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Novel genes for potential ligand-binding proteins in subregions of the olfactory mucosa

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Odorant detection is specifically mediated via receptor neurons in the olfactory mucosa but is a complex process involving a number of different cell types producing proteins of differing function. We have used the technique of subtractive hybridization cDNA cloning to identify novel genes expressed exclusively in the olfactory mucosa which may play a role in olfaction. Ten distinct groups of cDNA clones were identified which corresponded to mRNA transcripts highly expressed in rat olfactory mucosa but undetectable in thymus, kidney, lung, brain, spleen and liver. Some of these clones identify substructures in the mucosal tissue for which no other probes are currently available. Others identify novel mRNA species in the Bowman's glands. The predicted proteins for three of these clones are homologous to proteins which bind to either lipopolysaccharides (RYA3 and RY2G5) or to polychlorinated biphenyls (RYD5). In addition, while RYA3 and RY2G5 are highly homologous, they appear to be expressed in different parts of the mucosal tissue. The sequence homologies and subanatomical location of expression suggest that these proteins might interact with odorants before or after specific recognition by odorant receptors. Therefore, the olfactory mucosa may possess diverse, functionally-distinct odorant-binding proteins which recognize and bind separate classes of odorants.

Key words: lipopolysaccharide-binding protein/neutrophil bactericidal protein/odorant-binding protein/olfaction/subtraction

Introduction

The vertebrate olfactory system is a highly sensitive system capable of detecting and discriminating a large number of odorant molecules (Lancet, 1986). Comparatively little is understood of the molecular mechanisms by which a variety of odorants are recognized and processed by the olfactory neurons. Accumulated evidence suggests that odorant transduction occurs via an adenylyl cyclase cascade mediated by a G protein. The olfactory receptor neurons possess unique forms of a G_α subunit termed $G_{olf\alpha}$ (Jones and Reed, 1989), adenylyl cyclase (Bakalyar and Reed, 1990) and a cAMP-gated ion channel (Dhallen *et al.*, 1990; Ludwig *et al.*, 1990). In addition, a novel multigene family of G protein-coupled receptors has recently been identified which may encode the odorant receptor molecules (Buck and Axel, 1991). These predicted proteins are highly diverse, a prerequisite for the identification of a wide variety of odorant molecules.

Although this potential class of specific odorant receptors has now been identified, other odorant-binding proteins were previously shown to be present in olfactory mucosa preparations. For example, odorant binding protein (OBP), identified in rat, frog and bovine nasal glands (Lee *et al.*, 1987; Pevsner *et al.*, 1986, 1988a), is most likely secreted and binds odorants without apparent specificity (Pevsner *et al.*, 1990). Nonetheless, the recent identification of diverse odorant-binding proteins in Lepidoptera (Vogt *et al.*, 1991) and a homologue of rat OBP, term OBP_{II} (T.N.Dear, K.Campbell and T.H.Rabbitts, submitted for publication), show that distinct odorant-binding proteins co-exist. Therefore, these odorant-binding proteins may exhibit some degree of odorant-discriminating ability and may act as carrier molecules, transporting odorants across the mucus layer to access the receptor sites (Pevsner *et al.*, 1986).

Using the technique of subtractive hybridization, we have isolated cDNA clones which identify structures within the olfactory mucosa for which no other probes are currently available. In addition, we have identified three novel genes exclusively expressed in the olfactory mucosa, the predicted proteins of which are homologous to a variety of ligand-binding proteins. These proteins appear secretory in nature suggesting that they may be involved in binding small hydrophobic odorants in the mucus layer. Therefore the olfactory mucosa may possess a diverse collection of odorant-binding proteins, each of which is capable of recognizing and binding specific classes of odorants.

Results

Identification of olfactory mucosa-specific mRNAs

In order to identify mRNAs that are selectively expressed in the olfactory mucosa (OM), we constructed a cDNA library enriched for OM-specific sequences by hybridizing OM cDNA with excess Rat-2 fibroblast poly(A)$^+$ RNA, resulting in a final enrichment of 9.9-fold. This value was confirmed by comparative screening of the difference and a complete OM cDNA library with OMP and $G_{olf\alpha}$ probes (data not shown). After cloning into λgt10, the enriched library contained 8×10^6 independent clones with insert sizes ranging from 0.15–1.0 kb in length (average = 0.44 kb). Approximately 28 000 clones were screened by differential hybridization to ^{32}P-labelled OM and Rat-2 cDNAs. Two types of clones were analysed; those hybridizing exclusively to OM cDNA and those clones representing low abundance species in OM cDNA as suggested by their inability to hybridize with either of the ^{32}P-labelled cDNAs. After two further rounds of rescreening, 372 clones were obtained of which 282 gave a hybridization signal with OM [^{32}P]cDNA while 90 did not give a

