Multiple roles of secretory lipocalins (Mup, Obp) in mice

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A b s t r a c t . Many biological processes involve globular transport proteins belonging to a family called lipocalins. The prominent feature in lipocalin structure is their specific tertiary conformation forming eight-stranded beta barrel with capacity to bind various ligands inside. The importance of lipocalins is evident from the list of vital substances (hydrophobic ligands including vitamin A, steroids, bilins, lipids, pheromones etc.) that these proteins transport and from their high expression levels in various tissues. Among wide spectrum of lipocalins, Major Urinary Proteins (Mup) and Odorant Binding Proteins (Obp) are well known for their capacity to bind and carry odorants / pheromones and have been studied to detail in various mammalian models including mice, rats, and hamsters. However, many lipocalins (also including Mups) have previously been described with respect to their protective function in mammalian organism where they transport potentially harmful molecules to a degradation site (e.g. lysozomes) or straight out of the body. As most of lipocalins share similar tertiary structure, their potential role in both transport and excretion processes may be additive or complementary. In addition to a role of lipocalins in chemical communication this review presents lipocalins from the point of view of the "toxic waste hypothesis". This hypothesis assumes that members of lipocalins that are linked to a metabolic degradation of their ligands were an ideal source for natural selection during evolution due to an ability of potential receivers to detect lipocalin ligands levels as a signal by other individuals.

Key words: urinary proteins, chemical communication

Introduction

Lipocalins are a family of globular proteins that bind and transport various ligands of different size and structure such as volatile pheromones/odorants, fatty acids, lipids, steroids, bilins and retinol. They are involved in many biological processes, such as immune system response, prostaglandin synthesis, retinoid binding, metabolism of progesterone, as well as protection of small hydrophobic pheromones against chemical damage in aqueous environment (F l o w e r 1996). Lipocalins may also play a role in cancer cell interactions (B r a t t 2000) and, for example, a role in the inhibition of sperm – egg binding in human (S e p p ä l ä et al. 2002) or maintaining pregnancy in mare (C r o s s e t et al. 1996). Furthermore, lipocalins are abundant in both, prokaryotic and eukaryotic cells. Bacterial lipocalins, however, are found only in gram-negative bacteria. Eukaryotic cells have most likely acquired first lipocalin genes from the endosymbiotic α-proteobacterial (gram-negative or a common ancestor of gram-negative and gram-positive) ancestors of the mitochondrion (B i s h o p 2000). Accordingly, ancestral

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lipocalins presumably share similar functions including (i) transport of harmful substances and theoretically also (ii) communication on both the cellular and individual levels.

Lipocalin taxonomy

Lipocalin genes share very little sequence homology, however, on the level of proteins they share several features that group them into this protein family. These features include typical globular conformation formed by an eight stranded beta barrel, 4–8 exon structure (i.e. typically having 7 exons and 6 introns, Fig. 1D), with the fixed 2–5 exon size (S a l i e r 2000) and a typical lipocalin signature in amino acid sequence (GxW). More detailed analysis revealed that lipocalins may be divided into sub-families that share common motifs on the level of amino acid sequences. For example, based on the specific structure of three structurally conserved regions (SCR), lipocalins fall onto either kernel or outlier lipocalin family (F l o w e r 1996). The earliest evolutionary ancestor of lipocalins presumably belongs to outlier lipocalins family according to a fact that only these lipocalins were found in bacteria and plants. S a l i e r (2000) further suggested that the evolutionary appearance of kernel lipocalins preceded the arthropod / chordate divergence and their consequent evolutionary radiation.

Phylogenetic analyses based on amino acid sequences and exon/intron organization (G u t i é r r e z et al. 2000, G a n f o r n i n a et al. 2000, S á n c h e z et al. 2003) further segregated lipocalins into fourteen monophyletic clades. VIII.–XIV. clades contain lipocalins found so far only in mammals. For example, monophyletic clade VIII includes mouse Mups, clade IX involves rat alpha 2u microglobulins whilst clade X link together aphrodisin, probasin and Obp (G u t i é r r e z et al. 2000, G a n f o r n i n a et al. 2000).

