

cDNA cloning and regulation of two sex-hormone-repressed hamster tear lipocalins having homology with odorant/pheromone-binding proteins[☆]

Subramanya Srikantan, Vishwas Parekh¹, Prabir K. De^{*}

Centre for Cellular and Molecular Biology, Uppal Road, Hyderabad 500007, Andhra Pradesh, India

Received 9 November 2004; received in revised form 11 April 2005; accepted 27 April 2005

Available online 17 May 2005

Abstract

A major 20-kDa protein is female-specifically expressed in exorbital lacrimal gland (LG) of hamsters and secreted in tears. Here, we identify this female-specific LG protein (FLP) as a lipocalin, having 85% protein sequence identity with male-specific submandibular salivary gland proteins (MSP) secreted in saliva and urine of male hamsters. MSP is also female-specifically expressed in LG and secreted in tears but FLP was undetectable in submandibular gland (SMG). FLP and MSP have similar sex-hormonal regulation in LG, which is different from regulation of MSP in SMG. Female-specific expression of FLP and MSP in LG is due to their incomplete repression by endogenous estrogens and gonadectomy in both sexes and lactation in females resulted in their marked induction, which was prevented by estrogen or androgen treatment. FLP and MSP show best sequence identity with odorant/pheromone-binding lipocalins (58–29%). Maximum identity (58%) is with rat odorant-binding protein (OBP) expressed in lateral nasal glands, followed by aphrodisin of hamster vaginal discharge (39%). Cognate transcript and a cross-reacting 20-kDa protein were detected in nasal glands of rat in both sexes but not in hamsters. Results suggest that two closely related lipocalin genes encode FLP and MSP, which are evolutionarily closer to rat OBP than to hamster aphrodisin and these have evolved different tissue-specificity and sex-hormonal regulation. Possible functions for FLP and MSP are suggested, considering their homology to odorant/pheromone-binding lipocalins, their presence in tears, saliva and urine as well as their sex-specific and lactation-induced expression.

© 2005 Elsevier B.V. All rights reserved.

Keywords: Lacrimal; Tear lipocalin; Submandibular; Odorant-binding; Pheromone-binding; Sex-specific; Sex-hormone

1. Introduction

The lipocalin protein superfamily consists of abundant, secretory, usually acidic pI carrier proteins of Mr ~20 kDa [1–3], which usually have low sequence similarity but whenever investigated, were found to have a conserved 3D structure with a binding pocket for hydrophobic ligands. Lipids, odorants, pheromones, retinoids, porphyrins, side-

rophores, steroids are some of the ligands bound by different lipocalins [1–6]. Odorant-binding lipocalins in nasal gland secretions in both sexes of several mammalian species are believed to transport hydrophobic odorants across the aqueous nasal mucus to and from their receptors on the olfactory epithelium [7–11]. Sex-specific pheromone-binding lipocalins in urine of male mouse and rat, vaginal discharge of female hamster and saliva of boar are believed to deliver pheromones to the environment and serve as protein reservoirs for their sustained dissemination [12–17]. Lacrimal glands (LG) of human and pig, in both sexes, secrete lipocalins in tears [4,18–22], which do not bind odorants or pheromones. Human tear lipocalin binds a broad array of lipid ligands in tears [4,20,23]. In vitro, it binds retinol, microbial siderophores and harmful lipophilic compounds and has endonuclease, cystatin-like and anti-microbial activity [4,22,24–26]. Human tear lipocalin is

[☆] The novel nucleotide sequence of full-length FLP cDNA and the extended full-length cDNA sequence of MSP, reported here, have been submitted to the GenBank sequence database under accession numbers AF345648 and AF183407, respectively.

^{*} Corresponding author. Tel.: +91 40 7192560; fax: +91 40 7160591.

E-mail address: pkde@ccmb.res.in (P.K. De).

URL: www.ccmbindia.org (P.K. De).

¹ Present Address: Department of Hematology/Oncology, St. Jude Children's Research Hospital, Memphis, TN, USA.

also expressed in von Ebner's lingual salivary gland (VEG), trachea, prostate, pituitary and sweat [4,22,27]. Function(s) of human tear lipocalin is unclear although it has been proposed to act as a physiological scavenger and also help in stabilizing the tear film by binding lipids present in the outer layer of tears [20,22,23].

In Syrian hamster submandibular gland (SMG), we had reported the expression, regulation and partial cDNA of an abundant male-specific protein (MSP), expressed as N-glycosylated (~24 kDa) and non-glycosylated (~20.5 kDa) products of a single gene, which are secreted in saliva and also excreted in urine of males [28,29]. Additionally, in the exorbital LG, a major, secretory, female-specific lacrimal protein of Mr 20 kDa (hereafter referred to as FLP) was identified, which crossreacted with MSP antiserum [29,30]. MSP protein sequence, deduced from its partial cDNA, showed that it is a lipocalin [29]. However, the identity of FLP, its exact relationship with MSP, human tear lipocalin or any other protein in hamster or rat was not known.

Here we purify from hamster LG both FLP and MSP, the latter being found to be additionally expressed in LG. The full-length cDNAs of FLP and MSP were cloned and their relationship with each other and with other proteins was established by homology studies. cDNA sequence comparison and Southern analysis suggested that FLP and MSP are most likely encoded by two non-allelic genes. Sex hormone-regulated expression pattern of FLP and MSP in LG, tears and SMG was compared. Expression of related proteins in other hamster tissues and across other species was investigated. Possible functions of differentially regulated hamster FLP and MSP gene products secreted in tears, saliva and urine are discussed.

2. Materials and methods

2.1. Animal experimentation

Syrian hamsters, Wistar rats and BALB/c mice were bred and maintained in our Institute's animal house. All experiments involving animals were approved by our Institutional animal experimentation ethics committee and were done on groups of animals. Representative results are reported. Hamsters of both sexes were gonadectomized at 2 months of age. For hormonal studies, 5 µg estradiol-17-β or 50 µg dihydrotestosterone (both from Sigma) dissolved in 10% ethanol/saline were daily administered for fifteen days by subcutaneous injection to 15-day gonadectomized or normal hamsters of both sexes. Lactating hamsters were similarly treated from 1-day post-partum. Hamsters were sacrificed on the day after the last injection along with appropriate controls and tissues collected and weighed. Timed-pregnant hamsters were sacrificed on 15th day of gestation (late-pregnancy). Water extract of estrous vaginal discharge of hamster was prepared as described elsewhere [14]. Diluted tears were collected from hamsters by a simple ocular

surface washing procedure. Briefly, after anesthesia, 40 µl of sterile saline was placed on the ocular surface of eye by a micropipette and withdrawn after 20 s. Whole saliva was collected as described previously [29] from oral cavity, 1 min after subcutaneous injection of 1 mg pilocarpine nitrate to anesthetized hamsters. Urine (freshly voided) was collected on a clean plastic sheet or from bladder upon sacrifice. Stimulated human tears from healthy donors were collected as previously described [20].

2.2. Purification of 20 kDa FLP and its immunorelated proteins from LG

Purification fractions were monitored by SDS-PAGE [31] and Western blot analysis using MSP antiserum. Absorbance at 280 nm and the method of Lowry et al. [32] were used to monitor protein concentration. Pooled exorbital LGs (~5 g) from ovariectomized hamsters were homogenized (5% w/v) in 20 mM Tris-HCl, pH 8.0 (buffer-A) and centrifuged at 105,000×g for 1 h at 4 °C. The supernatant was fractionated with (NH₄)₂SO₄ and proteins precipitated within 30–95% saturation were dissolved in buffer-A containing 0.5 M NaCl and passed through a Concanavalin A-Sepharose (Amersham) column (1.6 × 10 cm). Proteins in the Con-A unbound pool containing most of the 20- and 24-kDa crossreacting proteins were precipitated at 45–95% (NH₄)₂SO₄ saturation, dialyzed against buffer-A and loaded on a DE-52 (Whatman) column (1.6 × 7 cm). Bound proteins were eluted with 500 ml linear gradient of NaCl (0–300 mM) in buffer-A. Fractions of a broad protein peak eluting between 60 and 100 mM NaCl, containing two incompletely resolved crossreacting proteins of Mr 20.5 and 24 kDa, were pooled to obtain pool-A. Fractions containing the 20-kDa protein (FLP) eluting with a peak at 175 mM were pooled to obtain pool-B. (NH₄)₂SO₄ was added to pool-A to a final concentration of 1.5 M and loaded on a phenyl Sepharose (Sigma) column (1.6 × 9 cm) equilibrated with buffer-A containing 1.5 M (NH₄)₂SO₄. Elution was carried out with a linear gradient of 1.5 to 0 M (NH₄)₂SO₄ in buffer-A. Fractions eluting between 1.2 and 0.5 M (NH₄)₂SO₄ containing both 20.5- and 24-kDa proteins were pooled (pool-A₁). Pool-A₁ was concentrated to 1.0 ml and then loaded on a precalibrated Sephadex G-75 superfine (Amersham) column (1.6 × 95 cm), eluted at 9 ml/h with buffer-A/150 mM NaCl and 1.0 ml fractions collected. Pure 20.5- and 24-kDa proteins, eluting as separate peaks, were individually pooled. Similar concentration and gel-filtration of pool-B (of DE-52) gave a single major peak containing pure 20 kDa FLP.

