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# The Vomeronasal Receptor $V2R_2$ Does Not Require Escort Molecules for Expression in Heterologous Systems

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## Abstract

In rodents, many behavioural responses are triggered by pheromones. These molecules are believed to bind and activate two families of G-protein coupled receptors, namely V1Rs and V2Rs, which are specifically expressed in the chemosensory neurons of the vomeronasal organ. V2Rs are homologous with Group 3 of G-protein-coupled receptors, which includes metabotropic glutamate receptors, calcium-sensing receptors, fish olfactory receptors, and taste receptors for sweet molecules and amino acids. The large extracellular region of these receptors is folded as a dimer and, in this form, binds agonists that in many cases are amino acids. It has recently been reported that V2Rs must be physically associated with specific major histocompatibility complex class Ib molecules (MHC) for their expression in both mouse vomeronasal neurons and heterologous cell lines. Here, we show that in contrast to the other V2Rs, $V2R_2$, an atypical member of this receptor family, can be successfully and abundantly expressed by insect cells without the requirement of escort molecules like MHC. Moreover, the extracellular binding domain of $V2R_2$, secreted as a soluble product, forms dimers via cysteine-mediated sulphur bridges. Overall, the data presented in this paper confirm that $V2R_2$ diverges from the other members of the V2R family and suggest a different role for this receptor in pheromonal communication.

**Key words:** expression, baculovirus, insect cells, G-protein coupled receptors, pheromones



## Introduction

Pheromones are molecules that consistently elicit a variety of stereotyped behaviours in animals of the same species. The importance of this type of chemical communication is emphasized by the existence of a chemosensory system parallel to the main olfactory one that works as a detector and integrator of intraspecific signals (Halpern and Martinez-Marcos, 2003). Pheromone detection begins in a sub-septal organ, the vomeronasal organ (VNO), where two families of G-protein coupled receptors (GPCRs), V1Rs and V2Rs (~100 members each), are differently expressed in two distinct neuronal layers of the VNO. V1Rs are expressed in the apical neurons (luminal side), whereas V2Rs are expressed in the more basal ones (Dulac and Axel, 1995; Herrada and Dulac, 1997; Matsunami and Buck, 1997; Ryba and Tirindelli, 1997). Projecting axons from these two neuronal populations also remain segregated into two distinct zones of the accessory olfactory bulb (Del Punta *et al*., 2002). As for the main olfactory epithelium, any individual neuron of the apical layer expresses only one receptor type among the V1Rs (Malnic *et al*., 1999). Similarly, only one V2R is expressed in each basal neuron. All basal neurons, however, express a supplementary and divergent V2R, namely $V2R_2$ (Martini *et al*., 2001). It has been reported that V2Rs, but not V1Rs, require the intracellular interaction with specific major histocompatibility complex (MHC) class Ib molecules for correct expression on the plasma membrane of the vomeronasal neurons and transfected cell lines (Ishii *et al*., 2003; Loconto *et al*., 2003). Despite this intriguing pattern of expression, knowledge about the signalling specificity in the basal neurons of the VNO remains elusive, nor have ligands been identified for V2Rs.

All V2Rs belong to the Group 3 of GPCRs (Pin *et al*., 2003) and are homologous with metabotropic glutamate receptors (mGluRs) (Pin and Duvoisin, 1995), calcium-sensing receptor (CaSR) (Brown *et al*. 1993), γ-amino-butyric acid type B ($GABA_B$) receptors (Kaupmann *et al*., 1997), taste receptors (T1Rs) (Hoon *et al*., 1999) and fish olfactory receptors (Speca *et al*., 1999). The main feature of this receptor group is a large extracellular region that in the absence of the transmembrane domain is responsible for binding with their

 

respective agonists (Pin *et al.*, 2003). In Group 3 GPCRs, dimerization represents an important mechanism that dramatically affects the glycosylation, the correct expression on the cell surface and the binding properties (Kaupmann *et al.*, 1997; Ray and Hauschild, 2000; Calver *et al.*, 2001; Margeta-Mitrovic *et al.*, 2001; Pagano *et al.*, 2001). Moreover, most Group 3 GPCRs bind amino acids with a variable range of affinities. Crystallographic data on the extracellular region of mGluR1 have lead to the identification of the residues interacting with the α-amino and α-carboxyl groups of glutamate in the ligand-binding pocket (Kunishima *et al.*, 2000). Interestingly, these residues are all conserved in CaSR (Reyes-Cruz *et al.*, 2001) and in the fish olfactory receptor R5.24 (Kuang *et al.*, 2003), which both bind amino acids (Speca *et al.*, 1999; Conigrave *et al.*, 2002).

Based on the divergence of $V2R_2$ from the V2R family and on its atypical expression pattern in the vomeronasal organ, we asked whether this receptor undergoes the same modalities of expression as required for the other V2Rs. In this work, we have successfully attempted to express both full-length $V2R_2$ and its extracellular region, as a secreted product, in heterologous systems. Thus, we have demonstrated that escort molecules, like those of the MHC complex, are not necessary for the correct expression of $V2R_2$. Moreover, the purification and characterization of the extracellular region of $V2R_2$, presented here, lays the basis for searching for ligands, and for the study of the structural features of this receptor.

