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Review

Vertebrate odorant receptors

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Abstract. Olfactory transduction begins with the binding of an odorous molecule to a protein receptor—odorant receptor—on the cell surface of olfactory neuron. Odorant receptors are encoded by a large gene family belonging to the superfamily of G-protein-coupled, seven-transmembrane-domain receptors. Since the identification of the receptor gene family in 1991, a considerable amount of progress has been made in the study of odorant receptors, including aspects of spatial and temporal expression pattern, the genomic organization of the receptor genes, regulation of expression, and receptor function. These studies are of critical importance in understanding how the olfactory system recognizes and distinguishes thousands of odors.

Key words. Olfactory; olfaction; odor; G-protein-coupled receptor.

Introduction

The olfactory system recognizes and discriminates a vast number and variety of odorous molecules (odorants). Olfactory transduction begins with the binding of an odorous molecule to a protein receptor, the OR (odorant receptor), on the cell surface of an olfactory neuron, thus initiating a cascade of enzymatic reactions that results in the production of a second messenger, and leading to the eventual depolarization of the cell membrane [1, 2]. As the first molecule in the transduction process, the OR places critical limits on the affinity and discriminability of odorants. ORs are encoded by a large gene family belonging to the superfamily of GPCRs (G-protein-coupled, seven-transmembrane-domain receptors). In mammals, for example, the gene family consists of a few hundred to around 1000 members, and each of them is encoded by an individual gene, making it the largest gene family in genome. The OR gene family was first identified by Buck and Axel in 1991 [3]. Since then, considerable effort has been directed at understanding many aspects of OR biology, including the spatial and temporal expression pattern, the genomic organization of OR genes, regulation of expression, and receptor function. Many of these studies are of critical importance in understanding how the olfactory system recognizes and distinguishes thousands of odors. This review discusses the studies carried out on ORs in vertebrates, whereas studies on the putative pheromone receptors identified from the vomeronasal organ [4–7] and ORs in invertebrates [8–10] are not included.

Molecular cloning of OR genes

The cloning of the genes for ORs was inspired by the studies that led to the finding that odor stimuli are transduced through a G-protein-dependent, cAMP (cyclic AMP)-mediated signal transduction pathway in

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olfactory neurons. It had been predicted that ORs should belong to the superfamily of GPCRs [11, 12], which are structurally characterized by traversing the cell membrane seven times. Based on this assumption, in 1991, Buck and Axel [3] conducted a series of PCRs (polymerase chain reactions) on complementary DNAs (cDNAs) obtained from rat olfactory epithelium. Using different combinations of degenerate oligonucleotide primers, which were designed according to conserved sequences in TM (transmembrane domain) 2 and TM 7 of a variety of GPCRs, they eventually identified a multigene family from olfactory epithelium which may encode ORs [3]. In the original report 18 different members (10 full-length and 8 partial length) of the olfactory multigene family were cloned and characterized. They share clearly the sequence and structural properties with receptors of the GPCR superfamily, but also exhibit features different from any other GPCRs.

Several lines of evidence lent strong support to the likelihood that this multigene family encoded ORs. First, messenger RNA Northern blot experiments showed that they were specifically expressed in olfactory epithelium, but not in other tissues. Second, the proteins encoded by the olfactory multigene family were clearly members of the GPCR superfamily. Third, the size of the olfactory multigene family was very large. Initial analyses of genomic Southern blots and screens of genomic libraries indicated that it comprised at least 100 to several hundred genes in rat. Sequence analysis further showed that the olfactory multigene family exhibited striking divergence within the TM 3, TM 4 and TM 5. These transmembrane regions had been suggested to be involved in the ligand-binding GPCRs [13]. The large size of the olfactory multigene family and the variability in regions thought to be important in ligand binding are suitable to accommodate the extremely large number and diverse structures of odor molecules. However, the most critical criterion, the ability to mediate an odor-induced response, was not experimentally satisfied at the time.

Since the original identification in rat [3], OR genes with full-length or partial coding sequences have been cloned from many species ranging from mammals to fishes, including rat [14–18]; mouse [18–21]; dog [22, 23]; human [22, 24–29]; pig [30]; chick [31, 32]; frog [33]; salamander [34, 35]; catfish [36]; zebrafish [37–40]; goldfish [41]; lamprey [42]. The cloning procedures are nearly all initiated by PCR with primers designed from the OR sequences cloned by Buck and Axel [3], and the other OR sequences characterized later on.

A very useful Internet database of ORs, the Olfactory Receptor DataBase (ORDB) [43, 44], has been established which houses over 400 published and many unpublished OR sequences from different species as well as analysis tools and models. This database can be accessed at http://senselab.med.yale.edu/ORDB/html/

Sequence characteristics

The ORs consist of between 300 and 350 amino acids. Analysis of many sequences reveals that they contain seven hydrophobic stretches (19–26 amino acids each) that represent potential transmembrane domains.

Sequence comparisons [3, 17, 36, 45] show the common features of ORs, some of which are shared by many other GPCRs. ORs lack a definable N-terminal signal sequence, and the short N-terminal segment contains a potential N-linked glycosylation site. There are several potential phosphorylation sites in the intracellular region. Among the many conserved amino acid residues that ORs share with other GPCRs are the conserved cysteines at the first and second extracellular loops, and the NPxIY sequence motif at the beginning of TM 7.