Genomic organization

Most of mouse lipocalin genes are sited within clusters on three chromosomes depicted in Fig. 1 and 2. These are chromosome 2 (Lcn, Ptgds), 4 (Mups, Orosomucoid, Ambp,) and X (Obp, probasin) and this corresponds to rat chromosome 3, 5 and X. The synteny of lipocalin clusters from chromosome 2, 4 can be found on human chromosome 9 (S a l i e r 2000) but the syntenic region on human chromosome X reveals no evidence of functional Obp-like genes (E m e s et al. 2004). Here we describe the genomic organization of particular lipocalins that play a role in chemical communication in mice and other rodents.

Chromosome 4
Major urinary protein (Mup) gene cluster on chromosome 4 (cca 59.9Mb to 61.9Mb) (Fig. 1C) is the most abundant and compacted lipocalin cluster in mice. A 2Mb cluster contains 40 genes but at least 4 more can be predicted inside the three sequencing gaps. The cluster accounts for 21 intact protein coding genes each consisting of 179–185 amino acids (probably two hidden in the second and third sequencing gaps) and 19 pseudogenes (probably two hidden in the first and third sequencing gaps). As indicated by the zig-zag organization of genes inside the cluster (M3 to M26P) the massive amplification of gene/pseudogene pairs (at least 18x) is evident from the presence of large retroviral sequences upstream and downstream of each gene/pseudogene pair at precisely the same localization. Also the sequence similarity shows that the pseudogenes are more related to each other than to any other intact gene. The same applies for the intact genes in the same region inside the cluster. Interestingly, as a

result of this reduplication, all the pseudogenes except one are in the sense organization. Four pseudogenes (M39P, M31P, M24P and M23P) also contain retroviral-like sequences inside one of their respective introns. Both M41P and M36P have only the first exon preserved and thus are prematurely shortened in comparison with other genes and pseudogenes. Consequently, the whole mouse Mup cluster can be divided into a „recent" C57BL/6 specific and massively reduplicated central part of a cluster and into an „ancient" multiple rodent specific region or ancient Mup core. The data presented here (see Fig. 1 and 2), to some extent, complement recent findings by L o g a n et al. (2008) who described genomic organization of Major Urinary Proteins in C57BL/J6 mice using also NCBI database. Similarly, L o g a n et al. (2008) divided Mups into two groups; an older and divergent class of genes (group A) and a novel recently re-duplicated group of genes (group B) with a high sequence and, thus, functional homology.

Orosomucoid (Orm) gene cluster depicted in (Fig. 1A) is also located on chromosome 4 just beyond the Mup cluster and contains three active genes and one pseudogene. The pseudogene has a G to T mutation in the splice acceptor site of the third intron and premature stop codon (Q186X) which is missing in the functional genes from this cluster. Although this gene cluster is sited just beyond the Mup cluster, to our knowledge, there is no evidence for its involvement in chemical communication.

