Cloning of an olfactory sensory neuron-specific protein in the land snail (*Eobania vermiculata*)

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We have isolated a gene encoding for an olfactory sensory neuron (OSN)-specific protein in an invertebrate, the land snail *Eobania vermiculata* (GenBank accession number AY147909.). Using *in situ* hybridization, we detected expression of its mRNA in the dendrite, cell body and axon of OSNs. By neural tracing, using the lipophilic tracer DiI and *in situ* hybridization, we have revealed the organization of OSNs and their connections with olfactory glomeruli in the land snail. Sequence and expression pattern analogy of land snail protein with olfactory marker protein (OMP) from vertebrates suggest that the land snail protein is an OMP-like protein. This protein could represent a plesiomorphic character in the evolution of olfactory proteins.

Keywords: olfactory marker protein; neural tracing; olfactory sensory neuron; olfaction; land snail; mollusc

1. INTRODUCTION

The olfactory system in molluscs is poorly characterized as compared to that in other invertebrates and vertebrates. While the morphology of the olfactory system in land snail species has been described (Wright 1974; Chase & Tolloczko 1989, 1993; Zaitseva 1991; Mazzatenta *et al.* 2003), the neural network and molecular components remain unknown.

In vertebrates, it has been proved that olfactory marker protein (OMP) is the only specific molecular marker of olfactory sensory neurons (OSNs) (Margolis 1972; Keller & Margolis 1976; Sydor *et al.* 1986; Krishna *et al.* 1992; Buiakova *et al.* 1994; Rössler *et al.* 1998; Horowitz *et al.* 1999; Baldisseri *et al.* 2002; Celik *et al.* 2002).

In the present study we investigated the presence of specific proteins in OSNs of the land snail (*Eobania vermiculata*). We report the cloning and sequencing of the corresponding cDNA and its expression in OSNs detected by *in situ* hybridization. Combining the results of *in situ* hybridization and neural tracing with DiI, we have revealed the organization of OSNs and their connections with olfactory glomeruli in the land snail.

2. MATERIAL AND METHODS

Specimens of the land snail *E. vermiculata* were obtained from the Istituto Internazionale di Elicicoltura (Cherasco, Italy). Animals were treated before sampling by injecting 1.2 mg succinylcholine in 4 M magnesium chloride solution into the haemocoel.

Genomic DNA was extracted by proteinase K digestion and purified by phenol-chloroform extraction. RNA was extracted using liquid nitrogen and purified following standard procedures with Trizol Reagent (GIBCO, Life Technology). cDNA was generated using a kit based on Moloney murine leukaemia virus reverse transcriptase (GIBCO, Life Technology). OMP degenerate primers (Operon Technologies) were designed based on the mammalian common regions MAED(G/S/R)PR (OMP1 sense 5'-ATG GC(T/C/A/G) GA(A/G) GA(T/C) (A/G)G(T/C/A/G) CC(T/C/A/G) CA(A/G)-3') and SVVNFQL (OMP2 antisense 5'-(C/T)TG (G/A)TT (G/A)AA (C/T/G/A)AC (C/T/G/A)AC (G/A)CT (C/T/G/A)A(A/G)-3') corresponding to the first and the last seven amino acids. PCR conditions were as follows: first step at 94 °C for 5 min; second step 35 cycles at 94 °C for 1 min, 48 °C for 1 min, and 72 °C for 1 min; and third step at 72 °C for 5 min. Electrophoretic bands were purified using a QIAEX II (Qiagen) kit and ligated into a p-GEM plasmid. The crude ligation product was used to transform Bluescript competent cells. Positive colonies were checked for the presence of the insert by PCR, using the plasmid's primers SP6 and T7, and DNA was purified with a miniprep QIAquick (Qiagen) kit and sequenced. Sequence analyses were performed using the NCBI-BLAST and ExPasy Molecular Server (<http://www.ncbi.nlm.nih.gov>; <http://www.au.expasy.org>).

For *in situ* hybridization we dissected posterior rhinophores and fixed them in paraformaldehyde 4% in phosphate buffered saline (PBS) for 2 h. After washing, rhinophores were treated with proteinase K (0.25 mg ml⁻¹) then incubated overnight at 37 °C with a 5' biotinylated oligonucleotide corresponding to the first 51 nucleotides: 5'-CTG AAA CGA CTT GTG TGG AGA AGA TTT CAG CTG CGG ACT ATC CTC AGC CAT-3' (MWG Biotech AG, Germany). Control samples (pedal muscle, salivary and mucipares glands) were incubated overnight with biotinylated oligonucleotide and 30 times molar excess of unmarked oligonucleotide. Unspecific binding sites were blocked by overnight incubation with 25% goat serum, 1% Triton, 10% bovine serum albumin (BSA), washed in PBS, and incubated in 1% BSA, 0.1% Triton and 1 : 400 avidin-fluorescein isothiocyanate. Tissue samples were cryoprotected and embedded in TissueTek, 25 µm slices were sectioned using a cryostat.

