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Characteristic features and ligand specificity of the two olfactory receptor classes from *Xenopus laevis*

Mario Mezler[1,2], Jörg Fleischer[1] and Heinz Breer[1,*]

[1]*University of Hohenheim, Institute of Physiology, 70593 Stuttgart, Germany* and [2]*Bayer AG, Agricultural Centre, MWF, Geb. 6240, Monheim, Germany*

*Author for correspondence (e-mail: breer@uni-hohenheim.de)

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Summary

Amphibia have two classes of olfactory receptors (ORs), class I (fish-like receptors) and class II (mammalian-like receptors). These two receptor classes correspond to the two classes identified in other vertebrates, and amphibians thus provide a unique opportunity to compare olfactory receptors of both classes in one animal species, without the constraints of evolutionary distance between different vertebrate orders, such as fish and mammals. We therefore identified the complete open reading frames of class I and class II ORs in *Xenopus laevis*. In addition to allowing a representative comparison of the deduced amino acid sequences between both receptor classes, we were also able to perform differential functional analysis. These studies revealed distinct class-specific motifs, particularly in the extracellular loops 2 and 3, which might be of importance for the interaction with odorants, as well as in the intracellular loops 2 and 3, which might be responsible for interactions with specific G-proteins. The results of functional expression studies in *Xenopus* oocytes, comparing distinct receptor types, support the idea that class I receptors are activated by water-soluble odorants, whereas class II receptors are activated by volatile compounds.

Key words: olfactory receptor, *Xenopus laevis*, sequence analysis, oocyte, heterologous expression.

Introduction

In vertebrates, perception of distinct odors results from the interaction of odorous molecules with defined sets of olfactory sensory cells in the nasal neuroepithelium (Sicard and Holley, 1984). The chemospecificity of olfactory sensory cells is supposed to be determined by the olfactory receptor types that they express (Buck and Axel, 1991). Recently the selective interaction of olfactory receptors with distinct odorants has been demonstrated in heterologous expression studies employing divergent expression systems (Raming et al., 1993; Kiefer et al., 1996; Wellerdieck et al., 1997; Krautwurst et al., 1998; Zhao et al., 1998; Wetzel et al., 1999; Touhara et al., 1999; Murrell and Hunter, 1999).

Comparison of the amino acid sequences of olfactory receptors expressed in different vertebrate groups revealed the existence of two classes of olfactory receptors (Freitag et al., 1998); while fish express only class I receptors (Ngai et al., 1993a; Ngai et al., 1993b; Freitag et al., 1998), mammals express class II receptors (Buck and Axel, 1991; Lancet and Ben-Arie, 1993). Olfactory receptor cells of fish respond to water-soluble odorants, i.e. amino acids (Caprio et al., 1989; Restrepo et al., 1990; Kang and Caprio, 1991; Ivanova and Caprio, 1993; Friedrich and Korsching, 1997), whereas olfactory receptor cells of terrestrial vertebrates respond to volatile compounds (Firestein and Werblin, 1989; Kashiwayanagi and Kurihara, 1995; Tareilus et al., 1995; Duchamp-Viret and Duchamp, 1997; Bozza and Kauer, 1998), so it has been assumed that class I receptors may be specialized for the detection of water-soluble odorants, whereas class II receptors recognize volatiles (Freitag et al., 1998; Sun et al., 1999).

Amphibia live in aquatic as well as terrestrial environments, and perform the transition between the aquatic and terrestrial lifestyle during metamorphosis (Nieuwkoop and Faber, 1956). They are capable of detecting both water-soluble and volatile odorants (Elepfandt, 1996). In adult *Xenopus laevis* this capability is correlated to the functional morphology of the nasal cavity, as olfactory sensory epithelium is located in the water-filled lateral diverticulum (LD) as well as in the air-filled medial diverticulum (MD) (Föske, 1934; Altner, 1962; Weiß, 1986). Molecular studies have revealed the existence of both vertebrate olfactory receptor classes in *X. laevis* (XOR), and in situ hybridization studies demonstrated that the class I receptors are expressed in the LD, while the class II receptors are expressed in the MD (Freitag et al., 1995; Freitag et al., 1998).

The available sequence data from polymerase chain reaction (PCR) fragments provides only limited information towards the identification of class-specific sequence motifs of both

receptor classes from *X. laevis*. We therefore set out to decipher the complete coding sequences of receptor types from both classes, in order to analyze the peptide sequences of XORs with respect to putative class-specific motifs. In addition, experiments were performed to explore the functional properties of both receptor types by heterologous expression in a species-identical expression system, the *Xenopus* oocyte.

Materials and methods

cDNA library screening

Olfactory epithelium of 50 clawed frogs *Xenopus laevis* (Daudin, 1803) was isolated from the nasal cavities. The epithelium comprised the tissue from the medial diverticulum (MD), the lateral diverticulum (LD) and the vomeronasal organ (VNO). Poly(A)$^+$-RNA was isolated, and used for the construction of a directional (*Eco*RI, *Xho*I) oligo(dT)-primed olfactory cDNA-library in the phage Vector λ ZAP Express^{TM} (Stratagene, La Jolla, CA, USA), containing the sequence of the pBK-CMV phagemid. The phage cDNA-library was subsequently amplified once. Six independent screenings were performed with single DIG-labeled probes. These probes are PCR-fragments of putative olfactory receptors amplified from the cDNA library using the primers and procedures described in a previous study (Freitag et al., 1995). The DIG-labeling of isolated receptor-inserts was carried out by random hexamer priming using the DIG-DNA labeling kit (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer's protocols. Labeled DNA was dissolved in TE (10 mmol l^{-1} Tris, 1 mmol l^{-1} EDTA, pH 7.4) at a concentration of 10 ng μl^{-1} and stored at –20 °C. For each screening, phage lifts of 3.6×10^5 independent phage clones were performed on Hybond N$^+$ membranes (Amersham, Freiburg, Germany). The hybridization procedure was done according to standard protocols (Sambrook et al., 1989). Briefly, the phage DNA on the filters was denatured (0.5 mol l^{-1} NaOH, 1.5 mol l^{-1} NaCl, 10 min), neutralized (0.5 mol l^{-1} Tris, 1.5 mol l^{-1} NaCl, pH 8.0, 2× 10 min), air-dried for 15 min and the DNA fixed by baking at 80 °C for 2 h. The filters were prehybridized in prehybridization solution (45 % formamide, 5× standard saline citrate SSC, pH 7.5, 0.02 % SDS, 2 % Blocking Reagent (Roche Molecular Biochemicals, Mannheim, Germany), 0.1 % laurylsarcosine and 100 μg ml^{-1} herring sperm DNA) between 42 °C and 60 °C for 3 h, depending on the stringency demanded, and hybridized with the same solution containing 10 ng ml^{-1} of DIG-labeled DNA of the respective olfactory receptor overnight. After hybridization, filters were rinsed with 2× SSC, 0.1 % SDS at room temperature for 5 min, and then 4× at 42–60 °C in 0.1× SSC, 0.1 % SDS for 20 min. The detection of the chemiluminescent signal was performed as described in the 'DIG system user's guide for filter hybridization' from Roche Molecular Biochemicals using CSPD as substrate, and the signals were visualized with X-ray film (Fuji RX). Positive single-phage plaques were then isolated in 500 μl of lambda-dilution buffer (100 mmol l^{-1} NaCl, 50 mmol l^{-1} Tris-HCl, 10 mmol l^{-1} $MgSO_4$, 0.01 % gelatine, pH 7.5) and eluted overnight at 4 °C, then an in vivo excision (IVE) was performed as described (Stratagene, La Jolla, CA, USA). Single bacterial colonies (bacterial strain XLOLR) were then isolated, the phagemid-DNA extracted using standard procedures, and the identity of the phagemid insert verified by Southern blotting. The DNA was then sequenced on an ABI 310 genetic analyzer (Perkin Elmer, Weiterstadt, Germany) using the RR dye Deoxy Terminator Cycle Sequencing Kit (Perkin Elmer) and the primer-walking technique.