Chromosome X
To date, only Obp1a and Obp1b genes were described on mouse X chromosome. However, our NCBI search revealed that there is a 0.5Mb Odorant-binding protein (Obp) gene cluster on chromosome X (Fig. 1B) containing 7 intact genes (here described as O1-O8) and one pseudogene annotated as O2P, which is oriented in the opposite direction. Probasin (Pbsn) gene is located at the 5'region of Obp cluster and it is not a true member of the Obp cluster. This fact is indicated by the sequence differences of the Probasin (Pbsn) gene and its lower compaction in comparison with the Obp genes. Based on the sequence comparisons, these 8 Obp genes of the cluster emerged by repeated duplication of three ancient genes and can be divided into three structural groups. The first one belongs to the highly related O1-like genes (O1, O2P, O3), which indicates very recent duplication event, the Obp1a-like group (O4, O5, O6) and Obp1b-like group (O7 and O8). In this region (i.e. between O6 and O7 in the Obp cluster), there are presumably wide sequencing errors because of the presence of multiple retroviral sequences that occur within this cluster. From the point of view of the amino acid sequence homology in other species, we would stress that the group of 7 Obp coding genes contains two extra cystein residues forming typical amino-acid signature CxxxC. Moreover three aminoacids between these two cysteins can be diagnostic for the three structural groups defined above (i.e. O1-like group having CNDDC, Obp1a-like group CEKEC and Obp1b-like group CDEGC). This fixed CxxxC motif emerges also in mouse probasin (CGRRC) located near the Obp cluster and is also found in hamster lipocalins probably linked to X chromosome such as aphrodisin (CYKNC), probasin (CLKKC), Msp (CSDEC) and Flp (CIDEC). Contrary to mouse, the genes for Obp in rat are localized on three different chromosomes: the chromosome X (Obp1f – NM_138903.1 and Obp- ENSRNOG032359), the chromosome 5 (Obp3 – NM_001033958.2) and the chromosome 3 (Obp2b – M76734.1). Interestingly, only X-linked Obps contain motif CxxxC (CGDEC) whilst the other two have no such sequence motif and show higher similarity with Mup or Lcn like mouse lipocalins. Therefore, we think that in some cases the nomenclature is misleading and there is a need for more precise revision. The presence of common stable motif in various lipocalins linked to X
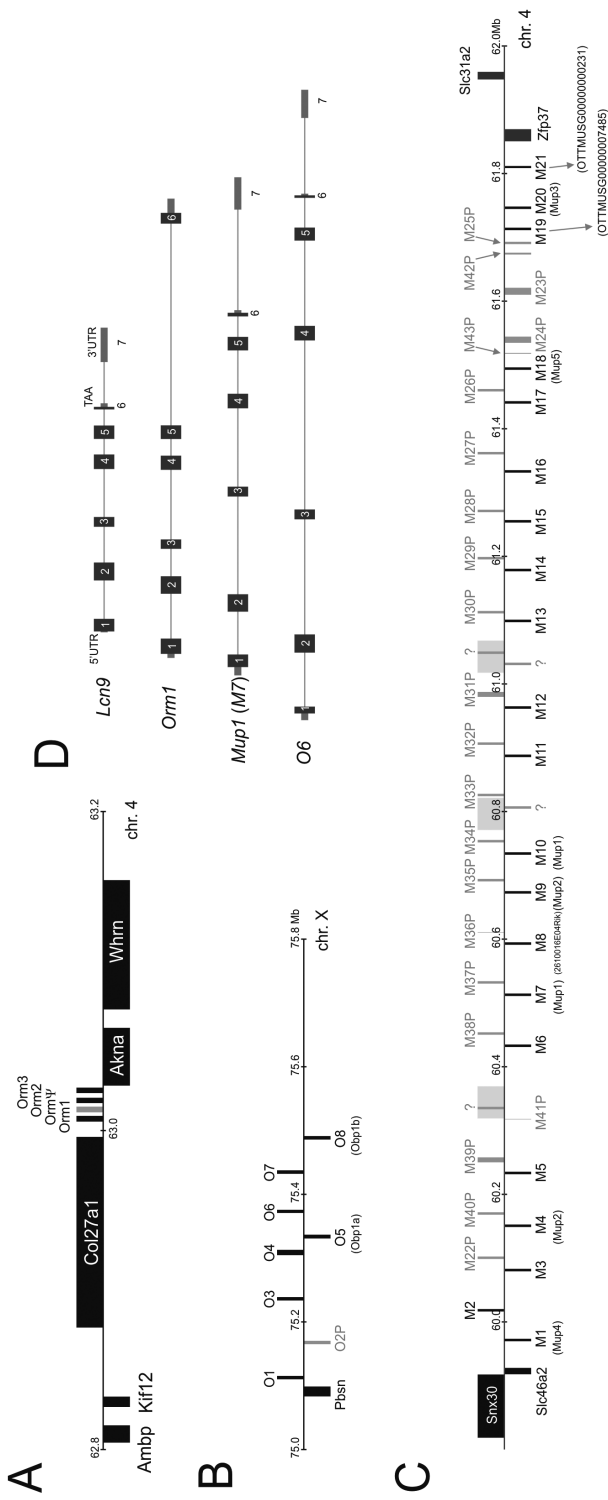
Fig. 1. Mouse Mup-like lipocalin gene clusters and their representatives. Functional protein coding genes printed black whilst pseudogenes are grey - with proposed annotations and relative length and position in respect to each other and cluster neighbours. Genes in antisence orientation are depicted below the line indicating the reference sequence (in brackets). A) Topology of mouse Orosomucoid (Orm) gene cluster is located on chromosome 4 (62.8Mb to 63.2Mb), B) Odorant-binding protein (Obp) gene cluster is located on chromosome X (75.0Mb to 75.6Mb), C) Major urinary protein (Mup) gene cluster is located on chromosome 4 (59.8Mb to 62.0Mb), D) Exon/intron structure of Mup-like lipocalin representatives, Lipocalin 9 (Lcn9), Orosomucoid 1 (Orm1), Major urinary protein 1 (Mup1) described here as M7, Odorant-binding protein gene reported here as O6. All sequences were acquired from mouse Reference assembly (C57BL/6J), built 37.1, July, 2007, www.ncbi.nlm.nih.gov.

chromosome may be necessary for their specific conformation and/or may form unique site for binding specific ligands.

