Chem. Senses 27: 691–701, 2002

Porcine Odorant-binding Protein Selectively Binds to a Human Olfactory Receptor

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Abstract

Odorant-binding proteins (OBPs) represent a highly abundant class of proteins secreted in the nasal mucus by the olfactory neuroepithelium. These proteins display binding affinity for a variety of odorant molecules, thereby assuming the role of carrier during olfactory perception. However, no specific interaction between OBP and olfactory receptors (ORs) has yet been shown and early events in olfaction remain so far poorly understood at a molecular level. Two human ORs, OR 17–209 and OR 17–210, were fused to a Green Fluorescent Protein and stably expressed in COS-7 cell lines. Interaction with OBP was investigated using a highly purified radioiodinated porcine OBP (pOBP) preparation, devoid of any ligand in its binding cavity. No specific binding of the pOBP tracer could be detected with OR 17–209. In contrast, OR 17–210 exhibited specific saturable binding (K_d = 9.48 nM) corresponding to the presence of a single class of high-affinity binding sites (B_{max} = 65.8 fmol/mg prot). Association and dissociation kinetics further confirmed high-affinity interaction between pOBP and OR 17–210. Autoradiographic studies of labeled pOBP to newborn mouse slices revealed the presence of multiple specific binding sites located mainly in olfactory tissue but also in several other peripheral tissues. Our data thus demonstrate a high-affinity interaction between OBP and OR, indicating that under physiological conditions, ORs may be specifically associated with an OBP partner in the absence of odorant. This provides further evidence of a novel role for OBP in the mechanism of olfactory perception.

Introduction

The sense of smell allows the perception and discrimination of thousands of odorant molecules in living organisms from invertebrates to mammals. Such chemical signalling modulates the social behaviour of most animal species which rely on odorant compounds to identify kin, mate, to locate food or to recognize territory. Olfactory perception is based on the activation by odorant molecules of G-coupled receptors, designed as olfactory receptors (ORs) and located at the cilia of olfactory neuronal endings (Breer, 1994). Odorants, which are commonly hydrophobic molecules, have to be conveyed from the air through the aqueous nasal mucus by carriers. Odorant-binding proteins (OBPs) have been thought to play such a functional role (Pelosi, 1996). These low-molecular soluble molecules are similar to retinol-binding protein and belong to the lipocalin family [for review, see Flower (Flower, 1994)]. They are physiologically secreted in high amounts in the nasal mucus layer [for review, see Flower (Flower, 1996)] but, despite a putative odorant carrier function, their precise role in early olfactory perception remains unclear (Tegoni *et al.*, 2000). In particular, a potential recognition between OBP and OR has not yet been clearly established and therefore could be an important key in the understanding of this complex mechanism.

Olfactory receptors are encoded by several hundreds of genes in mammals, possibly representing 3% of their genome (Mombaerts, 2001). Full or partial OR sequences have been cloned from genomic DNA or olfactory epithelium cDNA in humans and in other vertebrates (dog, pig, rodents, chicken, catfish) as well as in invertebrates (*C. elegans* and *Drosophila*) [for review, see Mombaerts (Mombaerts, 1999)]. Interestingly, OR expression is not restricted to the olfactory

epithelium but has also been observed in other tissues like testis (Parmentier *et al.*, 1992; Thomas *et al.*, 1996), notochord (Nef and Nef, 1997), insulin-secreting beta cells, spleen and heart (Blache *et al.*, 1998), where their function remains to be deciphered. Pioneering studies have attempted to elucidate OR function by expressing gene sequences in either insect or mammalian cells and screening for odorant signalling (Raming *et al.*, 1993; Krautwurst *et al.*, 1998; Wetzel *et al.*, 1999). Specific recognition of odorant molecules has been shown to be lilial and lyral for rat OR 5 receptor (Raming *et al.*, 1993), heptanal for mouse OR 17 (Krautwurst *et al.*, 1998) and helional for human OR 17–40 (Wetzel *et al.*, 1999). Even though heterologous OR expression appeared to suffer from limited cell surface targeting, these data formally demonstrated a direct recognition of the odorant ligand by the receptor that occurred in the absence of an OBP. More recently, a survey of mammalian OR specificity based on a set of structurally related odorants demonstrated that a single OR could recognize different odorant molecules sharing the same odotope and that a single odorant is recognized by multiple ORs (Malnic *et al.*, 1999). Such a broad recognition mechanism in perception events suggests the existence of a combinatorial receptor coding scheme to encode odor identities, contributing to olfactory discrimination.

The cascade of perireceptor events encompassing the recognition of odorant by the intermediate of a carrier protein has also remained very elusive. OBPs are very abundant in the olfactory mucus, suggesting that these molecules may transport odorants to ORs (Pevsner *et al.*, 1985). Bovine OBP (bOBP) was first discovered for its ability to bind 2-isobutyl-3-metoxypyrazine (IBMP), described as 'bell pepper smell' (Pelosi *et al.*, 1982). Its crystallographic structure consists of an homodimeric protein, exhibiting a domain-swapping mechanism and containing an endogenous ligand in its internal binding cavity (Bianchet *et al.*, 1996; Tegoni *et al.*, 1996). Recently, the X-ray structure of pOBP has been also determined (Spinelli *et al.*, 1998). Despite the fact that pOBP and bOBP are folded in the typical β-barrel lipocalin structure, pOBP is monomeric and devoid of naturally occurring bound ligand. However, a conformational stability study of pOBP further revealed the existence of a monomer–dimer equilibrium depending on experimental conditions (Burova *et al.*, 1999).

Interestingly, a wide variety of odorant compounds were shown to bind to bOBP (Pevsner *et al.*, 1990) as well as pOBP, within a micromolar range of affinity. It has been recently demonstrated that odorants of unrelated chemical structure can bind to pOBP with similar affinities by interacting with different amino acid residues in the binding pocket (Vincent *et al.*, 2000). As a result, OBPs may trap unrelated odorants and function as scavengers for lipophilic ligands [reviewed by Pelosi (Pelosi, 1994)]. Therefore, OBP is currently thought to exert its function *in vivo* by regulating odorant delivery across the hydrophilic mucosal barrier to the membrane receptors located at the cilia tips of the olfactory neuron dendritic knob. A role for OBP in odorant removal to terminate the odorant response has also been proposed (Boudjelal *et al.*, 1996). In this case, OBPs would be involved in a detoxification role, common to several members of the lipocalin family, and would thereby prevent receptor desensitization. To assess further such a dual role for OBPs, it would be of importance to determine whether these mechanisms occur sequentially or are controlled by binding specificity and odorant concentration at the OR level.