Several features are distinct to the ORs. The second extracellular loop that contains about 38 amino acids, including three highly conserved cysteines, is substantially longer than most other GPCRs. The third cytoplasmic loop, which contains only 15 to 17 amino acids, is relatively short. Among ORs, the most conserved region is in the second intracellular and extracellular loops as well as within TM 2, TM 6 and TM 7. TM 3, TM 4 and TM 5, however, exhibit striking divergence. There are a few 'landmark' consensus sequences that are unique to ORs: for example, the mayDRyvAiCx-PlxY motif (letters in capital case are highly conserved residues) at the C-terminal of TM 3 and the second intracellular loop, and the KafsTCxsh motif at the N-terminal of TM 6.

Subfamilies within the OR gene family

When comparing the sequences between ORs, some receptors are more homologous to each other than to others. The OR gene family therefore can be divided into subfamilies such that the members of a given subfamily share significant sequence conservation [3]. The criterion of 60% sequence identity has been used to group ORs into different subfamilies [45]. The range of divergence is considerable, with some ORs in different subfamilies sharing less than 20% identity, whereas in many cases ORs in the same subfamily may differ from each other by only a few residues.

The size of a given subfamily is usually determined by genomic Southern blot analysis. Because there is no intron in the short 1-kb coding region of the OR, with probes from the coding region each band detected is likely to represent a different gene. The receptors detected under stringent conditions are grouped in the same subfamily. The subfamilies range in size from a single member to as many as over 20 members [3, 46]. Subfamilies defined by DNA cross-hybridization (i.e. Southern blot) are more stringent that the members in a subfamily share at least 80% identity [45].

Sequence comparisons further indicate that the ORs from fish (teleost) and the ORs from mammals are distinct from each other [33, 36, 45]. The difference may reflect the fact that fishes detect water-soluble molecules, whereas terrestrial vertebrates smell volatile, hydrophobic compounds. *Xenopus* possesses a gene repertoire encoding both 'fishlike' receptors and 'mammalian-like' receptors [33]. The two classes of receptors are expressed in different compartments of the frog's nasal cavity. The 'fishlike' ORs are exclusively expressed in the lateral diverticulum, whereas mammalian-like ORs are expressed in the main diverticulum. The recently cloned ORs from the ancient vertebrate lamprey represent another distinct class [42].

A large gene family

The OR gene family is composed of a large number of distinct OR genes in the genome. Although the exact number of OR genes in any species is currently not known, estimates of the size of an OR gene family are determined by genomic Southern blot analysis and screening of genomic libraries.

In mammals, the repertoire of OR genes has been estimated to consist of 500–1000 genes [47–49], making the OR gene family the largest gene family in the genome yet identified. Buck and Axel [3] originally estimated a minimum of 100–200 genes in the rat genome, but this was a lower limit [3] because the probes used did not include representatives of all existing subfamilies. Screening of rat genomic phage libraries at low stringency with a complex probe generated by PCR with the OR family-specific degenerate primers resulted in 0.1% positive phage [14], suggesting the presence of as many as 1000 receptor-related sequences per haploid genome [1]. In the dog, the size of the family was estimated to be more than 400 genes by the screening of a dog genomic library with a mixture of PCR clones, and it was considered that the number was an underestimate [22].

In mouse and rat, quantitative in situ hybridization studies [15, 19, 50, 51] indicate that the number of OR genes is in the range of 1000 and may be even larger. For example, in mouse [19], probes for three different receptors, K18, K4 and K7, labeled 0.08 (one-fourth of 1/150), 0.08, and 0.16% (one-fourth of 1/300) of olfactory neurons, respectively. K18, K4 and K7 belong to different subfamilies. Each of these three subfamilies contains about 10–15 member genes, and each probe might recognize multiple ORs in the subfamily. In rat [51], probes for receptor OR14, OR18 and OR37 labeled 0.37, 0.23, and 0.4% of olfactory neurons, respectively. It is known that the OR37 subfamily consists of at least five receptors in rat [18].

In fish, the repertoire of ORs is significantly smaller than in mammals. In catfish, the size of the OR gene family was first estimated to contain about 100 genes, though the maximum number of OR genes that could be detected by all means was only about 20 [36]. In situ hybridization results, however, were consistent with the estimated size of OR genes in that each probe hybridized to only 0.5–2% of olfactory neurons [36, 52]. In zebrafish, the size of the OR gene family was estimated to be in a similar range [38, 39]. The individual probes of six different receptors each identified 0.5–2.5% of olfactory neurons [38]. In situ hybridization with probes for 10 different receptors was reported to label maximally 10% of olfactory neurons [39]. The significantly smaller number of receptors in fish may reflect the smaller repertoire of aquatic odorants.

Regardless of the large repertoire of OR genes, humans possess a significant number of OR pseudogenes in their genome [24–26, 28, 29]. Analysis of an OR gene cluster which contained 14 OR genes on chromosome 17 revealed that more than 37.5% of OR genes in this cluster were pseudogenes [25, 26]. As many as 75% (21/28) of the OR genes identified on chromosome 11 are pseudogenes [29]. The sequences of 87 ORs from 16 chromosomes indicate that 72% of the sequences are pseudogenes [28]. Expression of the OR pseudogenes in human olfactory epithelium has been observed [27].

Spatial expression

With a few exceptions, the expression of ORs is restricted to olfactory neurons of olfactory epithelium. The expression pattern of OR genes in olfactory epithelium has been extensively investigated by using in situ hybridization. A particular receptor probe hybridizes to only a small fraction of olfactory neurons that are not clustered, but rather scattered in the olfactory epithelium.

