

Reverse Chemical Ecology Suggests Putative Primate Pheromones

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Abstract

Pheromonal communication is widespread among living organisms, but in apes and particularly in humans there is currently no strong evidence for such phenomenon. Among primates, lemurs use pheromones to communicate within members of the same species, whereas in some monkeys such capabilities seem to be lost. Chemical communication in humans appears to be impaired by the lack or malfunctioning of biochemical tools and anatomical structures mediating detection of pheromones. Here, we report on a pheromone-carrier protein (SAL) adopting a “reverse chemical ecology” approach to get insights on the structures of potential pheromones in a representative species of lemurs (*Microcebus murinus*) known to use pheromones, Old-World monkeys (*Cercocebus atys*) for which chemical communication has been observed, and humans (*Homo sapiens*), where pheromones and chemical communication are still questioned. We have expressed the SAL orthologous proteins of these primate species, after reconstructing the gene encoding the human SAL, which is disrupted due to a single base mutation preventing its translation into RNA. Ligand-binding experiments with the recombinant SALs revealed macrocyclic ketones and lactones as the best ligands for all three proteins, suggesting cyclopentadecanone, pentadecanolide, and closely related compounds as the best candidates for potential pheromones. Such hypothesis agrees with the presence of a chemical very similar to hexadecanolide in the gland secretions of *Mandrillus sphinx*, a species closely related to *C. atys*. Our results indicate that the function of this carrier protein has not changed much during evolution from lemurs to humans, although its physiological role has been certainly impaired in humans.

Key words: salivary proteins, odorant-binding protein, human pheromones, primates, evolution, disulfide bridges, ligand-binding assays.

Introduction

In mammals, detection of pheromones is mediated by specialized receptors (Dulac and Axel 1995; Herrada and Dulac 1997; Matsunami and Buck 1997; Ryba and Tirindelli 1997; Tirindelli 2021) in the vomeronasal organ and soluble carrier proteins (Pelosi et al. 1982; Pelosi 1994; Tegoni et al. 2000). These latter polypeptides, named OBPs (odorant-binding proteins), belong to the larger superfamily of lipocalins (Åkerstrom et al. 2000), and are able to carry hydrophobic ligands in aqueous biological fluids. All mammalian OBPs share with lipocalins the typical β -barrel structure (Flower et al. 2000), which is composed of eight β -strands and an α -helical segment (Bianchet et al. 1996; Tegoni et al. 1996). The name of OBPs was assigned in early studies because these proteins were assumed to bind all types of odorants and to be involved in general olfaction (Bignetti et al. 1985; Pevsner et al. 1986). However, current knowledge strongly suggests that OBPs of mammals should be regarded as specific carriers

for pheromones rather than being involved in the detection of general odorants (Pelosi 2001; Pelosi and Knoll 2021). This assumption is motivated by several pieces of biochemical, anatomical, and physiological evidence:

- (1) the number of these proteins (a handful in most species) is very small when compared with the hundreds of olfactory receptors, as well as to the several dozens of OBPs in insects, where they mediate detection of both pheromones and general odors (Pelosi, Iovinella, et al. 2018);
- (2) despite their wide spectra of binding, best ligands are always species-specific pheromones;
- (3) they are secreted in the vomeronasal organ and in other parts of the nasal cavity, such as the respiratory and the nasal septum regions, but never in the olfactory area; incidentally, they are almost completely absent in the human nose, where a single OBP is expressed at barely detectable levels (Lacazette et al. 2000; Briand et al. 2002);

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- (4) several OBPs expressed in the nasal cavity are also produced by glands and secreted in body fluids, where they have been found to be tightly bound to species-specific pheromones. Examples include the major urinary proteins (MUPs) present at high concentrations in the urine of rodents (Böcskei et al. 1992; Cavaggioni and Mucignat-Caretta 2000; Perez-Miller et al. 2010; Beynon et al. 2014), salivary lipocalins (SALs) secreted in the saliva of pig and other mammals (Marchese et al. 1998; Spinelli et al. 2002; Pelosi and Knoll 2021), as well as other members found in different body fluids (supplementary fig. S1, Supplementary Material online). All above-mentioned proteins belong to the same subgroup of OBPs and are structurally related to each other with amino acid identities around 50% or more, even between members belonging to phylogenetically distant species (Marchese et al. 1998; Spinelli et al. 2002; Pelosi and Knoll 2021).

In humans, the SAL-MUP ortholog gene contains a mutation that prevents splicing and assembling of the RNA. This mutation is not present in other primates, including apes, but had already appeared in *Homo neanderthalensis* (Zhang et al. 2010; Meslin et al. 2011). The disruption of a gene encoding a pheromone-binding protein in humans would not be unexpected based on the assumption that we do not produce or use pheromones to communicate. Although this issue is still a matter of debate, the absence of a vomeronasal organ in adults (in the few individuals in which it can be detected, it is not innervated and vestigial), as well as of an accessory bulb, which is the area in the brain dedicated to the first processing of signals originated by pheromones in the vomeronasal organ, seem to support such hypothesis (Wysocki and Preti 2004; Trotier 2011; Wyatt 2020). However, it has been observed that some receptors expressed in the vomeronasal organ in other mammals and dedicated to detection of pheromones have migrated to the main olfactory epithelium in humans, where they might perform a similar function (Rodriguez et al. 2000). On the other hand, behavioral observations and reports on the existence of a vomeronasal organ in infants make the hypothesis of pheromonal communication between mother and newborn more likely and worth investigating (Schaal et al. 2020).