Lipocalins involved in chemical communication

Molecular mechanisms underlying chemical communication involve several steps: (i) volatile ligands (pheromones) are encapsulated by secretory lipocalins (by e.g. major urinary proteins, Mup) in the liver and excreted out of the body via glomerular filtration and deposited as a urine mark, (ii) similarly pheromones are encapsulated by lipocalins in salivary glands and secreted into oral cavity; these proteins are consequently spread onto the fur where they dry and release pheromones; (iii) the activity of pheromones that enter the nasal cavity during breathing may be prolonged by salivary and nasal lipocalins. These pheromone-lipocalin complexes are likely to be consecutively passed to the proximity of chemosensory neurons, where they cause prolonged or more intense (iv) signal transduction when pheromones bind to an extra-cellular part of G-protein coupled receptors and, then, the pheromone-lipocalin complexes can pass the information from chemosensory neurons to CNS via long axons.

Major Urinary Proteins (Mups) are the most studied group of lipocalins associated with chemical communication. In *Mus musculus* they are abundant in number as well as in quantity. However, the polymorphism on the level of amino acid sequences is very low. They often differ in single nucleotide substation, triplet (i.e. amino acid) deletion, which is why their role in individual recognition is still in question. Nevertheless, there is an evidence that these proteins – with or without ligand – can induce physiological and behavioural responses (C h a m e r o et al. 2007). The level of Mup production is sexually dimorphic on both mRNA and protein levels, with males producing more Mups than females. This dimorphism has also been documented in *Mus musculus* and *Mus domesticus* as species-specific (S t o p k o v a et al. 2007). Because of that we assume that Mups contain component carrying sex-specific and species-specific information.

It has been demonstrated that various pheromones that are carried and presented by Major Urinary Proteins have a specific effect upon a receiver's physiology. For example, 2,5-dimethylpyrazine, which was found in female urine is responsible for puberty delay in juvenile females (N o v o t n y et al. 1986). The opposite effect, puberty acceleration, is triggered by several male pheromones that include α- and β-farnesenes, 6-hydroxy-6-methyl-3-heptanone (N o v o t n y at al. 1999a), 2-sec-butyl-4,5-dihydrothiazole and 2,3-dehydro-exo-brevicomin have a similar effect (N o v o t n y et al. 1999b). Last two mentioned semiochemicals are also known to promote estrus synchronization (J e m i o l o et al. 1986). In the context of specificity of pheromone action, it has been shown that such stimuli selectively stimulate different areas of chemosensory neurons with co-localised specific receptors in the VNO (L e i n d e r s - Z u f a l l et al. 2000). This information is further passed on to 'fire-up' different centres in the brain. The resulting effect of chemical communication has recently been documented on the level of social regulation of Mup expression. In an experiment where males were separated from females by a grid such that only olfactory and limited tactile interactions were allowed, individuals of both sex in C57Bl/6 upregulated the expression level of all detected urinary Mups when compared to controls (S t o p k a et al. 2007). In this experiment the level of sexual dimorphism in this inbred mouse line was also evident with levels of female Mup excretion significantly lower than the levels typical for males. However, female expression levels significantly correlated with their estrus cycles such that the level of Mup expression was highest just at

the beginning of estrus (S t o p k a e t al. 2007). Furthermore, Mups are involved in many behavioural processes including aggression, dominance and social regulation of reproduction (N o v o t n y et al. 1990, M a r c h l e w s k a j a - K o j et al. 2000, C h a m e r o et al. 2007, R u s u et al. 2008, see also J a n o t o v a & S t o p k a 2009 – in this issue). To conclude, the evidence suggests that mate choice –generating sex-specific variation – was a strong selective force shaping the evolution of given lipocalins involved in pheromonal action that trigger specific responses on the level of reproductive physiology.