In mouse [19, 21] and rat [15, 46, 51, 53], the olfactory neurons expressing a specific receptor are not distributed across the entire epithelium. They are spatially restricted in one of three or four connected but nonoverlapping zones within the olfactory epithelium. Within a given zone, however, the distribution is in an apparently random pattern. The expression zones are bilaterally symmetrical and are organized along the dorsal-ventral and medial-lateral axes, extending along the entire anterior-posterior axis of olfactory epithelium. Highly homologous ORs in the same subfamily are expressed in the same zone.

Although most ORs are expressed in one of the broad zones, some ORs exhibit unique expression patterns. One particular OR subfamily, the OR37 subfamily [18, 51, 53, 54], is expressed exclusively in a small area on

the tip of central turbinates with a very high cell density. It is possible that other OR genes will also show unusual expression patterns. The functional relevance of the zonal expression patterns is unknown.

The olfactory epithelium is pseudostratified with a single layer of supporting cells on the top close to the surface, and several layers of olfactory neurons in the middle region, and one or two layers of basal cells at the bottom along the basal membrane. A detailed analysis in the rat revealed that for some ORs, neurons expressing a distinct receptor were preferentially located in a particular laminar zone of the epithelium [55]. If the position for the basal lamina is set to be 0 and the apical surface to be 1, the olfactory neurons [olfactory marker protein (OMP)-positive] are located in the region between 0.1 and 0.8. OR14-expressing neurons were found to be restricted within the region between 0.1 and 0.6, and the majority of them were concentrated in layers at position 0.3. The OR133-expressing neurons, however, were located in a laminar zone between 0.3 and 0.8, and on average at 0.7. The OR105-expressing neurons were preferentially located around 0.6, whereas OR18-expressing cells were mainly present around 0.4 [55]. Within an OR subfamily, neurons expressing different subtypes may have different laminar distributions. In the mouse [18], the OR37 subfamily contains three highly homologous members that are all expressed in the same unique area of olfactory epithelium. The mOR37a-expressing neurons are randomly distributed throughout the cellular layers of the epithelium, but the mOR37b and mOR37c-expressing neurons are preferentially located in the upper one-third of the epithelium. In the goldfish [14], two populations of neurons apparently exist in olfactory epithelium. One expresses ORs; the other expresses the putative pheromone receptors that are, in mammals, expressed in the neurons located in the vomeronasal organ. The cells expressing the putative pheromone receptors are abundantly located near the apical surface. In contrast, the cells expressing ORs are randomly distributed in the olfactory epithelium but well below the layer near the apical surface.

In catfish, olfactory neurons expressing a particular OR appear to be distributed randomly throughout the olfactory epithelium [52]. In zebrafish, however, some spatial pattern has been found. For four ORs analyzed in detail [39], olfactory neurons expressing a particular receptor can be found along the whole extent of the olfactory epithelium, but they are located with higher frequency in one of three concentric, ringlike domains around median raphe. The diameter of these rings was different for different receptors. However, a spatial pattern of distribution was not found by another observation of three different ORs in zebrafish [56].

In chick, during the embryonic stages and in adult, in situ hybridization showed that there is no spatial organization of the olfactory neurons expressing particular ORs. The neurons appeared to be randomly distributed throughout the olfactory epithelium [31, 32].

Temporal expression during ontogenesis

The expression of OR genes during development has been examined extensively by in situ hybridization. Nef et al. [15] first examined the expression of an OR gene, OR3, in embryonic mice. OR3 was first detected at day E (embryonic day) 10. At E10 and E11, OR3-positive cells were distributed along the curvature of the base of the telencephalic epithelium and were not in the primitive olfactory epithelium (or nasal pit). From E12 to E13 until adult stages, OR3 transcripts were detected only in the developing or mature olfactory epithelium. Sullivan et al. [57] reported that in mouse, OR gene expression was first observed in the olfactory epithelium at E11.5 for some ORs in some embryos. The number of cells expressing OR gene increases dramatically between E12.5 and E13.5. This is well before E14, the time reported that the first synapses between olfactory axons begin to form in the mouse olfactory bulb [58, 59]. The expression of a set of 12 ORs was examined on embryos at E13 and E13.5. All 12 ORs are found to be expressed at these stages, indicating that developmental onset of expression of different OR genes is similar [57]. In rat [60], the OR expression can be detected at E14. The zonal pattern of OR gene expression is apparent as soon as ORs are first detected in the developing olfactory epithelium [15, 57, 60].

In zebrafish, the ontogeny of OR gene expression has been studied by in situ hybridization [37, 38, 40]. The olfactory placodes in zebrafish first appear at 14–16 h (6–10 somites) postfertilization. By 24 h (20 somites) axonal projections between olfactory placodes and the developing forebrain are apparent; hatching occurs at about 72 h [61]. Specific hybridization to a probe of the olfactory cyclic nucleotide-gated channel was observed over the olfactory placode beginning at 24 h [38], and evident expression of OR genes was detected at 30 h postfertilization [38, 40]. During development, an asynchronous onset of OR expression was observed [38, 40]. More remarkably, different ORs in the same subfamily, and which are tightly linked in the genome, are activated at different times of development [40]. The OR 2/6 subfamily contains genes sharing between 71 and 84 amino acid identity that are clustered within 60 kb of genomic DNA and are all transcribed in the same orientation. Their relative positions in genomic DNA from the transcription initiation side are 6, 2, 2.5, 2.2, 2.7, 2.3, 2.4 and 2.6. However, receptors 2, 2.2, 2.3 and

2.5 are expressed earlier than the other receptors. They are first expressed between 24 and 36 h of development. Receptor 2.6 (i.e. ZF1I [37]) is expressed at 38 h, receptor 2.4 is expressed at 48 h, and receptor 6 is expressed the latest, at 120 h.