Pheromonal communication is certainly active in lemurs, as documented by several behavioral studies. These primates use secretions of the antibrachial and brachial glands to scent mark and communicate with conspecifics (Palagi and Dapporto 2006; Scordato et al. 2007). In a recent report, aldehydes of medium-long length isolated from gland secretions of the ring-tailed lemur, *Lemur catta*, have been identified as chemosignaling molecules (Zhang and Webb 2003; Setchell et al. 2010; Shirasu et al. 2020), although there is no conclusive evidence for classifying them as true pheromones (Drea et al. 2020). In monkeys, the accepted view is that platyrrhines (New-World monkeys) use semiochemicals, whereas this form of communication may have been lost in cercopithecoids (Old-World monkeys). However, there is

increasing evidence that also some Old-World monkeys use scent marking and chemical signaling (Setchell et al. 2010; Charpentier et al. 2013; Drea 2015), whereas we have no indication so far of pheromonal communication in apes. In this respect, mandrills are the Old-World monkeys that have received most attention. The chemical composition of sternal gland secretions, which mandrill males use for scent marking, has been investigated, and several components that could represent putative pheromones have been identified (Setchell et al. 2010; Vaglio et al. 2016). However, behavior and chemical information in this field is scanty and erratic, whereas biochemical aspects have not yet been addressed. Even the annotation of OBPs in primates is poor and incomplete.

Applying a “reverse chemical ecology” approach, we have searched for chemical structures that could throw light on the nature of pheromones in primates, and how their detection may have evolved from lemurs to humans. We therefore have expressed the three SAL-MUP ortholog proteins, after restoring the human pseudogene. Using ligand-binding studies, we then have compared the biochemical behavior of these proteins in: 1) a lemur species (*Microcebus murinus*), in which chemical communication is well documented (Caspers et al. 2020; Kollikowski et al. 2020), 2) an Old-World monkey (*Cercocebus atys*) belonging to the same subfamily of mandrills, which are suspected to use pheromones (Vaglio et al. 2016), and 3) in human, where there is so far no strong evidence for pheromonal communication. The results of our reverse chemical ecology approach have proposed macrocyclic ketones and lactones as putative pheromones for all three representative proteins, suggesting that primates have retained the use of the same volatile molecules as pheromones until the genes for their binding proteins became nonfunctional in humans and perhaps no longer used also in apes.

Results

Protein Expression and Purification

Ortholog sequences of SAL-MUP from human, lemur, and Old-World monkey species were expressed in *Escherichia coli* from synthetic genes optimized for bacterial expression, and were obtained in good yields. As a representative of lemurs, we selected *M. murinus*, its genome being one of the best annotated. The species chosen as a representative of Old-World monkeys, *C. atys*, is closely related to mandrills, for which behavior studies have suggested an active pheromonal communication, and putative semiochemicals have been identified from the analysis of glands secretions (Setchell et al. 2010). For the human SAL-MUP ortholog, we reconstructed the specific gene by restoring the mutation that prevented RNA assembly (Zhang et al. 2010; Meslin et al. 2011). Human and *C. atys* proteins are very similar to each other (90% identity), but only about 50% identical with their lemur ortholog. Figure 1 reports the alignment of the human (HsapSAL), the monkey (CatySAL), and the lemur (MmurSAL) proteins. Purification was performed using well-established protocols of anion-exchange

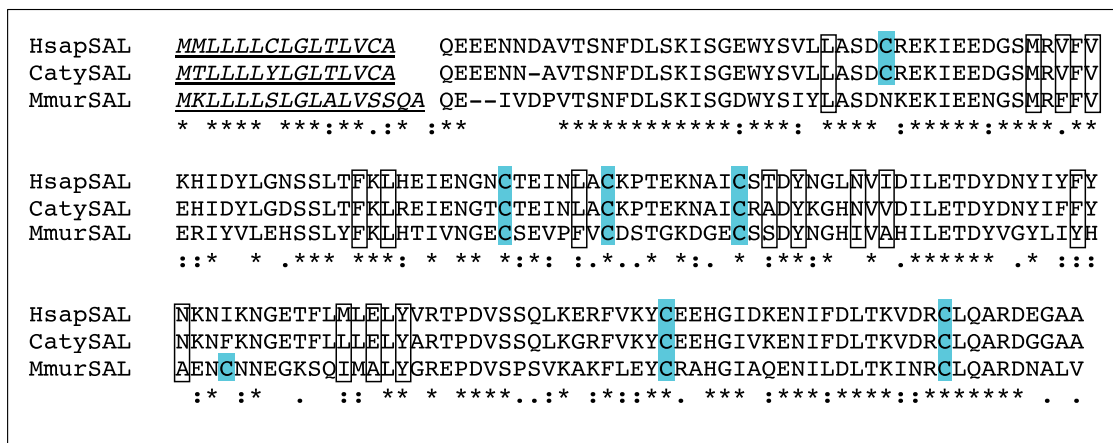


Fig. 1. Alignment of the amino acid sequence of the SAL proteins used in this work. Hsap, *Homo sapiens*; Caty, *Cercocebus atys*; Mmur, *Microcebus murinus*. The human and the monkey proteins share 90% of their amino acids, whereas they are about 50% identical with the lemur protein. All three SALs present six cysteines (highlighted) conserved between the human and monkey proteins, whereas one of them in the lemur sequence is not aligned. Boxed residues are those deduced by sequence alignment as present within the ligand-binding pocket of the protein, based on crystallographic data of other proteins binding pheromones, such as pig SAL (PDB: 1GM6), panda OBP3 (PDB: 5NGH), and mouse MUP1 (PDB: 1MUP) (supplementary fig. S1, Supplementary Material online).

chromatography. Supplementary figure S2, Supplementary Material online, summarizes the results for the expression and purification of the recombinant proteins.