## Materials and methods

### Materials

Culture media were purchased from Invitrogen (Milan, Italy). Enzymes and antibiotics were obtained from AmershamPharmaciaBiotech (Milan, Italy). Primers were purchased from MWG (Ebersberg, Germany). Chemicals were from Sigma (St Louis, MO) or AmershamPharmacia-Biotech.

### Cell culture

*Spodoptera frugiperda* cells (Sf9 cells) were propagated in a monolayer at 27°C in SF-900II medium, supplemented with 10% fetal bovine serum or in suspension without addition of serum. Human embryonic kidney cells (HEK) were cultured in a monolayer at 37°C, 5% $CO_2$ in Dulbecco's modified Eagle medium with the addition of 10% serum and antibiotics. Cells were transiently transfected by the calcium phosphate method.

### Construction of transfer vectors for receptor expression

Full-length c-*myc*-tagged $V2R_2$ was constructed from $V2R_2$-pCI by sequential overlap extension polymerase chain reaction (PCR). A fragment was made with primers 70F (ACTCGAGCCG CCATGTCTA) and 70R (CAAGTCTTCTTCGCTGATAAGCTTCTGTTCTTGAGCACCCAATACAGC). Another fragment was made with primers 71F (GAACAGAAGCTTATCAGCGAAGAAGACTTGAACAAAACAGAGGAAGTA) and 67R (TTCTCATTGGAGTAGACTTTGGGAAT). A second PCR was made with primers 70F and 67R and the product was digested with *Xho*I and *Acc*I and ligated to $V2R_2$-pCI. The resultant plasmid, MYC-$V2R_2$-pCI contained the c-*myc* tag after the predicted signal peptide sequence. For insect cell expression, MYC-$V2R_2$-pCI was digested with *Xho*I–*Not*I and introduced in pFastBac (Invitrogen) to generate MYC-$V2R_2$-pFB.

The plasmid pFastBac (Invitrogen) was digested with *Eco*RI and *Hin*dIII and ligated to an adaptor containing a *Nhe*I, *Not*I and *Sal*I site to generate pFBM. pFBM was digested with *Not*I and *Hin*dIII and ligated to six consecutive histidine codons followed by the termination codon TAA to generate pFBMhis.

The extracellular region of $V2R_2$ was made as follows. The plasmids MYC-$V2R_2$-pCI and $V2R_2$-pCI were used as a template for PCR amplification. Primers 90F (CCCAAAGTCTACTCCAATGAG) and 90R (AGTCGACTAAGCGGCCGCCTCTTCATAAGCGAGGAA) were used to amplify a 1100 bp fragment of the N-terminal region of the extracellular domain. Primer 90R was modified to include an additional *Not*I site followed by a stop codon and a *Sal*I site. The *Acc*I–*Sal*I fragment of the PCR product was ligated with the *Nhe*I–*Acc*I fragment of MYC-$V2R_2$-pCI or $V2R_2$-pCI and with the *Nhe*I–*Sal*I digested pFBM in a three-piece ligation to generate MYC-sR2 and $sR_2$.

A three-piece ligation was also used to generate the histidine-tagged $V2R_2$ constructs sR2-HIS and MYC-sR2-HIS. The first piece was obtained by digestion of $V2R_2$-pCI or MYC-$V2R_2$-pCI with *Nhe*I–*Acc*I. The second piece was the *Acc*I–*Not*I fragment of the final PCR product described above. The digestion of pFBMhis with *Nhe*I–*Not*I produced the third fragment.

The EGFP-$sR_2$ construct was prepared by generating a PCR product containing a 5′ *Xho*I site in place of the predicted signal peptide of $V2R_2$. The product was cut with *Xho*I–*Acc*I and inserted into $sR_2$ that was previously subcloned in *Xho*I–*Not*I of pCI. This construct represented the truncated receptor without the signal peptide (tSP-sR2-pCI). Then, pEGFP-C1 was cut with *Nhe*I–*Sal*I and ligated in frame to tSP-sR2-pCI to give EGFP-$sR_2$-pCI. The $V2R_2$ signal peptide was amplified from $V2R_2$-pCI, cut with *Nhe*I–*Age*I and cloned in EGFP-$sR_2$-pCI. The tagged receptor sequence was then cut with *Nhe*I–*Not*I and cloned into pFBM to generate EGFP-sR2.

### Generation of recombinant baculoviruses for $V2R_2$

Transfer vectors containing the different receptor constructs were used to transform DH10Bac *Escherichia coli* according to the manufacturer's instructions (Invitrogen). Viral DNA from positive colonies was tested by PCR to verify the

correct recombination. Recombinant baculoviruses encoding the receptor cDNAs were produced after transfection by calcium phosphate precipitation of Sf9 cells. The viruses were harvested after 5 days and used to infect Sf9 cells for a second round of virus amplification. Titration of the viral stocks was always performed before use. For the expression assay, Sf9 cells were infected with each baculovirus at a multiplicity of infection of 1 or 2. Cells and media were harvested 48–72 h after infection.