Low-affinity binding sites for bOBP have been described in nasal and respiratory epithelia (Boudjelal *et al.*, 1996), supporting the hypothesis that OBP binds outside the olfactory tissue, but evidence that these sites account for ORs has not yet been ascertained. Our understanding of the molecular interaction of odorants with OBPs and ORs is thus rather limited at a time when molecular cloning of ORs and genome sequencing are providing us with hundreds of orphan genes which require functional identification. In this study, we addressed a putative OBP–OR recognition by taking advantage of known full-length gene sequences of ORs from human genome. Among the 16 OR genes present in the locus p13.3 of chromosome 17, we first identified two orphan genomic sequences as being expressed specifically in human nasal epithelium. Using purified radiolabelled porcine OBP and genetically engineered human ORs, we have been able to show a differential pOBP binding to the receptors. To our knowledge, these data provide the first evidence of a selective recognition between OBP and OR. Interestingly, this interaction was found to be of high affinity, suggesting that the OBP–OR complex may very well occur under physiological conditions.

Materials and methods

Animals and chemicals

BALB/c mice were obtained from IffaCredo (Orléans, France) and were housed in a temperature-, humidity- and light-controlled room with free access to food and water. The pOBP (also called pOBP-I, major component of porcine nasal mucus) was kindly provided by Professor Paolo Pelosi. The protein was obtained and purified according to the procedure described by Dal Monte *et al.* (Dal Monte *et al.*, 1991). Molecular biology products (restriction enzymes and synthetic oligonucleotides) were purchased from Eurogentec Belgium; polymerase enzymes from Roche Molecular Biochemicals; polynucleotide kinases from Promega; human testis cDNA and plasmids from Clontech. Cell culture reagents; DMEM, fetal calf serum, trypsine/EDTA, geneticin, gentamicin and fungizone were purchased from Invitrogen. Biochemical products were from Sigma-Aldrich (1,10-phenantroline), ICN Biomedicals ([^{125}I]Na) and Calbiochem (lactoperoxidase).

Expression of ORs in human olfactory epithelium and testis

Genomic DNA was isolated from blood cells (kindly provided by Dr Francis Castet, CNRS, Marseille). Olfactory cDNA library was prepared from human middle turbinate tissue using the Stratagene vector lZAPII (Crowe *et al*., 1996). Specific internal primers for PCR amplification and for sequencing were designed to amplify specific OR genes from a human chromosome 17 cluster (17p13.3), based on the available sequence (Rouquier *et al*., 1998) as follows: OR 17–201, 5′-CTCTTGTCCCACAAGTCC-3′ and 5′-TTATCCTTGTCTGAAA-3′ (370 bp); OR 17–209, 5′-GACTGCTACGTGGGCATA-3′ and 5′-TGTGAGCT-GCAGGTGGAA-3′ (373 bp); OR 17–210, 5′-ATGGCT-TATCACTGCTAT-3′ and 5′-GTGGAGAAAGCCTTC-TGG-3′ (370 bp). One nanogram of DNA was used in each of a series of polymerase chain reaction (PCR) experiments containing 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 5 mM dNTP, 1.5 U of Taq DNA polymerase (Boehringer-Mannheim) and 100 pmol of each primer. PCR mixtures using the olfactory or testis cDNA (Human testis Quick-Clone^{TM} cDNA, Clontech) library has been carried out in the presence of 3mM $MgCl_2$ according to the following schedule: 94°C (90 s, 1 cycle), 94°C (20 s), 50°C (25 s), 72°C (30 s, 40 cycles) and 72°C (120 s, 1 cycle). PCR reactions were analysed by gel electrophoresis in 1% agarose, further purified and sequenced to verify the specific amplification of ORs.

Construction of p-EGFP/OR 17–209 and p-EGFP/OR 17–210

The intronless genes encoding OR 17–209 and OR 17–210 putative olfactory receptors from human chromosome 17 (p13.3) were amplified by PCR using the cosmid no. ICRF105cF06137 (GenBank HSU53583). In order to create a GFP C-terminal fusion protein, OR 17–209 and OR 17–210 genes were mutated to eliminate the stop codon. The substitution of the stop codon with a *Bam*HI restriction site allowed in-frame subcloning of the new sequence with GFP cDNA, in the pEGFP-N1 mammalian expression plasmid (Clontech). The following oligonucleotidic sense (S) and antisense (AS) primers were used (Eurogentec): OR-209 *Hin*dIII S: 5′-TAA GAA GCT TGC CAC CAT GGA GGG GAA AAA TCT G-3′; OR-209 stop/*Bam*HI: 5′-ATT AGG ATC CCC AGG GGA ATG AAT TTT CCG-3′; OR-210 *Hin*dIII S: 5′-CTG TAG GTG TTA AGG TGC ATT-3′; OR-210 stop/*Bam*HI: 5′-ATT AGG ATC CCC AGC CAC TGA TTT AGA GTG-3′. PCR experiments containing 10 ng of cosmid DNA preparation, 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 0.001% gelatin, 0.2 mM dNTP, 1.5 U of high-fidelity Expand^{TM} mix of polymerases (Roche Molecular Biochemicals) and 100 pmol of each primer were performed according to the following schedule: 94°C (90 s, 1 cycle), 94°C (20 s), 50°C (25 s), 72°C (90 s, 40 cycles) and 72°C (120 s, 1 cycle). The pEGFP-N1 recombinant clones were isolated using *Hin*dIII/*Bam*HI restriction enzymes (Eurogentec) and were fully sequenced to assess the expected inserted DNA sequence.

Construction of pS 5HT1$_C$

The membrane import sequence of the human 5H-T_{1C} receptor was synthetically reconstituted. Four sense oligonucleotides and four antisense oligonucleotides corresponding to the complete 5H-T_{1C} membrane import sequence were synthesized. One microgram of each internal oligonucleotides was phosporylated using 1 U of polynucleotide kinase (Promega) in a kinase buffer containing 2 mM of ATP. Reaction was performed for 60 min at 37°C, and the incubation finally inactivated for 10 min at 65°C. All the oligonucleotides were denatured for 10 min at 80°C and then mixed for matching. Matching was performed overnight with a decreasing temperature gradient from 80 to 20°C. The resulting fragment was then ligated into the *Bgl*2 and *Hin*dIII sites of the pEGFP-N1 plasmid in frame with the N-terminal OR sequences.