Table I. OM-specific cDNA clones isolated via subtractive hybridization

Clone	Number of isolates[a]	RNA[b] (size in kb)	Relative abundance[c]	Sublocalization in olfactory mucosa
RYA9[d]	7	2.2	9.7	olfactory neurons
RYA3	5	2.0	7.1	subepithelial layer
RYD5	54	0.6	6.3	Bowman's glands
RYF3	27	2.2	5.1	lateral nasal gland
RYH2	1	0.6	2.8	n.d.[e]
RY2D1[f]	4	1.3	2.5	Bowman's glands
RY2G12[f]	5	0.9	2.5	lateral nasal gland
RY2G5	1	2.0, 1.7	2.1	Bowman's glands
$G_{olf\alpha}$[g]	n.a.[e]	3.5, 2.7	1	olfactory neurons
RY2G7	4	1.1, 0.5	0.7	n.d.
RY2E10	2	0.6	<0.01[h]	n.d.

[a]cDNA clones were grouped together on the basis of cross-hybridization at high stringency or by sequence analysis of full-length cDNA clones.
[b]As determined by Northern blot analysis.
[c]Estimate based on the number of positive signals obtained by screening 5×10^5 clones of an OM cDNA library in λgt10. Levels are relative to $G_{olf\alpha}$.
[d]This cDNA has complete identity with the nucleotide sequence of rat OMP cDNA (Rogers *et al.*, 1987).
[e]n.d., not determined; n.a., not applicable.
[f]Detailed characterization of these clones is reported elsewhere (T.N.Dear, K.Campbell and T.H.Rabbits, submitted for publication).
[g]This G_α protein subunit is reported to be olfactory neuron-specific (Jones and Reed, 1989). It was not detected in the differential library screening but is included as a reference standard.
[h]No positive signals were observed in two independent screenings of an OM cDNA library.

hybridization signal with either cDNA. Cross-hybridization at high stringency and, in some cases, DNA sequence analysis, resolved the 372 clones into 79 separate groups, of which 19 came from the 282 OM cDNA-positive clones. An individual member from each group was used to screen a panel of total RNAs from rat tissues and the cell lines Rat-2, SNIF-12,11 and 6 (Coon *et al.*, 1989) by Northern hybridization analysis. Of the 19 OM cDNA-positive groups, 10 were designated OM-specific based on their ability to detect mRNA species exclusively in the OM while seven groups also exhibited a low level of expression in lung tissue and two groups also exhibited expression in brain, spleen, liver, lung and kidney. Of the 60 groups corresponding to OM and Rat-2 cDNA-negative clones, 10 groups were expressed in all tissues and cell lines while 50 groups did not reveal any expression in the tissues or cell lines examined. These latter groups of clones were not examined further and, consequently, their origin remains uncertain.

The 10 groups specific for OM are listed in Table I along with the sizes of the corresponding mRNAs. The relative abundance of each clone within an OM cDNA library is also shown. One of these clones (RYA9) corresponded to the previously identified gene for olfactory marker protein (OMP) (Rogers *et al.*, 1987). Four of these clones, RYA3, RYF3, RY2G5 and RYD5 were characterized in detail.

mRNA expression analysis

The tissue distribution of mRNAs hybridizing to the RYA3, RYF3, RY2G5 and RYD5 cDNAs is shown in Figure 1. The RYA3, RYF3 and RYD5 cDNA clones detect transcripts of approximately 2.0, 2.2 and 0.6 kb respectively in Northern blots of OM total RNA. The RY2G5 cDNA clone detects two transcripts of 2.0 and 1.7 kb. There was no detectable expression for any of these mRNAs in rat thymus, lung, brain, liver, kidney and spleen. In addition there was no expression observed in the neuronal cell lines SNIF-12,11 and 6 (Coon *et al.*, 1989), nor in the transformed fibroblast cell line Rat-2 (which was used as the source of competitor RNA for subtraction).

Precise sublocalization of the various mRNAs, within the olfactory mucosa, was achieved using *in situ* hybridization. Anatomical identity within the mucosa was defined by thionine-staining (Figure 2H) in association with *in situ*