Membrane extracts were prepared as previously described (Tirindelli and Ryba, 1996). Proteins were separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and analysed by immunoblotting.

### Purification of the extracellular domain of $V2R_2$

The cell culture medium of infected Sf9 cells (100 ml) was collected and centrifuged at 800 *g* for 10 min and the supernatant filtered onto nitrocellulose (Millipore 0.22 μm). Medium was then concentrated up to 15-fold by pressure filtration (Amicon). After being dialysed against 50 mM Tris–HCl, pH 7.5, medium was filtered and loaded onto a metal affinity column (Talon, Clontech) to retain histidine-tagged receptors. The column was washed with 150 mM NaCl, 50 mM of Tris–HCl, pH 8.0 and the bound material was eluted with 200 mM imidazole, pH 8.0. The eluate was dialysed against 10 mM Tris–HCl, 150 mM NaCl, pH 7.5, and further purified by immunoaffinity chromatography using an anti-$V2R_2$ conjugated sepharose column. After an overnight incubation at 4°C, the column was extensively washed with 10 mM Tris–HCl, 150 mM NaCl, pH 7.5 and the bound material eluted with 100 mM glycine, pH 2.5 and quickly buffered with Tris–HCl, pH 8.0. Aliquots of the fractions were analysed by SDS–PAGE, silver staining and immunoblotting.

### Western blot analysis

Proteins were separated by SDS–PAGE and electroblotted onto a nitrocellulose membrane (AmershamPharmacia Biotech). The membrane was then blocked for 1 h in TTBS (10 mM Tris–HCl, pH 8.0, 150 mM NaCl, and 0.05% Tween 20) with 5% bovine serum albumin and 1% dry milk at room temperature. Antibodies were incubated overnight at 4°C. Anti-$V2R_2$ (Martini *et al.*, 2001) and anti-His (Roche) were used at 1:1000 dilution; anti-c-*myc* (Invitrogen) at 1:5000. After being washed, the membrane was incubated with a goat anti-mouse IgG or a goat anti-rabbit IgG conjugated with peroxidase (Pierce). Development was performed by a commercial detection kit (Pierce).

### Reducing treatments

The constructs MYC-sR2-HIS and EGFP-$sR_2$ were used to co-infect Sf9 cells. After 72 h, centrifuged and filtered medium was treated with the reducing agent dithiothreitol (DTT) at 100 mM for 3 h at room temperature. Following a short dialysis, the medium was treated with 100 mM iodoacetamide for 3 h and then extensively dialysed overnight against 10 mM Tris–HCl, pH 7.5. Medium was then loaded onto a Talon column and eluted with 200 mM imidazole, pH 8.0. All steps were analysed by SDS–PAGE in the presence or without 3% β-mercaptoethanol in the sample buffer.

## Results

To facilitate the monitoring and purification of $V2R_2$, different epitopes were incorporated into the amino and carboxyl terminus of the receptor. Figure 1 shows a schematic view of the constructs that were used for expression of $V2R_2$. For the full-length receptor, a c-*myc* epitope was added after the predicted signal peptide, at Gln-23. The constructs encoding the extracellular region of $V2R_2$ were truncated after Gly-621 of the $V2R_2$ sequence, this being the last residue preceding the first transmembrane loop. In the constructs MYC-sR2 and MYC-sR2-HIS, a c-*myc* epitope was added after the predicted signal peptide sequence; in the construct EGFP-$sR_2$, EGFP was introduced after the signal peptide of $V2R_2$. Six histidines were added to the terminal Gly-621 in the MYC-sR2-HIS and sR2-HIS constructs. All constructs contained the cysteine-rich region at end of the extracellular domain.

Figure 2 shows the Western analysis of membrane and medium proteins after infection of Sf9 cells with recombinant viruses encoding the full-length or the truncated (extracellular) $V2R_2$. This latter was found to be abundantly secreted in the culture medium of insect cells. The specificity of the products was tested with an antibody raised against the C-terminal region of the extracellular domain of $V2R_2$ (Martini *et al.*, 2001) and yielded a broad band at ~94–116 kDa (predicted size 100 kDa) for the full-length receptor and 76 kDa (predicted size 71 KDa) for its extracellular region (Figure 2A,B). In order to verify that the secreted receptor was not truncated at the amino terminus or at the

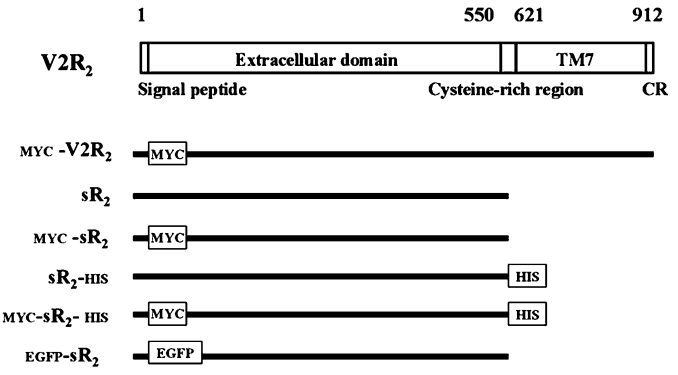


**Figure 1** Diagram of the constructs employed for insect cell expression of $V2R_2$. Full-length $V2R_2$ is represented according to its primary amino acid sequence. The numerical position of amino acids and functional regions are indicated. All products include the cysteine-rich region preceding the seven transmembrane domain (TM7). CR is the cytoplasmic region.

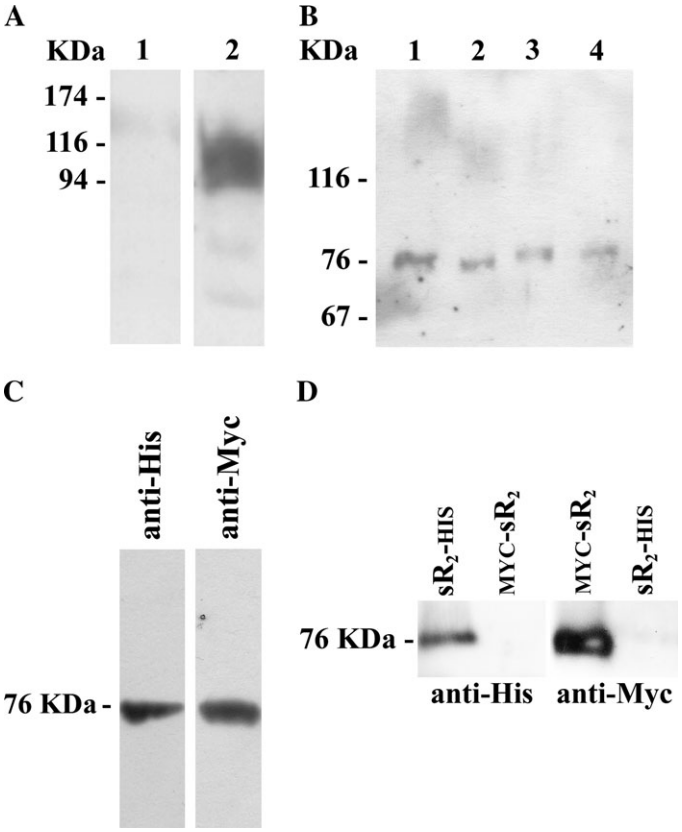


**Figure 2** Western analysis of $V2R_2$ in Sf9 cells. **(A)** Membrane preparation (40 μg) of insect cells infected with baculovirus for full-length $V2R_2$ (MYC-$V2R_2$, lane 2) or mock infected as a control (lane 1) was loaded onto SDS–PAGE and the blot was probed with anti-$V2R_2$ antibody. **(B)** Culture medium (20 μl) of insect cells infected with baculoviruses for MYC-sR2 (lane1), $sR_2$ (lane 2), MYC-sR2-HIS (lane 3) and sR2-HIS (lane 4) was loaded onto SDS–PAGE. The blot was probed with the anti-$V2R_2$ antibody. **(C)** Western blot of culture medium of Sf9 infected with baculovirus for MYC-sR2-HIS was immunoblotted with the anti-His or anti-c-Myc antibody as indicated. **(D)** Western blot of culture medium of Sf9 infected with baculovirus for MYC-sR2 or sR2-HIS was immunoblotted with the anti-His or anti-c-*myc* antibody as indicated.

carboxyl terminus, the culture medium of Sf9 cells, infected with recombinant virus for MYC-sR2-HIS, was separately immunoblotted with the anti-c-*myc* and anti-His antibody. Figure 2C shows that product was intensely stained by the two antibodies, indicating that degradation did not occur. In general, receptor degradation was never observed more than 72 h post-infection by Western analysis with all antibodies employed in this work. Figure 2D also shows the specificity of the antibodies that were used.

Similar experiments were performed with two other V2Rs, namely $V2R_1$ and VN2, either alone or in combination with $V2R_2$. In all cases, no secretion or expression on the cell membrane was detected (data not shown). Some constructs (e.g. EGFP-$V2R_1$) were found to be abundantly expressed in insoluble inclusion bodies of insect cells (data not shown). No expression was observed when these constructs were transfected in HEK cells (data not shown).

Dimerization is a fundamental prerequisite for the correct expression and ligand-binding properties of Group 3 GPCRs, and normally occurs via the extracellular region of these receptors. Therefore, we performed electrophoresis of the culture medium or cell membranes of Sf9 infected with recombinant baculovirus for the full-length and truncated $V2R_2$ under non-reduced condition. Figure 3A (lanes 1 and 2) shows that the band corresponding to the full-length receptor was converted to a doublet of ~200 kDa, indicating the formation of dimeric species. Similarly, dimerization and oligomerization also occurred for the truncated $V2R_2$ when the reducing treatment was omitted (Figure 3A, lanes 3 and 4). Interestingly, the monomeric form of the receptor was never observed under non-reduced conditions. In order to exclude the possibility that dimerization was due to electrophoretic artefacts, dimer formation was also demonstrated by co-infecting insect cells with recombinant baculovirus for MYC-sR2 and sR2-HIS (Figure 3B, lanes 1 and 2). Following chromatography with a metal affinity resin (Talon) to separate His-tagged proteins, products were electroblotted with the anti-c-*myc* and anti-His antibodies (Figure 3B, lanes 3 and 4). The result of this experiment shows that the two differently tagged receptors co-eluted from Talon as both antibodies revealed them. The eluates from Talon, immunoblotted with anti-c-*myc*, often show a high molecular weight band (probably a dimer) that is not recognized by the anti-His antibody. This band, however, stains with the anti-$V2R_2$ antibodies (data not shown). We believe that this is the result of a partial reduction (by the sample buffer) of oligomers mainly consisting of MYC-sR2 and that are efficiently retained by Talon. Indeed, MYC-sR2 was found to be more abundantly expressed than sR2-HIS in co-expression experiments when supernatant were tested with the same antibody (anti-$V2R_2$, data not shown).

We next asked whether dimerization exclusively occurred via intermolecular disulphide linkages or whether other modalities, such as hydrophobic interactions, should also be taken into account. With this in mind, the recombinant viruses for MYC-sR2-HIS and EGFP-$sR_2$ were used to co-infect Sf9 cells and half of the medium was treated with the cysteine-reducing agent DTT. The EGFP-tagged truncated receptor was chosen in this experiment as its greater size (100 kDa) allows an easy identification of the two products according to their molecular weight. Reduced and non-reduced culture media were then affinity purified through a Talon column and the eluates were immunoblotted with the anti-$V2R_2$ antibody. Figure 3C shows that, following affinity chromatography, co-elution of the two tagged receptors was only observed when the reducing treatment was omitted. Figure 3D shows the specificity of the column to exclusively retain the His-tagged receptors. Overall, this result suggests that the truncated receptor does not retain the dimeric form after reduction of the cysteine linkages.

Next, we wanted to ascertain whether $V2R_2$ expression was a specific property of insect cells or whether other