In chick, OR expression was first detected in olfactory placodes at day E5 [31, 32] when the first olfactory axons emerge and begin growing toward the telencephalon where the bulb will later form. The OR-positive cells were also found in a stream of cells along the forming olfactory nerve between the placode and the telencephalic vesicle. After E6 [31] or E7 [32], the expression of ORs was detected only within the olfactory epithelium, except that one OR gene, COR7b, was found transiently expressed in the notochord from E2 to E6 [62]. Interestingly, COR7b shares 98.5% identity at the nucleic acid level in the coding region with another member of the subfamily, COR7a, which is expressed only in the olfactory epithelium from E6.

One neuron, one receptor

In most vertebrates, a single olfactory neuron is believed to express only one OR gene [11, 20, 63], i.e. one neuron, one receptor [64]. Experimental evidence to support this view is, however, mostly indirect. From in situ hybridization studies, a probe for a given receptor (or an OR subfamily) detects fewer than 0.1–0.5% of olfactory neurons in mouse and rat [15, 19, 50, 51, 53], and 0.5 to 2 of olfactory neurons in catfish [52, 65], indicating that a single olfactory neuron may express only one, or at most a few, receptors (considering the size of OR gene family). Additionally, different OR probes label distinct, nonoverlapping subpopulations of olfactory neurons. In catfish, hybridization with a mixed probe of two [65] or four [52] different ORs which do not cross-hybridize to each other labels a similar number of olfactory neurons as the sum of the olfactory neurons detected by each probe used individually, indicating that ORs in different subfamilies are not expressed in the same neurons. A similar result was obtained in the mouse [19], and in the chick [32]. Finally, receptors in the same subfamily that are highly homologous and are closely linked in the genome are expressed in different neurons. In the zebrafish [40], a series of double-label in situ hybridization, each with probes for two members of the OR 2/6 subfamily, showed clearly that the frequency of signal colocalization was from 0 to at most 5%. The 5% colocalization observed was considered within the noise level of the investigation. In the mouse [18], for example, three highly homologous genes of the OR37 subfamily are all expressed in the same unique area of olfactory epithelium and are tightly linked in the genome. However, in situ hybridization shows that the number of neurons detected by using a probe which detects all OR37 subtypes correlated very well with the sum of hybridization signals obtained using the subtype-specific probes individually. In the chick [62] the COR7 subfamily contains two ORs, COR7a and COR7b, that encode receptors which share 96.6% amino acid identity. In situ hybridization with the probe that recognizes both COR7a and COR7b labels a similar number of cells in the olfactory epithelium during development as the sum of the cells labeled with the specific probes for COR7a or COR7b used individually. These results all strongly suggest that each olfactory neuron expresses just one member of an OR gene subfamily.

In the chick, Leibovici et al. [32] showed, by double-label in situ hybridization, that a single olfactory neuron did not coexpress different ORs (or subsets of ORs). However, Nef et al. [31] reported that, in the chick, the probes of mixed ORs labeled a significantly smaller number (13–52%) of olfactory neurons than the sum of the neurons labeled with individual probes in the mix, indicating that some olfactory neurons express more than one receptor.

Further evidence that receptors that are highly homologous are expressed in different neurons has also been obtained from the observations that olfactory neurons expressing the same receptor project to the same pair of glomeruli in olfactory bulb [66, 67]. Receptors P2 and P3 were found to project to different pairs of glomeruli, indicating that P2 and P3 are expressed in different neurons [67]. The P2 and P3 receptors share 75% amino acid identity, and both genes reside in the same OR gene cluster.

Somewhat more direct evidence supporting the single-receptor model came from a recent publication [68], in which Malnic et al. used a two-step, single-cell RT-PCR procedure to identify the OR genes expressed by individual olfactory neurons isolated from mouse. In this procedure, the cDNAs of each single olfactory neuron were first obtained by reverse transcription with oligo-dT primers and then amplified by PCR. The amplified cDNAs were then subjected to a second PCR with degenerate primers matching conserved amino acid sequence motifs in mammalian ORs. The OR cDNA products of this second PCR reaction were then isolated and sequenced. In this study, analysis of the OR cDNA products of the second PCR showed that only a single OR cDNA sequence was obtained from each cell. Preliminary data from single-cell PCR studies from salamander [34] were also consistent with this notion. Receptor sequences amplified from the cytoplasmic contents obtained by a whole-cell patch clamp electrode pipette from a given single olfactory neuron were either identical or they differed only in a few nucleotides, which could be ascribed to PCR error.

Further, a single olfactory neuron not only is likely to express an exclusive OR gene, but it appears to express only one of two OR alleles. In the mouse [20], the expression of an OR gene, I7, was examined in olfactory neurons from the F1 progeny cross between *Mus spretus* and *Mus musculus* mice, which contain polymorphisms in the I7 gene. A limiting dilution was performed on dissociated olfactory neurons to obtain pools of 200 cells. After the cDNAs from each pool were amplified by PCR for I7, only 1 in 10 pools was shown to contain an I7-expressing neuron, indicating that each I7-positive pool contains a single I7-expressing neuron. The PCR products were then subcloned and hybridized with oligonucleotide probes designed to distinguish between the maternal and paternal transcripts. The results demonstrated that I7 expression derives exclusively from only one of two alleles. The same result was obtained from analysis of another OR gene, I54. This mono-allelic expression of OR genes is also confirmed by observations from mice in which the receptor P2 gene is genetically altered [66]. In heterozygotes, which carry one wild-type P2 allele and one modified P2-IRES-tau-lacZ allele, the number of lacZ-positive cells is about half of the total number of P2-expressing cells as determined by in situ hybridization with P2 probes. In homozygotes, however, the number of P2-expressing cells equals the number of lacZ-positive cells.