Cysteine Pairing Assessment

HsapSAL, CatySAL, and MmurSAL contain six cysteines (fig. 1), which occur at conserved positions in the former two proteins, whereas in the lemur, only five of them can be aligned. Conversely, pig SAL and murine MUPs have four and three cysteines, respectively, of which two are involved in a disulfide bond (supplementary fig. S1, Supplementary Material online). To ascertain the occurrence of disulfide bridges, HsapSAL, CatySAL, and MmurSAL were submitted to extensive alkylation with iodoacetamide under nonreducing denaturing conditions, and recovered, purified proteins were then digested with chymotrypsin. Resulting digests were finally analyzed by nanoLC-ESI-Q-Orbitrap-MS/MS assigning S-S-crosslinked and carboxyamidomethylated peptides by dedicated bioinformatics procedures (supplementary table S1, Supplementary Material online). Examples of mass spectra of the digestion products are reported in figure 2. Results demonstrated the presence of two conserved disulfide bridges in HsapSAL, CatySAL, and MmurSAL (fig. 3). One coincided with that already determined in most mammalian OBPs (including pig SAL, murine MUPs, hamster aphrodisin, and others, supplementary fig. S1, Supplementary Material online), whereas the other was peculiar of HsapSAL, CatySAL, and MmurSAL, due to the unique occurrence in these proteins of a cysteine residue not present in the other orthologous lipocalins (supplementary fig. S1, Supplementary Material online). The remaining two cysteines were found to be present in their reduced form.

Ligand-Binding Experiments

Purified HsapSAL, CatySAL, and MmurSAL were then used in ligand-binding assays to evaluate their affinities to putative

pheromones and other volatiles. In order to adopt the competitive binding approach, we firstly measured the binding of the three SALs to a fluorescent reporter. All of them bound N-phenyl-1-naphthylamine (1-NPN) with good to moderate strength, whereas the other commonly used fluorescent reporter for OBPs, 1-aminoanthracene (1-AMA), did not show significant affinity to any of the three proteins. In particular, with 1-NPN, we measured dissociation constants of 2.3, 7.9, and 6.2 μ M for MmurSAL, CatySAL, and HsapSAL protein, respectively. The relative binding curves are reported in figure 4A.

Next, using 1-NPN as a reporter, we tested a number of volatile chemicals for their ability to displace the fluorescent ligand from the above-mentioned molecular complexes. Figure 4B shows some representative examples of competitive binding curves obtained with the lemur SAL, which exhibited the strongest binding activity. Figure 4C reports experiments performed with the same protein and the best ligands using a narrow range of concentrations. All the results relative to MmurSAL, CatySAL, and HsapSAL proteins are graphically summarized and compared in Figure 4D, whereas the original binding curves are reported in supplementary figure S3, Supplementary Material online. The structures of all the ligands are reported in supplementary figure S4, Supplementary Material online. We can incidentally observe that some ligands reduce the initial fluorescence at low concentration values, but then the fluorescence increases at higher levels. This behavior has been described before and explained with the ability of some chemicals to form micelles entrapping the probe, thus increasing the fluorescence observed (Sun et al. 2012; Leal and Leal 2015; Pelosi, Zhu, et al. 2018). We noticed large differences in the strength of binding between the MmurSAL and the other two proteins, with dissociation constants of the lemur protein about one order of magnitude lower. At the same time, the three proteins presented similar spectra of binding, and appeared to be all tuned to macrocyclic ketones and lactones of 15–16 carbon