Cell culture and transfection

COS-7 cells (ATCC reference: CRL-1651) were cultured in DMEM supplemented with 10% heat-inactivated fetal calf serum and containing 50 μg/ml gentamicin and 2.5 μg/ml fungizone, in a humidified incubator (95% O_2, 5% CO_2) at 37°C. Stable transfection (with either 5HT1$_C$-OR-209-GFP or 5HT1$_C$-OR-210-GFP construct) was performed onto semiconfluent COS-7 cells grown in 100 mm cell culture dishes with 2 μg of recombinant pEGFP-N1 plasmid using the lipofectAMINE^{TM} (Gibco) reagent according to the manufacturer's recommandations. After 2 days, cells were cultivated with 0.5 g/l of geneticin.

Confocal microscopy

All fixation steps were performed on ice to prevent receptor internalization. COS-7 cells were fixed in 3% paraformaldehyde buffer for 20 min. Fixed cells were washed with 0.05 M NH_4Cl for 10 min, and then twice with PBS for 5 min. Cells were fixed to slides with Mowiol (Vector) and dried for 12 h. Images of fluorescent (GFP) cells were obtained using a Leica TCS 4D microscope with excitation at 488 nm.

Iodination of pOBP

The pOBP was submitted to iodination using the lactoperoxidase method (Marchalonis, 1969). Briefly, 2 nmol of purified pOBP in 50 mM borate/phosphate buffer (pH 7.5) were incubated with 2 mCi of [^{125}I]Na, 10 μg of lactoperoxidase and 12 μl of H_2O_2 (100% diluted 800 times). After 15 min of incubation, the reaction was stopped by dilution in 0.1% trifluoroacetic acid (TFA). The iodination mixture was purified by HPLC onto a nucleosil column (C18, 300 A, 5 μm) eluted at a flow rate of 1 ml/min in 0.1% TFA with an initial 5 min isocratic 20% acetonitrile step, followed by a 45 min linear gradient from 20 to 60%

acetonitrile. The chromatography was followed by automatic recording of absorbance at 275 nm and radioactivity. Fractions were collected into low absorption tubes, diluted in 50 mM Tris–HCl buffer (pH 7.5), containing 10% BSA, aliquoted and stored at –20°C until use.

SDS–PAGE analysis of the radiolabelled pOBP fractions

Fractions of each radiolabeled pOBP (25 000 c.p.m.) were heated at 95°C for 5 min in the presence of 5 % of β-mercaptoethanol. Samples were submitted to SDS–PAGE analysis according to the procedure of Laemmli (Laemmli, 1970) on a 14 % polyacrylamide resolving gel. The coloured wide-range prestained protein marker from Sigma was used as a standard. The gel was fixed for 10 min in 10% (v/v) acetic acid and dried under vacuum before radioautography on Kodak X-OMAT AR film for 1 week.

Binding of ^{125}I-OBP on cells expressing OR 17–209 or OR 17–210 receptors

Untransfected cells and transfected cells, with either $5HT1_C$-OR-209-GFP or $5HT1_C$-OR-210-GFP construct, were grown in 24-well plates until confluence, and were washed twice with 0.5 ml of binding buffer (Earle–HEPES buffer pH 7.4, supplemented with 0.09% glucose and 2% BSA) and then incubated for 15 min at 37°C in the same buffer. Association kinetics were followed by measuring the binding of 0.4 nM ^{125}I-labelled OBP to OR-expressing cells at different times. After 60 min of association, the dissociation kinetic was initiated by adding 10 µM unlabelled pOBP to the incubation medium. In saturation experiments, whole cells were incubated for 25 min at 37°C with increasing concentration of ^{125}I-labelled OBP (0.05–18.4 nM). At the end of the incubation period, cells were then washed twice with 0.5 ml of ice-cold binding buffer and harvested in 1 ml 0.1 M NaOH. The radioactivity bound to the cells was measured with a gamma counter (Packard, counting efficiency 80%). Non-specific binding was measured in the presence of a excess of unlabelled OBP (10 µM) and substracted from total binding to obtain specific binding. No specific binding was observed on untransfected COS-7 cells (data not shown). k_a, k_d and K_d (k_a/k_d) values were calculated as previously described (Dal Fara *et al.*, 2000).

Binding and radioautography on whole mouse slice

Slide-mounted sections of newborn (P15) BALB/c mice (IffaCredo, Orléans, France) were prepared as previously described (Gaudriault *et al.*, 1994). Preincubation was performed for 15 min at 4°C in a 50 mM Tris–HCl buffer (pH 7.4) containing 5% BSA and 0.8 mM 1,10-phenantroline (Sigma-Aldrich), a metalloprotease inhibitor. Slices were incubated with 2×10^6 c.p.m. (1.4 nM) of radiolabelled pOBP in a 400 µl final volume of the same buffer for 30 min at room temperature. Non-specific binding was determined in the presence of an excess (10 µM) of unlabelled pOBP. Sections were then washed three times for 5 min in the ice-cold buffer containing 2% BSA and 0.8 mM 1,10-phenantroline, and finally washed in ice-cold distilled water (without BSA) for 5 min. Slide-mounted sections were apposed to a beta Max Hyperfilm (Amersham) in a Kodak X-ray film cassette for 8 weeks at –70°C.

Results

Screening cDNA libraries for OR expression

Putative olfactory sequences reported from earlier studies have been cloned from human testis (Parmentier *et al.*, 1992) while most available OR sequences have been deduced from genomic DNA. Many of them have been located in clusters, and of these, the p13–3 locus of chromosome 17 has been extensively studied (Ben-Arie *et al.*, 1994; Rouquier *et al.*, 1998). Three human genes distributed in this locus and encoding putative olfactory receptors (OR 17–201, OR 17–209 and OR 17–210) were selected to initiate this study. Their full-length sequence was intronless like all other mammalian OR genes reported so far, and presented an open reading frame suited for heterologous protein expression.