Sequence analysis

All sequences were analyzed using the HUSAR 3.0 software package (Heidelberg Unix Sequence Analysis Resources HUSAR, EMBL, Heidelberg, Germany), which is based on the software package 7.2 of the Genetic Computer Group (GCG, Madison, Wisconsin, USA) and the phylogenetic analysis software PHYLIP 3.5c (J. Felsenstein, University of Washington, USA).

Preparation of Xenopus *oocytes*

Female *Xenopus* were anaesthetized in a solution containing 0.2 % tricain (Sigma) and $NaHCO_3$ (2 g l^{-1}). An incision was made in the abdomen, and ovary lobes containing a total of approx. 500–1000 oocytes were dissected free. The lobes were cut into small pieces, containing 20–50 oocytes each, transferred into OR-Mg medium (82 mmol l^{-1} NaCl, 20 mmol l^{-1} $MgCl_2$, 5 mmol l^{-1} Hepes, 2 mmol l^{-1} KCl, pH 7.5), washed 2× in fresh OR-Mg medium, and digested in collagenase solution consisting of OR-Mg and 2 mg ml^{-1} collagenase A (Roche Molecular Biochemicals, Mannheim, Charge 840 892 22) at 18 °C for 2-3 h, until the oocytes were released and the follicle cells could be removed easily by gentle shaking (Quick and Lester, 1994). After 5 washes in OR-Mg, vital oocytes of stages V and VI were isolated, washed once in ND96 medium [96 mmol l^{-1} NaCl, 5 mmol l^{-1} Hepes, 2 mmol l^{-1} KCl, 1.8 mmol l^{-1} $CaCl_2$, 1 mmol l^{-1} $MgCl_2$, 50 μg ml^{-1} Gentamicin (Gibco BRL), pH 7.5], and finally stored in fresh ND96 medium at 18 °C.

Injection and electrophysiological measurements

The coding sequence of the olfactory receptors was amplified with specific primers and PWO polymerase under standard conditions. The primers contained appropriate restriction sites, allowing a directional cloning of the receptors into special expression vectors, pGemHe (Liman et al., 1992) or pcDNA3.1(+) (Invitrogen, Gronigen, The Netherlands). Thereafter the orientations and sequences were controlled by sequencing the complete clone. The pGemHe plasmids were subsequently linearized at the 3′ end with appropriate restriction enzymes, transcribed in the sense orientation with a Cap-RNA-Kit as described by the manufacturer (Roche Molecular Biochemicals, Mannheim, Germany) using T3 polymerase, the cRNA resuspended in RNase-free water and the concentration set to 100 ng μl^{-1}. To ensure that no degradation had occurred during preparation and handling, each RNA was finally analyzed on an agarose gel. The procedure for the GIRK1 (Accession number P48549), GIRK4 (Accession number P48544), green

fluorescent protein (GFP, derived from pGreen Lantern, Gibco BRL), and the prostanoid receptor EP_3F (accession number HS13214; Schmid et al., 1995) were equivalent. For the cRNA of the cDNA-library of the olfactory epithelium from *Xenopus laevis* a total of 10 μg of the library DNA in the pBK-CMV-phagemid was isolated by IVE, linearized at the 3′ end in independent reactions using five different restriction enzymes (*Apa*I, *Kpn*I, *Xba*I, *Not*I and *Xho*I), the cRNA prepared as described, and dissolved at a concentration of 500 ng μl^{-1} in RNase-free water. In most experiments 50 nl (about 5 ng) of the receptor, Girk1, GIRK4, EP_3F or GFP cRNA solution, or 150 nl (approximately 75 ng) of the cDNA library cRNA solution, were injected into the cytoplasm of the oocytes. Alternatively, 20 nl of plasmid DNA (100 ng μl^{-1}) were injected directly into the nucleus of the oocyte. After an incubation period of 4–7 days in 35 mm culture dishes (TC-Dish 35×10, Nunc, Denmark) at 18 °C in batches of 20–50, the oocytes were transferred individually into the recording chamber, and clamped in the two-electrode voltage-clamp mode at +30 mV or −80 mV with an oocyte amplifier (Turbo-TEC03, NPI electronics, Germany). The oocytes were continuously superfused with frog Ringer solution (hNa; 115 mmol l^{-1} NaCl, 2.5 mmol l^{-1} KCl, 1.8 mmol l^{-1} $CaCl_2$, 10 mmol l^{-1} Hepes, pH 7.2), Rimland-Ringer solution (115 mmol l^{-1} KCl, 2.5 mmol l^{-1} NaCl, 1.8 mmol l^{-1} $CaCl_2$, 10 mmol l^{-1} Hepes, pH 7.2) or Rimland with barium (3 mmol l^{-1} or 0.3 mmol l^{-1}, respectively) by gravity flow, stimulated with odorants diluted in these solutions, and the resulting currents measured as described (Methfessel et al., 1986; Stühmer et al., 1992). For the functional expression studies of *Xenopus* olfactory receptors (XORs) a total of 155 oocytes were analyzed. Of these, 39 cells were water-injected control oocytes and 116 cells were expressing XORs (N=105) or the olfactory cRNA (N=11).

Stimulations

The oocytes were stimulated with complex stimulants, such as extracts of the fish food tetramarine (Tetra, Germany), minced beef heart (1:100; filtered through a 0.45 μm membrane, Nytran, Schleicher Schüll), coffee aroma (stock solution 1:10 in DMSO; final concentration 1:500 to 1:1000, Nestlé, Mainz, Germany), or cocktails of amino acids.

Tetramarine extract, prepared at a concentration of 33 mg ml^{-1} in H_2O, stirred for 3 h at 4 °C, then filtered through a 0.45 μm membrane and diluted to a final concentration of 0.33–3.3 mg ml^{-1}, caused general reactions in all oocytes; it was therefore used as a test for vitality after each series of stimulation in each oocyte. For stimulation of transfected and water-injected oocytes a selective fraction of tetramarine was prepared, consisting of small (<3 kDa), heat-stable, water-soluble compounds.

Cocktails of amino acids were prepared as follows. The amino acids were dissolved as stock in double-distilled H_2O, 0.1 mol l^{-1} NaOH or 0.1 mol l^{-1} HCl at a concentration of 100 mmol l^{-1} and stored at –20 °C. The amino acids were then diluted to concentrations of 0.10–3 mmol l^{-1} each in normal frog Ringer solution, and pH adjusted to 7.2. The amino acids were used either as a cocktail of 22 amino acids (alanine, arginine, asparagine, aspartate, cysteine, cystine, glutamate, glutamine, glycine, histidine, hydroxy-proline, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophane, tyrosine, valine) or as cocktails of amino acids with particular physicochemical characteristics (Kang and Caprio, 1991), including long-chain neutral (LCN), short-chain neutral (SCN), basic, acidic and other amino acids. The human prostanoid receptor EP_3F was stimulated with 1 μmol l^{-1} prostaglandin E_2 (in Rimland–Ringer solution).

Western blots

To test the oocyte system for expression of olfactory receptor protein (OR), the *Xenopus* receptor XB107 was cloned into an expression vector containing in-frame the nucleotide sequence of an N-terminal Flag-Tag (Hopp et al., 1988; Chubet and Brizzard, 1996), producing a peptide representing a highly efficient epitope. With a specific antibody (Anti-Flag-M1 monoclonal antibody, Eastman Kodak Company, Rochester, NY, USA) the receptor protein could be made visible. Three oocytes per batch were solubilized in solubilization buffer (50 mmol l^{-1} imidazole, 100 mmol l^{-1} NaCl, 3 mmol l^{-1} EGTA, 5 mmol l^{-1} EDTA, 0.1 mol l^{-1} PMSF, 0.02 % sodium azide, 1 % sodium deoxycholate, 20 mmol l^{-1} Tris-HCl, pH 7.5), homogenized for 10 min in a water bath with ultrasound at room temperature and then incubated on ice for 1 h. The samples were centrifuged at 14000 r.p.m. at 4 °C (Eppendorf, 5417R), the pellet of undissolvable material discarded, 5× sample buffer (625 mmol l^{-1} Tris-HCl, pH 6.8, 50 % glycerol, 0.05 % Bromophenol Blue, 5 % SDS, 7.5 mmol l^{-1} DTT) added to the supernatant and these samples stored at 4 °C (Breer and Benke, 1986) until use.

One-dimensional SDS–PAGE gel electrophoresis was performed essentially as described by Laemmli (Laemmli, 1970). The gels were blotted onto nitrocellulose membranes using a semidry blotting sytem (Pharmacia, Freiburg, Germany), stained with Ponceau S, dried and stored at 4 °C until use. For western blot analysis non-specific binding sites were blocked with 5 % non-fat milk powder (Nutraflor, Dietmannsriel, Germany) in TBST (10 mmol l^{-1} Tris-HCl, pH 8.0, 150 mmol l^{-1} NaCl, 0.05 % Tween 20). The blots were incubated overnight at 4 °C with a specific antibody (Anti-Flag M1 monoclonal antibody, Eastman Kodak Company, Rochester, NY, USA) at a concentration of 1:5000 and after three washes with TBST a horseradish peroxidase-conjugated anti-mouse IgG (1:10000 in TBST with 3 % milk powder) was applied. Following three washes an TBST an ECL-System was used to visualize bound antibody as described by the manufacturer (Amersham).

Results

Identification and sequence analysis of odorant receptors

In a previous study PCR fragments of olfactory receptors from *Xenopus laevis* were identified, representing two distinct classes of receptors (Freitag et al., 1995). Since these PCR

fragments comprise only 50–70 % of the complete coding sequence, excluding the N- and C-terminal regions of the receptor, we expected that a comprehensive comparison of the *Xenopus* receptors with those from other vertebrate species, as well as the identification of class-specific sequence motifs would be difficult (Freitag et al., 1998). Such analyses, as well as functional expression studies, require complete coding sequences. Receptor clones were therefore isolated from a *Xenopus* olfactory cDNA library, and for ten different olfactory receptors the full sequence was identified and analyzed. Hydrophobicity plots indicated the existence of seven hydrophobic putative transmembrane domains (TM), connected by extracellular (EC) and intracellular (IC) loops. 40 amino acids are conserved in all ten receptors, five of which are proline

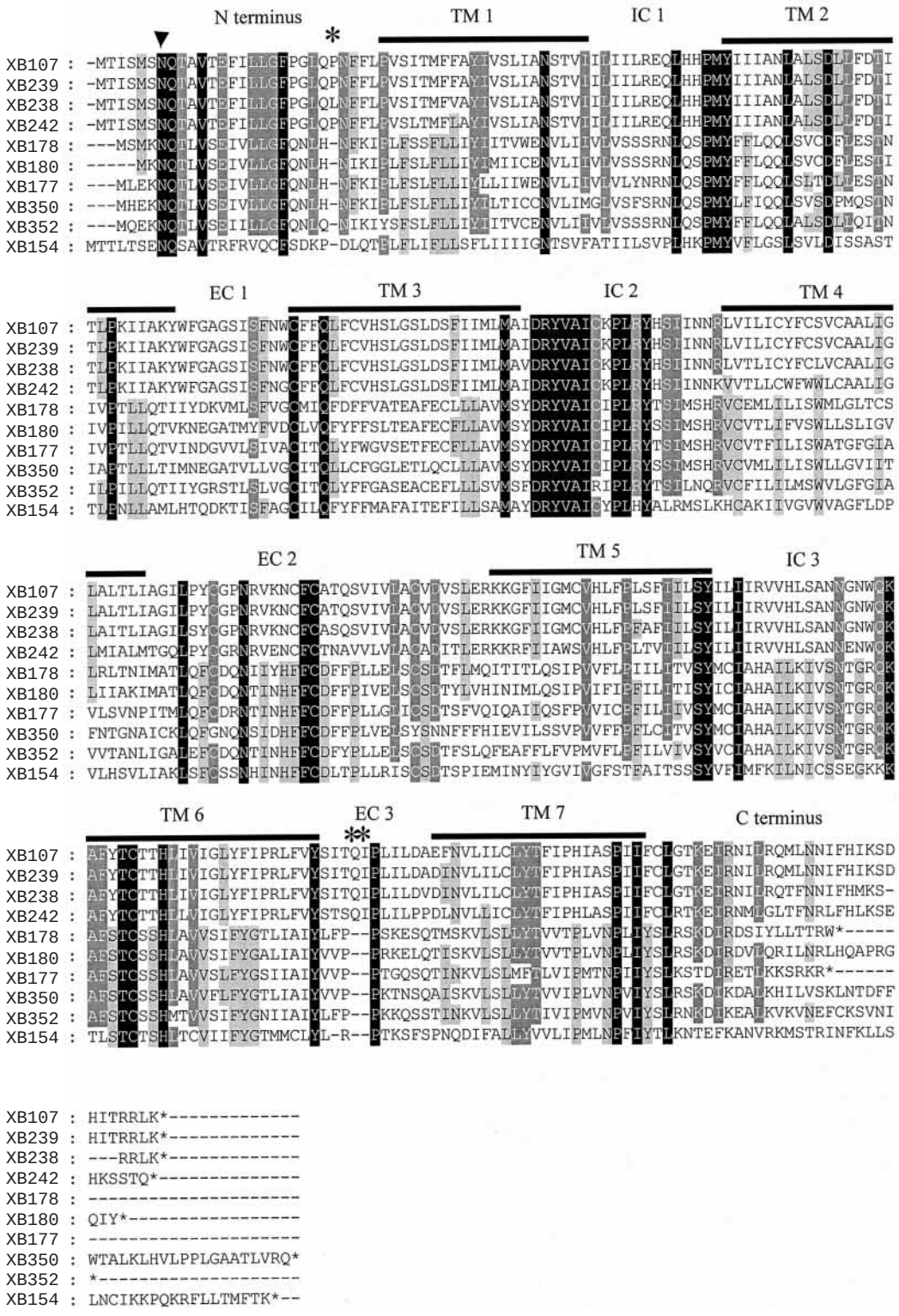

Fig. 1. Alignment of olfactory receptors from *Xenopus leavis*. Alignment of the deduced amino acid sequences for ten olfactory receptors from *Xenopus laevis*. Identical amino acid residues are highlighted in black, residues present in >80 % of the sequences are highlighted in dark grey, those with >60 % conservation in light grey. Putative transmembrane domains (TM) are highlighted by black bars, and extracellular domains (EC) and intracellular domains (IC) are indicated. The putative N-linked glycosylation site is marked by an arrowhead. Additional class-specific amino acids within the amino acid chain are highlighted by asterisks. Class I receptors: XB107, 239, 238, 242; class II receptors: XB178, 180, 177, 350, 352 and 154.

(Fig. 1). Conserved amino acids were primarily found in the N-terminal region as well as in IC2, EC2 and TM6. The proximal part of IC2 [M^{122}(AS)XDRYVAI(C_R)XPL(R_H)Y^{136}] and the proximal part of TM6 [$Q_K{}^{239}KA_TF_L$(SY)TC(TS)(TS)$HL_M{}^{249}$] showed a particularly high degree of sequence conservation. In contrast, the domains EC1, TM3, TM4 and the C terminus seem to be rather divergent. In contrast to the class II receptors XB178 to XB154, the class I receptors XB107 to XB242 comprise an insertion within the EC3 loop (Freitag et al., 1998), and an extra amino acid in the N-terminal domain ($P_L{}^{25}$). A putative N-glycosylation site at N^8 (arrow) is generally conserved, whereas putative O-glycosylation sites diverge. The identity dendrogram (Fig. 2A) demonstrates that the ten *Xenopus* olfactory receptors (XORs) belong to two classes, as described previously for PCR fragments (Freitag et al., 1995). While the four class I receptors (XB107, XB239, XB238 and XB242) share 82–99 % amino acid sequence identity, the six class II receptors (XB178, XB180, XB177, XB350, XB352 and XB154) are more diverse, showing sequence identities from 78 % (XB180/XB178) to 32 % (XB154/XB350). A phylogenetic analysis revealed that receptors XB239, XB107, XB238 and XB242 are phylogenetically related to the receptors from fish, whereas receptors XB154, XB180, XB178, XB350, XB177 and XB352 are grouped with olfactory receptors from mammals and birds, and receptor XB154 is the most 'mammalian-like' (Fig. 2B).

It has been suggested that domains TM3 to TM5, and possibly the connecting extracellular loops, might participate in ligand binding (Lancet and Ben-Arie, 1993; Singer and Shepherd, 1994, Singer et al., 1995; Floriano et al., 2000). An

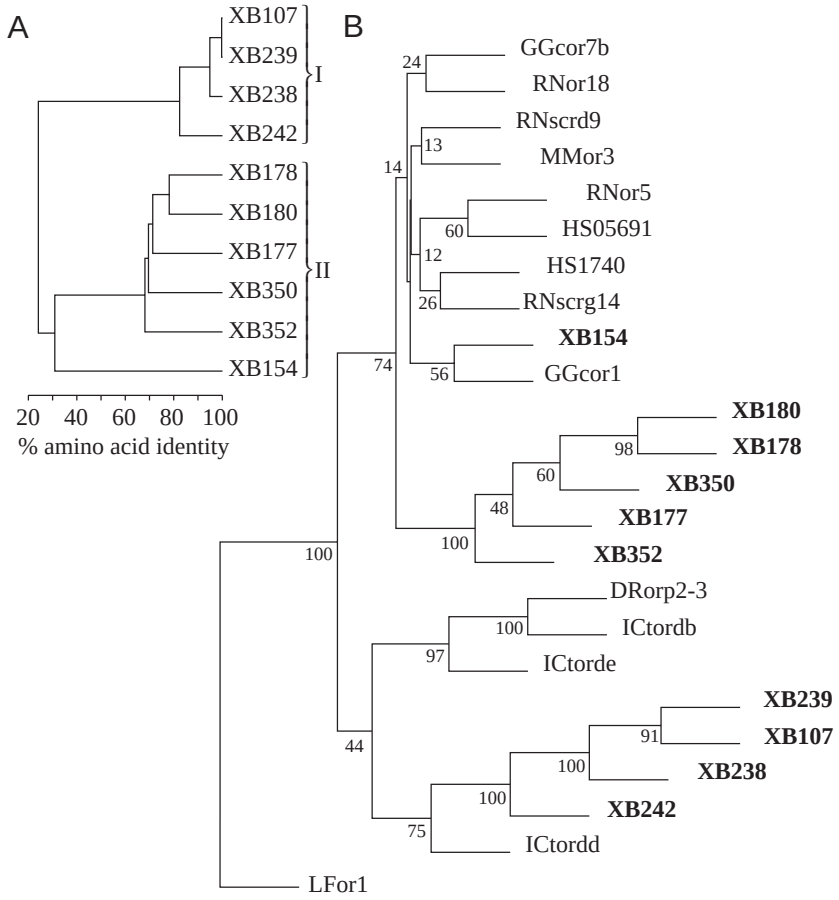

Fig. 2. Identity dendrogram and phylogenetic relationships between divergent olfactory receptor sequences. (A) The complete deduced amino acid sequences of the ten XORs (see Fig. 1) were aligned in Pileup, and an identity dendrogram was constructed. Two classes of receptors are clearly distinguishable: class I, comprising the receptors XB107 to XB242 and class II, including the receptors XB178 to XB154. **(**B) Phylogenetic tree of the amino acid sequences of ORs from different species. The open-reading frames (ORFs) of 100 known olfactory receptors were initially aligned together with the ten XOR-receptors. 14 representative receptors from chicken (GGcor7b, S. Nef, I. Allaman, E. De Castro and P. Nef, unpublished material; GGcor1, Leibovici et al., 1996), rat (RNor18, RNor5, Raming et al., 1993; RNscrd9, RNscrg14, Walensky et al., 1998), mouse (MMor3, Nef et al., 1992), human (HS05691, J. E. Lamerdin, P. M. McCready, E. Skowronski, A. W. Adamson, K. Burkhart-Schultz, L. Gordon, A. Kyle, M. Ramirez, S. Stilwagen, H. Phan et al., unpublished material; HS1740, Ben-Arie et al., 1994), catfish *Ictalurus punctatus* (ICtordb,d,e, Ngai et al., 1993a), zebrafish *Danio rerio* (DRorp2-3, Barth et al., 1997) and the ten receptors from *Xenopus* (XB, this study) together with the receptor LFor1 from the lamprey *Lampetra fluviatilis* (Freitag et al., 1999) as outgroups were then realigned and analyzed by parsimony analysis with the PHYLIP package (Joseph Felsenstein, Version 3.5c, 1995). The bootstrap-support of 100 replicates is indicated.

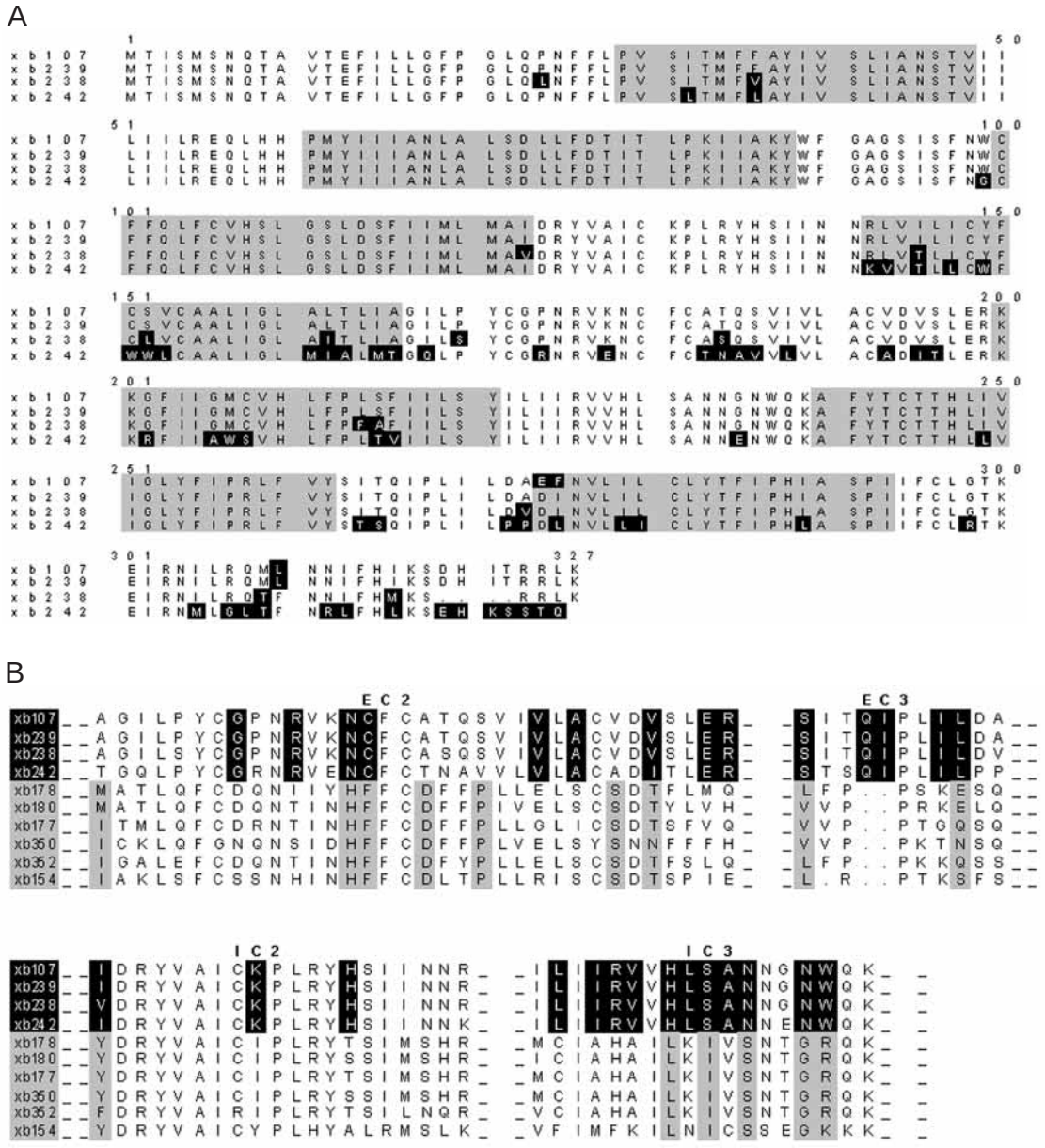

Fig. 3. Sequence analysis of XORs. (A) Amino acid substitutions in receptor sequences of class I receptors. Substituted amino acids are highlighted in black. Transmembrane domains are shaded in grey. A high number of amino acid substitutions can be found in the TM4, EC2 and TM5 domains (not shown). (B) Class-specific sequence motifs in ORs from *Xenopus laevis*. XB107, XB239, XBb238, XB242 represent class I receptors, whereas XB178, XB180, XB177, XB350, XB352 and XB154 are class II receptors. The extracellular domains 2 (EC2) and 3 (EC3), as well as the intracellular domains 2 (IC2) and 3 (IC3) are shown. Class-specific amino acid locations, constituting physico-chemically comparable amino acids, are highlighted in black (class I-specific) and light grey (class II-specific). Connecting transmembrane regions, as well as N- and C-terminal domains, are omitted and symbolized by dashes.

alignment of the four class I receptors, which share more than 82 % sequence identity, revealed particularly divergent regions in the TM4, EC2 and TM5 domains; 32 of 64 substitutions (50 %) are located in these regions, which represent only 24 % of the sequence (Fig. 3A). A comparison of the two closely related class II receptors XB178 and XB180 elucidated an accumulation of non-synonymous nucleotide substitutions in TM4. 16 non-synonymous substitutions, leading to the expression of different amino acids, were detected in this domain, while synonymous nucleotide substitutions were not found. This corresponds to a K_A value for non-synonymous nucleotide substitutions of 0.454±0.1587 (mean ± S.D.), indicating a high positive selection pressure on this region (Ngai et al., 1993a).

No convincing evidence for class-specific sequence motifs characteristic of mammalian-like or fish-like receptors has yet been obtained. This may be because only receptors from distantly related species could be compared. A comparison of

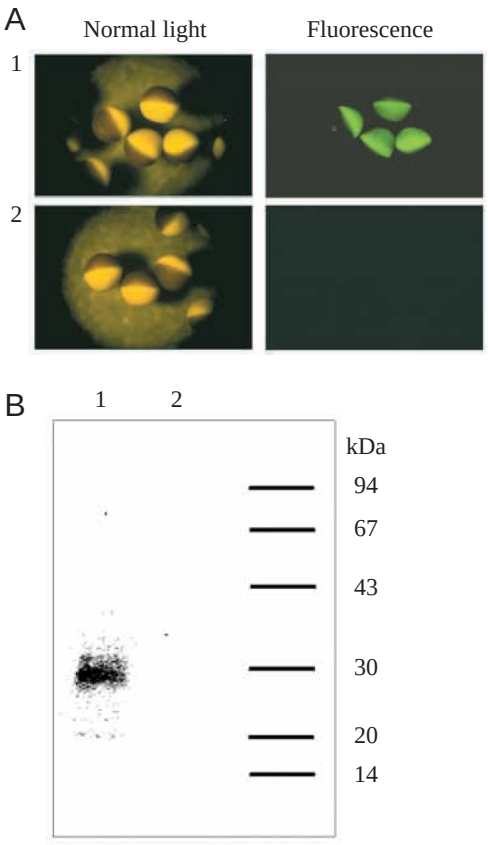

Fig. 4. Establishing the oocyte expression system. (A) Expression of GFP in *Xenopus* oocytes. Oocytes were injected with cRNA for GFP (1), or with water (2). Left, cells under normal light; right, fluorescence microscopy. (B) Expression of receptor XB107 in *Xenopus* oocytes. Oocytes were injected with an expression vector encoding receptor XB107 with an N-terminal Flag-Tag (lane 1) or water (lane 2) and incubated at 18 °C for 7 days. The oocyte proteins were subjected to western blot analysis. The positions of molecular mass marker proteins are shown.

the two XOR classes, however, revealed distinct class-specific sequence motifs. Class-specific amino acids were found at 87 positions within the receptor sequences. For 29 specific positions in class II XORs, physicochemically different amino acids were found in class I receptors. When focussing on the extracellular loops EC2 and EC3, as well as the intracellular loops IC2 and IC3, class-specific amino acid motifs were determined (Fig. 3B). The specific pattern of charged amino acids in the domains IC2 and IC3 may indicate divergent G-protein coupling for the two receptor classes (Iismaa et al., 1995; Ji et al., 1998).

Functional characterization of odorant receptors

To study functional determinants of the two olfactory receptor classes from *Xenopus*, and to explore a possible functional divergence, olfactory receptors from both classes were expressed in the *Xenopus* oocyte as a species-identical expression system. As a first step towards establishing the oocyte system for the expression of XORs, expression efficiency of oocyte batches was monitored using GFP (Fig. 4A). Only oocytes that have been transfected successfully show fluorescence. In addition, the G-protein-activated, inwardly rectifying potassium channel subunits GIRK1 and GIRK4 (Kubo et al., 1993; Doupnik et al., 1995; Kaprivinsky et al., 1995) were coexpressed with the G-protein-coupled receptors (GPCRs) in order to allow an electrophysiological monitoring of the activity elicited *via* the intrinsic $G\alpha_{q/11}$ pathway, which results in a Ca^{2+}-dependent chloride current (Miledi et al., 1987; Guttridge et al., 1995), and the $G\alpha_{o/i}$ pathway, which causes an increase in the potassium current *via* the coinjected GIRKs (Kubo et al., 1993). Coinjection of the GIRKs and the human prostanoid receptor EP_3F, which is known to couple to the $G\alpha_i$ subunit of heterotrimeric G proteins (Schmid et al., 1995), resulted in a significant potassium current in ooctyes stimulated with $1\,\mu mol\,l^{-1}$ prostaglandin E_2 (data not shown). As a first step towards the functional analysis of XORs in oocytes, the translation of XOR protein was assessed by western blot analysis. An immunoreactive protein of apparent molecular mass 29 kDa was visualized on western blots (Fig. 4B), indicating a significant expression of receptor protein.

Based on the similarity between the class I XORs and olfactory receptors of different fish species (see Fig. 2B), it was assumed that oocytes expressing this class of receptors might respond to water-soluble odorants. As the ligands for distinct receptor types are unknown, the oocytes were stimulated with subfractions of a water-soluble extract from fish food (Tetramarin, see Materials and methods). The fraction containing small (<3 kDa), heat-stable, water-soluble compounds induced a specific, concentration-dependent current only in oocytes expressing class I receptors, as shown in Fig. 5A for the class I receptor XB107, activating the Ca^{2+}-dependent chloride current (1.5±0.5 μA) in the oocytes (N=23, i.e. 69 % of tested cells). Water-injected control oocytes (N=12) did not respond to these stimuli (data not shown), nor did class II XOR-expressing cells (N=14), even at very high concentrations (Fig. 5B). A hydrophilic extract from minced beef heart, a preferred food of adult frogs, also induced specific currents only in cells expressing class I receptors (N=12; 50 %; data not shown).

Oocytes expressing class I XORs or class II XORs were exposed to coffee aroma, a very complex mixture of small, hydrophobic, organic compounds (Grosch, 1996). A significant outward current (7.3±1.48 μA) was induced only in class II XOR-expressing cells (N=31; 23 %; Fig. 5B). In contrast, cells expressing class I XORs (N=18; Fig. 5A) or control cells (N=4) showed no responses. The control stimulation of oocytes with frog-Ringer solution failed to induce a current in either case (Fig. 5A,B). The data indicate that receptors of both classes can in fact be functionally expressed in *Xenopus* oocytes, and that both receptor classes display a selective responsiveness. Since it was not possible to identify the active compounds from the complex odor mixtures, experiments were performed using defined odorants. In order to increase the probability that olfactory receptors

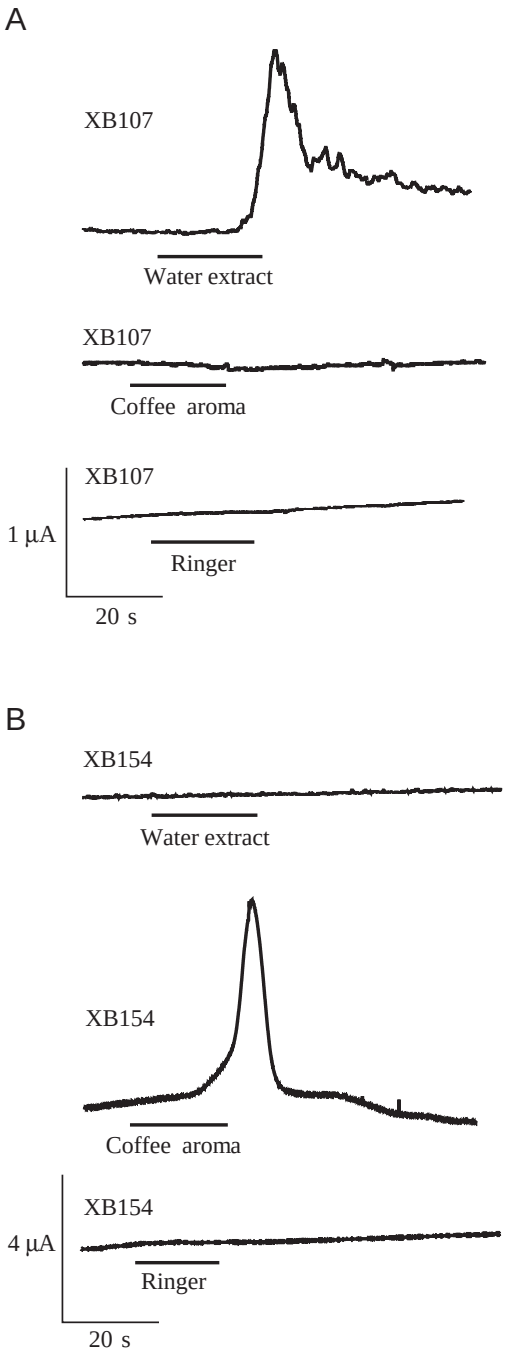

Fig. 5. Functional expression of both classes of XORs. (A) Oocytes expressing a representative class I receptor (XB107) and (B) cells expressing a representative class II receptor (BX154) were stimulated with tetramarin fractions (water extract, upper traces), coffee aroma (middle traces) or frog-Ringer solution (lower traces). Cells expressing XB107 responded with a typical current upon stimulation with the water extract, but showed no response with coffee aroma (A). (B) Oocytes expressing a representative class II receptor (XB154) did not react to water extract, but showed a strong outward current upon stimulation with coffee aroma. Neither cell type responded to the control-stimulation with frog-Ringer solution (Ringer). In all cases current plots were done at a holding potential of +30 mV; the times of stimuli are indicated by bars. Please note the different scaling in 5A and B.

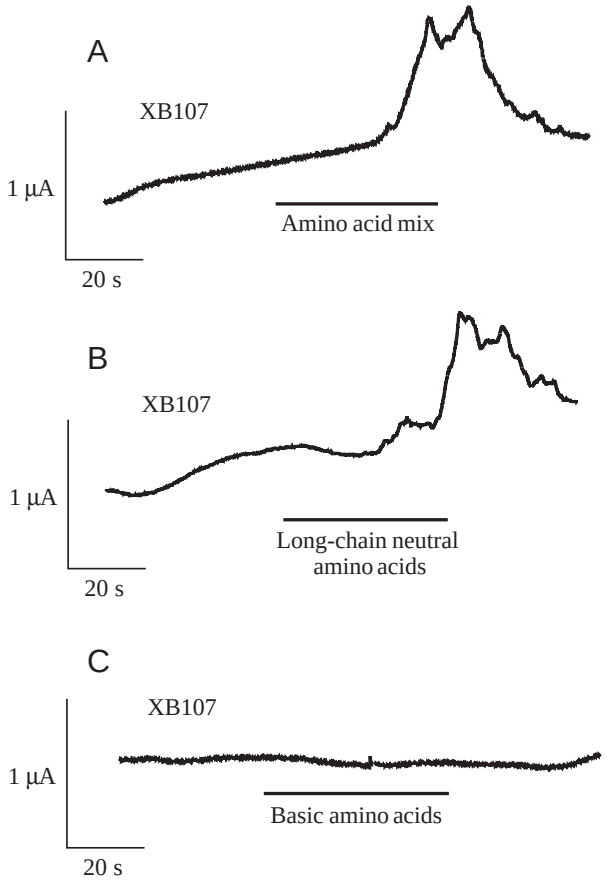

Fig. 6. Response of class I XOR expressing oocytes to stimulation with different amino acid fractions. Cells expressing the class I receptor XB107 were stimulated with 22 different amino acids (amino acid mix, A), long-chain neutral amino acids (B) and basic amino acids (C). Clear responses are seen in A and B, while the cells did not react to basic amino acids (C) or other amino acid fractions (see text). The concentration of single amino acids was 200 μmol l^{-1} each in all cases. As in Fig. 5 the holding potential was +30 mV; times of stimuli are indicated by bars.

responding to defined odorants are expressed in the oocytes, the complete set of mRNA from the olfactory epithelium of *Xenopus laevis* was expressed as cRNA (see Materials and methods), and the oocytes probed with defined odorants. A mixture of 22 amino acids (200 μmol l^{-1} each) caused specific outward currents (0.3±0.08 μA) in the injected cells (*N*=9; 33 %); control oocytes did not respond (*N*=4). Subsequently, oocytes expressing four different class I or six different class II XORs were stimulated with the amino acid mixture. A specific outward current (2.7±0.3 μA) was induced only in oocytes expressing class I receptors (*N*=15; 40 %), while cells expressing class II receptors showed no response (*N*=5). The amplitude of the induced current in cells expressing class I receptors was approximately 3 μA, and was thus much larger than in cells expressing the complete cRNA (data not shown). This is probably because upon injection of receptor-encoding mRNA more receptor protein is expressed in the cell, than upon injection of cRNA, which is a very heterogenous mixture

of RNAs. To provide more information about the identity of putative ligands for a distinct class I XOR subtype (XB107), mixtures of amino acids with similar physico-chemical properties were employed (Kang and Caprio, 1991). It was found that oocytes expressing this class I receptor XB107 responded as well to the complete amino acid mixture as to long-chain neutral amino acids, but other mixtures, for example basic amino acids, had no effect (Fig. 6).

Discussion

Based on the discovery that class I ORs exist in fish and amphibia and class II ORs are expressed in tetrapods, it has been speculated that class I receptors detect water-borne odorants, whereas volatiles are detected by class II receptors. In amphibia, particularly in *Xenopus laevis*, this idea is further emphasized by the observation that class I receptors in the nose of the frog are expressed in the LD, which responds to water-soluble odorants, while class II ORs are abundant in the MD, specialized for detecting volatile odors. The data presented here combine for the first time a sequence analysis for both classes of vertebrate olfactory receptors and a functional analysis of heterologously expressed receptor types.

The functional analysis of ORs expressed in *Xenopus* oocytes showed that receptors of each class did indeed detect different categories of odorants. While class I receptors responded to complex mixtures of water-soluble substances as well as to amino acids, class II receptors responded to mixtures of hydrophobic compounds. Some of the class I receptors from *Xenopus* may thus be amino acid receptors. However, in fish, receptors with sequence similarities to calcium-sensing receptors (CaSR) and metabotropic glutamate receptors (mGluR) act as receptors for amino acids (Speca et al., 1999). It is conceivable that vertebrates possess different types of receptors that interact with this group of ligands. The two types of putative amino acid receptors, expressed in different vertebrate orders, could be tuned to different amino acids, or display high or low affinities for a given amino acid. Alternatively, it is conceivable that the two receptor types are expressed in different sensory cells, e.g. one receptor type in pheromone-sensing cells, in the fish olfactory epithelium, and the other receptor type in sensory cells detecting general odors.

Previous studies have shown that the length of the extracellular loop EC3 diverges between the two OR classes (Freitag et al., 1998). The present study underlines this trait. Significant differences regarding class-specific, particularly charged amino acids, were found both in this region and in the EC2 domain. Furthermore, throughout the XOR sequences of both classes, amino acids in certain positions, e.g. in TM4 between XB178 and XB180, and also in TM3 and TM5, show high variability. This observation agrees with a recent report that describes a highly variable region in the proposed inner cleft between the transmembrane domains TM4 and TM5, marking a complementarity-determining region (CDR), which seems to be specific for olfactory receptors (Sharon et al., 1998). While this highly variable region within the internal faces of the transmembrane domains TM3 to TM5 might reflect the structural diversity of appropriate odorous ligands (see also Floriano et al., 2000), the distinct class-specific motifs within the extracellular loops EC2 and EC3 (Fig. 3B) may determine which general physico physico-chemical features a compound must have to act as an appropriate ligand for a class I or class II receptor.

For rhodopsin-like GPCRs the intracellular domains IC2 and IC3 are responsible for G-protein coupling (Wess, 1997; Wess, 1998), and a distinct pattern of charged amino acids in these domains is considered to be fundamental for the G-protein specificity of a given GPCR (Iismaa et al., 1995; Ji et al., 1998). Interestingly, in XORs, characteristic patterns of charged amino acids were found within domains IC2 and IC3 for each of the two receptor classes. This could imply that each of the two receptor classes couples to a distinct G-protein subtype. This idea fits in with the results of *in situ* hybridization experiments that demonstrated a colocalization of G_{o1} α-subunits and class I XORs in the LD, whereas a novel G_s-like α-subunit is colocalized with class II XORs in the MD (Mezler et al., 2001).

Taken together our results demonstrate that the two receptor classes identified in *Xenopus laevis* show class-specific sequence motifs in both the putative ligand-binding, and the possible G-protein interaction domains. Furthermore, we could demonstrate that class I and class II XORs are activated by water-soluble and volatile ligands, respectively. Future studies combining detailed sequence comparisons of both receptor classes with functional expression studies of a large array of ORs from both classes in one standardized expression system are necessary to find the structural basis for divergent receptor function, not only for ligand binding but also G-protein coupling. If this were followed by a mutagenic approach to these receptors and advanced molecular modeling studies (Floriano et al., 2000), a much more complete picture of olfactory receptor functioning might emerge. Use of the *Xenopus* oocyte system to express both classes of receptors from a single species, *Xenopus laevis*, may prove an ideal model system in which to shed new light on the structural features of receptor proteins interacting with distinct chemical compounds.

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The GenBank accession numbers of the sequences reported here are: XB3, AJ249488; XB6, AJ249489; XB107, AJ249404; XB238, AJ250750; XB239, AJ250751; XB242, AJ250752; XB154, AJ250753; XB177, AJ250754; XB178, AJ250755; XB180, AJ250756; XB350, AJ250757; XB352, AJ250758.

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