For deglycosylation experiments, pure 24-, 20.5- and 20-kDa LG proteins were incubated with N-glycosidase F or O-glycosidase (Boehringer) as described previously [29] and analyzed by SDS-PAGE (10.5% gel) followed by Coomassie staining.

For N-terminal sequencing, purified proteins were resolved in SDS-PAGE and transferred onto PVDF mem-

branes for sequencing in a 473A Applied Biosystem automated protein sequencer.

2.3. Cloning of full-length cDNAs of FLP and MSP

2.3.1. Cloning of partial cDNAs

Total RNA was extracted from female LG and male SMG using Trizol reagent (Gibco BRL). First strand synthesis was done using a kit (Gibco BRL) and products were used as templates for subsequent polymerase chain reaction (PCR) using oligo-dT and MSP-primer (5'-GAAGTCAGTCCCTCAGAGGTTGACG-3'), which was designed from the SMG-MSP partial cDNA sequence [29] and corresponded to a consensus stretch found between the N-terminal sequences of 20 kDa FLP, 20.5-/24-kDa LG proteins and 20.5/24 kDa MSP of SMG. PCR conditions were 94 °C for 5 min, followed by 25 cycles of 94 °C for 30 s, 42 °C for 30 s and 72 °C for 1 min; final extension at 72 °C for 10 min. Products of duplicate PCRs for LG and SMG were cloned in pMOS Blue (Amersham) and 8 clones from each PCR were sequenced for both strands using Big Dye Terminator ready reaction kit (Perkin Elmer) and ABI Prism 3700 DNA analyzer. All SMG clones and four LG clones had sequence identical with MSP partial cDNA [29], whereas remaining LG clones had identical partial cDNA sequence, which was unique but closely related (95% identity) to MSP partial cDNA.

2.3.2. 5'- RACE

Gene-specific primers, GSP-1 (5'-AAAGTTGATGTGG-GGAATATTCATG-3') and GSP-2 (5'-CACTATCGTCGACATTAACGTTGTG-3') were designed on consensus regions of the 2 types of partial cDNA sequences obtained above. 5'-RACE was carried out on total RNA of female LG and male SMG using a kit (Gibco BRL). Briefly, first strand cDNA syntheses were carried out using GSP-1 primer followed by dC tailing and then PCR using GSP-2 primer and oligo-dG anchor primer. PCR conditions were 94 °C for 5 min, followed by 25 cycles of 94 °C for 30 s, 56 °C for 30 s and 72 °C for 1 min; final extension at 72 °C for 10 min. Products of duplicate PCRs (for LG and SMG) were separately cloned and a total of 16 LG and 16 SMG RACE clones were sequenced. All SMG and ~20% of LG RACE clones were identical and had an unique 5'-sequence stretch, which was identified as 5'-extension of MSP cDNA cloned from SMG and LG on the basis of sequence overlaps and encoded amino acids. Remaining LG RACE clones were identical and encoded the unique N-terminal sequence of FLP and were identified as 5'-extension of the unique partial cDNA cloned from LG (see above).

2.3.3. Full-length cDNA

To amplify full-length cDNA from female LG and male SMG RT products, duplicate PCRs were done using oligo-dT and a degenerate FL primer (5'-AACAYSTGTCA-GATTTTGACCT-3') designed to bind specifically the 5'

ends of both FLP and MSP type transcripts. PCR conditions were as described above using MSP primer. PCR products were separately cloned and 5 clones from each PCR were sequenced. Seven clones from LG contained a unique full-length cDNA sequence, which encoded the N-terminal sequence of FLP and were identified as FLP cDNA. Three LG clones and all SMG clones contained full-length cDNA of MSP and encoded the N-terminal sequence of LG 20.5/24-kDa proteins.

2.4. Southern hybridization

High molecular-mass genomic DNA was isolated from liver of female hamster using standard protocols. Aliquots of the genomic DNA (45 µg) were digested with different restriction endonucleases and duplicate aliquots (20 µg) of each digest were size fractionated in agarose gel (0.8%) and transferred to Hybond N⁺ membrane (Amersham). Blots were hybridized overnight at 65 °C with ³²P-labelled MSP or FLP partial cDNAs (nucleotides 68–508), which were amplified by PCR. Blots were once washed for 10 min with 0.2× SSC, 0.2% SDS at room temperature and then twice with 0.1× SSC, 0.1% SDS at 65 °C for 10 min each. Hybridization was detected by autoradiography.

2.5. Expression analysis

Tissues were homogenized in 20 mM Tris-HCl, pH 8.0 (2.5% w/v) and centrifuged at 30,000×g for 30 min. SDS-PAGE was done on 10.5 or 15% polyacrylamide gels after loading equal volumes of the soluble extracts of tissues, ocular surface washings or saliva. The gels were stained with Coomassie R-250 (Sigma) for protein profile comparisons or used for Western blotting. Blots were probed with rabbit antiserum raised previously against purified 24 kDa SMG-MSP [29] or against the purified 20 kDa FLP. Crossreaction with antibody was detected using either ¹²⁵I-labelled protein-A or alkaline-phosphatase conjugated anti-rabbit IgG goat antibodies.

For Northern blotting, total RNA of different tissues were electrophoresed in formaldehyde/agarose (1.2%) gels, transferred to Hybond N⁺ membranes and hybridized overnight at 42 °C with ³²P-labelled FLP full-length cDNA [33]. Blots were twice washed for 15 min at 60 °C in 2× SSC, 0.1% SDS (low stringency) and then twice for 30 min at 60 °C in 0.2× SSC, 0.1% SDS (high stringency) [33]. Hybridization was detected using a phosphorimager.

3. Results

3.1. Expression pattern of major 20-kDa LG/tear protein and MSP of SMG and their immunological similarity

LG secretion (tears) and SMG secretion (saliva) are released on the ocular surface and in the oral cavity,

respectively. Protein profiles of exorbital LG, ocular surface washings (diluted tears), SMG and saliva of hamsters in different hormonal states are shown in Fig. 1A to compare expression pattern of 20-kDa LG protein with MSP of SMG. A major 20-kDa protein is seen in LG extracts from normal females (lane 2) but not males (lane 1). Ovariectomized (lane 4) and lactating females (lane 5), both very low-estrogen states [29,34], show several-fold increased levels of this LG protein. Similar levels were induced in males after castration (lane 3). Female tears (lane 13) have a major 20-kDa protein, which is undetectable in male tears (lane 12). Levels of this tear protein are elevated several folds in ovariectomized (lane 15) and lactating (lane 16) females and similar levels are seen in castrated males (lane 14). Thus, although LG 20-kDa protein shows female-specific expression, it is maximally expressed in sex-hormone deficient states in both sexes. In contrast to this female-specific expression, male SMG (lane 6) and male saliva (lane 8) but not female SMG (lane 7) or female saliva (not shown) express abundant 24 and 20.5 kDa MSP, with the latter having slightly slower mobility than LG 20-kDa. Moreover, while MSP is induced to very high levels in female SMG after ovariectomy (lane 9) and during the natural low-estrogen state of lactation (lane 11), its levels are high and unaltered in castrated males (lane 10).