For histology, tissues were fixed by immersion in saline formalin (4% formalin in 0.9% NaCl solution), dehydrated in an ethanol series and embedded in paraffin. Transverse sections and longitudinal sections were cut on a microtome (15 µm thickness), dewaxed, and stained with cresyl violet, hematoxylin and eosin (Pabisch Top-Rapidstain).

For tracing of OSNs we used DiI C18 (Molecular Probe, NL). Dissected posterior rhinophores were fixed in paraformaldehyde 4%. Microinjection was performed under a dissecting microscope using a micromanipulator with 1 µm tip diameter glass microcapillaries filled with saturated solution of DiI in ethanol. Tissues were incubated at 37 °C for one week. They were first observed and photographed as whole mounts and then embedded in 5% agarose low melting temperature. Cross-sections of 100 µm thickness were obtained using a vibratome.

3. RESULTS

(a) Molecular cloning

We designed OMP degenerated primers based on the mammalian common regions and used them to perform a PCR on land snail genomic DNA. A band between 450–500 bp was amplified. PCR was, therefore, performed with cDNA prepared from the olfactory sensory epithelium, with pedal muscle tissue as a control. A band of the same size was obtained from the olfactory sensory epithelium, but not from the pedal muscle. This band was purified, cloned and sequenced. The resulting nucleotide sequence is composed of 465 bp and encodes a mature protein of 155 amino acids (figure 1). It lacks introns and the entire sequence is an open reading frame.

(b) Tissue expression

General anatomy and morphology of the olfactory sensory epithelium is revealed with cresyl violet, hematoxylin and eosin staining (see figure 5 in electronic Appendix A,

atggctgaggatagtcgcgagctgaaatcttctccacacaagtcgtttcagttctaccaacttctgatctgcgtactttgtgcattgtactg
M A E D S P Q L K S S P H K S F Q F Y Q L L I C M R T L C I V L
gtcctgctggccgccacattcgccatttctgatgctacagaggttgacaagagacttataggaggtcttgacttgagtgcggtttttaactgggtg
V L L A A T F A I S D A T E V D K R L I G G L D L S A V F K L V
gctagcgttcctacgttattacaacaatcagtcaaaagtgttcaggaccttctgccagtcgtgttgacgccgtcactagcctgggtgcgggatgctc
A S V P T L L Q Q S V K S V Q D L L P S L L D A V T S L V R D V
acgggcgctgccaccgggtattgtctacaaagccctagagactgttctagagtgactaaggcacttctcctggtaggcaataatgtcttggggctg
T G A A T G I V Y K A L E T V L G V T K A L L L V G N N V L G L
atccttttcggctgtaaactccagttctgtggctccttactagtcttgtgacatctattctcagcgtcgtgtttaaccagctc
I L S A V N S S S G L L T S L V T S I L S V V F N Q L

Figure 1. Nucleotide and amino acid sequence of an OSN-specific protein in the land snail, *Eobania vermiculata*. The land snail nucleotide sequence has been cloned by cDNA, using as a template OSN mRNA. The GenBank accession number is AY147909.

Figure 2. (a,b) *In situ* hybridization of a 25 µm cryosection and (c) a whole mount of the land snail olfactory cup expressing mRNA for a specific OSN protein. (a,b) Posterior rhinophore cut and mounted invaginated for comparison of the olfactory sensory epithelium (ose), where OSNs are specifically labelled, and non-sensory epithelium (nse) covers the rhinophore; ax indicates the OSN axon bundles. (c) A photomicrograph of olfactory glomeruli (g1–g5), positive stain due to terminating OSN axons. The scale bar in (a) is 100 µm, and in (b,c) 200 µm.

available on The Royal Society's Publications Web site). The olfactory region is localized at the tip of the rhinophore, below the eye. It is composed of an olfactory sensory epithelium, where the bipolar OSNs are located.

OSNs project their axons to the glomeruli and their dendrites to the external environment in the spongiform layer.