Since no information was available on physiological expression of these genes, we first investigated the presence of such sequences in cDNA libraries of both human olfactory epithelium and testis. Using specific primers designed to map the whole set of ORs located in the locus 17p13.3, we showed that OR 17–209 and 17–210 are actually expressed in the olfactory library, described elsewhere (Crowe *et al.*, 1996) but not in the testis library. Indeed, gel analysis (Figure 1) of the PCR products revealed amplified material for OR 17–209 and OR 17–210 only in the olfactory cDNA library with a single band at the expected size (370 bp). Sequencing PCR products revealed a single sequence per band corresponding to OR 17–209 and OR 17–210 respectively, while OR 17–201 could not be identified. Of note, OR 17–24 and OR 17–40 were also identified in the olfactory

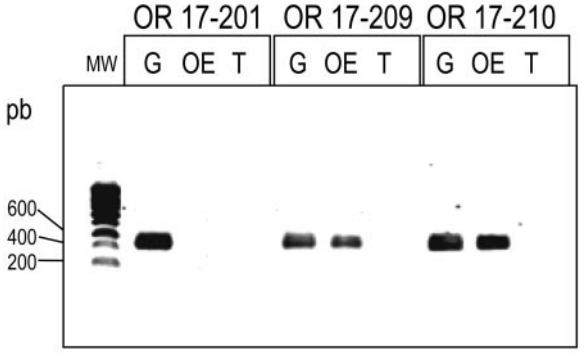


Figure 1 Expression of human ORs 17–201, 17–209 and 17–210 in olfactory epithelium and testis. Internal primers of OR 17 genes have been designed to amplify PCR products of ~370 bp to screen a human cDNA library of olfactory mucosa or testis. G, genomic control; MW, low mol. wt marker (bp); OE, olfactory epithelium cDNA library; T, testis library).

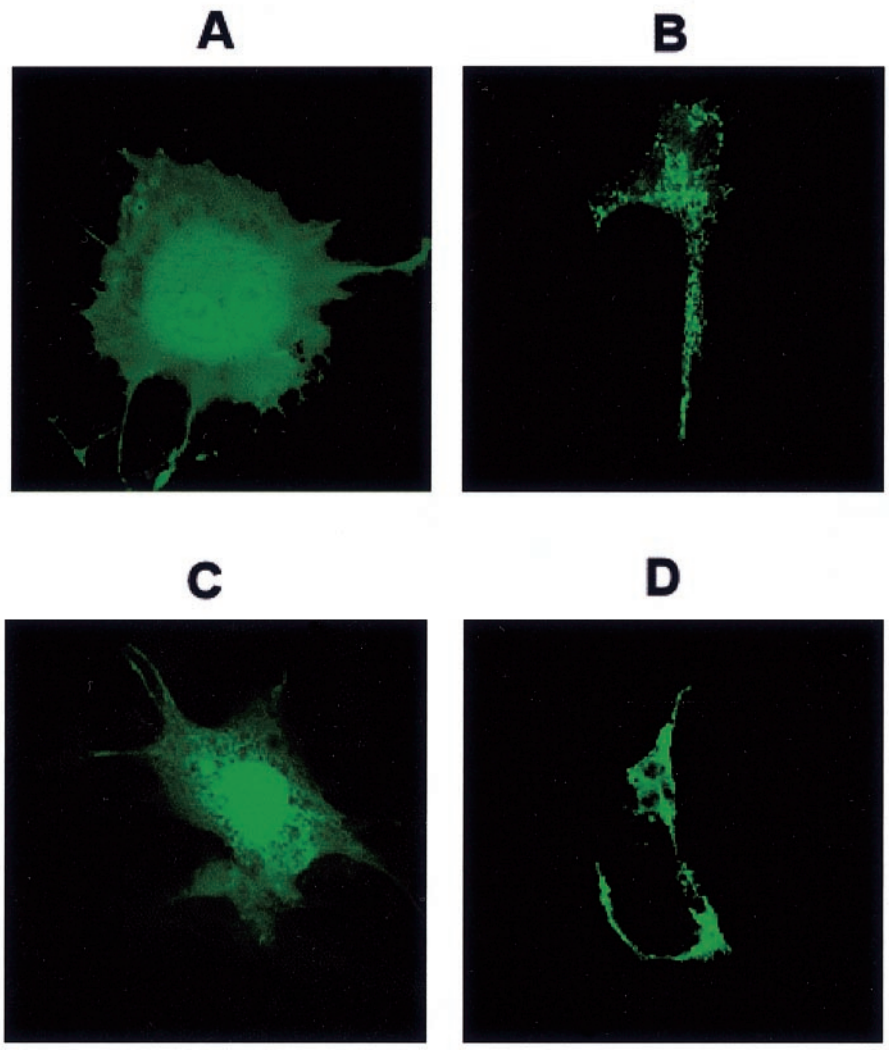


Figure 2 Expression of OR 17–209 and OR 17–210 proteins in COS-7 cells. Stably transfected cells expressing OR 17–209 **(A, B)** and OR 17–210 **(C, D)** fused to GFP at the C-terminus, and constructed without **(A, C)** or with **(B, D)** an N-terminal 5HT1c signal sequence at the N-terminus, were visualized by fluorescence microscopy after fixation.

library. Since these latter could also been found in testis (Vanderhaeghen *et al*., 1997), our findings further support the view of a differential expression of the OR repertoire in the olfactory epithelium.

Both OR 17–209 and OR 17–210 receptors have not yet been characterized for biological function and odorant recognition. Their protein sequences share 45% homology and showed a very distinct hydropathy profile with seven putative transmembrane domains (data not shown). When aligned with bovine rhodopsin, whose structure has been recently elucidated (Palczewski *et al*., 2000), OR 17–209 showed 50% identity with the photoreceptor while OR 17–210 exhibited only 36% identity with a remarkably short N-terminus. These data strengthen the bioinformatic analysis indicating that both proteins belong to two distinct OR subfamilies.

Heterologous expression of OR 17–209 and OR 17–210

Plasmids containing full-length OR sequences were then constructed for protein expression in mammalian cells. Earlier work from another group reported that expression of ORs at the surface of host cells is limited by unknown factors (Gimelbrant *et al*., 1999). A significant improvement could be observed by adding an heterologous import signal peptide to address receptor biosynthesis to the endoplasmic reticulum and facilitate proper intracellular sorting (Wetzel *et al*., 1999). Stably transfected COS-7 cells expressing the OR 17–209 and OR 17–210 gene sequences fused with the GFP protein revealed abundant fluorescent protein material, apparently addressed to the perinucleus region of the cells and probably retained in the endoplasmic reticulum (Figure 2A,C). To address OR proteins to the plasma membrane, we took advantage of the 5H-T_{1c} signal peptide, reported to

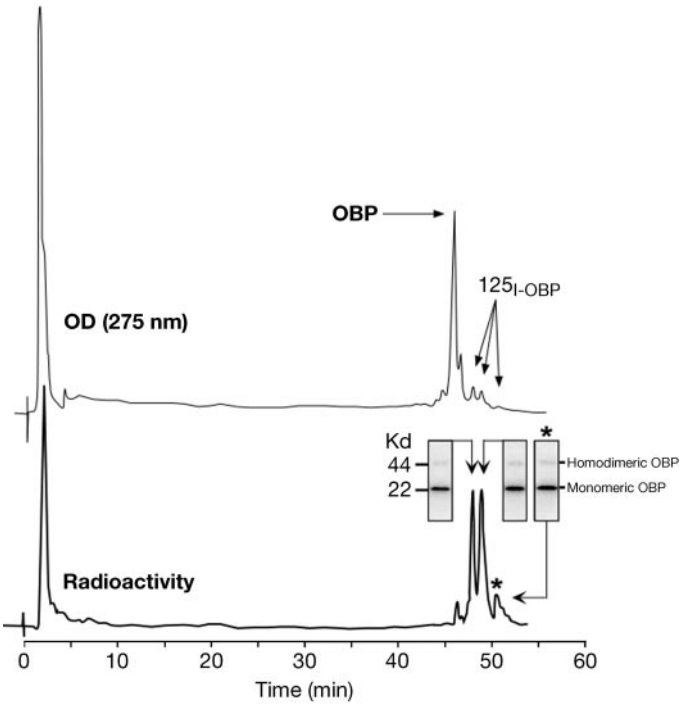


Figure 3 HPLC separation of [^{125}I]pOBP. Reverse-phase HPLC of iodinated pOBP was carried out as described in Materials and methods. Porcine OBP fractions were monitored by recording optical density at 275 nm and measurement of radioactivity. Inserts show SDS–PAGE analysis of radiolabelled pOBP fractions (25 000 c.p.m.) after disulphide bond reduction and (*) indicates the fraction with biological activity.

facilitate intracellular trafficking of unrelated receptors (Wetzel *et al*., 1999). As shown in Figure 2B,D, the presence of such heterologous peptide remarkably shifted the routing of OR chimeric proteins toward the cell surface. In addition to the labelling of intracellular compartments, numerous clusters of fluorescent patches could be observed at the cell surface and in the periplasmic space, indicating that both receptor proteins now followed a more efficient migration within the cell. Since no import sequence is originally present in both OR genes, it is likely that in olfactory neurons, some accessory mechanism must be involved for these proteins to reach the dentritic knob endings (Gimelbrant *et al*., 2001).

Iodination and purification of radiolabelled pOBP

According to amino acid sequence analysis (Paolini *et al*., 1998), five tyrosine residues are potentially available for iodination. To prevent protein denaturation, derivatization of pOBP preparation was carried out under experimental conditions limiting iodine supply, in order to obtain a single iodinated tyrosine residue per molecule. Three distinct labelled products were thereby obtained and separated by reverse-phase HPLC (Figure 3). The ratio of one iodine atom per mole of protein was assessed in each fraction by optical density coupled to radioactivity measurements. This labelling procedure was reproducible, and monoiodination of the ligand was achieved in consecutive experiments. Purified, radioiodinated products were further analysed by gel electrophoresis followed by autoradiography. Two protein bands of apparent mol. wt 22 and 44 kDa were identified at the expected molecular size for monomeric and homodimeric pOBP respectively (Figure 3, inserts). Both protein forms were present in each fraction with the monomeric form significantly predominant over the dimeric form. Since gel analysis was carried out under denaturing conditions, it appeared that pOBP dimers can partially resist reduction by forming strong hydrophobic interactions, a molecular feature reported by Burova *et al.* (Burova *et al.*, 1999).

Binding experiments

In preliminary experiments, we tested [^{125}I]OBP binding to membranes isolated from COS-7 cell clones expressing either OR 17–209 or OR17–210. Isolating radiolabelled membranes onto various filters in the presence or absence of polyethylenimine always led to a high, non-specific binding, and therefore filtration procedures were not retained for further binding assays (data not shown). Instead, experiments performed on whole cells revealed a much lower level of non-specific binding. It has been recently observed that tagging another olfactory receptor (OR-17) by GFP located in C-terminal position does not affect ligand binding or downstream signaling (Ivic *et al*., 2002). Figure 4A shows that radiolabelled peaks 1 and 2 did not exhibit any detectable interaction with OR 17–209 or OR 17–210 transfected cells, indicating that these two iodinated OBP fractions probably lost their binding activity upon derivatization. In contrast, fraction 3 was found to bind to a much higher extent to OR 17–210 expressing cells compared to the OR 17–209 clone or untransfected cells (Figure 4A). Binding of the tracer could be totally displaced with a micromolar concentration of unlabelled OBP, thereby demonstrating that the iodinated product and the native protein display similar recognition of OR 17–210. This fraction was likely to contain an active iodinated form of pOBP and was therefore selected for binding experiments.

We then examined the kinetic parameters of [^{125}I]OBP interaction with OR 17–210 expressing cells. The association kinetics shown in Figure 4B indicated that [^{125}I]OBP specific binding was time-dependent and reached an apparent equilibrium at ~30 min with a half-maximal binding at 4–6 min. After 60 min of association, [^{125}I]OBP binding could be reversed by addition of 10 μM of unlabelled OBP to the cells. Half-maximal release of the tracer was also ~5 min. The calculated rate constants for association (k_a) and dissociation (k_d) were about 8 and 7 pM^{-1} min^{-1} respectively. These kinetic constants allowed us to determine an apparent K_d (k_d/k_a) of 8.77 nM.

As shown in Figure 5, saturation experiments demonstrated that the specific binding of OBP to OR 17–210

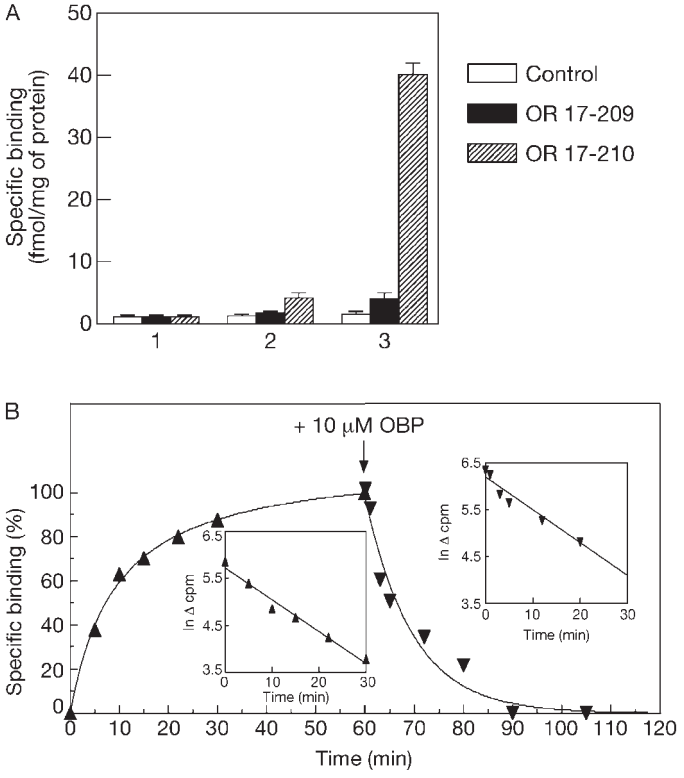


Figure 4 **(A)** Specific binding of radiolabelled fractions to untransfected (control) or transfected COS-7 cell line. **(B)** Representative experiments of the kinetics binding parameters of [^{125}I]OBP binding to OR 17–210 expressed in COS-7 cells. Main figure: Time course of association (▲) and dissociation (▼) of [^{125}I]pOBP (O.4 nM) to OR 17–210 expressing cells (160 μg protein/well). After 60 min of association at 37°C, 10 μM of unlabelled pOBP was added (arrow). Inserts: Pseudo-first order representation of data for both kinetics (▲, expressed in c.p.m., represents the difference between the curve and its asymptote).

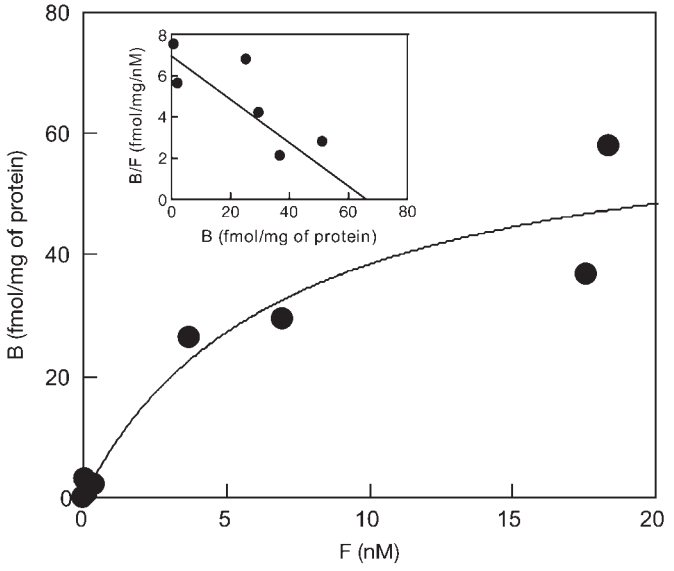


Figure 5 Representative experiments of the binding of [^{125}I]pOBP to OR 17–210 receptors expressed in COS-7 cells. Cells (160 μg protein/well) were incubated with increasing concentrations of pOBP (from 0.1 to 20 nM) containing a constant amount of tracer (100 000 c.p.m.). After 30 min at 37°C, the cells were washed and radioactivity was counted. Non-specific binding was determined in the presence of 10 μM of unlabelled OBP. Insert: Scatchard plots of [^{125}I]OBP specific binding to OR 17–210 expressed in COS-7 cells (B, bound; F, free ligand).

expressing cells was saturable. Scatchard analysis of binding data revealed a single population of high-affinity binding sites with an apparent K_d of 9.5 nM and a maximal binding capacity of 66 fmol/mg prot. Therefore, K_d values deduced from kinetic experiments (Figure 4B) and saturation experiments (Figure 5) were in good agreement. Successive preparations of iodinated pOBP show similar results to those represented in Figures 4 and 5.

Mass spectrometry was also performed in non-denaturing condition in order to ascertain whether our pOBP preparation contained any endogenous ligand. The protein was given a molecular mass of 17 690.27 ± 0.61 Da, which is in good agreement with the molecular mass deduced from the pOBP sequence. Porcine OBP preparation was also capable of binding odorant since its size increased after incubation with IBMP (S. Canarelli and O. Clot-Faybesse, unpublished data). It was thus concluded that pOBP was unliganded and functional. These findings demonstrate also that interaction between OBP and OR can physiologically occur in the absence of odorant.

Expression of [^{125}I]pOBP binding sites in mouse tissues

To further comfort the biological activity of the iodinated pOBP and potentially to assess binding sites in other tissues, we performed *in situ* binding on whole postnatal mice saggital sections. Slices were incubated with each of three radiolabeled pOBP fractions and non-specific binding was measured by displacing the tracer with 10 μM of unlabeled pOBP. As shown in Figure 6, only fraction 3 exhibited specific binding in these experiments. Non-specific binding was very weak with fraction 3 and specific binding was thus comparable to total binding. Again, fractions 1 and 2 failed to present any significant physiological binding, indicating that iodination resulted in a loss of activity for these two forms. In contrast, a strong signal was present with fraction 3 at the level of nasal fossa turbinates, as expected for a ligand that should bind to ORs located in the olfactory epithelium. When autoradiography was carried out at intermediate time intervals, the labelling intensity of nasal cavity was found to reach saturation rapidly, while the labelling of regions outside the nasal epithelium increased consistently. A 2 month exposure (Figure 6) allowed the visualization of specific peripheral binding in the following regions: nasal epithelium, tracheal, intervertebral, intercostal and hit joint cartilages, liver, stomach and intestinal surface, thymus, dorsal muscles, heart and possibly coronaries. These findings show that pOBP preferentially binds to nasal fossea but can also bind to several other regions, suggesting that

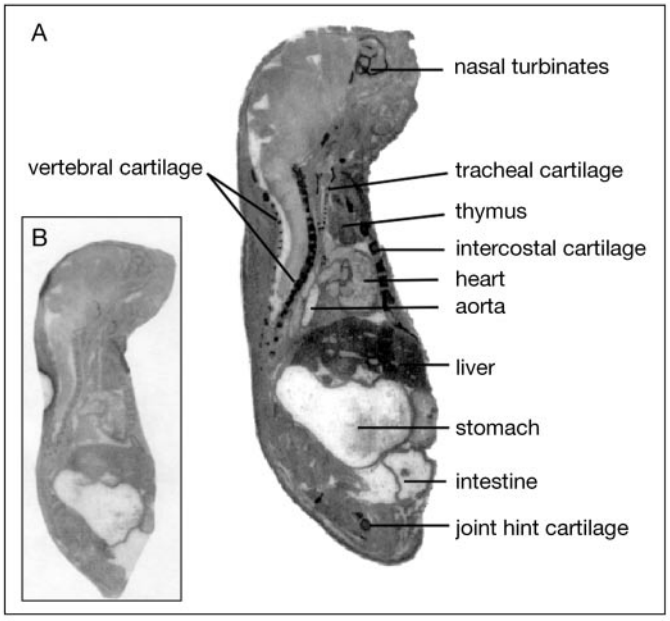


Figure 6 Distribution of [^{125}I]pOBP-binding sites in 15 day old mice. Autoradiograms were obtained from parasagittal (15 μm) sections of whole mice incubated with 1.4 nM of [^{125}I]pOBP for 30 min at room temperature. Total binding **(A)** and non-specific binding **(B)** were determined by incubation of excess of unlabelled pOBP (10 μM) on adjacent sections.

various tissues may express secondary binding sites for this protein.

Discussion

Odor perception occurs as a result of a cascade of biochemical and electrophysiological reactions in which OBP and OR molecules are key partners in odorant uptake. Despite a general agreement that OBPs are important to the signalling process, clear experimental evidence of their role in olfactory perception has not yet been produced and their precise function still remains elusive. In a attempt to elucidate if they are part of these early events, we decided to investigate a putative OBP–OR interaction using cell lines that express human ORs.

OR gene sequences are found at >25 locations in the human genome covering all chromosomes except chromosomes 20 and Y (Rouquier *et al.*, 1998). OR clusters have been identified on several chromosomes and ~100 genes have been partially or fully sequenced. So far, this large repertoire of protein receptors has not yet been fully elucidated for expression and specificity. Located on chromosome 17, one cluster of 16 OR genes, designated as the 17p13.3 locus, has been studied in considerable detail by several groups, including one of ours (Ben-Arie *et al.*, 1994; Rouquier *et al.*, 1998; Sharon *et al.*, 1999). In this cluster, OR genes were found to be distributed among two families and are thought to originate from duplication mechanisms. A large part (72%) of total human OR sequences, however, appeared to be pseudogenes (Rouquier *et al.*, 1998). Since it has been previously reported that a 17–40 OR gene transcript has been found to be expressed in adult nasal epithelium (Crowe *et al.*, 1996), it was of importance to determine for each OR originating from the human 17p13.3 cluster whether or not they also potentially display the same location. PCR screening in both adult nasal tissue and testis showed that OR 17–209 and OR 17–210 were found to be specifically present in an olfactory cDNA library. This suggested to us that both gene transcripts were putative candidates for odorant recognition. Using a chimeric construction, we have been able to obtain OR proteins successfully expressed at the surface of host cells and could thereby engineer stable cell lines.

To show a potential interaction between OBP- and OR-expressing cell lines, an OBP partner had to be selected at a time when no human OBP was cloned and thus available for homologous binding. We hypothesized that heterologous OBP–OR binding could happen, provided receptor recognition was an intrinsic function of OBP. This hypothesis was based on the observation that amino acid sequences are more conserved within OBPs from the same class, but different animal species, than between proteins of the same species [for review see Pelosi (Pelosi, 1996)], suggesting that structural differences of OBPs could be related to odorant-binding specificities, not to OR recognition. pOBP was an excellent candidate to address this issue because it was available in a highly purified form, its binding properties have been extensively studied (Burova *et al.*, 1999; Vincent *et al.*, 2000), and both its sequence (Paolini *et al.*, 1998) and its structure (Spinelli *et al.*, 1998; Perduca *et al.*, 2001) have been determined. The X-ray crystallography of pOBP (Spinelli *et al.*, 1998) revealed that this protein is monomeric and is devoid of naturally occurring bound ligand. In contrast, recent work based on the crystal structure of a monoclinic form of pOBP revealed the presence of an endogenous ligand (Perduca *et al.*, 2001). This discrepancy could be accounted for by the protocol used to purify pOBP from porcine mucus. An additional stage of extraction with organic solvent is needed to eliminate potential endogenous ligand in the binding cavity of the pOBP sample (Burova *et al.*, 1999). In this study, we assessed by mass spectrometry that the pOBP preparation used for binding studies was devoid of ligand.

