanillin stimulation, as a function of odorant concentration (redrawn from reference 6). Differential SPR response to odorants relative to controls obtained by replacing odorant with water.

Fig. 2. Visualization by immunoblotting of the cmyc-OR1740 receptor heterologously expressed in Saccharomyces cerevisiae. Expression of the receptor was induced by galactose during 108h at 15°C. The receptor was detected using a monoclonal anti-cmyc antibody. The bands at near 34 kDa and 55 kDa correspond respectively to the monomer and dimer forms of the OR1740 receptor.

Fig. 3. OR1740 receptor homo-oligomerization monitored by co-immunoprecipitation. Crude membranes from S. cerevisiae co-expressing cmyc-OR1740 and HA-OR1740 (lane 1), or expressing cmyc-OR1740 alone (lane 2), were immunoprecipitated using anti-cmyc antibody. The immunoprecipitated proteins were resolved by SDS-PAGE and immunblotted using anti-HA antibody. IP: immunoprecipitation, IB: immunoblotting, Mw: molecular weight (kDa).

Fig. 4. Investigation of OR1740 receptor homo-oligomerization by BRET. A, BRET level was measured either from disrupted yeast cells co-expressing OR1740-Rluc (donor) and OR1740-EYFP (acceptor), or from crude membranes prepared from these cells, with 5 µM of coelenterazine h. The data are shown as the normalized BRET ratio. B, BRET assays performed from clones with variable acceptor (OR1740-EYFP) to donor (OR1740-Rluc) ratios. BRET levels are plotted as a
function of acceptor to donor ratio, estimated from immunoblots as described in the Materials and Methods section. Data are representative of at least three independent experiments.

Fig. 5. Investigation of the OR1740 receptor homo-oligomerization at the subcellular level by BRET. A, BRET level was measured in each cellular membrane subfraction, obtained by sucrose gradient fractionation from crude membranes of yeast co-expressing OR1740-Rluc and OR1740-YFP. B, all subfractions were analysed by immunoblotting with antibodies targeting the yeast ER and plasma membrane markers, Dpm1p (Mw = 30 kDa) and Pma1 (Mw = kDa) respectively.

Fig. 6. Odorant-induced BRET modulation. A, crude membranes from three different clones co-expressing OR1740-Rluc and OR1740-EYFP were used to perform BRET assays without or with odorants (helional as an OR1740 agonist, octanal and vanillin as negative controls). B, The BRET level was measured in membrane subfractions 1, 4, 5 (ER) and 11, 12 (plasma membrane) from clone 2 with helional and without odorant as a reference. (**) shows significant differences estimated by Student test (P < 0.05). Data are representative of three independent experiments.

Fig. 7. BRET level variation upon OR1740 stimulation with various helional concentrations. BRET measurements were performed using crude membranes from yeast cells co-expressing OR1740-Rluc and OR1740-EYFP, or expressing OR1740-Rluc alone. Receptors were stimulated with various concentrations of helional. Results are expressed as the relative variation of BRET level at the various odorant concentrations. Data are representative of three independent experiments.
Figure 5

A

B

mBRET ratio vs. fraction number

1 2 3 4 5 6 7 8 9 10 11 12

Pma1

Dpm1p
Figure 6

A

![Graph A showing mBret ratio for different clones with and without helional, octanal, and vanillin.](image)

B

![Graph B showing mBRET ratio for different fraction numbers with and without helional.](image)

yeast clones co-expressing OR1740-RLuc and OR1740-EYFP
Figure 7
Relationship between homo-oligomerization of a mammalian olfactory receptor and its activation state demonstrated by bioluminescence resonance energy transfer (BRET)

Fallou Wade, Agathe Espagne, Marie-Annick Persuy, Jasmina Vidić, Régine Monnerie, Fabienne Merola, Edith Pajot-Augy and Guenhaël Sanz

J. Biol. Chem. published online March 17, 2011

Access the most updated version of this article at doi: 10.1074/jbc.M110.184580

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts