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Xenopus V1R Vomeronasal Receptor Family Is Expressed in the Main Olfactory System

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Abstract

To date, over 100 vomeronasal receptor type 1 (V1R) genes have been identified in rodents. V1R is specifically expressed in the rodent vomeronasal organ (VNO) and is thought to be responsible for pheromone reception. Recently, 21 putatively functional V1R genes were identified in the genome database of the amphibian *Xenopus tropicalis*. Amphibians are the first vertebrates to possess a VNO. In order to determine at which point during evolution the vertebrate V1R genes began to function in the vomeronasal system, we analyzed the expression of all putatively functional V1R genes in *Xenopus* olfactory organs. We found that V1R expression was not detected in the VNO but was specifically detected in the main olfactory epithelium (MOE). We also observed that V1R-expressing cells in the MOE coexpressed Gi2, thus suggesting that the V1R-Gi2–mediated signal transduction pathway, which is considered to play an important role in pheromone reception in the rodent VNO, exists in the amphibian MOE. These results suggest that V1R-mediated signal transduction pathway functions in *Xenopus* main olfactory system.

Key words: amphibian, G-protein, olfactory epithelium, pheromone receptor, vomeronasal organ

Introduction

The majority of vertebrates possess 2 anatomically distinct sensory organs: the olfactory organ and the vomeronasal organ (VNO) (Bargmann 1997). The olfactory organ is generally considered to detect conventional odors, whereas the VNO primarily detects pheromones (Estes 1972; Halpern and Martinez-Marcos 2003).

In the rodent VNO, 2 large and divergent families of G-protein–coupled receptors with 7 transmembrane domains (vomeronasal receptor type 1 [V1R] and vomeronasal receptor type 1 [V2R]) are specifically expressed (Dulac and Axel 1995; Herrada and Dulac 1997; Matsunami and Buck 1997; Ryba and Tirindelli 1997). The mouse V1R family has been shown to respond to the known mouse pheromones, thus suggesting that V1R is actually a pheromone receptor (Boschat et al. 2002; Del Punta et al. 2002). V2R is also likely to mediate pheromone responses, although the function of this gene family has not been confirmed (Kumar et al. 1999). Neurons that express either V1R or V2R coexpress Gi2 or Go, respectively, and these G-proteins are thought to play an important role in signal transduction (Jia and Halpern 1996).

To date, 191 putatively functional V1R genes have been identified in mice (Zhang et al. 2007). Putatively functional V1R gene families have also been identified in other mammals, such as goats, humans, dogs, cows, and opossums (Rodriguez et al. 2000; Rodriguez and Mombaerts 2002; Wakabayashi et al. 2002; Grus et al. 2005). Some of goat and human V1Rs are expressed in the main olfactory epithelium (MOE) (Rodriguez et al. 2000; Rodriguez and Mombaerts 2002; Wakabayashi et al. 2002), but the function of these V1Rs is yet to be analyzed.

Recent reports have found that a small number of V1R genes exist in teleost species, despite their lack of a VNO (Pfister and Rodriguez 2005; Shi and Zhang 2007). Shi and Zhang (2007) identified 21 putatively functional amphibian V1R genes in the genome database of *Xenopus tropicalis*. Thus, there is a question regarding the evolutionary

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points at which V1Rs began to be expressed in the VNO and to function as pheromone receptors.

Phylogenetically, the VNO first appeared in amphibians (Bertmar 1981; Bruner 1984; Eisthen 2000), whereas some sex pheromones, such as sodefrin and splendipherin, have been identified in newts (Kikuyama et al. 1995) and frogs (Wabnitz et al. 1999), respectively. Newt vomeronasal receptor cells recognize sodefrin (Kikuyama and Toyoda 1999), which suggests that vomeronasal receptor cells function in amphibian pheromone sensing. Amphibians are phylogenetic intermediates between fish and mammals and are adapted for both aquatic and terrestrial life. Characterizing amphibian V1Rs should therefore provide new insights into the evolution and function of this gene family. We identified and isolated *Xenopus* V1R genes and analyzed their expression in these olfactory organs.

Materials and methods

Frogs

Xenopus tropicalis was kindly provided by Dr A. Kashiwagi of Hiroshima University, and *Xenopus laevis* was obtained from a commercial source (Copacetic, Aomori, Japan). Animals were cooled to 0 °C to anesthetize completely and then sacrificed by decapitation. All frogs were treated in accordance with the institutional guidelines of Tokyo Metropolitan Institute of Medical Science. Tissue samples were prepared as described elsewhere (Hagino-Yamagishi et al. 2004). Briefly, *Xenopus* adult olfactory organs were fixed in 4% paraformaldehyde/phosphate-buffered saline (pH 7.4) at 4 °C overnight and were decalcified in 0.27 M EDTA (pH 7.0) for 2 days. Fixed samples were embedded in O.C.T. compound and were rapidly frozen at −80 °C.

Cell counting

Cryostat sections (6 μm) of the middle cavity (MC), principal cavity (PC), and VNO of *X. laevis* were stained with hematoxylin, and total cells in the olfactory and vomeronasal epithelia were counted. At the same time, areas of the epithelia were measured with image processing software (ImageJ ver. 1.38; http://rsb.info.nih.gov/ij/index.html), and cell number in 1 mm^2 of MC, PC, and VNO epithelia was calculated (7294.7 ± 268.0 [n = 5], 7053.3 ± 350.3 [n = 6], and 8502.3 ± 177.0 [n = 6], respectively). For subsequent calculations, we adopted approximate cell numbers of 7300, 7000, and 8500 per 1 mm^2 of PC, MC, and VNO epithelia, respectively. We then counted the number of the V1R-expressing cells, measured the area of epithelium in each section, and estimated the V1R-expressing cells per 10 000 epithelial cells. Approximately 75 000 to 157 000, 55 000 to 160 000, and 106 000 to 286 000 cells in the PC, MC, and VNO epithelia were analyzed, respectively.

Identification of *X. tropicalis* V1R genes

In order to obtain full-length sequences of *Xenopus* V1R genes, we used the TBLASTN program to screen Xenbase, an *X. tropicalis* genome database (http://www.xenbase.org/, assembly v4.1, released in August 2005). The amino acid sequence of the 7 transmembrane domains (from TM1 to TM7) of Zebrafish (*Danio rerio*) V1R (AAQ88216) was used to query the database. Putative V1R sequences were identified with an E value cutoff of 10^{-5}. These sequences were then used as queries to search for other putative V1R sequences in Xenbase and GenBank. V1R genes were considered to be pseudogenes if they contained premature termination codons or had deletions in their coding sequences.

Cloning of *Xenopus* V1R and G-protein gene sequences

The putatively functional *X. tropicalis* V1R gene were isolated from genomic DNA by PCR. Genomic DNA was prepared from the livers of adult *X. tropicalis*, as described previously (Hagino-Yamagishi et al. 2004), and was used as a PCR template. A cDNA fragment coding for *X. laevis* Golf (AJ296281) was isolated from a cDNA library derived from the *X. laevis* olfactory and VNOs (Hagino-Yamagishi et al. 2004) by PCR with the primers AGCACGATTGT GAAACAGATG and TGCATTCTCTGGATGATGTC. PCR amplification was carried out for 30 s at 94 °C, 1 min at 55 °C, and 30 s at 72 °C for 30 cycles. DNA fragments coding for *X. laevis* Go (X14636) and Gi2 (AF086606), olfactory receptors class 1 (OR1) (AJ250750), olfactory receptors class 2 (OR2) (AB294143), and the vomeronasal receptor xV2RE were as described previously (Hagino-Yamagishi et al. 2004; Kashiwagi et al. 2006). Amplified DNA fragments were cloned into a pBluescript SK vector (Stratagene, La Jolla, CA), and used for the preparation of riboprobes.

In situ hybridization

Adult *Xenopus* olfactory organs were sectioned coronally (at 6 μm), and sections were hybridized with digoxigenin (DIG)-labeled cRNA probes, as described previously (Kashiwagi et al. 2006). Briefly, sense and antisense DIG-labeled riboprobes were synthesized with a DIG RNA labeling mix according to the manufacturer's protocol (Roche Diagnostics, Mannheim, Germany). Sections were treated with 2% H_2O_2 for 15 min, followed by 1 μg/mL proteinase K for 10 min. After fixation with 4% paraformaldehyde for 10 min, sections were treated with 0.2 N HCl for 20 min and neutralized with 0.1 M Tris–HCl (pH 8.0) for 1 min. Hybridization was carried out in a hybridization solution containing 50% formamide, 10 mM Tris–HCl (pH 7.6), 200 μg/mL tRNA, 1× Denhardt's solution, 600 mM NaCl, 0.25% SDS, 1 mM EDTA (pH 8.0), and 5 μg/mL cRNA probe at 55 °C overnight. After hybridization, sections were washed with 5× SSC/50% formamide at 55 °C for 30 min and were treated with 2 μg/mL RNase

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for 30 min. Samples were then washed twice with 2× SSC and twice with 0.2× SSC at 55 °C. After treatment with blocking reagent (Roche Diagnostics), sections were incubated with an alkaline phosphatase–conjugated anti-DIG Fab fragment antibody (Roche Diagnostics). Positive signals were visualized with the chromogenic substrates 4-nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl-phosphatase (Roche Diagnostics).

Double in situ hybridization

Two-color in situ hybridization was carried out using DIG- and fluorescein-labeled cRNA probes. The fluorescein-labeled probes were synthesized using Fluorescein Labeling Mix according to the manufacturer's protocol (Roche Diagnostics). In situ procedures were essentially as described above. Sections were treated with 0.5% Triton-X 100 for 10 min, followed by 0.3% H_2O_2 for 15 min, and with 5 μg/mL proteinase K for 10 min. After fixation with 4% paraformaldehyde for 10 min, sections were treated with 0.2 N HCl for 20 min and were neutralized with 0.1 M Tris–HCl (pH 8.0) for 1 min. Hybridization was carried out at 55 °C overnight. Hybridized RNAs were detected with an alkaline phosphatase–conjugated anti-DIG Fab fragment antibody (Roche Diagnostics) followed by visualization with FastRed (DAKO Cytomation) and with a horseradish peroxidase–conjugated anti-fluorescein antibody (Roche Diagnostics) followed by visualization with the TSA Biotin System (PerkinElmer, Boston, MA) and streptavidin-Alexa 488 (Molecular Probes, Eugene, OR).

Results

Characterization of V1R sequences in *X. tropicalis*

Twenty-one putatively functional V1R sequences, 2 V1R pseudogenes, and 1 incomplete V1R sequence were identified. The amino acid sequences of the putatively functional V1R genes were the same as those identified by Shi and Zhang (2007), except that the present V1R2 gene corresponds to their V1R2 and V1R3 genes (amino acid sequences of their V1R2 and V1R3 are the same) (Shi and Zhang 2007), and the present V1R3 gene is novel (amino acid sequences of the 21 V1R genes are shown in Supplementary Material online). All V1R genes are intron-less. Twenty-three of these V1R sequences were tightly clustered within a 300-kb region of one scaffold (Figure 1). There were 12 conserved amino acids in the predicted amino acid sequences of the putatively functional 21 V1R genes (areas shaded gray in Supplementary Material online). These amino acids are also highly conserved among mouse (Rodriguez et al. 2002) and fish (Pfister and Rodriguez 2005) V1Rs. The potential N-linked glycosylation site in extracellular loop 2, which is present in mouse (Rodriguez et al. 2002) and fish (Pfister and Rodriguez 2005; Shi and Zhang 2007) V1Rs, was also conserved in all *X. tropicalis* V1Rs, with the exception of V1R17 (boxed sequences in Supplementary Material online).

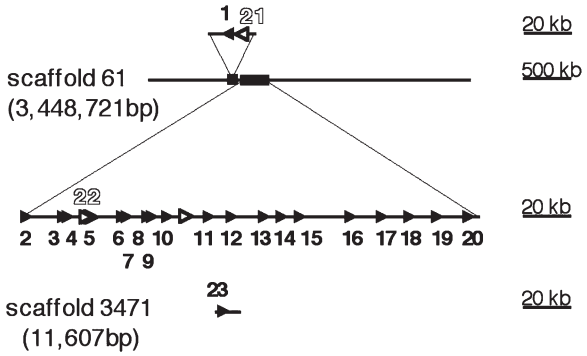

Figure 1 Distribution of *Xenopus tropicalis* V1R genes in 2 genomic clusters. Position and direction of each V1R gene are indicated by triangles. Putatively functional genes are represented by filled triangles and pseudogenes and partial sequences by open triangles.

Expression of V1R genes in *Xenopus* species

Xenopus possesses 2 olfactory organs, the MOE and VNO, although the MOE is subdivided into the PC and the MC (Foske 1934; Altner 1962; Scalia et al. 1991). The PC is responsible for detecting airborne odorants, and the MC detects water-soluble odorants (Altner 1962). The structure of a coronal section of the *Xenopus* olfactory organs is depicted in Figure 2A. The PC, MC, and VNO are located at the dorsal, lateral, and ventral regions of the nose, respectively. The fish-like olfactory receptor (OR1) and the mammalian-like olfactory receptor (OR2) are specifically expressed in the MC and PC, respectively (Freitag et al. 1995). *Xenopus* V2R is predominantly expressed in the VNO but is also expressed in some MC cells (Hagino-Yamagishi et al. 2004). The expression of OR1 (Figure 3A-5, arrows), OR2 (Figure 3B-3, arrows), V2R in the VNO (Figure 3C-1, an arrow), and in the MC (Figure 3A-3, arrows) was shown.

We isolated all 21 of the putatively functional V1R genes from the genome of *X. tropicalis* and investigated their selective expression in one or more of the *Xenopus* olfactory organs in adults. Although rodent V1Rs are specifically expressed in the VNO (Dulac and Axel 1995), in situ hybridization analysis confirmed that *Xenopus* V1R gene expression (V1R2 and V1R16) was not present in the VNO (Figure 2B-3 and 2B-6) or PC (Figure 2B-2 and 2B-5) but was present in the MC (Figure 2B-1 and 2B-4, arrows). We also analyzed the expression of V1R17 and V1R18 and found that V1R-expressing cells were mostly present in the MC (Figure 2B-7 and 2B-10, arrows) but not in the VNO (Figure 2B-9 and 2B-12). We observe a small number of the V1R-expressing cells in the PC (Figure 2B-8 and 2B-11, an arrow). These results suggest that these *tropicalis* V1R genes are largely expressed in the MOE of the MC, and in some cases in the MOE of the PC.

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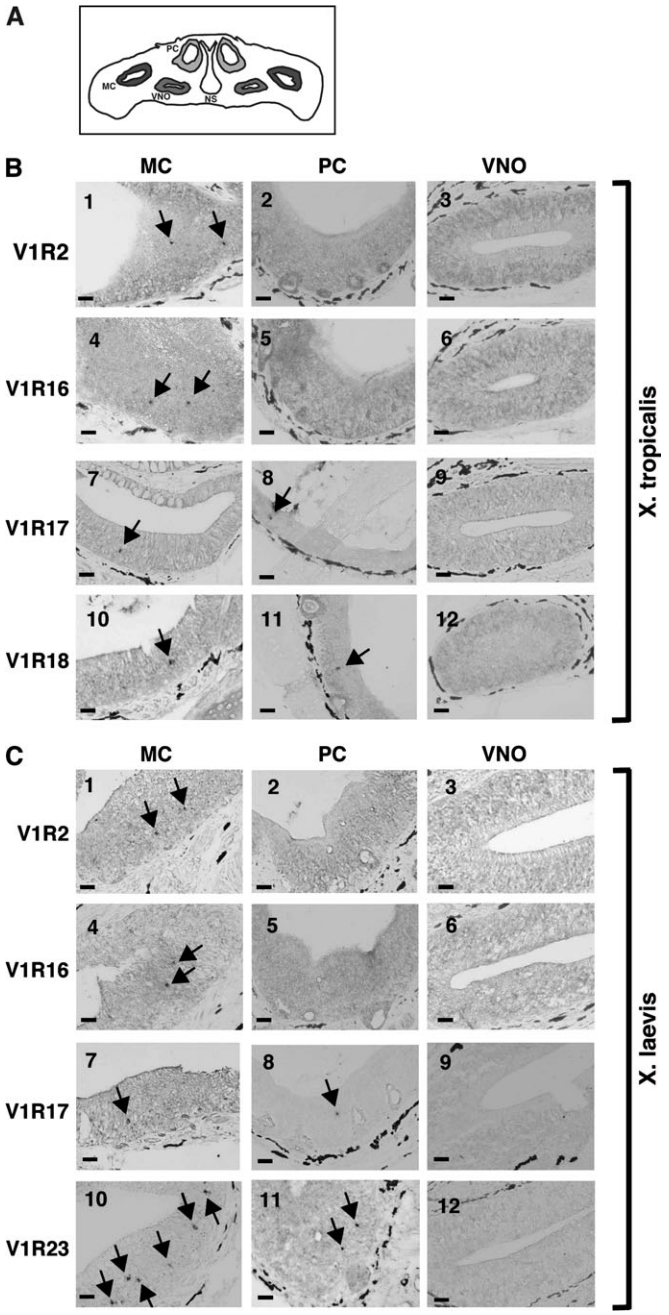

Figure 2 Expression of V1R genes in *Xenopus* olfactory organs. (Panel A) Schematic illustration of coronal section of adult *Xenopus* olfactory organs. MC, middle cavity; PC, principal cavity; VNO, vomeronasal organ; and NS, nasal septum. (Panel B) Coronal sections of adult MC (1, 4, 7, and 10), PC (2, 5, 8, and 11), and VNO (3, 6, 9, and 12) for *Xenopus tropicalis*. Sections of the *X. tropicalis* olfactory organs were hybridized with DIG-labeled V1R2 (1, 2, and 3), V1R16 (4, 5, and 6), V1R17 (7, 8, and 9), and V1R18 (10, 11, and 12) antisense cRNA probes. (Panel C) Coronal sections of adult MC (1, 4, 7, and 10), PC (2, 5, 8, and 11), and VNO (3, 6, 9, and 12) for *Xenopus laevis*. Sections of the *X. laevis* olfactory organs were hybridized with DIG-labeled V1R2 (1, 2, and 3), V1R16 (4, 5, and 6), V1R17 (7, 8, and 9), and V1R23 (10, 11, and 12) antisense cRNA probes. V1R-expressing cells are indicated by arrows. Black spots on the periphery of the MOE and VNO in panel B and C are melanocyte aggregates. Bar represents 50 μm.

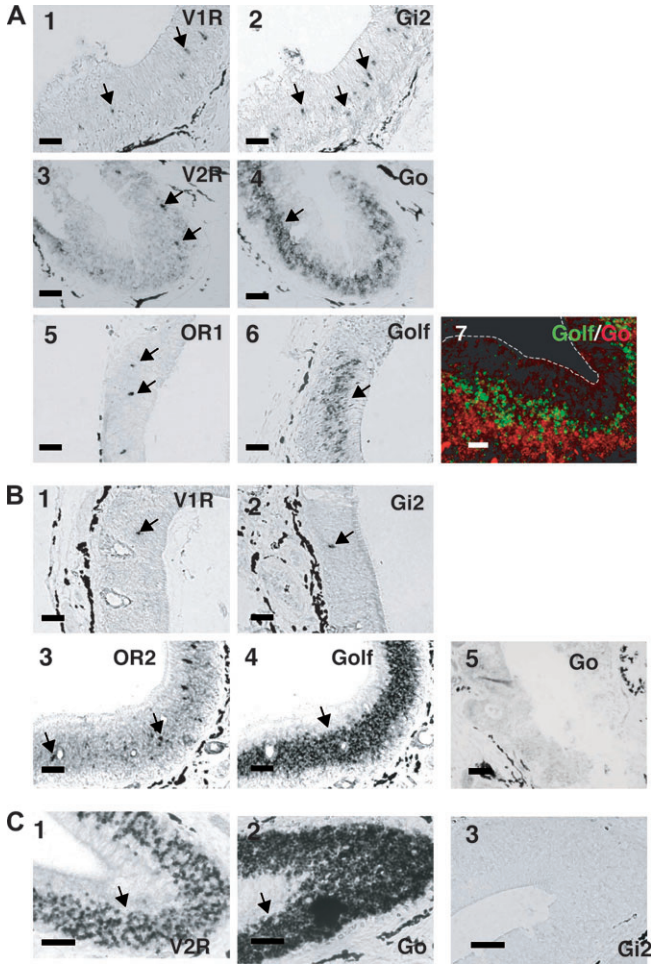

Figure 3 Expression of receptors and G-proteins in *Xenopus* olfactory organs. Coronal sections of the MC (panel A), PC (panel B), and VNO (panel C) of adult *Xenopus laevis* were hybridized with DIG-labeled antisense cRNA probes: V1R13 (A-1); Gi2 (A-2, B-2, and C-3); xV2RE (A-3 and C-1); Go (A-4, B-5, and C-2); OR1 (A-5); Golf (A-6 and B-4); V1R17 (B-1); and OR2 (B-3). For experiment A-7, DIG-labeled antisense cRNA probe for Go (red) and fluorescein-labeled antisense cRNA probe for Golf (green) were used. Bar represents 50 μm.

Next, we examined the expression of V1R genes (V1R2, V1R16, V1R17, and V1R23) in a closely related species, *X. laevis*. V1R2- and V1R16-expressing cells were not detected in the VNO (Figure 2C-3 and 2C-6) or PC (Figure 2C-2 and 2C-5) but were detected in the MC (Figure 2C-1 and 2C-4). V1R17- and V1R23-expressing cells were not detected in the VNO (Figure 2C-9 and 2C-12) but were detected in the MC (Figure 2C-7 and 2C-10, arrows). In the PC, we detected some V1R-expressing cells (Figure 2C-8 and 2C-11, arrows). These results also suggest that these *laevis* V1R genes are mostly expressed in the MOE of the MC, and in some cases in the MOE of the PC. We observed no obvious difference in the distribution of the V1R23-expressing cells along the anterior–posterior axis of the *Xenopus* MC (data not shown). Table 1 lists the expression of all V1R genes in the olfactory organs of *X. laevis*. The

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Table 1 Expression of V1R genes in the olfactory organs of *Xenopus laevis*

Gene	MOE		VNO
	MC	PC	
1	0.0	0.0	0.0
2	3.3	0.0	0.0
3, 4	0.1	0.0	0.0
5	3.0	0.0	0.0
6, 7	0.0	0.0	0.0
8, 9	2.5	0.0	0.0
10	6.5	0.0	0.0
11, 12	2.7	0.0	0.0
13	4.0	0.0	0.0
14	0.8	0.0	0.0
15	3.5	0.0	0.0
16	2.8	0.0	0.0
17	0.7	0.1	0.0
18	0.1	0.0	0.0
19	0.1	0.1	0.0
20	0.6	0.0	0.0
23	33.1	0.2	0.0

The number of V1R-expressing cells per 10 000 cells in the epithelium was indicated.

expression of V1R genes was indicated as the number of the V1R-expressing cells per 10 000 epithelial cells (see Materials and methods). We found that cells expressing all V1R genes were mostly detected in the MC, except those expressing V1R1 and V1R6/7. Expression of some V1R genes (V1R17, V1R19, and V1R23) was also detected in the PC. However, we observed no V1R-expressing cells in the VNO. The similar pattern of the expression of the V1R genes was observed in *X. tropicalis* (data not shown). These results demonstrate that *Xenopus* V1R genes are largely expressed in the MOE.

Expression of receptors and G-proteins in *Xenopus* MC

From our data, it is clear that 3 receptor families, V1R, V2R, and OR1, are expressed in the MC. We then investigated the presence of spatial segregation in the expression of these receptor families in the MC and found that V1R-expressing cells were primarily localized basally, although some cells were detected in the middle and apical layer of the MOE (Figure 3A-1 arrows). Most of the V2R-expressing cells were localized in the basal layer (Figure 3A-3 arrows), whereas OR1-expressing cells were mainly located in the apical layer (Figure 3A-5 arrows). Thus, V1R is expressed sparsely throughout the MC, whereas V2R and OR1 are expressed in relatively specific layer.

These data raised the possibility that expression of G-proteins is similarly localized in the *Xenopus* MC, particularly as rodent V1R and V2R are coexpressed with Gi2 and Go, and Gi2 and Go are, respectively, localized in the apical and the basal regions of the vomeronasal neuroepithelial layer (Jia and Halpern 1996; Dulac 2000). We thus analyzed the expression of *Xenopus* G-proteins (Gi2, Go, and Golf). We found that Gi2 was expressed sparsely throughout in the MOE (Figure 3A-2 arrows), whereas most of the Go-expressing cells were localized in the basal layer (Figure 3A-4 an arrow and Figure 3A-7 red signals) and the Golf-expressing cells were mainly located in the apical layer (Figure 3A-6 an arrow and Figure 3A-7 green signals). Therefore, the expression patterns of Gi2, Go, and Golf resemble those of V1R, V2R, and OR1, respectively. Subsequently, double in situ hybridization was performed and coexpression of V1R and Gi2 (Figure 4A-1 to 4A-3), V2R and Go (Figure 4B-1 to 4B-3), and OR1 and Golf (Figure 4C-1 to 4C-3), respectively, was identified. At present, we have observed no cells coexpressing V1R and Golf (Figure 4A-1′ to 4A-3′), V1R and Go (Figure 4A-1″ to 4A-3″), V2R and Gi2 (Figure 4B-1′ to 4B-3′), V2R and Golf (Figure 4B-1″ to 4B-3″), OR1 and Gi2 (Figure 4C-1′ to 4C-3′), and OR1 and Go (Figure 4C-1″∼3″).

These results suggest that the V1R-Gi2-mediated signal transduction pathway, which is thought to play an important role in rodents, is also present in amphibians. They also suggest that 3 distinct signal transduction pathways, mediated by V1R-Gi2, V2R-Go, and OR1-Golf, function in the MC.

Expression of receptors and G-proteins in *Xenopus* PC

In the PC, OR2-expressing (Figure 3B-3 arrows) and Golf-expressing cells (Figure 3B-4 an arrow) were seen throughout the MOE. However, a small number of V1R-expressing (Figure 3B-1 an arrow) and Gi2-expressing (Figure 3B-2 an arrow) cells were also present. The expression pattern of V1R thus resembles that of Gi2. Using double in situ hybridization, we confirmed the coexpression of V1R and Gi2 (Figure 4D-1 to 4D-3), and OR2 and Golf (Figure 4E-1 to 4E-3). At present, we have observed no cells coexpressing V1R and Golf (Figure 4D-1′∼3′) and OR2 and Gi2 (Figure 4E-1′ to 4E-3′). We have not observed the distinct expression of Go in the olfactory neurons of the PC (Figure 3B-5). These data suggest that the V1R-Gi2–mediated signal transduction pathway is also present in the PC and that 2 distinctive transduction pathways, mediated by V1R-Gi2 and OR2-Golf, are likely to function.

Discussion

We found that 23 of the 24 *Xenopus* V1R genes were tightly clustered in one scaffold (Figure 1) and that there was considerable sequence variation (data not shown). These data

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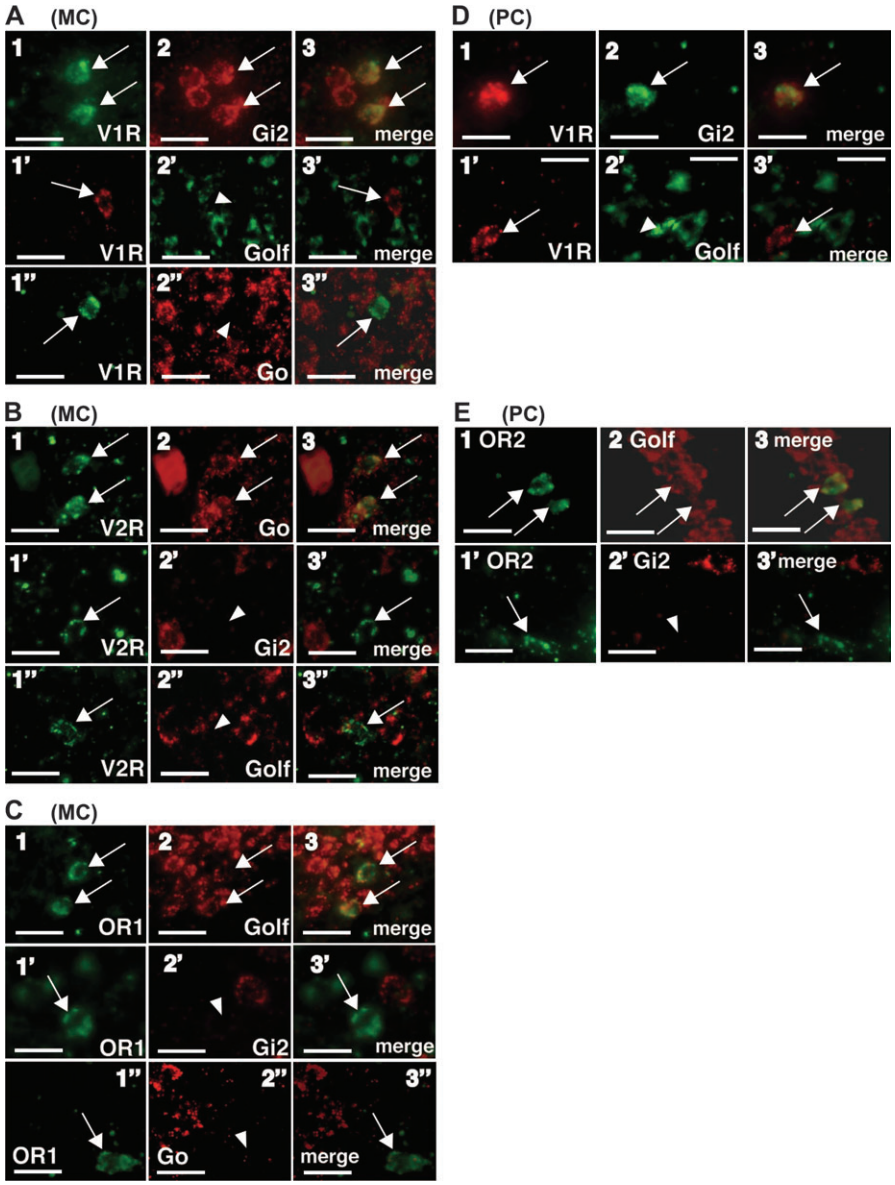

Figure 4 Double-label in situ hybridization of the receptor and the G-protein. Coronal sections of the MC **(A–C)** and PC **(D–E)** of adult *Xenopus laevis* were hybridized with antisense cRNA probes: V1R23 (A-1, A-1′, A-1″, D-1, and D-1′); xV2RE (B-1, B-1′, and B-1″); OR1 (C-1, C-1′, and C-1″); OR2 (E-1 and E-1′); Gi2 (A-2, B-2′, C-2′, D-2, and E-2′); Go (A-2″, B-2 and C-2″); and Golf (A-2′, B-2″, C-2, D-2′, and E-2). Merged images of the receptor and the G-protein are shown (panels A–E, 3–3″). Arrows indicate cells expressing the corresponding receptors or G-proteins, and arrowheads indicate the sites where the receptor-expressing cells exist. Bar represents 10 μm.

suggest that amphibian V1R genes arose from an ancestral gene through many rounds of gene duplication, after the divergence of tetrapods from fish. Such gene multiplication and sequence diversification may have provided amphibian V1Rs with the potential to discriminate between a range of chemical signals.