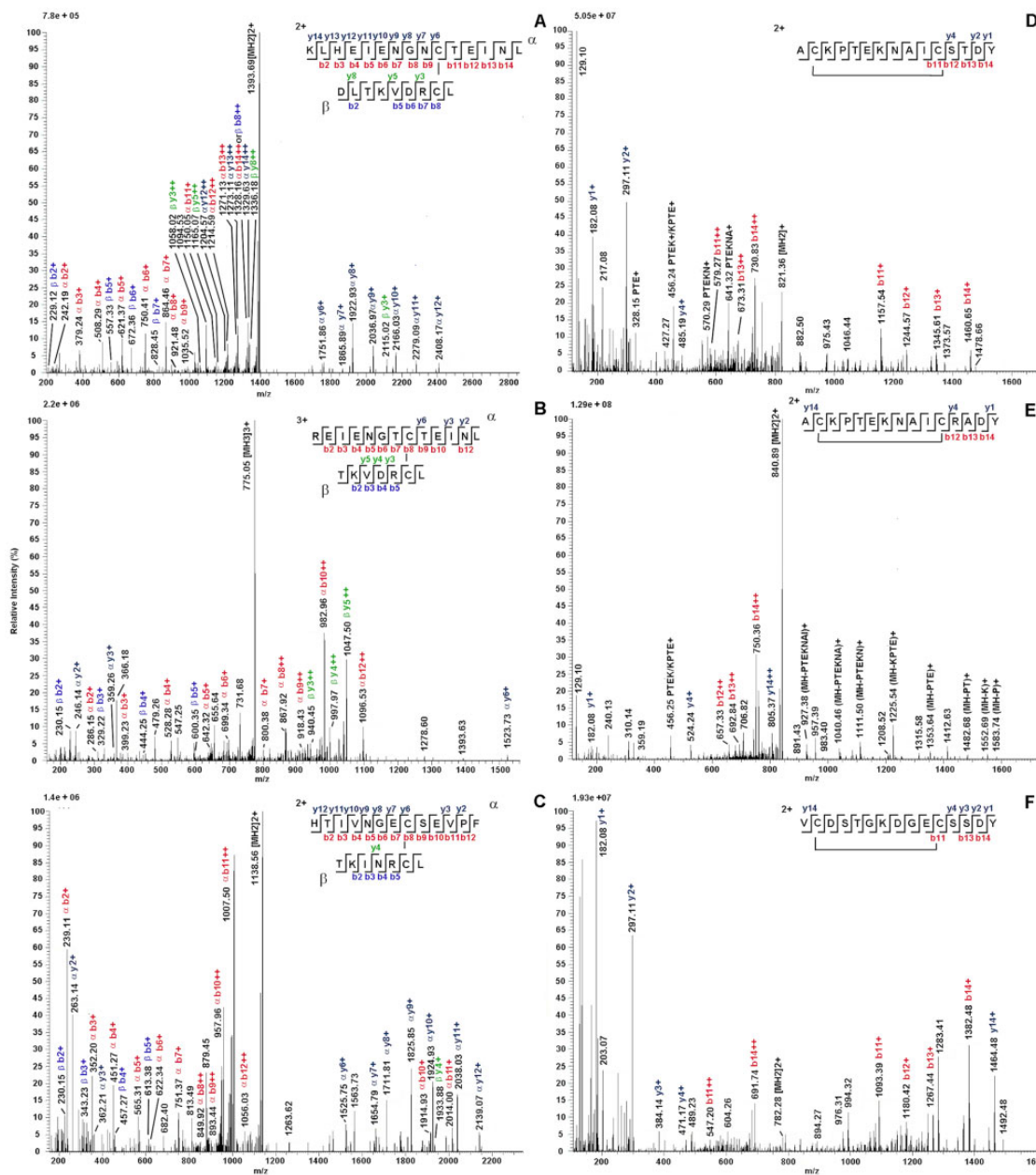


Fig. 2. Exemplificative fragmentation spectra of disulfide-bridged peptides identified in the chymotryptic digest of HsapSAL, CatySAL, and MmurSAL by nanoLC-ESI-Q-Orbitrap-MS/MS analysis. Panels (A) and (D), (B) and (E), and (C) and (F) report peptides from HsapSAL, CatySAL, and MmurSAL, respectively. The fragments are reported in different color depending on peptides present in S-S-linked species, and corresponding b and y ion series. Complete data on disulfide-bridged and carboxyamidomethylated peptides are reported in [supplementary table S1, Supplementary Material](#) online.

atoms. In particular, MmurSAL exhibited a broader spectrum of binding, which included, among others, some steroids, γ -lactones, and long-medium linear aldehydes ([supplementary fig. S4, Supplementary Material](#) online). In particular, the structural similarity between steroids and macrocyclic ketones/lactones has been recognized a long time ago ([Amoore et al. 1977](#)) and can be easily appreciated when folding the flexible structure of hexadecanolide into a shape reproducing that of 5α -androst-16-en-3-one ([supplementary fig. S4, Supplementary Material](#) online). Incidentally, we can observe that a synthetic compound, *cis*-4-(4-*tert*-butylcyclohexyl)-4-methylpentan-2-one (reported here as *cis*-ketone),

with a smell very similar to that of 5α -androst-16-en-3-one ([Beets and Theimer 1970](#); [Amoore et al. 1977](#)), also exhibited strong affinity to MmurSAL. Curiously, although its structure appears quite different from that of a steroid, their space-filling models are extremely similar ([Beets and Theimer 1970](#)).

The main classes of compounds showing good affinity to MmurSAL, namely macrocyclic ketones and lactones, steroids, γ -lactones, and long-chain aldehydes, were already reported as pheromones for mammals and insects. In particular, muscone (3-methylcyclopentadecanone) is the pheromone of the musk deer ([Walbaum 1906](#)), and civetone (9-cycloheptadecanone) is the pheromone of the civet cat ([van](#)