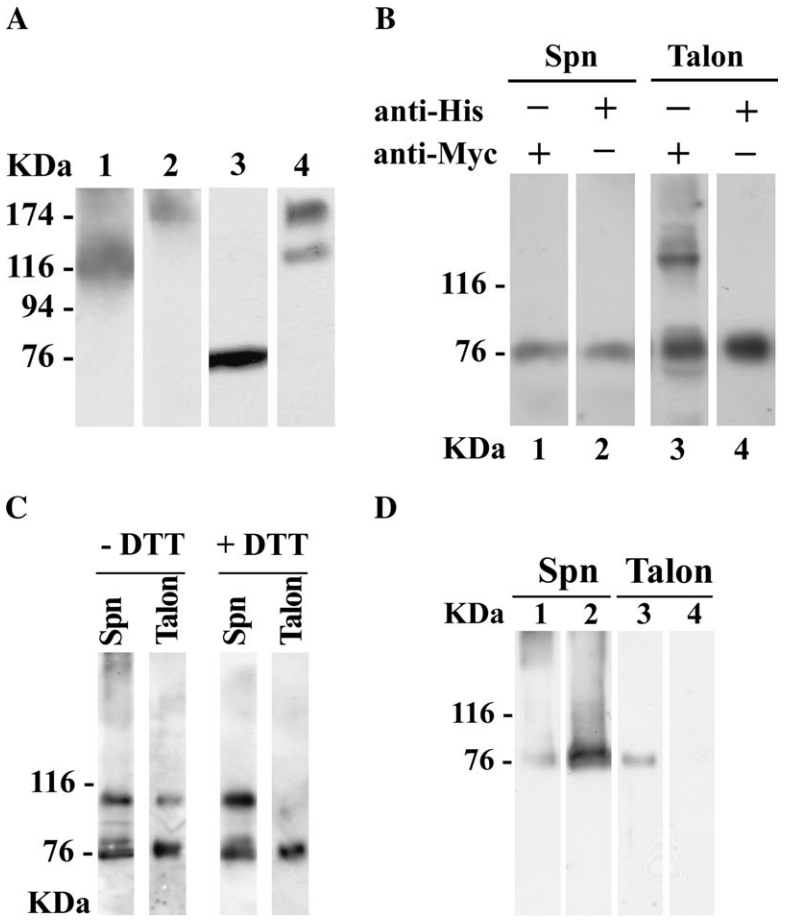


**Figure 3** Homodimeric interactions of $V2R_2$. **(A)** membrane extract of MYC-$V2R_2$ infected insect cells (lanes 1 and 2) and culture medium of MYC-sR2 infected insect cells (lanes 3 and 4) was loaded onto 7% SDS–PAGE under reduced (3% β-mercaptoethanol in the sample buffer, lanes 1 and 3) or non-reduced conditions (lanes 2 and 4). Immunoblotting was performed with the anti-c-*myc* antibody. **(B)** Western blot probed with the anti-His (lanes 2 and 4) and anti-*myc* (lanes 1 and 3) antibodies of culture medium of Sf9 cells co-infected with MYC-sR2 and sR2-HIS before (Spn) and after elution from a Talon column (Talon). **(C)** Western blot of reduced (+DTT) and non-reduced (−DTT) culture medium of Sf9 cells co-infected with MYC-sR2-HIS and EGFP-$sR_2$ before (Spn) and after (Talon) affinity chromatography. Revelation was performed with the anti-$V2R_2$ antibody. **(D)** Supernatants and Talon eluates from insect cell preparations, respectively infected with sR2-HIS (lanes 1 and 3) and MYC-sR2 (lanes 2 and 4), were probed with anti-$V2R_2$ antibody.

heterologous systems also display this ability. The construct MYC-sR2-HIS was cloned into a mammalian expression vector and used to transfect HEK cells. Culture medium was affinity purified to remove serum proteins and then concentrated up to 15-fold. This step was required before Western analysis, given the low level of receptor expression under these conditions. Immunoblotting of the truncated receptor expressed by HEK cells yielded a major band at a molecular mass of 76 kDa, comparable with that secreted by Sf9 cells. A minor product banded at ~70 kDa, thus suggesting the presence of a non-glycosylated or slightly degraded receptor (Figure 4). Similarly, we could detect a very low level of expression when HEK cells were transfected with the construct encoding the full-length receptor (data not shown). These data suggest that, although with quantitative differences, $V2R_2$ can be generally expressed in different eukaryotic cell systems.

Finally, we wanted to verify if insect cells could produce sufficient amounts of truncated $V2R_2$ for further structural

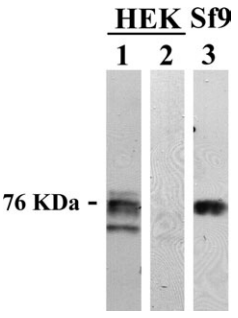


**Figure 4** Truncated $V2R_2$ expression in mammalian cells. The construct encoding MYC-sR2-HIS was cloned into pCI and used to transfect HEK cells. The culture medium (10 ml) of MYC-sR2-HIS (lane 1) or mock transfected (lane 2) HEK cells were affinity purified with Talon, dialysed and concentrated up to 15-fold. Twenty microlitres of solution was then loaded onto SDS–PAGE and immunoblotted with the anti-c-*myc* antibody. Twenty microlitres of plain culture medium of Sf9 cells infected with recombinant virus for MYC-sR2-HIS were used as a control (lane 3).

studies. Abundant secretion would also confirm that expression and transport of this receptor from the endoplasmic reticulum to the cell membrane is highly efficient. Cell cultures (100 ml) were infected with the recombinant virus for MYC-sR2-HIS and harvested after 72 h. Medium was concentrated, dialysed and chromatographed through a Talon column. After elution with imidazole, the pooled fractions were dialysed and loaded onto the anti-$V2R_2$ immuno-affinity column. Fractions were eluted and analysed by silver staining and western blotting (Figure 5A,B). Based on an overloaded SDS–PAGE, we estimated a purity of our final product of ~98%. From a typical purification procedure, we usually obtained a final yield of ~0.5–1 mg of secreted receptor per liter of conditioned medium.

## Discussion

The lack of V2R expression in heterologous systems has thus far hindered the study of the V2R receptor family. Recently, however, it has been reported that V2Rs are directed to the plasma membranes exclusively via physical interactions with vomeronasal specific MHC class Ib molecules that are expressed in the same cell (Ishii *et al.*, 2003; Loconto *et al.*, 2003). This was elegantly demonstrated either in vomeronasal neurons or in heterologous cell systems. Here, we wanted to investigate if this was also true for an atypical V2R, namely $V2R_2$. $V2R_2$, in fact shows a relatively low homology and a different expression pattern with respect to the other V2R members (Ryba and Tirindelli, 1997; Martini *et al.*, 2001). Surprisingly we found that this receptor could be efficiently expressed in heterologous systems like mammalian and insect cell lines without the help of escort molecules. This appears to be an exclusive feature of $V2R_2$ as, in our hands, other V2Rs failed to show expression in all cell lines we have tested.