In situ hybridization experiments were performed using a 5' biotinylated 51-mer probe, corresponding to the first

Figure 3. Land snail OSN: DiI tracing and *in situ* hybridization of specific OSN protein. (a) A cross-section of the olfactory cup where the dye has traced OSNs and the olfactory glomerulus. d, dendrite; s, soma; ax, axon; g, glomerulus; l, lobuli. (b) High magnification of OSN axons expressing OMP mRNA spread inside the glomerulus, where they synapse with glomerular cells. bx, axon bundles. The scale bar in (a,b) is 25 µm.

51 nucleotides of the *E. vermiculata* protein sequence. Figure 2a,b shows cross-sections of the rinophore and figure 2c shows a whole-mount preparation. The labelling observed follows incubation with the biotinylated probe and fluorescent streptavidin is localized on OSNs in the olfactory sensory epithelium: no labelling is in the non-sensory epithelium, figure 2a. In figure 2b, higher magnification of the olfactory sensory epithelium, OSNs grouped in bundles projecting their axons to glomeruli are labelled by the probe. In figure 2c the positive stain is due to terminating OSN axons in the glomeruli. To test for the specificity of the labelling, sections were co-incubated with an excess of unlabelled oligonucleotide. No distinct labelling of OSNs and glomeruli was observed in the presence of an excess of unlabelled oligonucleotide and in control tissues (pedal muscle, salivary and mucipares glands).

To complement the results obtained by *in situ* hybridization, OSNs were labelled by tracing with the fluorescent dye, DiI. The lipophilic tracer was micro-injected directly into olfactory glomeruli. After one week of incubation, a limited number of OSNs were labelled retrogradely. The morphology of OSNs labelled by *in situ* hybridization and by DiI tracing are compared in figure 3, showing the selectivity and specificity of this protein expression.

4. DISCUSSION

We have isolated a gene encoding for a protein specifically and selectively expressed in the OSNs of the land

snail (*E. vermiculata*). This protein shares many distinctive characteristics with vertebrate OMPs. It lacks introns and the entire sequence is an open reading frame as for vertebrate OMPs (Margolis *et al.* 1993; Wang & Reed 1993; Buiakova *et al.* 1994), a common feature among other olfactory system proteins such as olfactory receptors and odorant binding proteins. Indeed, this may be a peculiar characteristic of proteins involved in chemoreception, indicative of an hypothetical common ancestral protein. Land snail protein is composed of 155 amino acids, while the OMP family ranges in size from 155 amino acids in fish, to 163 amino acids in mammals (Margolis 1972; Keller & Margolis 1976; Sydor *et al.* 1986; Krishna *et al.* 1992; Buiakova *et al.* 1994; Rössler *et al.* 1998; Celik *et al.* 2002). These little differences in protein length correspond to a progressive reduction of amino acid composition in the transitions among vertebrates and invertebrates. Further, the calculated molecular weights of the land snail protein and that of the vertebrate OMPs are comparable: 16.32931 kDa in the land snail and ranging from 14 kDa in fish (Celik *et al.* 2002) to 18 kDa in mammals (Margolis 1972; Keller & Margolis 1976; Sydor *et al.* 1986; Buiakova *et al.* 1994). Moreover, the land snail proteins' calculated isoelectric point is 7.01, which is similar to that of a frog. Sequence identity between the land snail protein and vertebrate OMPs is as follows: 12.9% with fish; 13.5% with *Xenopus* 2; 16.2% with *Xenopus* 1; 18.1% with human; 20% with mouse and rat (figure 4) and homology increases when looking at the consensus. In particular, among invertebrate and vertebrate sequences there are at least three well-conserved regions. Furthermore, land snail predicted secondary structure is similar to that of OMP's (see figure 6 in electronic Appendix A).

Moreover, using *in situ* hybridization experiments, we found that the land snail protein is expressed only in OSNs and not in other analysed tissues. The specificity and selectivity of the land snail protein are two of the principal characteristics of OMPs. In contrast to previous work (Chase & Tolloczko 1993), we were able to detect labelled fibres that project only to glomeruli; we were not able to detect any fibre that projects directly to the cerebral ganglia.

To date, a clear function for the OMP has not been clearly established (Smith *et al.* 2002). Therefore, it is not possible to confirm absolutely the presence of an OMP using a functional analysis. Instead, the main characteristic of an OMP is its unique expression restricted to OSNs. Thus, our data support the existence of an OMP-like protein in the land snail and also provide strong evidence that this protein is phylogenetically and functionally conserved among taxa with different evolutionary histories.

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