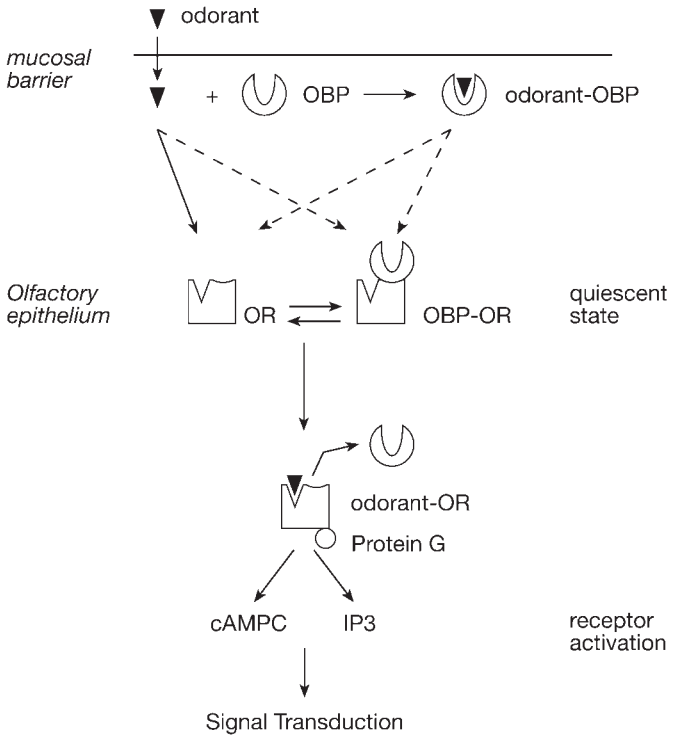
Based on sequence analysis, pOBP contains five tyrosine residues (Tyr^{20}, Tyr^{52}, Tyr^{78}, Tyr^{82} and Tyr^{92}) among the 157 amino acid residues. Upon monoiodination of the protein, three fractions differing in hydrophobicity were obtained, indicating that not all five tyrosine residues were accessible to derivatization in our experimental procedure. The two major labelled species (fractions 1 and 2) displayed no binding activity towards OR 17–209 or OR 17–210, while the minor peak (fraction 3) exhibited selective interaction with OR 17–210. These findings suggest that at least two tyrosine residues of pOBP are essential for receptor recognition. Regarding fraction 3, iodination is likely to occur on

a residue located distally from the OR binding site to generate to an active tracer.

In situ binding studies in mice further strongly supported the evidence of an heterologous recognition between OBP and target-binding sites. They confirmed the olfactory epithelium as the major target tissue for pOBP but also showed secondary binding sites. In mice, we found specific binding sites in many other locations such as tracheal, intervertebral, intercostal, hit joint cartilages, liver, stomach and intestinal surface, thymus, dorsal muscles, heart and coronaries. It is worth noting that OR expression has been also documented in many tissues outside the olfactory epithelium, e.g. germ cells and testis (Parmentier *et al*., 1992; Thomas *et al*., 1996), notochord (Nef and Nef, 1997), spleen and insulin-secreting β-cells (Blache *et al*., 1998), heart (Drutel *et al*., 1995; Ferrand *et al*., 1999) and possibly in distinct brain areas (Raming *et al*., 1998). Taken together, these findings suggest that the observed labelling of regions outside the olfactory epithelium may very well reflect the presence of peripheral OBP-binding sites. Since tissue specificity is of major relevance when defining the physiological role of a protein, the presence of ORs in these regions remains to be identified by *in situ* hybridization techniques. It would be also of interest to follow the distribution of these binding sites during ontogenesis to understand if OBP binding sites are dependent upon development.

Earlier work using radiolabelled bovine OBP (bOBP) (which like pOBP contains five tyrosine residues) showed binding to isolated membranes from olfactory and respiratory epithelium with an apparent K_d value of 2 μM (Boudjelal *et al*., 1996). Because such a low-affinity interaction is not encountered in the G-coupled receptor family, it was suggested that OBP may facilitate the transport of odorants rather than play a direct role in olfactory signal transduction. However, this study should be now reinvestigated in view of our current data since the major part of radiolabelled bOBP used at the time was probably inactive. Binding parameters have probably been largely underestimated in the absence of ligand purification. Indeed, binding experiments reported in the present study revealed that only a small fraction of pOBP is active upon iodination. With the use of this purified, radiolabelled ligand, we could demonstrate a high-affinity interaction between pOBP and OR. Furthermore, only one of the two expressed receptors displayed recognition of this ligand, suggesting that OBP may discriminate its OR partner. We are now investigating structurally related lipocalins of different origins to get a better understanding of the molecular basis supporting such an heterologous binding.

At the present time, the current understanding of olfactory perception is based on three main observations: (i) pOBP selectively binds a panel of odorants (Vincent *et al*., 2000); (ii) OR activation by odorant is not dependent upon the presence of OBP (Raming *et al*., 1993; Krautwurst *et al*., 1998; Malnic *et al*., 1999; Wetzel *et al*., 1999); and (iii) unliganded pOBP can selectively bind an OR as shown herein. The formation of an OBP–OR–odorant complex, to initiate olfactory response, is therefore questionable and remains to be established. However, the current data do not rule out the hypothesis that OBP may play a role in odorant removal to terminate the odorant response and prevent receptor desensitization. Scheme 1 summarizes a putative multistep mechanism for odorant capture, taking into account all the observations currently available. In the absence of odorant, unoccupied OBP selectively binds to a relevant receptor with high affinity and the OBP–OR binding stabilizes the receptor in a resting state. When the odorant concentration is very low, virtually no odorant can by itself reach the OR because its limited solubility prevents it from crossing the mucus layer, and its uptake by OBP is favoured instead. The odorant-loaded OBP may interact with an OR or an OBP–OR complex, depending on their relative affinity. The odorant is then released and could activate the OR. As the concentration increases, free odorant could also directly reach a specific OR or displace an OBP–OR complex. In either case, odorant–OR binding occurs and signal transduction can proceed. Under these conditions, any odorant can be addressed to and taken up specifically to a relevant OR, allowing discrimination and/or fine tuning of odorant recognition as a function of both the nature and the amount of odorant. When the ligand concentration is high, the scavenger role of OBP is dominant, thereby preventing binding site saturation and protecting the OR from desensi-



Scheme 1 Proposed mechanism for OBP and OR interaction in presence and absence of odorant.

tization. Elucidation of ligand specificity of OR 17–210 is now clearly needed to validate these complex events. It is also necessary to identify if OR and OBP subfamilies may respectively recognize structurally related odorants before it can be concluded that OBP–OR recognition is of general physiological relevance.

Acknowledgements

We are very thankful to Professor Paolo Pelosi for constructive discussion and reading of this manuscript. We are also indebted to Professor Anny Cupo for fruitful discussion and support. We acknowledge the contribution of Dr Valérie Navarro, Dr Stéphane Martin and Dr Philippe Sarret (IPMC, CNRS UMR 6097, Sophia Antipolis, France) in designing the binding experiments. We are especially grateful to Stephane Canarelli (EPFL, Lausanne, Switzerland) for mass sprectrometry analysis. This work was supported in part by the Association pour l'Enseignement et le Développement de la Recherche en Région Provence-Alpes-Côte-d'Azur (ADER PACA).

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Accepted July 16, 2002