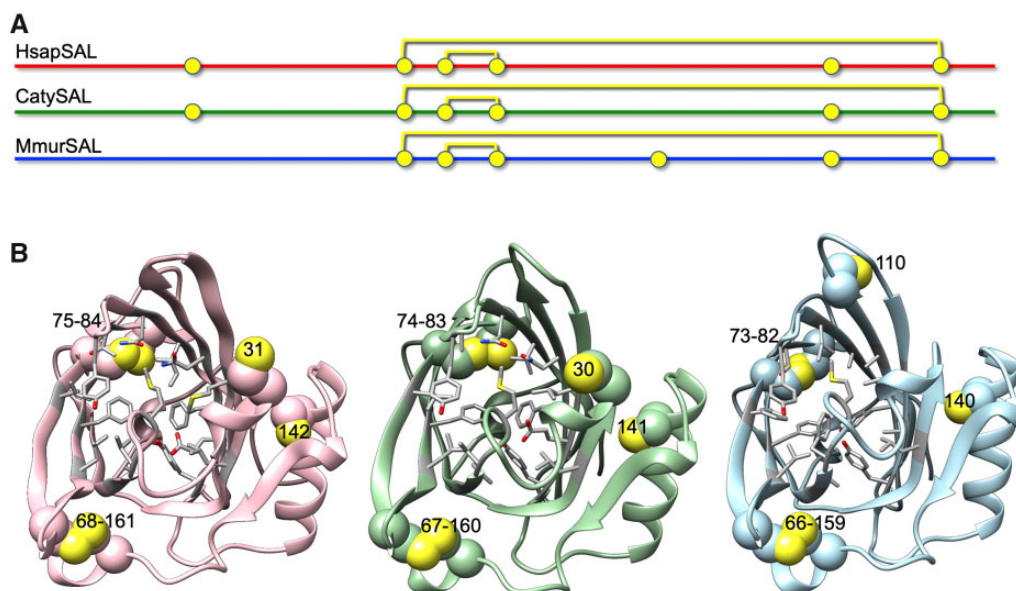


FIG. 3. (A) Schematic diagram showing the pairing of cysteines in HsapSAL, CatySAL, and MmurSAL. In all three proteins, the four conserved cysteines are joined by two disulfide bridges, whereas the remaining two cysteines are present in their reduced form. (B) Models of HsapSAL, CatySAL, and MmurSAL showing the six cysteines in space-filling mode and the residues lining the binding pocket. The latter amino acids were predicted being involved in ligand binding (boxed in the sequences of [fig. 1](#)) based on crystallographic structure of pig SAL, mouse MUPS, and other OBPs.

[Dorp et al. 2010](#)). The steroids 5α -androst-16-en-3-one and 5α -androst-16-en-3-ol are well known as the two components of the boar's sex pheromones ([Melrose et al. 1971](#); [Reed et al. 1974](#)). They were already described to be strongly bound to the boar's SAL, when this protein was isolated from the corresponding submaxillary glands ([Marchese et al. 1998](#); [Loebel et al. 2000](#)). On the other hand, aldehydes have been reported as pheromones of vertebrates, as well as insects. Dodecanal, tetradecanal, and 12-methyltridecanal have been recently identified as pheromones of the ring-tailed lemur, *L. catta* ([Shirasu et al. 2020](#)). Longer linear unsaturated aldehydes (14–18 carbon atoms) ([supplementary fig. S4, Supplementary Material](#) online) are known to be components of the pheromonal blend in a large number of moths, including species of the genera *Helicoverpa*, *Manduca*, *Plutella*, and many others (<https://www.pherobase.com>). These aldehydes were also suggested as putative pheromones for the giant panda, based on their strong affinity for the SAL ortholog (AimelOBP3) in this species ([Zhu et al. 2017](#)).

Apart from such interesting coincidences between insect and mammalian pheromones, the key information is that all three SALs are tuned to macrocyclic ketones and lactones of 15–16 carbon atoms, with only minor differences between them. This is particularly true when comparing the human with the *C. atys* protein, and reasonably understandable based on evolutionary considerations. Therefore, it appears that the ligand-binding spectrum of SALs is well conserved from lemurs to humans, and the above-reported cyclic molecules might represent putative ligands for the SAL of a human ancestor prior to the divergence of the Neanderthals.

3D Models

Structural models of the three SALs were generated using the dog allergen Canf6 (PDB: 5x7y) as a template for the three

proteins. The reliability of these models is supported by identity values higher than 50% between template and target, the position of four conserved cysteine residues, as well as by the spontaneous disulfide bridge formation between cysteines exclusively present in HsapSAL, CatySAL, and MmurSAL, which was de facto independently assigned by mass spectrometry analysis. [Figure 3](#) reports the models of the three proteins showing the six cysteines and the two conserved disulfide bridges, as well as the amino acids lining the binding pockets. These structural models confirmed the occurrence of residues boxed in the alignment of [figure 1](#), which were predicted being in the ligand-binding cavities of the proteins, based on the crystallographic structure of pig SAL and mouse MUPS.

Discussion

The approach of reverse chemical ecology assumes that the structure of a ligand can be hypothesized based on the shape of the binding pocket of the carrier protein and supported by binding affinities measured with the recombinant protein. Although being a relatively crude method, it can provide suggestions on the class of compounds that might be the natural ligands, in cases where other approaches cannot be applied. These include instances of extinct species for which biological samples cannot be obtained, but also of endangered and protected species, or else difficult to approach, for which biological samples are very difficult to obtain, particularly if they have to be collected in specific and narrow time windows. This was the case of the giant panda, to which we have recently applied this strategy ([Zhu et al. 2017](#)). Reverse chemical ecology was also successfully adopted with insects, helping to identify oviposition attractants for mosquitoes, as well as repellents for the Chaga disease vector