Fig. 1B shows Western blots using antiserum previously raised against the purified 24 kDa N-glycosylated MSP of male SMG [29]. As expected, in male SMG (lane 1) the 24 kDa MSP and the 20.5 kDa nonglycosylated MSP [29] show crossreactivity. A minor 30 kDa crossreactive band is also seen in male SMG, which is likely to be a higher N-glycosylated form of MSP [29,35]. Interestingly, in LG (lane 2) and tears (lane 3) of ovariectomized females, the major 20-kDa protein and a relatively minor 24-kDa protein show crossreactivity with MSP antiserum. Such cross-

reactions were also seen in LG and tears of normal or lactating females but were undetectable in normal males (data not shown).

3.2. Purification and partial characterization of 20-kDa FLP and MSP from LG

Since an obvious difference in hormonal regulation and a slight difference in mobility was seen between MSP in SMG and their immunorelated 20 and 24 kDa counterparts in LG, we purified these LG proteins to obtain their N-terminal sequences and investigate any interrelationship between them. Exorbital LG from ovariectomized hamsters was used as the starting material since they had higher levels of crossreacting 20- and 24-kDa proteins than intact females. Fig. 2 shows a flow chart of major purification steps (see Materials and methods for details). Coomassie stained protein profiles of major purification fractions are shown in Fig. 3A. In pool-A, obtained after NaCl gradient elution of DE-52 bound proteins, the 24-kDa protein and interestingly a 20.5-kDa crossreacting protein were recovered (Fig. 3A, lane 3; see also Fig. 3B, lane 1, for its Western blot). DE-52 fractions, containing the 20-kDa major protein (henceforth referred to as FLP), which eluted much later, were pooled to make pool-B (Fig. 3A, lane 4; see also Fig. 3B, lane 2). Pool-A was bound to phenyl Sepharose, gradient eluted and again pooled to get pool-A₁ (Fig. 3A, lane 5) consisting of the 20.5-/24-kDa proteins free from most other contaminants. Pool-A₁ was resolved on gel filtration to get final purified preparations of 24- and 20.5-kDa LG proteins shown in Fig. 3A, lanes 6 and 7, respectively. Pool-B of DE-52 was similarly resolved in gel-filtration to obtain purified FLP (Fig. 3A, lane 8). Silver staining (not shown) also confirmed that the final purified preparations of the three LG proteins were homogeneous. The estimated yield from 5 g of LG was 20 mg of FLP,

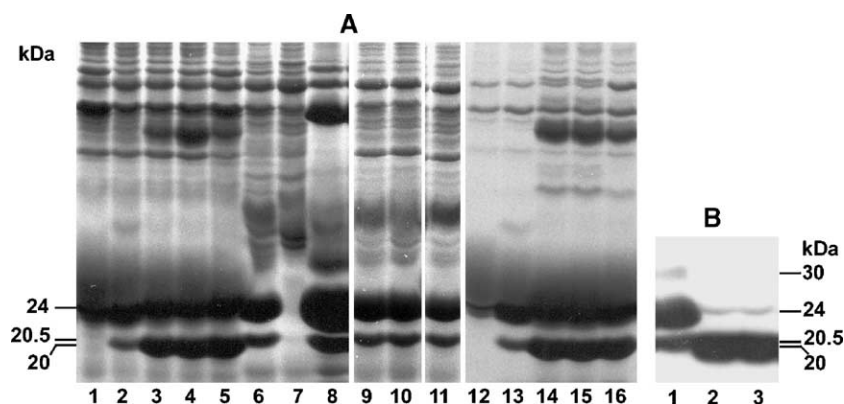


Fig. 1. Expression pattern of a major 20-kDa LG/tear protein and 24/20.5-kDa MSP of SMG and their immunological similarity. (A) Coomassie stained SDS-PAGE profiles of exorbital LG and SMG extracts, ocular surface washings (tears) and saliva. Lanes 1–5 are LG extracts: lane 1, male; lane 2, female; lane 3, castrated male; lane 4, ovariectomized female; and lane 5, lactating female. Lanes 6 and 7 are male and female SMG extracts, respectively; lane 8 is male saliva; lanes 9, 10 and 11 are SMG extracts of ovariectomized female, castrated male and lactating female, respectively. Lanes 12–16 are profiles of ocular surface washings; lane 12, male; lane 13, female; lane 14, castrated male; lane 15, ovariectomized female; lane 16, lactating female; (B) Western blot using MSP antiserum is shown. Lane 1, male SMG extract; lane 2, exorbital LG extract of ovariectomized female; and lane 3, diluted tears of ovariectomized female. SDS-PAGE was carried out on 10.5% polyacrylamide gels.

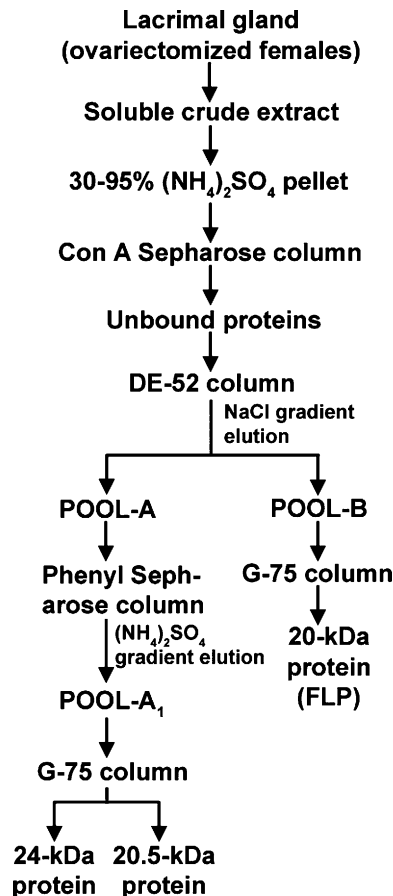


Fig. 2. Flow chart showing sequence of major steps involved in purification of FLP and its immunorelated 20.5- and 24-kDa proteins from hamster LG. See Materials and methods for details.

2 mg of 20.5 kDa and 5 mg of 24 kDa proteins. In gel filtration, the estimated native Mr of both purified FLP and 20.5-kDa LG protein was ~17 kDa while that of 24-kDa LG protein was ~23.4 kDa, indicating that they are monomers.

Fig. 3B shows that antiserum raised against purified FLP crossreacts with FLP (lane 5), 20.5-kDa (lane 4) and 24-kDa (lane 3) purified proteins and show crossreactions at ~Mr 20 and 24 kDa in crude LG cytosol (lane 6). Fig. 3C shows that N-glycosidase F treatment had no effect on 20.5-kDa protein (lane 4) but it deglycosylated most of the 24-kDa protein (lane 2) to a shorter species with mobility matching that of 20.5 kDa (lanes 3 and 4) but not of FLP (lane 5). Similar N-glycosidase F treatment of FLP and O-glycosidase treatment of three LG proteins had no effect on their mobility in SDS-PAGE (not shown). Moreover, FLP and 20.5-kDa LG protein did not stain for glycoprotein and also failed to bind different lectins (not shown). A unique N-terminal sequence HYQNLEVPSEVDG (14 residues) was obtained for FLP, while both LG 20.5- and 24-kDa proteins had N-terminal sequence AQHQNLEVPSEVDG (15 residues), which is identical to that of 20.5/24 kDa MSP expressed in male hamster SMG [29]. Notably, the above two sequences had considerable consensus but differed in

their initial 2/3 residues. Results of Western blots, glycosidase treatments and N-terminal sequencing indicated that FLP is a non-glycosylated protein, which is similar but distinct from 20.5- to 24-kDa LG proteins and the latter two proteins are non-glycosylated and N-glycosylated forms of MSP, which are additionally expressed in LG.