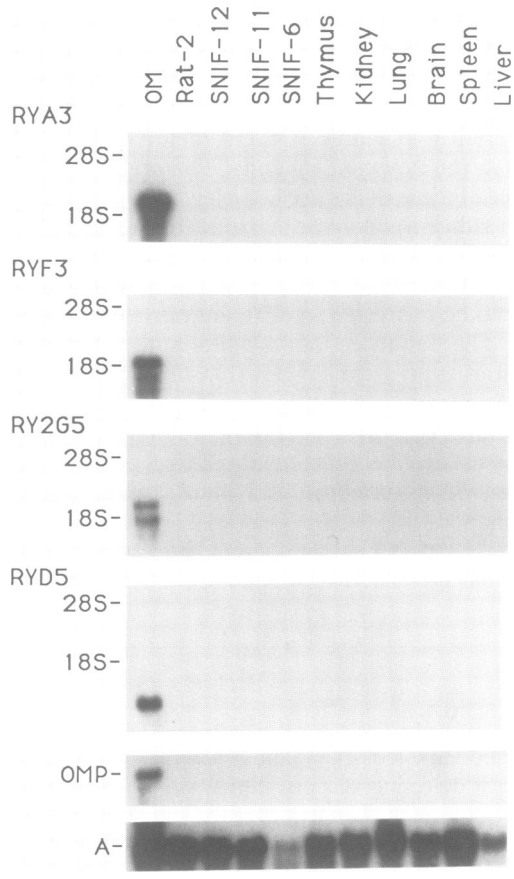

Fig. 1. Expression of RYA3, RYF3, RY2G5 and RYD5 mRNAs. Total RNA (10 μg) was separated by electrophoresis and blotted as described. The filter was sequentially hybridized to ^{32}P-labelled RYA3, RYF3, RY2G5, RYD5, OMP and actin cDNA probes. Origin of RNA samples is indicated above each lane. Positions of 28S and 18S rRNA size markers are indicated.

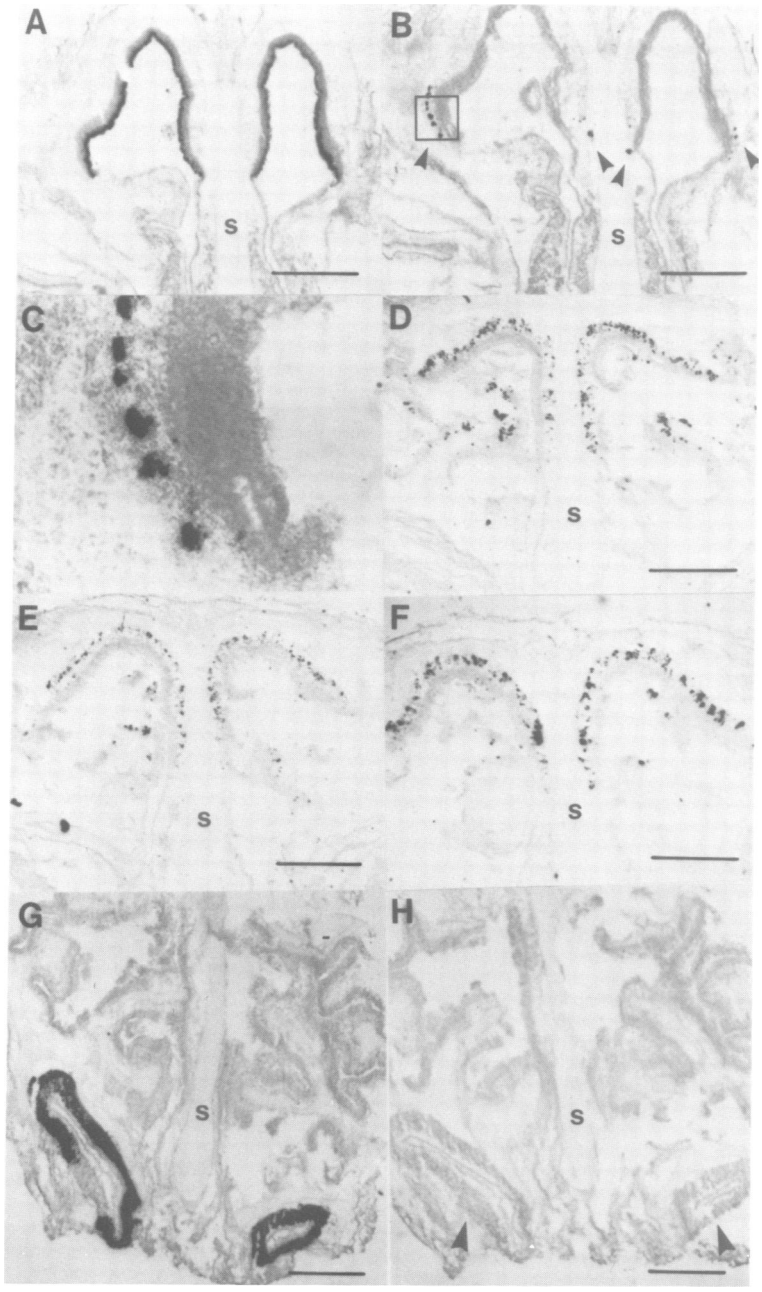

Fig. 2. Tissue localization of RYA3, RYF3, RY2G5 and RYD5 mRNAs. Coronal sections of Fischer rat olfactory epithelium were hybridized to ^{35}S-labelled antisense cDNA probes followed by exposure to autoradiographic emulsion and counterstaining with thionine. The septum(s) is indicated. Hybridization with ^{35}S-labelled sense cDNA did not reveal any specific signal (data not shown). Bar represents 1 mm. (**A**) Hybridization of OMP DNA. Signal is specific to the upper layers of the neuroepithelium. (**B**) Hybridization of RYA3 cDNA. Arrowheads indicate location of hybridization signal. (**C**) A 10× magnification of the boxed region in C. (**D**) Hybridization of RY2G5 cDNA. (**E**) Hybridization of RYD5 cDNA. (**F**) Hybridization of cytochrome P-450olf1 cDNA. D, E and F show a punctate pattern of expression in the subepithelial layer, characteristic of the Bowman's glands. (**G**) Hybridization of RYF3 cDNA. (**H**) Thionine-stained section equivalent to that shown in Figure 2G. Arrowheads indicate glands in which RYF3 mRNA is expressed.

hybridization of DNA probes corresponding to OMP (Figure 2A; see also Boehm *et al.*, 1991) and cytochrome P-450olf1 (Figure 2F). OMP is specifically expressed in the olfactory receptor neurons within the nasal mucosa and hybridization is limited to the upper layers of the neuroepithelium (Danciger *et al.*, 1989) while cytochrome P-450olf1 expression is restricted to the Bowman's glands in the subepithelial layer (Zupko *et al.*, 1991). RYD5 and RY2G5 mRNA expression appears to be restricted to the Bowman's glands based on the punctate pattern of hybridization in the subepithelial layer, similar to that of a cytochrome P-450olf1 probe (Figure 2D–F). RYA3 expression is restricted to a population of cells in the subepithelial layer present only at the base of the mucosa (Figure 2B,C). RYF3 mRNA is restricted to lateral nasal glands at the base of the nose (Figure 2G).

Nucleotide sequence analysis

The cDNA clones obtained in the PCR-based subtraction method used tended to be rather small, certainly less than the full size predicted from the Northern hybridization. Longer cDNA sequences were obtained by screening of a

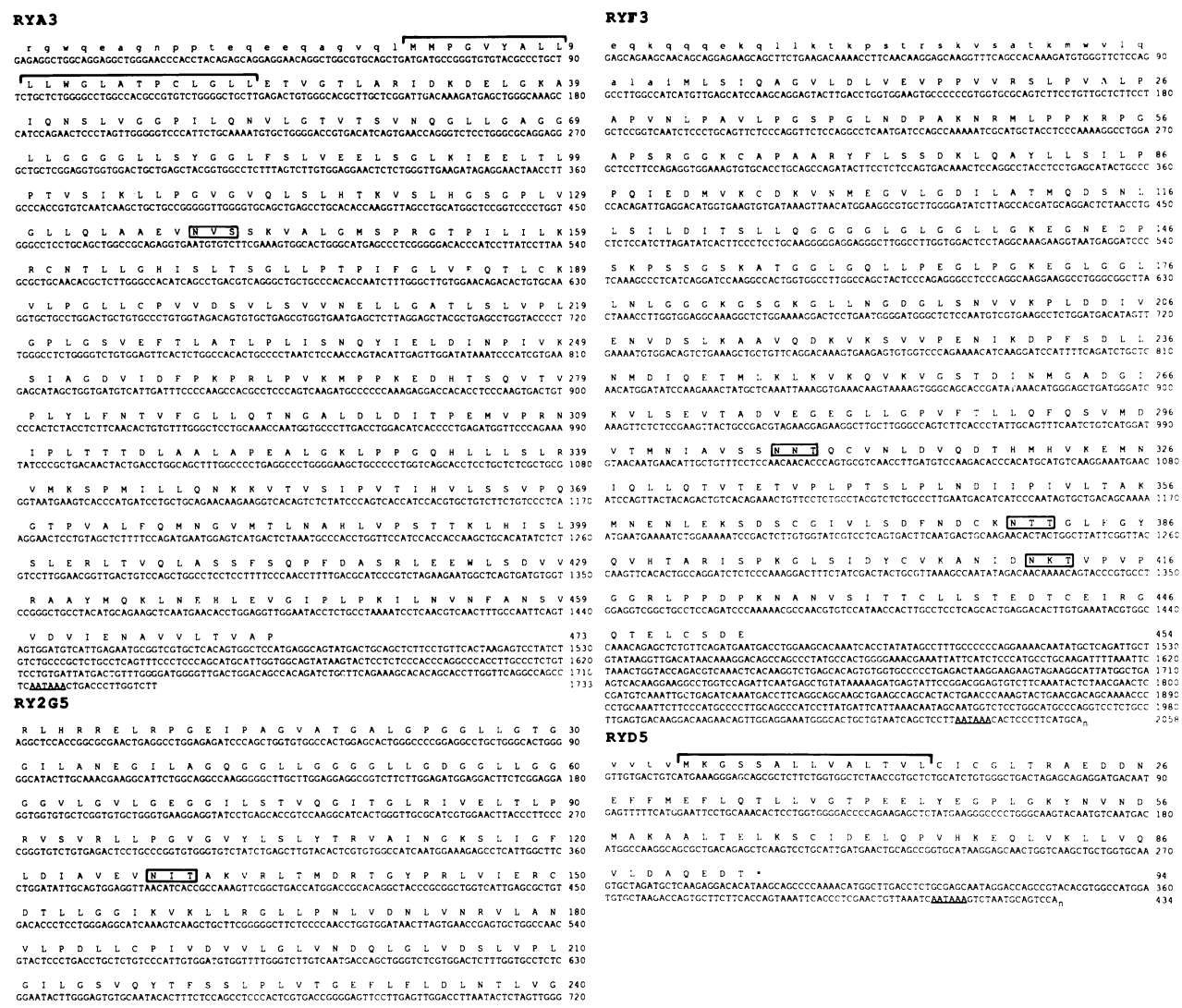

Fig. 3. Nucleotide and predicted amino acid sequences of RYA3, RYF3, RY2G5 and RYD5 cDNAs. Predicted amino acid sequences (one letter code) are shown above the nucleotide sequence. In-frame termination codons are indicated by asterisks. Putative polyadenylation signals are underlined and putative N-linked glycosylation sites (Winzler, 1983) are boxed. Potential hydrophobic leader sequences are indicated by brackets above the amino acid sequence. There are two potential initiating methionine codons in the RYF3 sequence, the second of which is closer in agreement with the consensus sequence for translation initiation (Kozak, 1987). In-frame amino acids upstream of the proposed initiating ATG codons in the RYA3, RYF3 and RYD5 cDNA sequences are indicated with lower case letters. The origin of the two distinct transcripts for RY2G5 (Figure 1) is unknown. One possibility is alternative 3′ termini as there is an internal potential polyadenylation signal at nucleotides 1635–1640. A_n, poly(A) tail.

cDNA library made from Fischer rat OM mRNA and by utilizing anchored PCR (Frohman *et al.*, 1988; Loh *et al.*, 1989). The cDNA and predicted amino acid sequences are shown in Figure 3.

A common feature is that the RYA3, RYF3 and RYD5 predicted proteins all possess hydrophobic N-terminal regions (Figure 3) which may encode signal peptides indicative of secretory proteins (Perlman and Halvorson, 1983). The RYA3, RYF3, RY2G5 and RYD5 cDNAs encode predicted proteins of 473, 454, 470 and 94 amino acids respectively. The predicted protein sequences for RYA3, RYF3 and RYD5 continue in-frame upstream of the