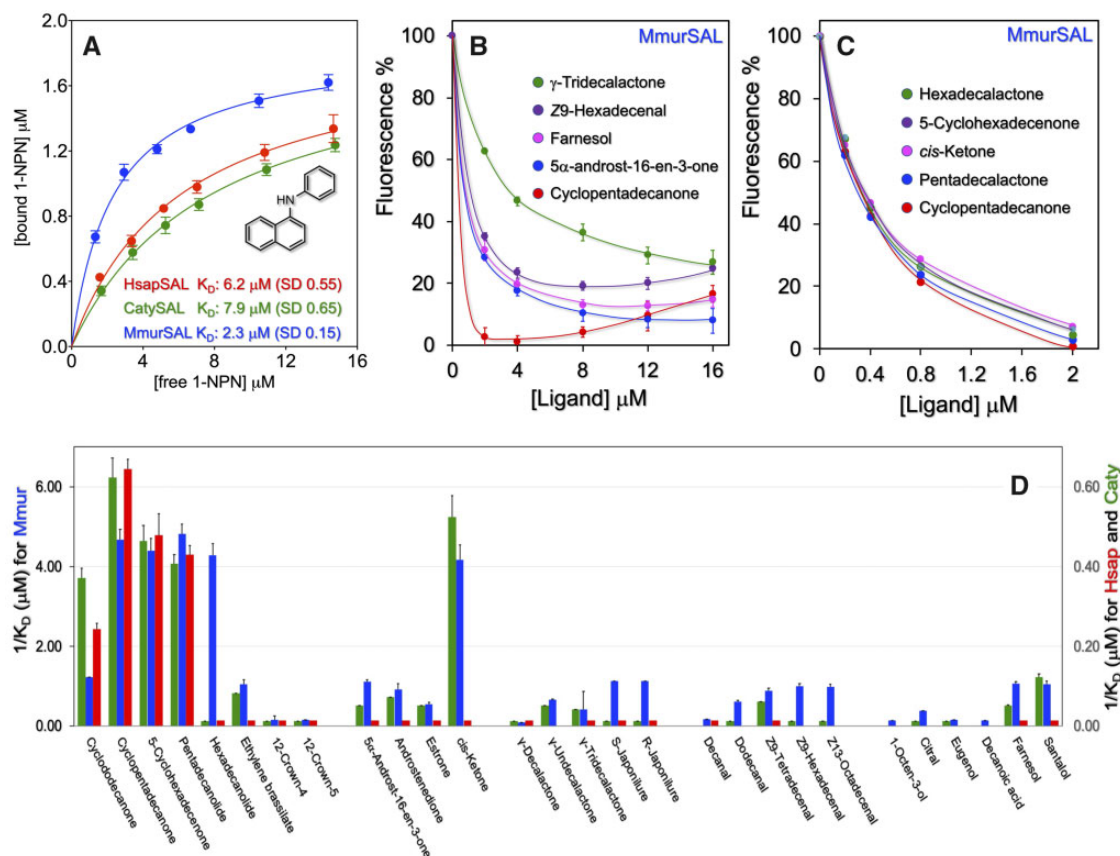


FIG. 4. Protein ligand-binding experiments. (A) HsapSAL, CatySAL, and MmurSAL bind the fluorescent probe with moderate affinities. The structure of phenyl-1-naphthylamine (1-NPN) is reported as an inset. (B) Competitive binding of selected ligands to MmurSAL. (C) Macrocyclic ketones and lactones bind to MmurSAL with affinities in the upper nanomolar range. (D) Graphic summary of the affinities of the used ligands to the three proteins. For a better appreciation of the strength of binding, the reciprocal of dissociation constants are reported. For the Y axis, two scales are used, differing by one order of magnitude, the left for MmurSAL, the right for the other two proteins. Despite quantitative differences in affinities between the lemur protein and the others, all three SALs appear to be tuned to the same group of chemicals comprising macrocyclic ketones and lactones of 14–16 carbon atoms.

Rhodnius prolixus (Leal et al. 2008; Choo et al. 2018; Franco et al. 2018).

The aim of the present study was to formulate hypotheses on the structure of potential pheromones for an Old-World monkey, in which behavioral observations already suggested the occurrence of an active chemical communication, as well as to speculate on putative semiochemicals that might have been used by early hominins, in which the SAL could have been still functional. To this purpose, we applied the approach of reverse chemical ecology within an evolutionary perspective, in which we compared the binding properties of a pheromone-binding protein (SAL) of a monkey with those of orthologs in a lemur and the reconstituted human protein. Our results indicated that the ligand-binding characteristics of the three orthologous SALs were conserved during evolution, suggesting large cyclic ketones and lactones as the best pheromone candidates.

In particular, members of 15–16 carbon atoms were the best ligands for all three proteins, with dissociation constants in the nanomolar range for MmurSAL, and significantly higher for the two other orthologs. This result is in agreement with the occurrence of a macrocyclic lactone, oxacycloheptadec-8-en-2-one, among the volatiles identified in the sternal gland

secretion of the mandrill (Vaglio et al. 2016), which is used by the animal to scent mark tree branches and other objects found in its environment. This chemical, whose structure is very similar to those of the best ligands found in our study, is the only compound, among about 80 gas-chromatographic peaks detected, whose origin cannot be traced to common sources, such as plants used by the animal, food, or contamination from plastics or other materials. Therefore, it represents the best candidate for being considered as a putative pheromone for the mandrill.

Although the use of pheromones in lemurs is widely accepted and supported also by the presence of a well-developed vomeronasal organ and accessory olfactory bulb (Meisami and Bhatnagar 1998; Meisami et al. 1998), in monkeys the issue is still a matter of debate. In particular, the current view is that chemical communication could still be active in New-World monkeys, but may have been abandoned in Old-World monkeys. In fact, a vomeronasal system with functioning accessory olfactory bulb has been reported in species of the former group, whereas it seems to be present only in fetuses of Old-World monkeys (Smith et al. 2001, 2002). On the other hand, behavioral studies have shown that at least some species of the latter group use scent-

marking (Setchell et al. 2010; Charpentier et al. 2013; Drea 2015), as mentioned above. Our results can add a new element to the puzzle, further supporting the idea that pheromonal communication might have survived at least in some Old-World monkeys.