3.3. cDNA cloning of FLP and MSP and sequence analysis

Using RT-PCR strategy, full-length cDNA of FLP was cloned from female exorbital LG (Fig. 4). Additionally, identical full-length cDNAs of MSP were also cloned from female LG and male SMG (Fig. 4). MSP cDNA cloned from LG must represent the cDNA for LG 20.5- and 24-kDa proteins, which are LG counterparts of MSP. FLP and MSP cDNAs are 789 bp each with 95% sequence identity and encode precursor proteins of 172 amino acids having 85% sequence identity. Both cDNAs have a 52 bp 5'-UTR preceding the translation start codon (ATG), which is surrounded by a consensus Kozak sequence (ACCATGG) (Fig. 4) typical of highly translated eukaryotic mRNAs [36]. The deduced 16 residue signal peptide of FLP is one residue (Alanine) longer at its C-terminus, but otherwise identical with that of MSP (Fig. 4). Thus, the difference in cleavage of the signal peptide from precursor proteins of FLP and MSP

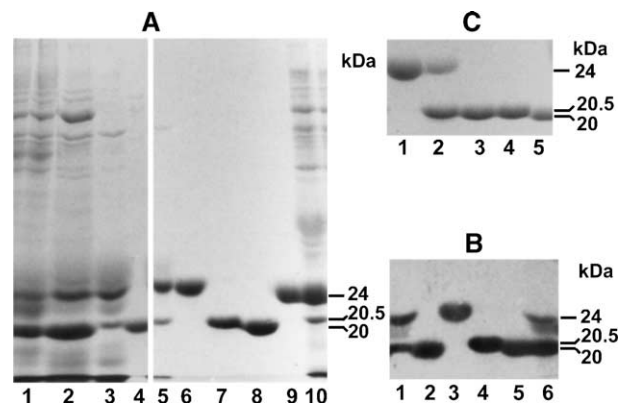


Fig. 3. Purification of FLP and MSP from LG. (A) Coomassie stained SDS-PAGE profiles of purification fractions (see text for details): Lane 1, crude soluble extract of exorbital LG of ovariectomized hamster; lane 2, Con A-Sepharose unbound pool; lanes 3 and 4 are pool-A and pool-B, respectively of DE-52 eluted proteins; lane 5, pool-A₁ of phenyl-Sepharose eluted proteins; lanes 6, 7 and 8 show final purified preparations of 24-kDa LG protein, 20.5-kDa LG protein and 20 kDa FLP, respectively, which were obtained after gel-filtration; lane 9, previously purified N-glycosylated MSP from SMG [29] and lane 10, crude soluble extract of male hamster SMG. (B) Western blot of purification fractions using antiserum raised against purified FLP: lane 1 is pool-A of DE-52 containing 20.5- and 24-kDa LG proteins and lane 2 is pool-B of DE-52 containing 20 kDa FLP; lanes 3, 4 and 5 are final purified pools of 24-kDa LG protein, 20.5-kDa LG protein and FLP, respectively, obtained after gel-filtration; lane 6, crude exorbital LG extract of ovariectomized hamster. (C) N-glycosidase F treatment of purified 24- and 20.5-kDa LG proteins. Lane 1, 24-kDa LG protein after control incubation; lane 2, 24-kDa LG protein after incubation with N-glycosidase F; lane 3, 20.5-kDa LG protein after control incubation; lane 4, 20.5 kDa LG protein after incubation with N-glycosidase F; lane 5 is purified FLP. Samples were run on 10.5% SDS-PAGE after incubation.

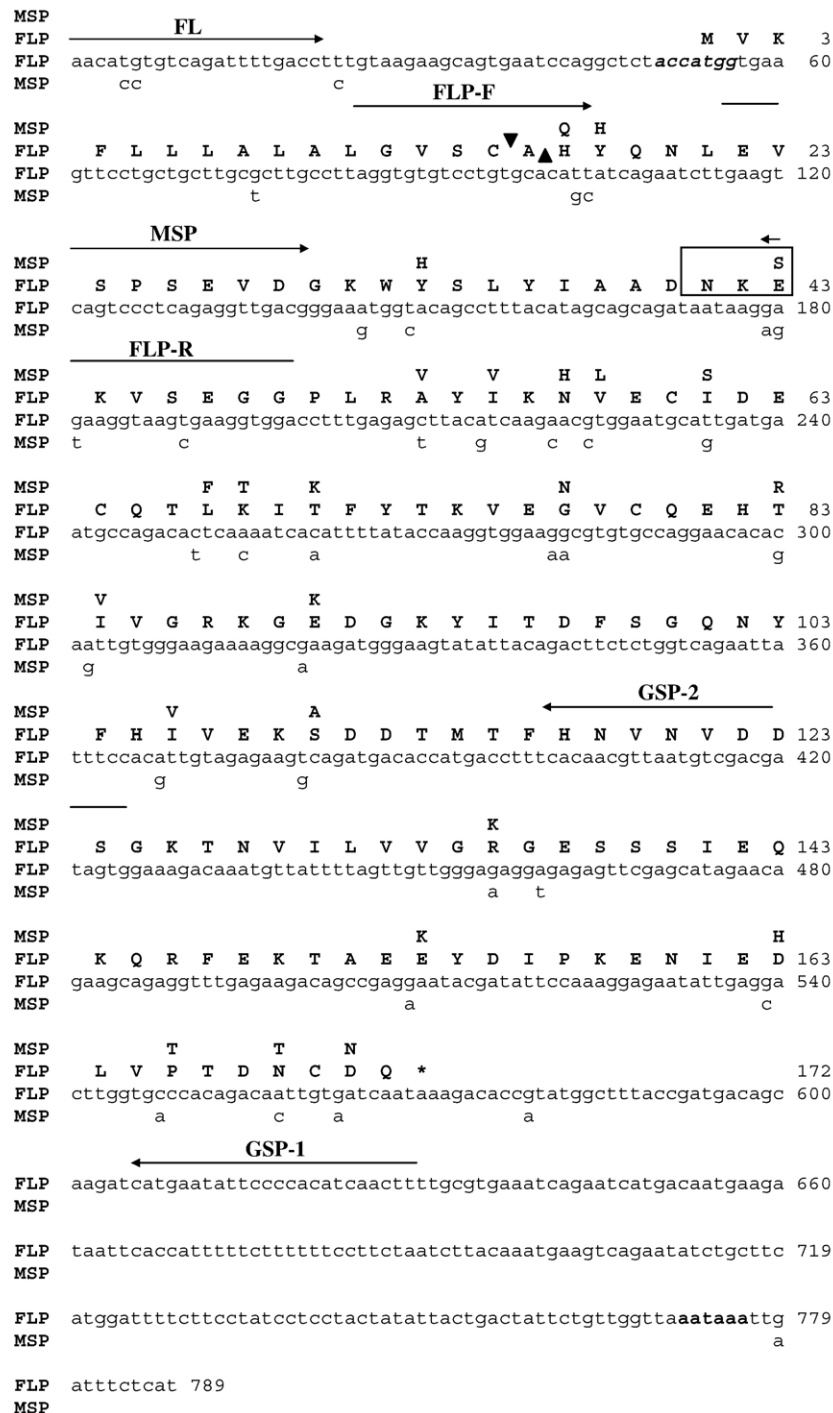


Fig. 4. Full-length cDNAs and deduced amino acid sequences of FLP and MSP. cDNA sequences are shown in lower case. Full-length cDNA sequence of FLP (789 bp) and only differences in MSP cDNA (789 bp) are shown. The consensus Kozak sequence (bold italics) and polyadenylation signal (in bold), identical in both the cDNAs are indicated in FLP sequence. Arrows shown above sequences indicate different primers used for PCRs (see Materials and methods for details). The deduced amino acid sequence for FLP precursor (172 residues) and only the differences in MSP are shown in upper case using single letter code. Asterisk indicates stop codon. Cleavage site for putative signal peptide of FLP (16 residue long) and that of MSP (15 residue long) are shown by arrowheads, which point up and down, respectively. These cleavages results in mature FLP (156 residues) and MSP (157 residues) having experimentally confirmed N-terminal sequences (see text). The putative N-glycosylation site in MSP (absent in FLP) is boxed. Note: The novel nucleotide sequence of full-length FLP cDNA and the extended full-length cDNA sequence of MSP, reported here, are submitted to GenBank with accession numbers AF345648 and AF183407, respectively.

must be influenced by the differences in their 17th and 18th residues [37]. This difference in cleavage results in mature FLP and MSP of 156 and 157 residues with calculated

masses of 17737 and 17815 kDa and calculated pI of 4.3 and 5.8, respectively, which is consistent with the slight difference in their mobility and considerable difference in their

elution from DE-52 anion-exchanger. As expected, the deduced amino acid sequence of MSP and not that of FLP has a putative N-glycosylation site. Interestingly, out of the 36 base mismatches between the FLP and MSP cDNAs, 31 are spread out in the coding region, out of which 27 are located in the 24 codons, which show amino acid changes (12 non-conserved) and these changes are all in the mature protein. Thus, the number of non-synonymous base substitutions in FLP and MSP is greater than synonymous substitutions. Similar observations are reported for genes involved in molecular recognition, on which positive selection is believed to operate [38–40]. Remarkably, the 221 bp 3'-UTRs of FLP and MSP were the most conserved stretches with only two mismatches (Fig. 4). Such closely similar 3'-UTRs in some paralogous genes is reported [38,39] and possible involvement of 3'-UTR in affecting stability of transcripts has also been suggested [41].