Genomic distribution and organization

The very large size of the OR gene family has motivated studies of genomic distribution and organization of the OR genes. These investigations may have important implications for understanding the mechanisms that regulate receptor expression, and may provide further insights into the evolution of the OR gene family

OR genes are widely distributed in the genome. In mouse, with 21 OR genes as a probe, 11 loci have been mapped on seven different chromosomes by Southern blot analysis [21]. In humans, through fluorescent in situ hybridization (FISH), 53 sites (28 with efficiencies greater than 5%) containing OR sequences were detected from all chromosomes except chromosomes 20 and Y. PCR-amplified OR sequences with degenerate probes were obtained from 16 flow-sorted chromosomes [28].

At discrete loci in the genome, the OR genes are organized as clusters [18, 21, 23, 25, 26, 29, 36, 40, 47, 67, 69]. In one OR gene cluster on human chromosome 17 that has been extensively analyzed [25, 26], at least 14 OR genes are physically mapped in one 0.35-Mb-long contiguous cluster within the chromosomal band 17p13.3, with an average intergenic separation of 15 kb. Three OR gene clusters were also physically mapped on human chromosome 11 at 11p15, 11q13 and 11q24 [29]. In zebrafish, a tight cluster of 10 OR genes within a 100-kb region of the genome has been characterized [40].

OR genes which are highly homologous in sequence are located in the same cluster and often adjacent to each other to form subclusters, but they can be interspersed by OR genes of different subfamilies [25, 47]. All clusters characterized so far contain OR genes belonging to multiple subfamilies. For example, the human chromosome 17 OR gene cluster [25] is composed of OR genes belonging to four subfamilies. The gene cluster in zebrafish [40] contains OR genes of two subfamilies. In a gene cluster, even within a subcluster, the transcriptional orientation of each gene can be in either direction.

OR gene structure

Like the genes for many GPCRs, OR genes have no introns in their coding region. All receptor genes analyzed, however, do have at least one intron following a single exon upstream of the coding-region exon [20, 26, 48, 70–72].

The gene structure of a mouse OR, MOR23, has been studied in detail [70]. The MOR23 gene is located in the H4–H5 region of chromosome 1. The genomic DNA fragments containing the MOR23 coding region were isolated, and a 10.7-kb region including the coding region and 8-kb 5′- and 1.7 kb 3′-flanking regions were sequenced. The MOR23 gene carries a 927-bp, intronless coding sequence; a polyadenylation signal, AATAAA, is present 155 bp downstream from the termination codon. An RNase protection assay revealed that it is expressed in both olfactory epithelium and testis. In olfactory epithelium, 5′ RACE (rapid amplification of the cDNA ends) analysis showed that the most MOR23 messenger RNAs (mRNAs) contained a 219-bp 5′-noncoding sequence. Except for the 16-bp sequence upstream of the ATG codon, the rest of the upstream noncoding sequence matched a region 4.5 kb upstream from the initiation codon in genomic DNA, indicating that a 4.5-kb intron was located closely upstream to the coding exon. The potential splice donor (GT) and acceptor (AC) sequences were found at junctions in the genomic sequence. Interestingly, MOR23 shows a tissue-specific differential initiation of transcription. The 5′ RACE in testis revealed that, unlike in olfactory epithelium, transcription was initiated in a region 90–170 bp directly upstream of the initiation codon, within the intron. The gene structure of a mouse OR, M4, has also been characterized [71] and was shown to have similar features for that of MOR23. A 300-bp exon is located 4 kb 5′ to the M4 initiation

codon, and a 3′splice site is located at 12 bp upstream of the initiation codon.

In rat, two OR subfamilies, SCR D and SCR G, were found to undergo 5′ splicing [72]. SCR D and SCR G were first identified from the cDNA of purified round spermatids, and it was found that the mRNAs were spliced upstream of their presumptive starting methionines. These genes were shown to be expressed also in olfactory epithelium and spleen. In one case, an identically spliced SCR G-14 (a member of SCR G subfamily) product was detected in all three tissues.

In humans, a 40-kb genomic region in chromosome 17 (17p13.3) which contains two OR genes (OR17-40 and OR17-228) and two OR pseudogenes has been sequenced [26]. Computational analysis suggested the presence of introns immediately (6 bp) 5′ to the coding regions, which are 6134 and 5433 bp for OR17-40 and OR17-228, respectively.

Regulation of expression

The mechanisms controlling gene expression of ORs are still largely unknown. Studies of spatial and temporal patterns of expression, of the genomic organization and the gene structure, as well as of promoter analysis of OR genes have all provided insight regarding the regulation of OR gene expression. A few models have been proposed to explain how the observed spatial and temporal expression pattern and the 'one neuron, one receptor' selection are accomplished, but currently there is no single model that satisfies all of the data.

The most comprehensive model includes an allelic inactivation and a cis-regulatory mechanism [20, 73]. In this model, the first step is the random inactivation of either the maternal or the paternal array of each olfactory receptor locus in all cells during early development. The second step is the choice of one chromosomal locus by each olfactory neuron during olfactory neurogenesis. In the third step, a cis-regulatory element directs the stochastic expression of only one gene from the one active allelic array. The zonal restriction of expression observed in mammals is achieved via a zone-specific transcription factor(s) that dictates which receptor genes are available within a linked array. This model does not include a mechanism for the asynchronous onset of OR expression observed in zebrafish, which may require the involvement of temporally restricted, specific trans-acting factor(s) in the regulation of OR expression [38, 40]. The actual processes of how each step in the model is accomplished remain to be elucidated.