The high similarity in the ligand-binding spectra of the SALs from lemur to monkey and human indicates that in those species where pheromonal communication is still active the same or very similar volatiles are used to communicate. It might also throw light on possible structure of putative pheromones that ancient hominins might have used. Such good correspondence in the binding properties of the three proteins matches their high sequence similarities particularly between the monkey and the human members (90% identity). These two proteins also share the majority of residues (13 out of 16) lining their binding pockets (figs. 1 and 3).

The recent report of linear aldehydes as putative pheromones for the lemur *L. catta* (Shirasu et al. 2020) seems to be in disagreement with the results of our study suggesting macrocyclic ketones and lactones as putative pheromone for the another lemur species *M. murinus*. However, it is widely documented that each species uses more than one volatile compound as pheromones. At least, male and female sex pheromones of very different structure have been reported in several species, but there are also pheromones mediating other behavior aspects, such as aggression, dominance, and aggregation (Wyatt 2014). Moreover, despite the relatively close phylogenetic distance between *L. catta* and *M. murinus*, we cannot exclude that the two species use pheromones belonging to different chemical classes.

In conclusion, this study has provided the following information and insights:

- (1) Based on the binding properties of SALs, our reverse chemical ecology approach has suggested macrocyclic ketones and lactones as putative pheromones for the lemur *M. murinus* and the monkey *C. atys*. Our hypothesis is supported by the presence in the gland secretion of another mandrill species, *Mandrillus sphinx*, of a chemical very similar to one of our best ligands (Setchell et al. 2010).
- (2) All three SALs are tuned to the same ligands, suggesting that macrocyclic ketones and lactones have been conserved as pheromones all across primates until pheromonal communication became inactive. Incidentally, the same compounds are known to act as pheromones for other mammals, such as the musk deer, the musk rat, and others.
- (3) The results of this study add a tile to the incomplete and still controversial picture of pheromonal communication among Old-World monkeys, and might stimulate further research in this debated field. They also suggest what could have been the ligands of the SAL in early hominins, in which this protein might still have been functional.

Materials and Methods

Chemicals

All chemicals for buffers and ligands for binding experiments were from Merck, Austria, and of analytical grade. Ligand solutions were prepared in spectroscopic grade (Uvasol) methanol. Oligonucleotides used as primers were custom synthesized at Eurofins Genomics, Germany. Enzymes and kits for DNA extraction and purification were purchased from New England Biolabs, USA.

Gene Cloning

Synthetic genes were custom synthesized at Eurofins Genomics, Germany, based on published amino acid sequences (HsapSAL: EAW50553, CatySAL: XP_011948226, MmurSAL: XP_012624360), and optimized for *E. coli* expression. For subcloning into the expression vector, the full-length gene was amplified using specific primers bearing NdeI and EcoRI restriction sites at the 5'- and 3'- ends, respectively. Amplification products were enzyme digested and cloned into pET-30 vector (Novagen, Darmstadt, Germany), and linearized with the same enzymes. The constructs encoded for the mature sequences with the only addition of an initial methionine. Positive colonies were used to transform *E. coli* BL-21 (DE3) competent cells for proteins expression. The primers used for subcloning were (restriction sites are underlined):

HsapSAL-fw: GGCATATGCAGGAGGAAGAGAACAATGA
 HsapSAL-rv: AAGAATTCCTAGGCTGCACCTTCATCACGAG
 CatySAL-fw: AACATATGCAGGAAGAAGAGAAT
 CatySAL-rv: TTGAATTCCTACGCAGCACCTCCATC
 MmurSAL-fw: GGCATATGCAGGAGATTGTCCGA
 MmurSAL-rv: TTGAATTCCTACACCAGAGCATTATC

Protein Expression and Purification

Bacterial cultures were grown to OD₆₀₀=0.6–0.8, then induced with 0.4 mM IPTG and harvested by centrifugation after additional 2 h, at 37 °C. After sonication, the proteins were obtained mostly in the pellet as inclusion bodies (supplementary fig. S2, Supplementary Material online), and were dissolved and denatured in 8 M urea containing 1 mM DTT. Refolding was obtained by extensive dialysis (three days with buffer change every day) against 50 mM Tris-HCl, pH 7.4. Purification was accomplished by anion-exchange chromatography on HiPrep-Q 16/10, 20 ml (Bio-Rad), along with standard protocols.

Protein Alkylation and Digestion, and Mass Spectrometry Analysis

CatySAL, HsapSAL, and MmurSAL pellets (100 µg) were dissolved in parallel in 0.1 M tetraethylammonium bicarbonate, pH 6.5, containing 4 M guanidinium chloride, alkylated with iodoacetamide (0.5 M final concentration) for 30 min, in the dark, and finally precipitated with 6 vol of cold acetone, overnight at –20 °C. After centrifugation at 16,000 xg for 20 min, at 4 °C, resulting pellets were vacuum-dried by SpeedVac (ThermoFisher Scientific, USA), dissolved in 0.05 M