Our extensive search of cDNA clones amplified from female exorbital LG using MSP/FLP consensus primers always showed a relative abundance of FLP clones over that of MSP (see Materials and methods). This indicated that the level of FLP expression is higher than MSP in LG. This is consistent with the observation that purification from LG gave much higher yield of FLP than MSP proteins. On the other hand, search for cDNA clones amplified from male SMG (see Materials and methods) revealed clones encoding only MSP and not FLP. Additionally, FLP cDNA-specific primers FLP-F and FLP-R (Fig. 4), when used in RT-PCR gave no product with male SMG mRNA, while a 115-bp product was obtained with female LG mRNA confirming lack of FLP expression in SMG (results not shown).

FLP and MSP cDNAs have significant identity only with cDNAs of odorant-binding protein (OBP; GenBank J03093)

(78%) expressed in rat lateral nasal glands in both sexes [7,10,11] and an OBP expressed in mouse head (GenBank AK017258) (76%), which are both lipocalins. Deduced amino acid sequence of FLP showed that it is a new member of the lipocalin protein superfamily with maximum identity (Fig. 5) with aforementioned rat and mouse OBPs (58–57%) followed by aphrodisin (39%), a pheromone/odorant-binding lipocalin of hamster vaginal discharge [5,13–16,42], which, however, had no significant cDNA match. Other lipocalins having significant amino acid sequence identity (39–29%) with FLP and MSP were OBPs of nasal glands of mouse, bovine and pig; putative pheromone-binding lipocalin of bovine sweat glands (Bos d2); pheromone-binding lipocalin of boar saliva (SAL1) and probasins of unknown function in rat and mouse prostate secretions [5,6,8,9]. Pheromone-binding urinary lipocalins, which are expressed in LG and SMG of rat and mouse in both sexes but male-specifically in their liver [12,13] and tear/VEG lipocalins of human and pig expressed in both sexes [4,21,22] had a more distant relationship with FLP and MSP (15–20% identity). As is characteristic of all lipocalins [1–3], FLP and MSP have a GXW motif near their N-terminal end (Fig. 5). Cysteines 63 and 154 of mature FLP and MSP (Fig. 5) are likely to be disulfide linked since they are conserved and disulfide linked in almost all known lipocalins [1–3]. The two other cysteines (residue 44 and 48), which constitute a CXXXC motif (Fig. 5), present only in a subset of odorant/pheromone-binding lipocalins (which includes rat/mouse OBPs and aphrodisin), are also likely to be disulfide linked as reported for aphrodisin [5]. 3D structure analysis of aphrodisin has identified 14 amino acids lining its ligand-binding pocket [5]. Interestingly, 12 of these residues are identical or conserved in FLP and MSP (Fig. 5).



Fig. 5. Multiple alignment of deduced amino acid sequence of hamster FLP and MSP with closely related lipocalins, rat OBP1f, mouse OBP and hamster aphrodisin. Mature protein sequences are aligned. Amino acids, which are identical or conserved in all sequences, are in bold. Cysteines are indicated by dots. The conserved lipocalin motif GXW and the CXXXC motif conserved in all the aligned sequences are boxed. The arrowheads indicate the 14 residues lining the odorant-binding pocket of aphrodisin [5]. The putative N-glycosylation site in MSP is underlined. Number of residues in each mature protein and percentage identity with FLP are indicated.

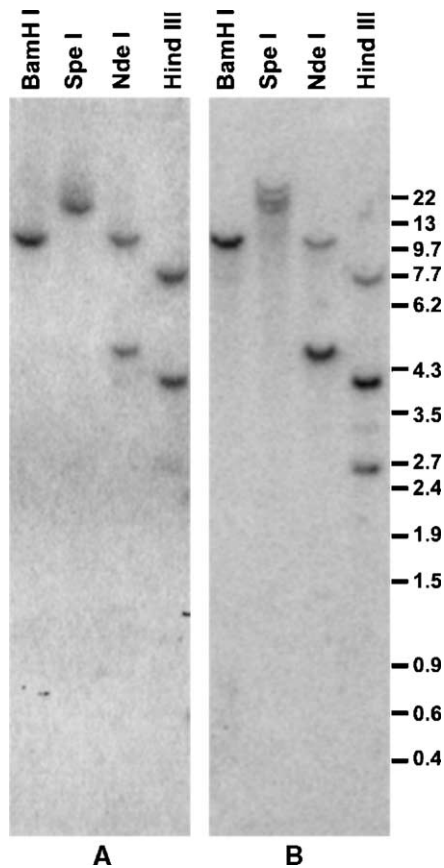


Fig. 6. Southern blot hybridization on hamster genomic DNA digested with restriction endonucleases. The blots were probed with ^{32}P -labelled FLP (panel-A) or MSP (panel-B) partial cDNAs (nucleotides 68–508) amplified by PCR. The restriction endonucleases used for digestion are indicated above each lane.

3.4. Southern hybridization

Fig. 6 shows the results of Southern hybridization of hamster genomic DNA using partial cDNAs of FLP (panel A) and MSP (panel B) as probes. *Bam*HI digested genomic DNA showed similar hybridization patterns with both the probes. However, for *Nde*I, *Hind*III and *Spe*I digests, differences in the relative intensity and pattern of hybridizing bands are seen depending on whether FLP or MSP probe is used. The 4.5 kb and 10 kb bands in *Nde*I digest are most likely to represent MSP and FLP gene fragments, respectively. In *Hind*III digest, the 7.7 kb band should represent FLP while the 2.6 kb (visible only in panel B) and 4 kb bands most likely represent MSP gene fragments. Similarly, *Spe*I digests also results in differential hybridization. The results indicate the presence of at least two distinct genomic fragments encoding FLP- and MSP-like cDNAs.

3.5. Expression analysis of FLP and MSP

To study the sex hormonal regulation of FLP and MSP transcripts in hamster exorbital LG, Northern analysis was

done using full-length FLP cDNA as a probe. Moreover, presence of similar transcripts in other hamster and rat tissues was also investigated. Fig. 7A shows that exorbital LG of females (lane 2) but not males (lane 1) have an apparently single band of hybridizing transcript, the levels of which increased in ovariectomized females (lane 4) and was markedly induced in castrated males (lane 3). A complete obliteration of this transcript was observed after estradiol treatment of normal and ovariectomized females (not shown) and also castrated males (lane 5). Similar disappearance was also seen after dihydrotestosterone treatment of ovariectomized females (lane 6) and castrated males (not shown). Fig. 7B shows that the hamster infraorbital LG, an accessory lacrimal gland [43], shows hybridizing transcript in females (lane 2) but not in males (lane 1). Fig. 7C shows that in hamster SMG, a hybridizing transcript of same size as the LG transcript was seen in normal males (lane 1) but was absent in females (lane 2). The hybridization seen in hamster LGs should be due to both FLP and MSP transcripts since their cDNAs have 95% identity and are of same size. However, the hybridization in SMG must be due to only MSP mRNA since FLP expression was not detected in SMG. Fig. 7D shows that lateral nasal glands of rat in both sexes (lanes 3 and 4) have a hybridizing transcript of same size as LG transcript. This hybridization, detected after low stringency washing, but barely detectable after high stringency washing (not shown), very likely represents rat OBP whose cDNA has ~78% match and is expressed in both sexes [7]. Interestingly, no hybridization was seen for lateral nasal glands of male or female hamsters (lanes 1 and 2). Moreover, LG and SMG of rat and mouse of both sexes showed no hybridization (not shown).