Another group of lipocalins thought to be involved in chemical communication includes odorant binding proteins (Obp). The first Obp was purified from cow nasal mucosa and has been shown to bind the specific "green smelling" compound 2-isobutyl-3-methoxypyrazine, which is why this Obp was originally named pyrazine binding protein (B i g n e t t i et al. 1985). Later study showed that at least bovine Obp interacts with a broad range of ligands of different structure (P e v s n e r et al. 1990). Since this time various Obp like sequences were reported from other mammalian species including rabbits, pigs (D a l M o n t e et al. 1991), elephants (L a z a r et al. 2002), rats (D e a r at al. 1991, B r i a n d et al. 2000a, L ö b e l et al. 2001,), mice (P e s et al. 1992, P e s & P e l o s i 1995), and humans (L a c a z e t t e et al. 2000). Although Obp is general name for these proteins, there is neither clear border between odorants and pheromones nor the evidence that specific pheromone binding lipocalins (such as Mups) specifically bind only pheromones and not odorants. Moreover, in hamsters another lipocalin belonging to Obps called "aphrodisin", evidently bind volatile pheromones (B r i a n d et al. 2000b) and seems to have taken the role of Mups that are absent in this species. The definition of Obp as lipocalins from nasal mucosa is also misleading as there is enough evidence that there are other tissues, which can express Obps.

The role of Obp in chemical communication is still under scientific debate. They are involved in the transport of odorants to the olfactory receptors (P e v s n e r & S n y d e r 1990), they may facilitate a transfer of ligands to the olfactory receptors from ligand-Obp complexes and consequently, they may act as deactivators by removing the odorants from the olfactory receptors and also scavange for potentially cytotoxic and genotoxic compounds. Furthermore, T a y l o r et al. (2008) suggested another hypothesis that the role of Obp may yield a prolongation of the activity of given signal by a prolonged retention and consecutive release of ligands that are encapsulated by these proteins. Such mechanisms may be essential in the amplification of the output signal of olfactory neurons in the brain.

Aphrodisin is another of lipocalin protein family (H e n z e l et al. 1988) highly similar to Obp and is specifically found in hamster vaginal discharge. Female hamsters advertise their sexual receptivity to attract males by depositing vaginal secrets (i.e. including this protein) onto the substrate during specific behaviour called vaginal marking. S i n g e r et al. (1986) applied this vaginal substrate to the hindquarters of an anesthetized male and they found that this substance reliably elicits another male's copulatory behaviour. However, it is also expressed by immature females before they reach fertility (M ä g e r t et al. 1999). The aphrodisin gene is known to be expressed in vagina, uterus, Bartholin's glands and also in female hamster parotid glands (K r u h ø f f e r et al. 1997, M ä g e r t et al. 1999, T h a v a t h i r u et al. 1999), however maximum expression of the Aphrodisin gene is encountered in glandular cells of the cervix uteri, from which the protein is secreted into the vaginal lumen (K r u h ø f f e r et al. 1997).

Like Mups, aphrodisin is a pheromone transporter whose activity is detected by receptors within the vomeronasal organ of the male hamsters. Recently, B r i a n d et al. (2004) described five aphrodisin-ligand-like compounds that specifically bind onto

natural aphrodisin and these described ligands are very similar to the substances that have a pheromonal activity in insects. This finding indicates a significant role of aphrodisin in chemical communication in hamsters. Interestingly, there is another female specific protein in hamsters expressed in lacrimal gland and secreted to tears, called Flp (female specific lacrimal protein, R a n g a n a t h a n et al. 1999). As a counterpart of this protein, a highly similar male specific protein Msp (male specific salivary protein) was identified with expression site in submandibular glands (T h a v a t h i r u et al. 1999). The structure of these proteins shows that they belong to the lipocalin family, and moreover they have specific Obp amino acid sequence motif CxxxC. A later paper (S r i k a n t a n et al. 2005) shows that Msp is secreted also in saliva and urine of males. The sequence analysis of these two similar genes (Msp and Flp) revealed a higher similarity with the rat Obp and mouse Obp (i.e. depicted as O1 and O3 in Figure 1) than with hamster Aphrodisin. Expression of Msp and Flp is under the regulation of sex hormones, however, the function of these tissue- and sex-specific lipocalins still remains unclear as well as their ability to bind some of the pheromone-like ligands.