We also managed to obtain abundant secretion of the extracellular region (putative ligand-binding region) of $V2R_2$ (see Figure 5). The secretion of the extracellular region of $V2R_2$ indirectly implies that the receptor is likely to be properly folded and functional, suggesting that binding and structural studies can be carried out in the future. Another indication that the receptor may be functional even in the absence of MHC class Ib molecules is indicated by its capacity to form dimers. We characterized this type of interaction and demonstrated that it occurs via cysteine linkages. In mGluR1, mutation at Cys-140 resulted in a significant amount of monomer in the absence of reducing agents (Ray and Hauschild, 2000). Interestingly, both CaSR and $V2R_2$, as reported here, dimerize but lack this cysteine. For CaSR, dimerization occurs at Cys-129, Cys-101 and Cys-236 (Pace *et al.*, 1999; Zhang *et al.*, 2001), the latter two corresponding to Cys-118 and Cys-248 of $V2R_2$ (Figure 6). Therefore, it is plausible that, in $V2R_2$, these residues are involved in disulphide linkages that anchor the two monomers. On the other hand, the extracellular domains of

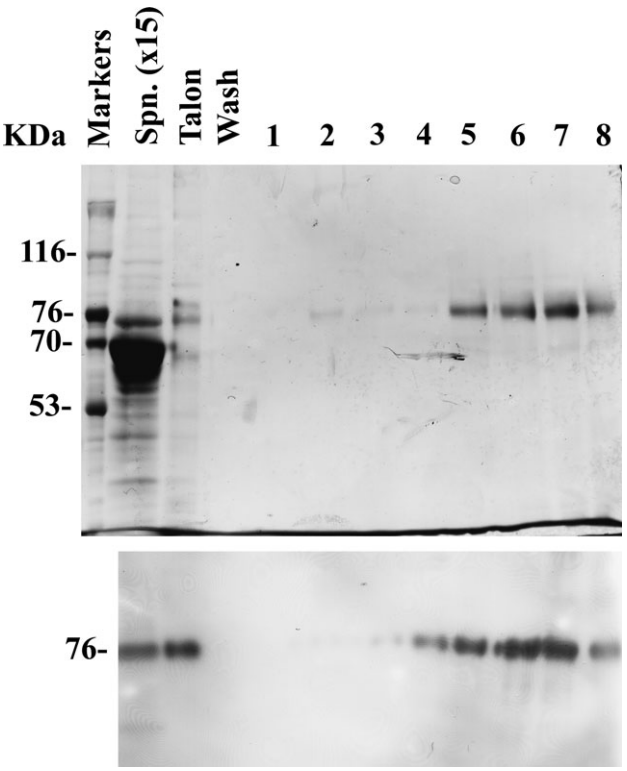


**Figure 5** Purification of the truncated receptor. Culture medium (100 ml) of Sf9 cells infected with recombinant virus for MYC-sR2-HIS was concentrated to 15-fold (Spn ×15), dialysed and loaded onto a Talon column. After washing with 50 mM Tris, 150 mM NaCl, pH 8.0, the bound material was eluted in 2 ml with 200 mM imidazole, pH 8.0 (Talon). The partially purified receptor was then dialysed and loaded onto anti-$V2R_2$ immunoaffinity column and eluted in 0.2 ml fractions with 100 mM glycine, pH 2.5. Twenty microlitres of the concentrated supernatant, the eluate from the Talon column and the fractions from the immunoaffinity column (1–8) were subjected to 7% SDS–PAGE, silver stained **(A)** and immunoblotted with anti-$V2R_2$ **(B)**.

$V2R_2$ and CaSR share 15 other cysteines in the equivalent position, raising the possibility that $V2R_2$ might also dimerize through these residues.

The molecular mass of full-length and truncated $V2R_2$, expressed by both insect and mammalian cells, determined by gel electrophoresis, was slightly higher than predicted, thus suggesting that modest post-translation modifications occur for this receptor. Modifications were also reported for both the truncated (extracellular) mGluR1 (Okamoto *et al.* 1998) and fish receptor 5.24 (Kuang *et al.*, 2003). In receptor 5.24 (Kuang *et al.*, 2003), the secreted polypeptide was ~40 kDa larger than predicted by the sequence. However, this extensive glycosylation did not affect the binding properties of the two receptors, suggesting that this could also be the case for $V2R_2$.

Mutational analysis coupled with information from the solved crystal structure has identified potential ligand-docking residues in mGluR1 (Kunishima *et al.*, 2000; Sato *et al.*, 2003). Further analysis of receptors related to mGluR1 has revealed an interesting pattern of functional conservation