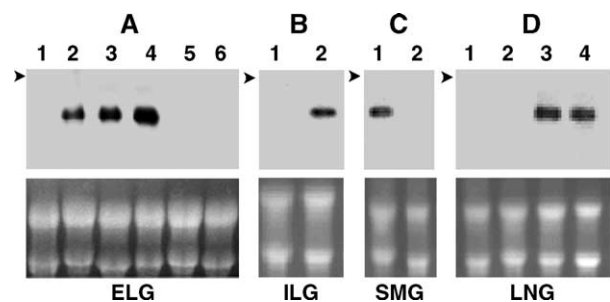


Fig. 7. Northern blots of exorbital LG (ELG), infraorbital LG (ILG) and SMG of hamster and lateral nasal gland (LNG) of hamster and rat. All blots were probed with ^{32}P -labelled FLP cDNA. (A) Total RNA from hamster ELG was loaded. Lane 1, male; lane 2, female; lane 3, castrated male; lane 4, ovariectomized female; lane 5, estradiol treated castrated male; and lane 6, dihydrotestosterone treated ovariectomized female. (B) Total RNA from male and female hamster ILG was loaded in lane 1 and lane 2, respectively. (C) Total RNA from male and female hamster SMG was loaded in lane 1 and lane 2, respectively. (D) Total RNA from LNG was loaded. Lane 1, male hamster; lane 2, female hamster; lanes 3, male rat and lane 4, female rat. Arrowhead indicates the position of 18S ribosomal RNA. Bottom panels show the ethidium bromide stained profiles of 28S and 18S ribosomal RNA.

Western blots were done using FLP antiserum to confirm whether FLP (LG 20-kDa) and MSP (LG 20.5-/24-kDa proteins) have similar sex hormonal regulation in exorbital LG. LG extracts from hamsters in different hormonal states and treatments were electrophoresed in 15% SDS-PAGE to resolve FLP from 20.5 kDa LG MSP. Fig. 8A shows that no crossreaction was found in LG of males (lane 1) while LG (lane 2) and tears (lane 9) of females had a major 20 kDa FLP and a minor 20.5 kDa MSP crossreactive bands. Additionally, the 24 kDa MSP was visible as a diffuse crossreaction. FLP and both forms of MSP were markedly induced in LG of castrated males (lane 3) and they were also induced in LG of ovariectomized (lane 4) and lactating females (lane 8) to levels higher than normal females. Such crossreactions were almost undetectable in LG of late-pregnant females (lane 7), which are known to have a high-estrogen state [29,34,44]. Treatment of ovariectomized females with either estradiol (lane 5) or dihydrotestosterone (lane 6) markedly reduced both FLP and MSP in LG and similar results were seen when normal and lactating females or castrated males were similarly treated (not shown). Like exorbital LG, the infraorbital LG of hamster also had 20, 20.5 and 24 kDa crossreacting proteins in females (lane 11) but not in males (lane 10). The expression pattern of FLP and MSP in infraorbital LG in different hormonal states and treatments (data not shown) was identical to that of exorbital LG. However, hamster Harderian gland (a large retro-orbital accessory LG), which is well known for its sexual dimorphism [45–48], had no crossreacting proteins in either sex (not shown). While FLP antiserum did not show any crossreaction with female SMG (lane 13), it crossreacted with major 24/20.5 kDa MSPs and a minor ~30 kDa protein in male SMG (lane 12) and male saliva (not shown). However, no crossreaction at 20 kDa was found, again confirming the absence of FLP expression in SMG.

Fig. 8B shows that while extracts of lateral nasal glands of hamsters of both sexes (lanes 1 and 2) do not show any cross-reaction with FLP antiserum, lateral nasal glands of rat from both sexes (lane 3 and 4) showed a single crossreaction at 20 kDa which very likely represents rat OBP which has 58% protein sequence identity with FLP and MSP. Hamster vaginal discharge, an abundant source of aphrodisin [14], however, showed no crossreaction with FLP antiserum (lane

5). Extracts of LG, SMG, VEG and urine of rat and mouse from both sexes (not shown) showed no crossreaction indicating lack of any FLP/MSP-like proteins. A variety of other hamster tissues from both sexes, e.g., parotid and sublingual salivary gland, pituitary, liver, kidney, lungs, brain, spleen and intestine and male-specific tissues like prostate, seminal vesicles showed no crossreaction with FLP antiserum. Moreover, human tears from both sexes also showed no crossreaction (not shown).

4. Discussion

Our results clearly demonstrate that FLP and MSP are two different but very similar tear lipocalins, which are co-expressed female-specifically, and have similar sex hormonal regulation in both exorbital and infraorbital LG of hamster. While FLP is non-glycosylated and lacks any consensus glycosylation site, MSP has a single N-glycosylation site and exists both as N-glycosylated and non-glycosylated forms with the latter having only a slightly higher mass than FLP and almost co-migrating with it. Such two forms of MSP, which are products of MSP gene [29], are expressed male-specifically in hamster SMG where we could not detect any expression of FLP. Notably, the expression level of MSP in LG was considerably lower than that of MSP in SMG or FLP in LG.

Expression analysis shows that androgens and estrogens display a relatively rare mode of sex-hormonal gene regulation in LG of both sexes wherein both hormones repress expression of FLP and MSP. The female expression of FLP and MSP in LGs is due to their incomplete repression by endogenous estrogens while their absence in male LG is due to complete repression by endogenous androgens. Thus, ovariectomy and also the natural, very low-estrogen state that prevails during lactation [29,34] results in their enhanced expression in female LG and castration results in massive induction in male LG and these inductions are prevented by exogenous administration of estrogen or androgen. However, this regulation of MSP (and FLP) in LG differs markedly from that of MSP in SMG, where MSP displays abundant, androgen-insensitive expression in male SMG, while it is undetectable in female SMG

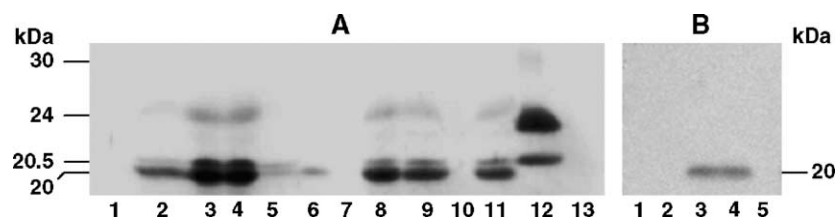


Fig. 8. Western blots of hamster and rat tissue extracts using FLP antiserum. (A) All lanes contained hamster tissue extracts. Lanes 1–8 are exorbital LG extracts of hamsters in different hormonal states: lane 1, normal male; lane 2, normal female; lane 3, castrated male; lane 4, ovariectomized female; lane 5, estradiol-treated ovariectomized female; lane 6, dihydrotestosterone-treated ovariectomized female; lane 7, late-pregnant female and lane 8, lactating female. Lane 9, is normal female tears; lanes 10 and 11 are male and female ILG extracts; and lanes 12 and 13 are male and female SMG extracts. (B) Western blots of nasal glands and hamster vaginal discharge using FLP antiserum. Lanes 1 and 2 are lateral nasal glands of female and male hamsters; lanes 3 and 4 are lateral nasal glands of female and male rat; and lane 5 contained extract of hamster vaginal discharge. All samples were run on 15% SDS-PAGE.

due to its complete repression by endogenous estrogens [28,29]. Like the repression of MSP in SMG of normal female, FLP and MSP in female LG are also completely repressed by endogenous estrogens during late-pregnancy when estrogen levels are extremely elevated [34,44]. After ovariectomy and also during the post-partum period when the lactating dam is acyclic [34] and nursing her litter, MSP in SMG [29] (and FLP/MSP in LG) are massively induced and copiously secreted. The lactational induction of MSP in SMG is, however, temporary and MSP is again undetectable post-weaning [29] when estrus cycle resumes [34] while in LG, FLP and MSP expression continues, although reduced to normal female levels.