To conclude, the absence or a variable presence of Major Urinary proteins in various rodent species has apparently been substituted by other members of lipocalin family. This can be seen in hamsters where, instead of Mup´s, the most important roles in chemical communication were (most likely) undertaken by other lipocalins like Aphrodisin, Msp or Flp.

P r o t e c t i v e   r o l e s   o f   l i p o c a l i n s

Lipocalins act as cellular nano-cleaners. They usually scavenge for potentially toxic and harmful molecules and thus their function is to reduce the deleterious effects of microbial toxins. Similarly, they bind and transport heavy metals such as iron that often emerges into an organism and its cells contained with food or as a product from catabolic degradation of own iron-containing metalloproteins. Iron is an essential metal occurring in all organisms. As a specifically protein-bound Fe^{2+} or Fe^{3+} cation (i.e. not as free iron) and due its ability of redox cycling, is iron an essential catalytical center of iron-containing metalloproteins and enzymes. The regulatory role of lipocalins in the iron transport and storage is a complex story beyond the scope of this review (see e.g. H e n t z e et al. 2004 for review). However, when an organism is overloaded with iron (and potentially other metals) there may be a pool of molecules, which are able to shift iron within the cell compartments. Among these iron –scavenging molecules, lipocalins may play an essential, but still little understood role. It is not surprising, that, due to inherent indispensability of iron, bacteria and other procaryotic cells use iron chelating molecules called siderophores to exploit this element from eukaryotic organisms. Furthermore, eukaryotic cells developed special lipocalins called siderocalins, compounds able to pick up bacterial siderophores and thus prevent bacterial growth (G o e t z et al. 2002, F i s c h b a c h et al. 2006, S m i t h 2007).

With respect to their protective role, lipocalins can be characterised on the basis of their affinity for a particular ligand type. One group of lipocalins primarily binds siderophores such as iron-binding lipocalin 2 (i.e. Lcn2), also called siderocalin or NGAL-neutrophil gelatinase associated lipocalin (G o e t z et al. 2002, M o r i et al. 2005) and lipocalin 1 (i.e. Lcn1) also called human tear lipocalin, i.e. Tl (F l u c k i n g e r et al. 2004). Second group of lipocalins with smaller beta barrels have no capacity to bind siderophores but their scavenger role involves binding of ligands connected to (iron catalyzed) Reactive Oxygen
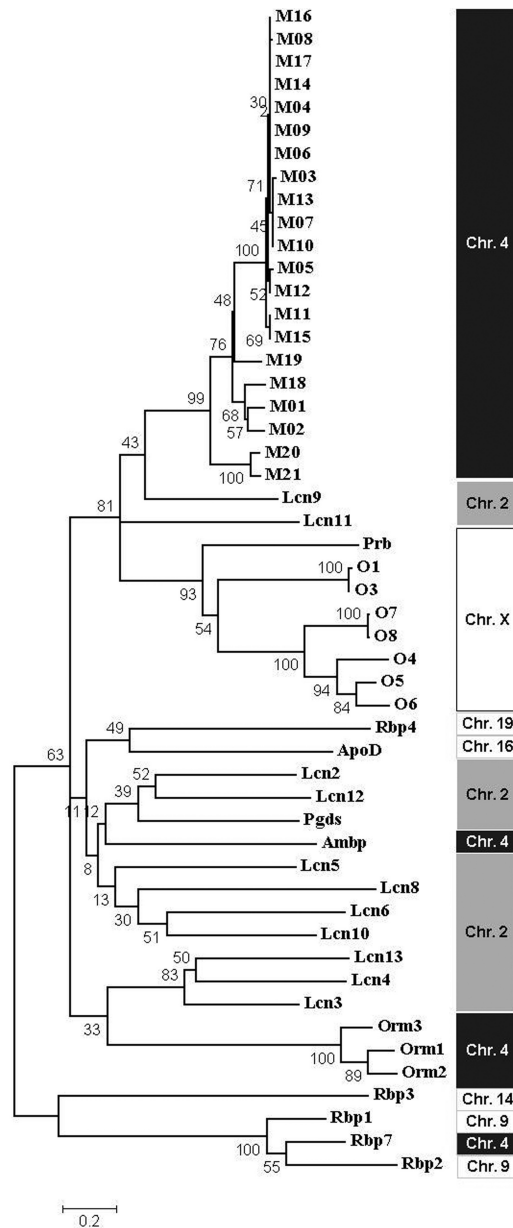
Fig. 2. Rapid reconstruction of lipocalin phylogeny. A bar on the right represents the chromosomal location for particular lipocalin genes from NCBI library, www.ncbi.nlm.nih.gov. The evolutionary history was inferred using the Neighbor-Joining method. The bootstrap consensus tree inferred from 2000 replicates is taken to represent the evolutionary history of the lipocalins analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated proteins clustered together in the bootstrap test (2000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 106 positions in the final dataset. Phylogenetic analyses including this figure were conducted in MEGA4 (T a m u r a et al. 2007).