The zonal expression of ORs in mammals may be regulated in part by the regions upstream of the OR coding region [71]. A 6.7-kb genomic fragment upstream of the receptor M4 coding region fused with a reporter sequence lacZ was inserted into the genome to generate transgenic mice. The 6.7-kb fragment includes a 4-kb intron immediately upstream of the M4 coding region, a 300-bp 5′-untranslated exon and a 2.4-kb region upstream of the 5′ exon. Expression of the transgene was obtained in three transgenic lines in which expression was restricted to olfactory tissue and testis. In each line, the expression within olfactory epithelium was within a receptor zone and occurred only in a subset of olfactory neurons distributed randomly within the zone. However, expression was detected in two different zones, one of which was identical to the endogenous M4 receptor. The transgenic lines examined had multiple-copy insertions, but not near any known OR loci. These findings indicated that some important regulatory elements, which are sufficient for providing zonal specification and permitting the stochastic selection of transgene expression in the appropriate cell type (i.e. olfactory neuron), are located in regions upstream of the OR coding region [71].

Function and functional expression

The principal function of ORs is to bind odorous molecules and to transduce this signal to the downstream protein (the G protein) of the signal transduction pathway. Our knowledge of ORs, however, has been gained largely through molecular studies at the level of DNA. Recent progress in functional expression of ORs allows us to begin exploring the function of OR proteins.

The large size of the OR family suggests that the initial discrimination between different odorants depends on the selective binding affinity of receptors, and that the high divergence at the TM 3, TM 4 and TM 5 may be the key for the discrimination. What is the relation between the protein sequence of an OR and its binding affinities for different odorants? Do ORs with similar sequences (members of the same subfamily) recognize related chemical ligands? On the one hand, because of the much larger size of the odorant repertoire, an OR must be able to bind several different—odorants-though some receptors may be tuned only to a few unique odorant ligands. On the other hand, a particular odorant may be recognized by different ORs. How specific or nonspecific are these receptors for their diverse ligands? For odorants, what are the critical molecular structures, or 'olfactophores' [2], that determine binding affinities and selectivity? Information on the relation of ORs to their odorant ligands is an essential element for understanding olfactory coding. In addition, the ORs may serve as a model for GPCRs. The OR gene family provides a large number of naturally

developed sequence variations which could contribute to a more thorough understanding of the relation between gene sequence, protein structure and ligand-binding specificity in GPCRs.

Physiological recording and calcium-imaging studies showed that individual olfactory neurons (therefore individual ORs, since one neuron, one receptor) frequently respond to multiple odorants [74–77], but the relation between the receptor and ligand could not be determined from these studies.

Experiments on the purification of OR proteins through overexpression, solubilization and chromatography have been conducted [17, 78, 79], and in one case, a purified receptor, the rat OR 5 receptor, was shown to have specific binding to the odorant lilial and lyral by a nontraditional ligand-binding assay [79].

The questions noted above could be profitably approached by the functional expression of ORs, in which individual receptors can be expressed and tested for odorant binding specificities through measuring the odorant-induced responses (e.g. second-messenger generation or electrical activity of the cell). Functional expression, at the most elemental, serves to provide the functional evidence that the olfactory multigene family indeed encodes ORs. It also pairs individual ORs while their appropriate odorant ligands. A system for functional expression would further provide a tool to experimentally test the critical residues of receptors for odorant binding by conducting site-directed mutagenesis experiments as well.

The functional expression of many GPCRs in heterologous systems has been routinely used to study the function of receptors. Attempts to functionally express ORs in heterologous systems, however, have encountered difficulties. The most recognized difficulty is that the expressed OR proteins fail to translocate efficiently to the cell membrane [80, 81]. Additionally, the receptors that do reach the membrane may be unable to efficiently couple with endogenous second messenger systems in heterologous systems, making a functional assay impossible. The extremely large repertoire of odorants and the large number of receptors also make the likelihood of pairing a particular receptor with its odorant ligand(s) rather small.

In the first reported attempt at expression of ORs that showed functional activity, Raming et al. [16] utilized the baculovirus system to show that the expression of an odorant receptor OR5 in sf9 cells resulted in generation of the second messenger IP_3 (inositol trisphosphate), but not cAMP, in response to a mixture of lyral and lilial. In this study, however, four mixtures of odors among a total of eight mixtures classified in different chemical groups elicited a significant increase in IP_3 concentration, and one other mixture also elicited an obvious increase in IP_3 concentration, although the increase is not statistically significant in the three duplicate experiments. The odorants that increased IP3 production show no structural relationship. The results indicated an extremely broad ligand affinity of the OR5 receptor. Expression of another receptor, OR12, that shares 75% identity with OR5, however, did not show a response to any of these odorants.

The functional expression of three fish ORs was reported in HEK (human embryonic kidney) 293 cells [82]. These receptors were expressed as fusion proteins with the N-terminal membrane import sequence of the guinea-pig 5-HT_3 receptor, and showed sensitivity to a commercial fish food mixture, but have not been paired with any specific ligand, leaving open the exact nature of their ligand specificity.