Inspection of mismatches between full-length cDNAs of FLP and MSP shows that they are spread throughout the coding region and also the UTRs. Thus, it is quite unlikely that their transcripts arise due to alternate splicing from a common precursor mRNA. The differential hybridization of genomic DNA fragments with FLP and MSP cDNA probes also suggests that FLP and MSP are transcribed from 2 distinct genomic fragments. Moreover, we could amplify from hamster genomic DNA, using a pair of FLP/MSP-consensus primers, 2 distinct genomic fragments, which after sequencing could be identified, based on their matches with cDNA sequences, as fragments of FLP and MSP genes containing their two putative introns (data not shown).

Are FLP and MSP two closely related genes or are they alleles of the same gene? FLP and MSP are most likely non-allelic for the following reasons: (1) In our extensive protein profile studies of LG and SMG, done on a very large population of hamsters (of our colony) and spanning many generations, we found all hamsters could express both proteins, FLP (in LG) and MSP (in SMG and LG) if their hormonal state was permissive (i.e., all gonadectomized or lactating hamsters) indicating lack of any segregation between FLP and MSP. (2) FLP and MSP have 5% and 15% differences between their cDNA and amino acid sequences which is unusually high for alleles. (3) They display different tissue- and sex-specific expression, unusual for alleles. (4) More than one copy of rat OBP gene and its mouse counterpart (the closest orthologues of FLP/MSP; see below) are located on rat and mouse X-chromosomes. Since similar gene arrangements have been seen in X-chromosomes of many eutherian mammals [49], and this is likely to be true for hamster, the expression of both FLP and MSP found in male hamster (which carries a single X-chromosome) after gonadectomy should rule out any possibility of these genes being allelic.

Lipocalins display considerable sequence divergence and are expressed in a variety of different tissues and can have different functions. Comparison with protein sequences in the database shows that mammalian lipocalins believed to bind odorants or pheromones with demonstrated or putative roles in chemical communication have maximum similarity with FLP and MSP. Although FLP is secreted in tears and MSP is secreted in tears, saliva and is also excreted in urine,

other known tear, salivary or urinary lipocalins are not among the ones having the best protein sequence match with FLP and MSP. Moreover, human tear/VEG lipocalin and its known counterpart in rat VEG and the urinary lipocalins of rat and mouse did not crossreact with FLP antiserum. Finally, except for OBPs of rat lateral nasal glands and their OBP counterpart in mouse, which had the best protein matches with FLP and MSP, all other lipocalins had no significant cDNA match with FLP and MSP. This also included hamster aphrodisin, which had the next best protein sequence match with FLP and MSP but did not crossreact with FLP antiserum. All of the above, when considered together with our results of Northern and Western analysis of rat lateral nasal glands strongly indicate that rat OBPs, expressed in lateral nasal glands of both sexes are closest orthologues of hamster FLP and MSP. Interestingly, rat-OBP (or FLP/MSP)-like crossreacting proteins/transcripts were undetectable in LG/SMG of rat and mouse and also in hamster lateral nasal gland. The close relationship shared by FLP, MSP and rat OBPs suggest that they must have diverged from an ancestral gene and acquired different tissue-specificities and sex hormonal regulation. Since, all lipocalins are believed to have diverged from a common ancestral gene [1–3], the aphrodisin gene must have diverged earlier and FLP and MSP genes could have resulted from a relatively recent gene duplication.

Hamster aphrodisin is the lipocalin closest to FLP and MSP whose 3D crystal structure is known [5]. Protein sequence comparison with aphrodisin suggests presence of a hydrophobic ligand-binding pocket in FLP and MSP. Moreover, *in vitro* studies have shown that both aphrodisin and rat-OBP can bind different odorant molecules [11,15] and recently, a putative pheromone was detected bound to natural aphrodisin [16]. Thus, considering the above and the sex-specific and lactation-enhanced expression of FLP and MSP and their presence in tears, saliva and urine suggests the possibility that they may also bind odorants and/or pheromones and have a role in chemical communication similar to OBPs and/or aphrodisin. Whether FLP and different forms of MSP bind odorants/pheromone, and if so, whether they display any differences in ligand-specificity between themselves and with aphrodisin, needs to be investigated, since specialized functions for these three hamster lipocalins are possible.

Human tear lipocalin, expressed abundantly in both sexes, is the only tear lipocalin whose function and crystal structure has been studied [4,20,22–27]. It has been suggested to have a role in stabilizing the outer lipid layer of tears and to also act as a physiological scavenger [20,22,23,26]. Similar protective roles for FLP and MSP in hamster tears cannot be ruled out but needs to be reconciled with their female-specific and lactation-enhanced expression. Striking sexual dimorphism in hamster tear composition is known, wherein only females have massive levels of free protoporphyrin IX [46,47] and some distinctly different lipids [45]. This is due to their abundant, female-

specific synthesis in Harderian gland and secretion into tears [46–48]. Free protoporphyrin IX is well known to cause photo-oxidative damage [46] and requires to be scavenged from the ocular surface. On the other hand, Harderian lipids have been suspected to have pheromonal functions [48]. Since certain lipocalins are known to bind protoporphyrin IX, heme and heme degradation products [1,50] and human tear lipocalin can bind a wide array of lipids [20], it needs to be investigated whether FLP and MSP of female hamster tears can bind such potential ligands.

Acknowledgements

The authors thank Dr. M.V. Jagannadham for help in N-terminal sequencing, Drs. J. Gowrishankar and T. Ramakrishna for discussions and suggestions and Mr. K. Ram for technical assistance. SS is supported by a Research Fellowship from Council for Scientific and Industrial Research, India and VP was supported by a post-doctoral fellowship from Department of Biotechnology, India.