Species (ROS). Efficiency of such a ligand (i.e. ROS) binding by lipocalins varies widely through out the spectrum of all described lipocalins including lipocalins from bacteria, plants, insects and mammals (C h a r r o n et al. 2008, G a n f o r n i n a et al. 2008).

Lipocalins from the first group have larger beta-barrels whilst those lipocalins that seem to have smaller barrels, such as bacterial YodA bind small molecules including cadmium, nickel and zinc on their surface (D a v i d et al. 2003). It is possible that even other lipocalins, with the ability to carry ligands in their beta barrel may also carry molecules on their surface, but this hypothesis is yet to be tested. The classification and description of these two groups based on the barrel size seems to have a biological relevance, however, so far there is no firm evidence that either member of these two categories cannot transport ligands of either size. Nevertheless, there seems to be a phylogenetic trend in the size of transported ligands and their binding affinity. It seems that during evolution lipocalins increase their specificity in ligand binding as modern lipocalins tend to bind smaller ligands with higher affinity. Furthermore, there seems to be higher frequency of repeated duplications in this group of recent lipocalins in particular species studied to date (G u t i é r r e z et al. 2000).

With this respect, a study by P e t r a k et al. (2007) is important as it shows that when laboratory mice are overloaded with iron, they up-regulate the expression of several members of Major Urinary Proteins in the liver. The function of these lipocalins is traditionally associated with pheromonal transport and it is still in question whether Mups are up-regulated simply to scavenge for hydroxyl radicals that often rise as a consequence of iron overload or whether Mups may also play a role in the removal of excessive amounts of iron by an active transport.

The scavenger role of (porcine and bovine) Obp was proved by G r o l l i et al. (2006) in an experiment where they demonstrated that Obps bind with high affinity HNE (4-hydroxy-2-nonenal), a toxic compound derived from lipid peroxidation, and therefore can protect living cells from damage caused by oxidative stress by removing cytotoxic compounds from the nasal mucosa. Nasal mucosa might drive the Obp-HNE complex into the first tract of the digestive system where they are deactivated. In humans, the similar scavenger role is proposed for tear lipocalin (Lcn1), which has significant sequence homology with human Obp variants. Althought, Lcn1 has different binding spectrum oriented towards compounds with higher molecular masses and hydrophobicity, it can also bind HNE.

To conclude, there seems to be a trend among modern lipocalins to increase binding specificity for their ligands over evolutionary time, trend to bind ligands smaller in molecular weight, and, trend to reduplicate those lipocalin members that are relevant either for the detoxification of harmful chemicals in organisms or for all the forms of chemical communication.

Conclusion

We suggest that, lipocalins originally functioned as scavengers of potentially harmful molecules whilst the role in chemical communication was most likely evolved later. Major Urinary Proteins are recently seen as favourite proteins in behavioural studies for their high capacity to bind, transport and transduce various pheromones that stimulate a myriad of physiological changes in the receiver of such signals. It is highly likely that the original function of lipocalins to transport harmful chemicals for detoxification was an ideal source for natural selection during evolution of chemical communication simply due to a link between the level of metabolic activity, pheromonal production and individual quality. Recent

duplication of genes for some of the mentioned lipocalins leads to their more specialized function, however, their original detoxification functions may still be exploited. Furthermore, in various behavioural studies Mups are often reported for their high level of variation. This variation, however, may be insufficient in hypothesis testing when other lipocalins excreted in different tissues are not taken into account.

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