A more successful attempt at functional expression of ORs in a heterologous system was reported recently in HEK 293 cells [81]. In this study, the N-terminal fragment (20 amino acids) of the bovine rhodopsin was fused upstream to the OR sequence to facilitate membrane targeting of the receptor. Expression vectors carrying the G protein subunits $G_{\alpha 15}$, and $G_{\alpha 16}$, which can promiscuously couple GPCRs that normally signal through other second messengers to the phosphatidylinositol bisphosphate (PIP_2) pathway, were cotransfected with the receptor constructs in order to efficiently generate an IP_3-mediated increase in intracellular calcium. The odorant-induced response was thus measured by calcium imaging. The authors generated an expression library of chimeric receptors to increase the likelihood of pairing a receptor with its odorant ligand(s). The expression library contained a large and diverse repertoire of mouse OR sequences from the beginning of TM 2 to the end of TM 7, which were flanked by the sequence from the N-terminal to TM 2 and the C-terminal sequence of a mouse odorant receptor M4. By testing 80 chimeric receptors against 26 odorants, three receptors were identified as responding to the odorants carvone, (–)citronellal and limonene, respectively. The authors further showed that the response profile of a receptor was conserved between the receptor chimera and the full-length receptor. In this study, functional expression of a rat receptor, I7, and its counterpart in mouse, the mouse I7, were also performed. The experiments showed that the rat I7 receptor responded to odorant octanal at a lower concentration than to odorant heptanal, whereas the mouse I7 did in the reverse. Remarkably, this difference in ligand specificity could apparently be attributed to a single-residue change in position 206 of the receptor sequence. The rat and mouse I7 receptors share 94% identity, with a total 15 different amino acids, of which only 3 (K90E in the first extracellular loop, V206I in TM 5 and F290L in TM 7) reside between TM 2 and TM 7. The substitution of valine with isoleucine at 206 led the rat I7 to preferen-

tially recognize heptanal, and the substitution of isoleucine with valine led the mouse I7 preferentially to recognize octanal. Interestingly, the nature of these changes, valine versus isoleucine and octanal versus heptanal, is consistent with compensatory alterations in the structures of ligand and receptor.

In an alternative approach, we have taken a strategy of using a homologous system, i.e. olfactory neurons in the olfactory epithelium, for functional expression of ORs [83]. Olfactory neurons provide both the entire machinery for proper folding and targeting the receptor protein to cilia membrane and the appropriate G protein system to couple the receptor for function. The approach relies on the large number of ORs, and their approximately equal and random expression in olfactory epithelium, to identify the increased response to odorant(s) in an epithelium in which a particular receptor is overexpressed. In rat, for example, each of the approximately 1000 ORs is expressed in roughly 0.1% of the total olfactory neurons (0.33 or 0.25% of olfactory neurons in one of the three or four expression zones). The overexpression of a particular OR in as few as 1–10% of the olfactory neurons in olfactory epithelium would lead to an increased response to its ligand(s). In this approach, the overexpression of a particular OR in an increased number of olfactory neurons is achieved via an adenovirus vector which can efficiently infect olfactory neurons [84, 85]. The odorant-induced responses are measured by recording of the EOG (electroolfactogram), a transepithelial potential due to the summed activity of many olfactory neurons.

In our studies [83], we generated a replication-incompetent adenovirus vector (i.e. Ad-I7 virus) containing an expression unit for an OR, the I7 receptor from rat, which was originally cloned by Buck and Axel [3], and GFP (green fluorescent protein), whose expression indicated both the location and the rate of infection in living tissue. In rat, the infection with Ad-I7 virus resulted in the expression of GFP, and therefore the I7 receptor, in as many as 20% of the cells in some regions of the epithelium. The responses to a panel of 74 odorants, including both aromatic and short-chain aliphatic compounds with various functional groups, were tested in the infected epithelia by EOG recording. Octanal, a 8-carbon aliphatic aldehyde and a set of other odorants with a similar chemical structure, heptanal, nonyl aldehyde, and decyl aldehyde, induced a significant increase in the response from the Ad-I7-infected epithelia versus the control epithelia, which were infected with an adenovirus carrying an expression unit for only the GFP. No increases were detected in aldehydes with less than 7 or more than 10 carbons. Other 8-carbon aliphatic compounds with different functional groups and 5 aromatic aldehydes also failed to elicit larger responses. Thus, the response profile of the I7 receptor, at least within the scope of the 74 odorants screened, was discovered to be specific for 7- to 10-carbon aliphatic aldehydes. Single-cell recordings from dissociated olfactory neurons by whole-cell patch clamp further revealed that all recorded Ad-I7 infected olfactory neurons responded to octanal, whereas only a small portion of uninfected neurons responded to octanal. The response of I7 to octanal was also confirmed by a study using HEK 293 cells [81] described above. The results strongly indicated that a member of the olfactory multigene family identified by Buck and Axel indeed encodes an OR that mediates a physiological response to a specific set of odorants.

Recently, a procedure that combined calcium imaging and single-cell RT-PCR [68, 86] led to a breakthrough in linking an odorant with its receptor(s). In this procedure, the response profiles of isolated, individual olfactory neurons to a panel of odorants were first examined by calcium imaging, and the OR in each neuron was then identified by a two-step, single-cell RT-PCR. In the study carried out by Malnic et al. [68], 13 ORs from 14 olfactory neurons were identified as responding to some of 17 aliphatic odorants. The results showed that an OR from an individual olfactory neuron recognized multiple odorants and that a single odorant was recognized by multiple receptors in different neurons, but that different odorants were recognized by different combinations of ORs. Touhara et al. [86] amplified a mouse OR, MOR23, from individual olfactory neurons responding to odorant lyral. They further confirmed the response of MOR23 to lyral by using an adenovirus-mediated expression system.