Surprisingly, V1R expression was not detected in the VNO, in which rodent V1R is specifically expressed (Figure 2 and Table 1). Although we cannot role out the possibility that minor V1R-expression may exist in the VNO, our results clearly show that *Xenopus* V1R genes are mostly expressed in the MOE of the MC and PC, which are generally considered to detect water-soluble and volatile odorants. Previously, we reported that Gi2 expression was not detected in the VNO (Figure 3C-3), although V2R (Figure 3C-1) and Go (Figure 3C-2) were predominantly expressed (Hagino-Yamagishi et al. 2004). The absence of the Gi2-expressing cells in the VNO supports our observation that expression of Gi2-coupling receptor, V1R, was not detected in the VNO. The difference in V1R-expression sites between amphibians and rodents indicates that, following the species divergence of terrestrial vertebrates from amphibians, there

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was a change in the principal expression site of V1R from the main olfactory system to the vomeronasal system.

Some mammals, including goats and humans, however, retain expression of V1R in the MOE (Rodriguez and Mombaerts 2002; Wakabayashi et al. 2002, 2007). Particularly in goats, V1R expression has been detected in the main olfactory bulb (MOB), which suggests that V1R-expressing neurons project their axons into this region (Wakabayashi et al. 2002). Furthermore, studies of V1R expression during olfactory development in mice showed that a limited subset of V1R genes are expressed in a small number of unidentified cells in the MOE (Karunadasa et al. 2006). Thus, it is likely that V1R-mediated chemoreception in the MOE has been conserved during the course of evolution. Recently, the role of the MOE for pheromone recognition has been discussed (Zufall and Leinders-Zufall 2007). It is possible that V1R plays a part in the MOE-mediated pheromone reception.

In rodents, Gi2 is expressed in V1R-expressing neurons (Jia and Halpern 1996) and is considered to play an important role in V1R-mediated signal transduction (Dulac 2000). The presence of cells coexpressing V1R and Gi2 in the *Xenopus* MC (Figure 4A) and PC (Figure 4D) suggest that V1R-Gi2 coexpression was already present in the amphibian MOE, although it might not be present in the VNO. We also observed the coexpression of V2R-Go (Figure 4B), OR1-Golf (Figure 4C), and OR2-Golf (Figure 4E) in the *Xenopus* MOE. These results suggest that multiple signal transduction pathways, mediated by V1R, V2R, OR1, and OR2, respectively, function in the *Xenopus* MOE and that these receptor families have distinct functions. Determination of the projection site for receptor-expressing cells and analysis of the processing system for this sensory information in the MOB and AOB will provide better understanding of these receptor-mediated sensory systems.

Although the MC is generally believed to detect water-soluble odorants, Iida and Kashiwayanagi (1999) demonstrated that the MC can respond to both water-soluble and volatile odorants; thus, both the PC and MC are able to detect volatile odorants. We found that *Xenopus* V1R genes are expressed not only in the MC but also in the PC (Figure 2 and Table 1). It is therefore possible that V1R detects volatile chemicals in the MC and PC. As rodent V1R is known to detect a volatile pheromone (Boschat et al. 2002), we cannot exclude the possibility that amphibian V1R detects volatile chemicals possessing pheromonal activity. If *Xenopus* V1R detects pheromones, the pheromonal information would be transmitted and processed by the main olfactory system.

The amphibian sex pheromones identified to date are either small water-soluble peptides or larger proteins (Kikuyama et al. 2002). For example, the newt sex pheromone sodefrin is a small water-soluble peptide and is reported to be detected by the vomeronasal cells (Toyoda and Kikuyama 2000). In *Xenopus*, V2R is predominantly expressed in the vomeronasal cells (Hagino-Yamagishi et al. 2004). Kimoto et al. (2005) reported that rodent V2R-expressing vomeronasal sensory neurons were stimulated by a peptide. Taken together, these data suggest that V2Rs are likely to recognize amphibian pheromones so far identified. Although fish V2R recognizes amino acids (Speca et al. 1999), it is possible that expression of V2R, which occurs in the fish olfactory epithelium, shifted to the VNO to act as a pheromone receptor in amphibians. On the other hand, the principal site of V1R expression shifted from the MOE to the VNO after the divergence of terrestrial vertebrates from amphibians. However, it is uncertain at what evolutionary stage V1R began to function as a pheromone receptor. As amphibians are adapted to both aquatic and terrestrial life, it is possible that amphibians use both water-soluble and volatile chemicals as pheromones, although volatile pheromones have not yet been identified in amphibians. To better understand the function of amphibian V1R, it is useful to analyze the pheromone receptors of other amphibian genera such as *Rana*. At the same time, characterization of ligands for V1R and other receptors, V2R, OR1, and OR2, in the MC and PC will help to clarify the function of the V1R and the functional differences between these receptors.

Supplementary Material

Alignment of deduced amino acid sequences of *X. tropicalis* V1R. The 12 residues conserved in *Xenopus*, mouse, and fish V1Rs are shaded in gray. Putative N-linked glycosylation sites (NXS/T) are boxed. Supplementary material can be found at http://www.chemse.oxfordjournals.org.

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