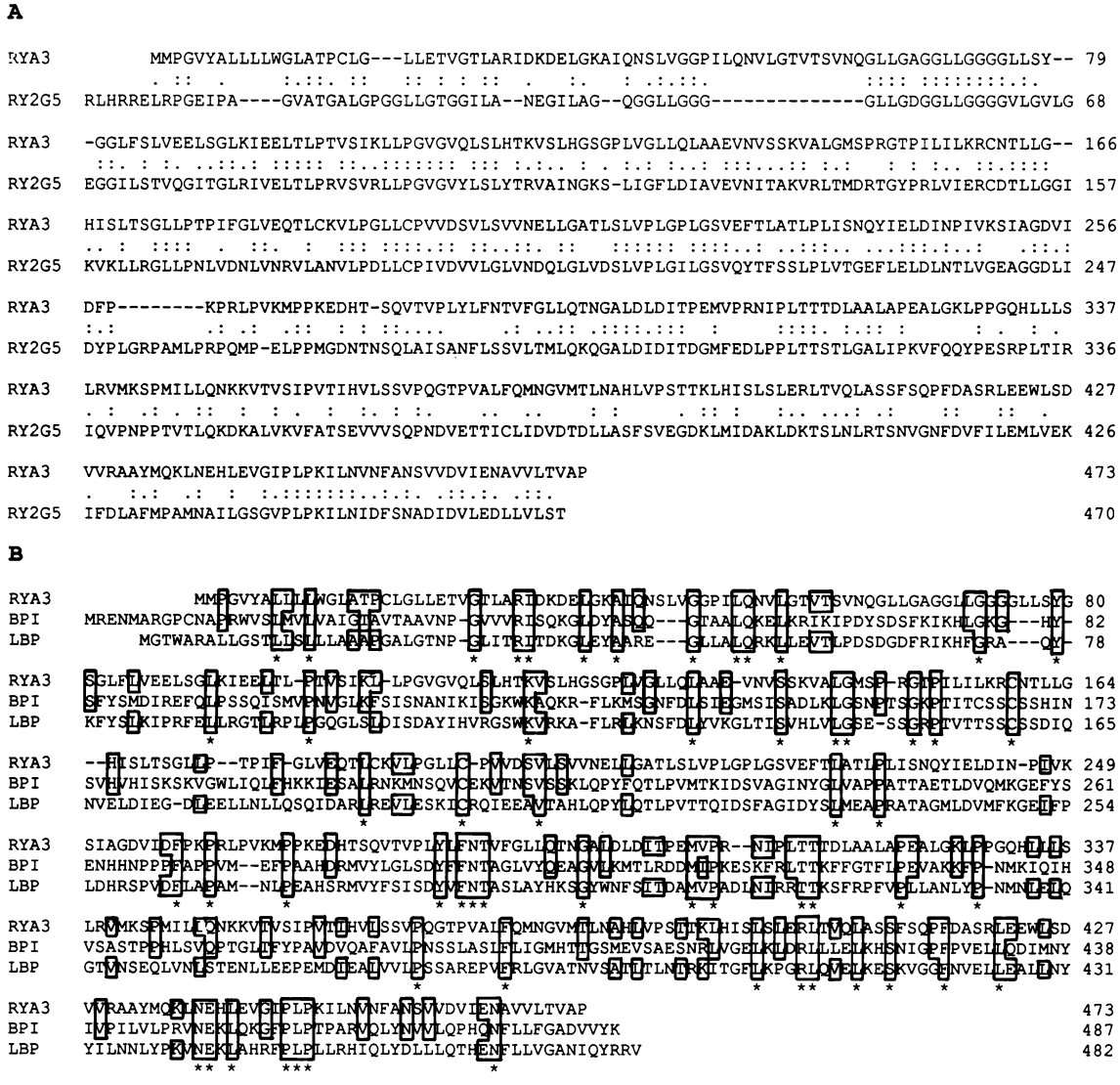

Fig. 4. Alignment of the predicted protein sequences for RYA3 and RY2G5 with homologous proteins. (**A**) Alignment of RYA3 and RY2G5 proteins. Amino acids in common are indicated by double dots while single dots denote conservative amino acid substitutions. Gaps, indicated by dashes, were introduced to maximize alignment. Conservative substitutions are classified as follows: D,E,N,Q; H,K,R; A,G,P,S,T; I,L,M,V; F,W,Y (**B**) Alignment of RYA3 predicted protein with the complete amino acids sequences for human neutrophil bactericidal protein (BPI) (Gray *et al.*, 1989) and rabbit lipopolysaccharide-binding protein (LBP) (Schumann *et al.*, 1990). Amino acids in common with RYA3 are boxed. Gaps, indicated by dashes, were introduced to maximize alignment. Asterisks denote fully conserved residues.

designated start sites in Figure 3 and, consequently, it is possible that the available nucleotide sequences do not encompass the entire protein coding regions. This is unlikely, however, as anchored PCR of cDNA for these transcripts terminated strongly at the first nucleotide shown in Figure 3 suggesting that these are the first nucleotides of the mRNAs. The RY2G5 protein coding sequence is probably incomplete as there is no potential initiating methionine in proximity of the 5′ end of the sequence.

A computer search of the PIR and EMBL databases did not reveal any sequences with significant homology to the RYF3 nucleotide or predicted protein sequence. The RYA3 and RY2G5 predicted proteins are, however, related to each other with 39.3% homology at the amino acid level (Figure 4A), each possessing a high proportion of leucine amino acids (19.5% and 18.1% for RYA3 and RY2G5 respectively). Both proteins demonstrate significant homology to human neutrophil bactericidal protein (BPI) (Gray *et al.*, 1990) (20.0% identity in a 476 amino acid overlap and 21.6% identity in a 407 amino acid overlap for RYA3 and RY2G5 respectively). In addition, the RYA3, predicted protein is also homologous to rabbit lipopolysaccharide-binding protein (LBP) (Schumann *et al.*, 1990) (20.4% identity in a 456 amino acid overlap). The alignment of BPI and LBP with RYA3 is shown in Figure 4B. BPI is a potent cytotoxic factor with strong affinity for lipopolysaccharides (Elsbach and Weiss, 1983). LBP, which is related by homology to BPI, is a plasma protein which also binds lipopolysaccharides (Tobias *et al.*, 1989).