Regulation of function

Protein function can be regulated by glycosylation and phosphorylation. There is accumulating though limited evidence that OR proteins undergo this sort of regulation. The OR proteins on olfactory cilia are likely to be glycosylated. Sequence analysis indicates that the short N-terminal segment contains a potential N-linked glycosylation site. Experimentally, Western blot analysis of olfactory cilia preparations with antibodies against the OR peptide sequences showed that the relative molecular mass of the immunoreactive proteins is higher than the deduced molecular weight (30–35 kDa) of the OR proteins. In one study [87], an antibody against the receptor OR5 recognized a band around 50-kDa. Upon deglycosylation with N-glycosidase-F, the band shifts to 30 kDa. In another study, two antibodies recognized a 54- and a 64-kDa band, respectively [88].

Ligand-dependent phosphorylation is essential to the desensitization of many GPCRs, in which the activated receptor is thought to be first phosphorylated and then

to bind a cytosolic protein, arrestin. A specific G-protein-coupled receptor kinase, GRK3 (i.e. βARK2), and an arrestin isoform, β-arrestin-2, were shown to be highly enriched in and localized to the dendritic knobs and cilia of olfactory neurons by immunohistochemistry and Western blot analysis [89]. Specific antibodies to GRK3 and to β-arrestin-2 [89] increased the odorant-induced elevation of cAMP and blocked the rapid decline of cAMP, which might be caused in part by desensitization of the receptors. Antibodies to GRK3 also inhibited odorant-induced phosphorylation of olfactory ciliary proteins [90]. Cilia preparations derived from GRK3-deficient mice lacked fast odorant-induced desensitization [91]. Antibodies against receptor OR5 specifically precipitated phosphorylated olfactory ciliary proteins upon odorant stimulation. The immunoprecipitated, phosphorylated protein appeared as a single band at 50 kDa after separation by electrophoresis [87].

Other functions

In addition to their role in binding odor molecules and mediating olfactory signal transduction, ORs may have other functions. ORs are thought to play a role in guiding axons to the correct glomeruli during the development and regeneration of olfactory neurons [49, 66, 67, 92, 93]. This is consistent with the finding that olfactory neurons expressing the same receptors project their axons to the same set of glomeruli in olfactory bulb [49, 66, 92]. This hypothesis was tested by a series of experiments utilizing gene knockout and gene replacement [66, 67], in which it was shown that the OR is required for convergence of olfactory neuron axons. The deletion of the coding region of the P2 receptor, or nonsense mutations in the P2 receptor, resulted in the failure of axons from these neurons to converge on the P2 glomeruli. Instead, they projected diffusely within the olfactory bulb [67]. The P2 coding region was then replaced with the coding regions of receptor M12 [66], M71, M50 and P3 [67], respectively. Substitution of the receptor resulted in the axons of the neurons expressing the new receptor converging to glomeruli, which are distinct from either the wild-type P2 glomeruli or the glomeruli of the wild-type replacing receptor, but in a position between them. The replacement of P2 with P3 resulted in the axons projecting to a glomerulus touching the wild-type P3 glomerulus. P2 and P3 share 75% identity and are located to the same gene cluster in the mouse genome. The wild-type P3 glomerulus resides about 200 μm (about three glomeruli) anterior to the wild-type P2 glomerulus. Computational analysis of OR sequences has led to the suggestion that the second extracellular loop of the receptor that is relatively long may play a role in guiding axons of olfactory neurons [93].

ORs may also be involved in chemotaxis of sperm during fertilization. Northern blot analysis first revealed that some OR genes are expressed in the testis of dog [22]. One receptor, DTMT, was expressed at a high level in a cell preparation enriched in late elongated spermatids and in a preparation enriched in round spermatids and pachytene spermatocytes. The expression of some OR genes in the testis of other species, including catfish [36] and rat [70, 72, 88], were also reported. Most ORs detected from testis are also expressed in olfactory epithelium [36, 70, 72, 88], but some receptors, DTPCRH09 in dog, for example [94], are apparently only expressed in testis, with little or no expression in olfactory epithelium. In dog, immunohistochemisty studies with the antibodies against the N-terminal fragment of the receptor DTMT showed positive staining in the late round and elongated spermatids, as well as in the cytoplasmic droplet of sperm cells, and on the tail midpiece of mature spermatozoa. Western blots further showed the presence of a 40-kDa immunoreactive protein in the membrane of mature sperm cells [94]. In rat, the OR subfamilies SCR D and SCR G were shown to be selectively expressed in spermatids by RT-PCR, RNase protection assays and in situ hybridization [72]. Antibodies against OR peptide sequences specifically recognized a 64-kDa band in the membrane fractions of sperm from rat, as well as from hamster and human by Western blot [88]. The immunohistochemistry further colocalized ORs and the desensitization proteins GRK3 and β-arrestin-2, which are also found in olfactory neurons [89], to elongating spermatids in rat testis and to the midpiece of rat mature sperm [88].

Finally, it has been proposed that some ORs may play roles which are currently unknown during development. In both mouse [15] and chick [31, 32], in situ hybridization experiments found that OR-positive cells transiently existed in the area between the olfactory placode and the telencephalic vesicle during early development of the olfactory system. In rat, one receptor, OL1, was shown to be expressed in the developing heart [95]. In chick, the OR gene COR7b was found to be transiently expressed in the notochord from day E2 to E6 during development [62].

Nomenclature

One serious deficiency in OR research is the undeveloped and inconsistent nomenclature currently in use. Naming receptors is now haphazard, with each of several laboratories using their own idiosyncratic system. A nomenclature that encompassed information regarding animal species, chromosomal localization of the gene, subfamily and ligand specificity would be desirable.

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