tetraethylammonium bicarbonate, pH 6.5 to a final protein concentration of $2 \mu\text{g}/\mu\text{l}$, and hydrolyzed with chymotrypsin (1:8 w/w enzyme/substrate). Protein digests were desalted with ZipTip C18 (Millipore, USA) and vacuum-dried. Peptides mixtures were dissolved in $20 \mu\text{l}$ of aqueous 0.1% v/v formic acid and analyzed with a nanoLC-ESI-Q-Orbitrap-MS/MS platform, consisting of an UltiMate 3000 HPLC RSLC nano-chromatographer (ThermoFisher Scientific) coupled to a Q-ExactivePlus mass spectrometer (Thermo Fisher Scientific) mounting a nano-Spray ion source (Thermo Fisher Scientific) (36). Peptide separation was achieved on an Acclaim PepMap RSLC C18 column ($150 \text{ mm} \times 75 \mu\text{m}$ ID; $2 \mu\text{m}$ particle size; 100 \AA pore size, Thermo Fisher Scientific), which was eluted with a gradient of solvent B (19.92/80/0.08 v/v/v water/acetonitrile/formic acid) in solvent A (99.9/0.1 v/v water/formic acid), at a flow rate of $300 \text{ nl}/\text{min}$. Solvent B started at 3%, raised linearly to 40% in 45 min, then reached 80% in 5 min, remaining at this percentage for 4 min, and finally returned to 3% in 1 min. The mass spectrometer worked in data-dependent mode in positive polarity, performing a full MS1 scan in the range m/z 345–1,350, at a nominal resolution of 70,000, followed by MS/MS scans of the ten most abundant ions in high-energy collisional dissociation mode. MS/MS spectra were acquired in a dynamic m/z range, with a nominal resolution of 17,500, a normalized collision energy of 28%, an automatic gain control target of 50,000, a maximum ion injection time of 110 ms, and an isolation window of $1.2 m/z$. Dynamic exclusion was set to 20 s (Arená et al. 2020).

Bioinformatics for Peptide Identification

For peptide assignment and protein mapping, raw mass data files were analyzed with Proteome Discoverer v. 2.4 package (Thermo Fisher Scientific), running by Mascot v. 2.6.1 (Matrix Science, UK) and ByonicTM v. 2.6 (Protein Metrics, USA) software. Database searching was performed against a customized database containing CatySAL, HsapSAL, and MmurSAL plus common protein contaminants and chymotrypsin. Parameters for database searching were variable oxidation at Met, deamidation at Asn/Gln, pyroglutamate formation at Gln and carbamidomethylation at Cys. Mass tolerance was set to $\pm 10 \text{ ppm}$ for precursors and to $\pm 0.05 \text{ Da}$ for MS/MS fragments. Proteolytic enzyme and maximum number of missed cleavages were set to chymotrypsin and 5, respectively. Proteome Discoverer peptide candidates were considered confidently identified only when the following criteria were satisfied: 1) protein and peptide false discovery rate confidence: high; 2) peptide Mascot score: > 30 ; 3) peptide spectrum matches (PSMs): unambiguous; 4) peptide rank (rank of the peptide match): 1; 5) Delta CN (normalized score difference between the selected PSM and the highest scoring PSM for that spectrum): 0. Byonic peptide candidates were considered confidently identified only when the following criteria were satisfied: 1) PEP 2D and PEP 1D: $< 10 \times 10^{-5}$; 2) false discovery rate: 0; 3) q value 2D and q value 1D: $< 10 \times 10^{-5}$. Disulfide bridge assignment

was performed by dedicated BioPharma Finder v. 4.0 (Thermo Fisher Scientific) and pLink v. 2.3.9 (Chen et al. 2019) software. Both programs were used for database searching enabling the specific function of disulfide bridge attribution, and applying the other parameters described for Proteome Discoverer and Byonic analyses. Confident disulfide-bridged peptide identification was considered when BioPharma Finder results showed a “confidence score” > 95 and/or pLink assignments had an “E-value” $< 1.0^{-10}$. Manual interpretation and verification of the candidate spectra were always performed.

Ligand-Binding Assays

Affinity constants of ligands to the three SAL proteins were evaluated by competitive binding experiments, using 1-NPN as the fluorescent reporter. Spectra were recorded on a PerkinElmer FL 6500 spectrofluorometer in a right-angle configuration, at room temperature, with slits of 5 nm for both excitation and emission, using 1 cm path quartz cuvettes.

Binding of 1-NPN was measured by titrating a $2 \mu\text{M}$ solution of the protein in 50 mM Tris–HCl, pH 7.4 with aliquots of 1 mM methanol solution of 1-NPN to final concentrations of 2–16 μM . The excitation wavelength was set at 337 nm and intensities were recorded in correspondence with the peak maximum, around 408–412 nm, depending on the protein. Binding curves and dissociation constants for 1-NPN were obtained using Prism software.

Affinities of other ligands were evaluated in competitive binding experiments by treating a solution of the protein and 1-NPN in 50 mM Tris–HCl, pH 7.4, both at the concentration of $2 \mu\text{M}$, with aliquots of 1 mM solutions in methanol of each chemical to final concentrations of 2–16 μM . Dissociation constants were calculated from the corresponding $[IC]_{50}$ values (the concentration of each ligand halving the initial value of fluorescence), from the equation: $K_d = [IC]_{50} / (1 + [1\text{-NPN}] / K_{\text{NPN}})$, where $[1\text{-NPN}]$ is the concentration of free 1-NPN and K_{NPN} the dissociation constant of the complex SAL/1-NPN.

Protein Models

3D model of HsapSAL, CatySAL, and MmurSAL proteins were obtained with the Swiss Model software (Peitsch 1995; Arnold et al. 2006; Kiefer et al. 2009), using the dog allergen Canf6 structure (PDB: 5x7y) as structural template for modeling protein sequences. Figures were generated with Chimera software (Pettersen et al. 2004).

Supplementary Material

Supplementary data are available at *Molecular Biology and Evolution* online.

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Data Availability

The data underlying this article are available in the article and in its [Supplementary Material](#) online.

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