The RYD5 predicted protein sequence is homologous to rat Clara cell secretory protein precursor (29.4% identity in an 85 amino acid overlap) (Nordlund-Muller *et al.*, 1990), the human equivalent (23.5% identity in an 85 amino acid overlap) (Singh *et al.*, 1988), and to the C1 subunit of rat prostatein (28.0% identity in an 82 amino acid overlap) (Parker *et al.*, 1982) (Figure 5). This homology may have relevance to possible interactions with odorants because the Clara cell secretory protein binds to polychlorinated

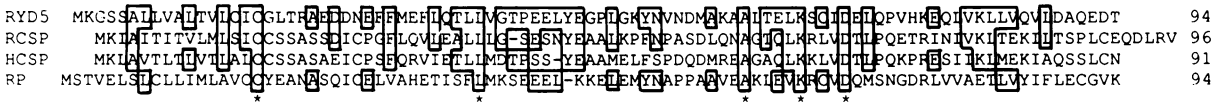

Fig. 5. Alignment of predicted protein sequence for RYD5 with homologous proteins. Alignment of RYD5 predicted protein with complete amino acid sequences of rat 10 kDa Clara cell secretory protein (RCSP) (Nordlund-Muller *et al.*, 1990), human 10 kDa Clara cell secretory protein (HCSP) (Singh *et al.*, 1988) and the C1 subunit of rat prostatein (RP) (Parker *et al.*, 1982). Amino acids in common with RYD5 are boxed. Gaps, indicated by dashes, were introduced to maximize homology. Asterisks denote fully conserved residues.

biphenyls (Nordlund-Muller *et al.*, 1990) while prostatein possesses steroid-binding ability (Lea *et al.*, 1979; Forsgren *et al.*, 1979).

Discussion

The use of cDNA subtractive hybridization as an aid to anatomical dissection

Subtractive hybridization cDNA cloning is a powerful technique that has been used to identify numerous differentially-expressed genes. Cell lines are more commonly used in subtraction experiments as they tend to represent homogenous populations unlike tissues which possess heterogeneous subpopulations. The nasal tissue, for example, consists of a variety of cell types including olfactory receptor neurons, basal stem cells, sustentacular cells, various mucosal glands and epithelial cells (Carr *et al.*, 1990) which are collectively referred to as the olfactory mucosa (OM). Consequently, any OM-specific mRNA will be derived from one or more of the various subpopulations.

A variety of cDNAs or proteins are available which serve as markers for subpopulations of the neuroepithelium. OMP, $G_{olf\alpha}$, olfactory-specific adenylyl cyclase and an olfactory-specific ion channel are expressed exclusively in the olfactory receptor neurons in the olfactory mucosa (Bakalyar and Reed, 1989; Danciger *et al.*, 1989; Jones and Reed, 1989; Dhallan *et al.*, 1990). OBP and OBP_{II} expression are restricted to the lateral nasal gland in rats (Pevsner *et al.*, 1988a; T.N.Dear, K.Campbell and T.H.Rabbitts, submitted for publication) while cytochrome P-450olf1, cytochrome P-450olf2 and UGT glucuronyl transferase are restricted to the Bowman's glands and surrounding tissue (Lazard *et al.*, 1991; Zupko *et al.*, 1991). We have identified 10 distinct cDNA clones for which the nasal epithelium is the major site of expression. In particular, two genes, RYD5 and RY2G5, are restricted in expression to the Bowman's glands. Furthermore, we have identified cDNA clones which correspond to mRNAs specific to subpopulations of the mucosa for which no other probes are available. RYA3 corresponds to an mRNA which is highly expressed in a population of cells in the subepithelial layer that appear to be present only at the base of the neuroepithelium while RYF3 mRNA is exclusively expressed in the large lateral glands at the base of the nose. No homology was found between the predicted RYF3 protein and known proteins and, therefore, possible function cannot be ascertained from the sequence. Nevertheless, the subtractive hybridization approach described herein has provided unique molecular markers, such as RYA3, for previously molecularly uncharacterized substructures within the olfactory mucosa.