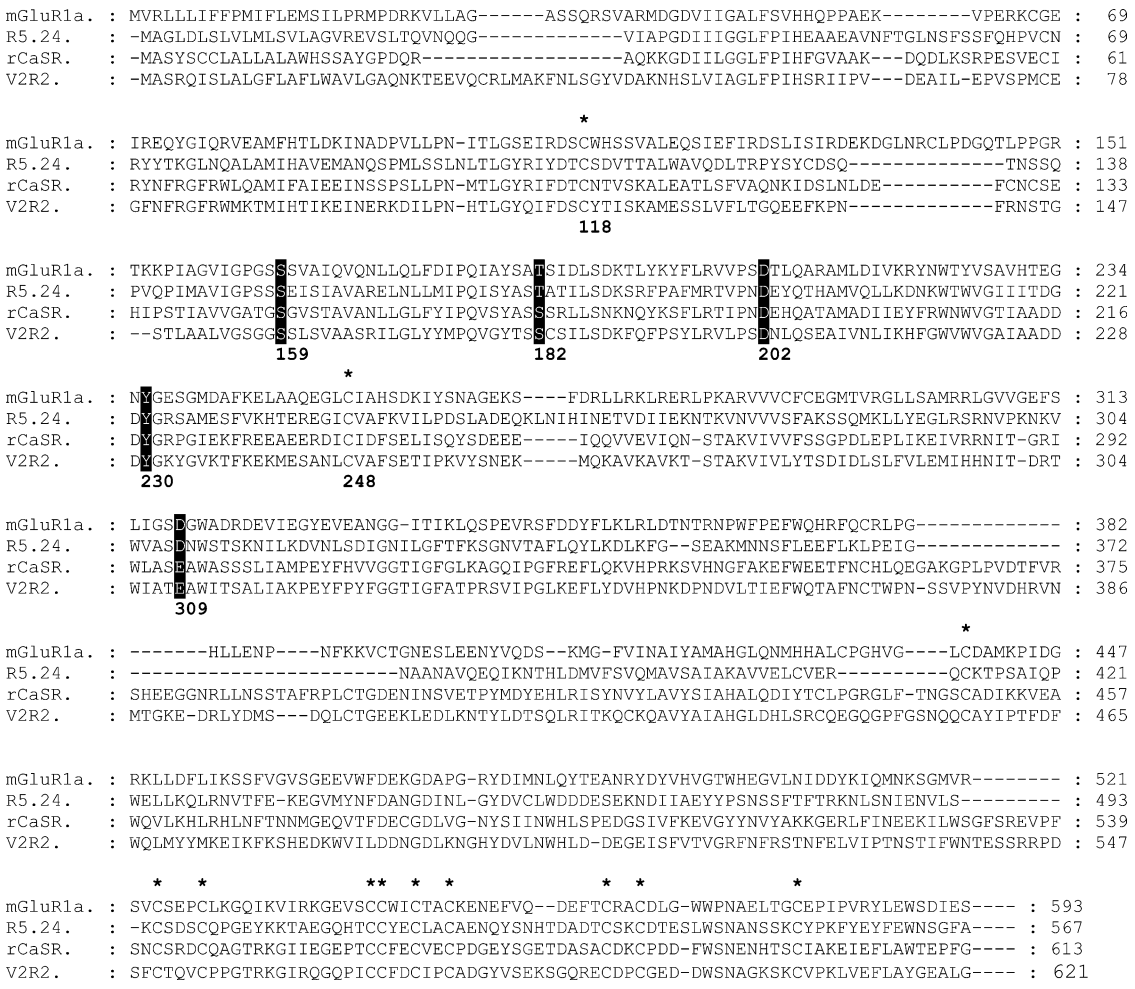


**Figure 6** Multiple sequence alignment of the extracellular amino-terminal domains of rat mGluR1, rat calcium-sensing receptor (rCaSR), goldfish R5.24 odorant receptor and mouse $V2R_2$. The alignment was made using clustalW 1.8. The alignment was modified to preserve the conserved cysteines. A few residues were manually adjusted to be consistent with the secondary structure in the mGluR1 crystal. Residues that interact with α-amino and α-carboxyl groups of ligands are highlighted with a black background. Asterisks at the top denote the conserved cysteines. Numbers in bold refer to the $V2R_2$ sequence.

(Jensen *et al.*, 2000; Rosemond *et al.*, 2002; Kuang *et al.*, 2003). The key residues that interact with α-amino and α-carboxyl groups are all conserved in the amino acid binding receptors CaSR, R5.24 and T1R1+T1R3. Curiously T1R2, which senses sweet molecules, lacks two of these residues (Hoon *et al.*, 1999). We aligned the extracellular region of $V2R_2$ with that of other receptors of Group 3 and showed that it also retains this characteristic signature (see Figure 6). This suggests that amino acids may bind to this receptor, thus implying a role for molecules with α-carboxyl-amino groups in pheromonal communication. Thus, one fascinating possibility is that $V2R_2$ is a sensor for a common amino acid or, more interestingly, for atypical amino acids such as end-products of the metabolism, not being reabsorbed by the renal tubules and that provided with pheromonal properties.

In conclusion, evidence that $V2R_2$ can be expressed by heterologous systems without interacting with MHC molecules is outlined in this report. It is possible that our speculations will be confirmed by studies on vomeronasal neurons. For example, it could be conceivable that one of the roles of MHC molecules is to interfere with the heterodimerization of $V2R_2$ with other V2Rs by interacting with the latter. This would imply independent roles for $V2R_2$ and the other V2Rs in pheromonal communication. In this context, it will be interesting to verify if, for example, $V2R_2$ is associated or not with MHC complex in VNO membrane extract, as has been reported for other V2Rs.

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