A possible function for RYA3, RY2G5 and RYD5

The RYA3 and RY2G5 predicted proteins exhibited homology to neutrophil bactericidal protien (BPI) and lipopolysaccharide-binding protein (LBP). BPI is a potent cytotoxic factor released from polymorphonuclear leukocytes that directs its activity solely towards Gram-negative bacteria (Elsbach and Weiss, 1983). The target cell specificity of BPI is effected by its strong affinity for lipolysaccharides, hydrophobic components of the Gram-negative bacteria envelope. LBP, related by homology to both BPI and RYA3 is a plasma protein synthesized in hepatocytes which, like BPI, binds to lipopolysaccharides of Gram-negative bacteria (Schumann *et al.*, 1990). These homologies suggest that the RYA3 and RY2G5 proteins may bind lipophilic molecules with some of the structural characteristics of lipopolysaccharides. Furthermore, the RYD5 predicted protein is homologous to the Clara cell secretory protein which binds polychlorinated biphenyls thereby protecting lung epithelial cells from damage by such molecules (Nordlund-Muller *et al.*, 1990). In addition, there is homology of the RYD5 protein to a subunit of rat prostatein, a secretory protein in rat prostatic fluid which binds a variety of steroids (Lea *et al.*, 1979; Forsgren *et al.*, 1979). Therefore, the RYD5 protein may also bind lipophilic molecules.

The common protein homology of RYA3 and RY2G5 is contrasted by the different sites of expression of these mRNAs within the OM. The RY2G5 and RYD5 mRNAs are localized to cells secreting components of the mucus layer suggesting that the encoded proteins may be present in the mucus bathing the chemosensory cilia of the receptor neurons. Therefore, these proteins might function by binding odorant molecules encountered in the mucus layer, thereby further increasing diversity in potential odorant-binding proteins, in addition to the previously identified OBP (Pevsner *et al.*, 1988b) and its homologue OBP_{II} (T.N.Dear, K.Campbell and T.H.Rabbitts, submitted for publication). Alternatively, in view of the homology of RYA3 and RY2G5 to BPI and RYD5 to a polychlorinated biphenyl-binding protein, these proteins might serve as a primary defence mechanism by recognizing and removing potentially harmful odorants or pathogenic microorganisms from the mucosa. Another possible function for such molecules is assisting in the clearance of excess odorant from the mucus layer to enable new odorant stimuli to be received.

The recently identified G protein-coupled receptor multigene family expressed in OM (Buck and Axel, 1991) may account for the specific odorant recognition potential of the olfactory system. Some of the odorant-binding proteins may have an ancillary function in carrying lipophilic odorants across the hydrophilic mucus layer in order to access the ciliary receptors. Further work will be necessary to determine if any of the odorant-binding proteins, or potential odorant-binding proteins, interact with the putative receptors.

Materials and methods

RNA preparation

Ten week old Fischer rats were killed and the OM was removed using forceps. Tissue samples were stored in liquid N_2 until required. Total RNA was extracted as described previously (Cathala *et al.*, 1983).

cDNA subtractive hybridization and library construction

cDNA preparation and subtraction were performed as previously described (Dear *et al.*, 1988). cDNA reverse transcribed from OM poly(A)$^+$ RNA was hybridized against a 100-fold excess of Rat-2 fibroblast poly(A)$^+$ RNA to a corrected R_0t of 1500. Hydroxylapatite chromatography of the hybridization mixture removed 88.5% of the input cDNA. A second round of subtraction removed 12% of the remaining cDNA. The enriched cDNA was tailed with dATP as described by Frohman *et al.* (1988) and amplified using the polymerase chain reaction with the oligomer 5′-GGAATTC-$TCGAGT_{17}$(G/C/A)-3′ as previously described (Saiki *et al.*, 1988). The resulting double-stranded cDNA was restricted with *Eco*RI and cloned into λgt10. A total cDNA library from OM poly(A)$^+$ RNA was constructed in λgt10 using a cDNA cloning kit (Pharmacia, Uppsala, Sweden) according to the manufacturers instructions. Differential screening of the enriched cDNA library was performed as previously described (Dear *et al.*, 1988).

Filter hybridization analysis

Northern and Southern hybridization procedures have been described (Boehm *et al.*, 1988). The murine OMP DNA probe was a 2.1 kb *Bam*HI fragment obtained from the λOMP6 genomic clone (Boehm *et al.*, 1991). The cytochrome P-450olf1 cDNA probe corresponds to nucleotides 443–858 in the published sequence (Nef *et al.*, 1989).

Sequence analysis

λgt10 cDNA clones were subcloned into the *Not*I site of pBluescript. DNA sequence was determined for both strands using the dideoxy-chain termination method with random sonicated clones (Bankier *et al.*, 1987) and in conjunction with sequence specific oligonucleotides. Complete cDNA sequences were assembled using the Staden sequence assembly program (Staden, 1990). Protein and nucleic acid alignments were made using the FASTA algorithm (Pearson and Lipman, 1988).

In situ hybridization

Six day-old rat OM was removed, embedded in Tissue Tek (Miles Elkhart, IN) and 20 μm sections were cut. The procedures for fixation, probe preparation and hybridization are reported elsewhere (Boehm *et al.*, 1991).

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