References

- [1] B. Åkerström, D.R. Flower, J.P. Salier, Lipocalins: unity in diversity, *Biochim. Biophys. Acta* 1482 (2000) 1–8.
- [2] D.R. Flower, A.C.T. North, C.E. Sansom, The lipocalin protein family: structural and sequence overview, *Biochim. Biophys. Acta* 1482 (2000) 9–24.
- [3] J.P. Salier, Chromosomal location, exon/intron organization and evolution of lipocalin genes, *Biochim. Biophys. Acta* 1482 (2000) 25–34.
- [4] D.A. Breustedt, I.P. Korndörfer, B. Redl, A. Skerra, The 1.8 Å crystal structure of human tear lipocalin reveals an extended branched cavity with capacity for multiple ligands, *J. Biol. Chem.* 280 (2005) 484–493.
- [5] F. Vincent, D. Lobel, K. Brown, S. Spinelli, P. Grote, H. Breer, C. Cambillau, M. Tegoni, Crystal structure of aphrodisin a sex pheromone from female hamster, *J. Mol. Biol.* 305 (2001) 459–469.
- [6] S. Spinelli, F. Vincent, P. Pelosi, M. Tegoni, C. Cambillau, Boar salivary lipocalin: three dimensional X-ray structure and androstenol/androstenone docking simulations, *Eur. J. Biochem.* 269 (2002) 2449–2456.
- [7] J. Pevsner, P.M. Hwang, P.B. Sklar, J.C. Venable, S.H. Snyder, Odorant-binding protein and its mRNA are localized to lateral nasal gland implying a carrier function, *Proc. Natl. Acad. Sci. U. S. A.* 85 (1988) 2383–2387.
- [8] M. Tegoni, P. Pelosi, F. Vincent, S. Spinelli, V. Campanacci, S. Grolli, R. Ramoni, C. Cambillau, Mammalian odorant binding proteins, *Biochim. Biophys. Acta* 1482 (2000) 229–240.
- [9] D. Pes, M. Mameli, I. Andreini, J. Krieger, M. Weber, H. Breer, P. Pelosi, Cloning and expression of odorant-binding proteins 1a and 1b from mouse nasal tissue, *Gene* 212 (1998) 49–55.
- [10] J. Pevsner, R.R. Reed, P.G. Feinstein, S.H. Snyder, Molecular cloning of odorant-binding protein: member of a ligand carrier family, *Science* 241 (1988) 336–339.
- [11] L. Briand, C. Nespoulous, V. Perez, J.J. Remy, J.C. Huet, J.C. Pernollet, Ligand-binding properties and structural characterization of a novel rat odorant-binding protein variant, *Eur. J. Biochem.* 267 (2000) 3079–3089.
- [12] R.J. Beynon, J.L. Hurst, Urinary proteins and the modulation of chemical scents in mice and rats, *Peptides* 25 (2004) 1553–1563.
- [13] A. Cavaggioni, C. Mucignat-Caretta, Major urinary proteins, α_{2U} -globulins and aphrodisin, *Biochim. Biophys. Acta* 1482 (2000) 218–228.
- [14] A.G. Singer, F. Macrides, A.N. Clancy, W.C. Agosta, Purification and analysis of a proteinaceous aphrodisiac pheromone from hamster vaginal discharge, *J. Biol. Chem.* 261 (1986) 13323–13326.
- [15] L. Briand, J.C. Huet, V. Perez, G. Lenoir, C. Nespoulous, Y. Boucher, D. Trotier, J.C. Pernollet, Odorant and pheromone binding by aphrodisin, a hamster aphrodisiac protein, *FEBS Lett.* 476 (2000) 179–185.
- [16] L. Briand, F. Blon, D. Trotier, J.C. Pernollet, Natural ligands of hamster aphrodisin, *Chem. Senses* 29 (2004) 425–430.
- [17] S. Marchese, D. Pes, A. Scaloni, V. Carbone, P. Pelosi, Lipocalins of boar salivary glands binding odours and pheromones, *Eur. J. Biochem.* 252 (1998) 563–568.
- [18] B. Redl, P. Holzfeind, F. Lottspeich, cDNA cloning and sequencing reveals human tear prealbumin to be a member of the lipophilic ligand carrier protein superfamily, *J. Biol. Chem.* 267 (1992) 20282–20287.
- [19] H. Lassagne, A.M.F. Gachon, Cloning of a human lacrimal lipocalin secreted in tears, *Exp. Eye Res.* 56 (1993) 605–607.
- [20] B.J. Glasgow, A.R. Abduragimov, Z.T. Farahbakhsh, K.F. Faull, W.L. Hubbell, Tear lipocalins bind a broad array of lipid ligands, *Curr. Eye Res.* 14 (1995) 363–372.
- [21] M. Garibotti, H. Christiansen, H. Schmale, P. Pelosi, Porcine VEG proteins and tear prealbumins, *Chem. Senses* 20 (1995) 69–76.
- [22] B. Redl, Human tear lipocalin, *Biochim. Biophys. Acta* 1482 (2000) 241–248.
- [23] B.J. Glasgow, G. Marshall, O.K. Gasymov, A.R. Abduragimov, T.N. Yusifov, C.M. Knobler, Tear lipocalins: potential lipid scavengers for the corneal surface, *Invest. Ophthalmol. Visual Sci.* 40 (1999) 3100–3107.
- [24] A.M.F. Gachon, E. Lacazette, Tear lipocalin and eye's front line of defence, *Br. J. Ophthalmol.* 82 (1998) 453–455.
- [25] M. Fluckinger, H. Haas, P. Merschak, B.J. Glasgow, B. Redl, Human tear lipocalin exhibits antimicrobial activity by scavenging microbial siderophores, *Antimicrob. Agents Chemother.* 48 (2004) 3367–3372.
- [26] M. Lechner, P. Wojnar, B. Redl, Human tear lipocalin acts as an oxidative stress induced scavenger of potentially harmful lipid peroxidation products in a cell culture system, *Biochem. J.* 356 (2001) 129–135.
- [27] B. Redl, P. Wojnar, H. Ellemunter, H. Feichtinger, Identification of a lipocalin in mucosal glands of the human tracheobronchial tree and its enhanced secretion in cystic fibrosis, *Lab. Invest.* 78 (1998) 1121–1129.
- [28] P.K. De, Sex-hormonal regulation of 20.5 and 24 kDa major male-specific proteins in Syrian hamster submandibular gland, *J. Steroid Biochem. Mol. Biol.* 58 (1996) 183–187.
- [29] E. Thavathiru, N.R. Jana, P.K. De, Abundant secretory lipocalins displaying male and lactation-specific expression in adult hamster submandibular gland: cDNA cloning and sex hormone-regulated repression, *Eur. J. Biochem.* 266 (1999) 467–476.
- [30] V. Ranganathan, P.K. De, Androgens and estrogens markedly inhibit expression of a 20-kDa major protein in hamster exorbital lacrimal gland, *Biochem. Biophys. Res. Commun.* 208 (1995) 412–417.
- [31] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, *Nature* 227 (1970) 680–685.
- [32] O.H. Lowry, N.J. Rosbrough, A.L. Farr, R.J. Randall, Protein measurement with the Folin phenol reagent, *J. Biol. Chem.* 193 (1951) 265–275.
- [33] J. Sambrook, E.F. Fritsch, T. Maniatis, *Molecular Cloning: a Laboratory Manual*, vol. 1, Cold Spring Harbour Laboratory Press, 1989, pp. 7.39–7.52.
- [34] R. Baranczuk, G.S. Greenwald, Plasma levels of estrogen and progesterone in pregnant and lactating hamsters, *J. Endocrinol.* 63 (1974) 125–135.

- [35] V. Ranganathan, P.K. De, Western blot of proteins from Coomassie-stained polyacrylamide gels, *Anal. Biochem.* 234 (1996) 102–104.
- [36] M. Kozak, An analysis of 5'-noncoding sequences from 699 vertebrate messenger RNAs, *Nucleic Acids Res.* 15 (1987) 8125–8148.
- [37] G. von Heijne, Patterns of amino acids near signal-sequence cleavage sites, *Eur. J. Biochem.* 133 (1983) 17–21.
- [38] S.G. Remington, J.D. Nelson, Secretoglobins: sexually dimorphic expression of androgen-binding protein mRNA in mouse lacrimal glands, *Invest. Ophthalmol. Visual Sci.* 46 (2005) 31–38.
- [39] J. Alvarez, J. Vinas, J.M. Alonso, J.P. Albar, K. Ashman, P. Dominguez, Characterization and cloning of two isoforms of heteroglobin, a novel heterodimeric glycoprotein of the secretoglobin–uteroglobin family showing tissue-specific and sex differential expression, *J. Biol. Chem.* 277 (2002) 233–242.
- [40] T. Endo, K. Ikeo, T. Gojobori, Large-scale search for genes on which positive selection may operate, *Mol. Biol. Evol.* 13 (1996) 685–690.
- [41] L.G. Sheflin, E.M. Brooks, B.P. Keegan, S.W. Spaulding, The cytosolic 47-kilodalton protein that binds to the 3'untranslated region of epidermal growth factor transcripts responds to orchiectomy in a tissue-specific fashion, *Endocrinology* 137 (1996) 5616–5623.
- [42] H.J. Magert, T. Hadrys, A. Cieslak, A. Groger, S. Feller, W.G. Forssmann, cDNA sequence and expression pattern of putative pheromone carrier aphrodosin, *Proc. Natl. Acad. Sci. U. S. A.* 92 (1995) 2091–2095.
- [43] A.A. Dunne, L. Steinke, A. Teymoortash, C. Kuropkat, B.J. Folz, J.A. Werner, The lymphatic system of the major head and neck glands in rats, *Otolaryngol. Pol.* 58 (2004) 121–130.
- [44] G.S. Greenwald, Endocrinology of the pregnant hamster, in: H.I. Siegel (Ed.), *The Hamster, Reproduction and Behaviour*, Plenum Press, New York, 1985, pp. 53–72.
- [45] A. Hida, Y. Uchijima, Y. Seyama, Sexual differences in branched chain amino acid metabolism into fatty acids and cholesterol in Harderian gland of golden hamster, *J. Biochem. (Tokyo)* 124 (1998) 648–653.
- [46] A. Coto-Montes, J.A. Boga, C. Tomas-Zapico, M.J. Rodriguez-Colunga, J. Martinez-Fraga, D. Tolivia-Cadreja, G. Menendez, R. Hardeland, D. Tolivia, Porphyrin enzymes in hamster Harderian gland, a model of damage by porphyrins and their precursors. A chronological study on the role of sex differences, *Chem. Biol. Interact.* 134 (2001) 135–149.
- [47] G.R. Buzzell, Sexual dimorphism in the Harderian gland of the Syrian hamster is controlled and maintained by hormones, despite seasonal fluctuations in hormone levels: functional implications, *Microsc. Res. Tech.* 34 (1996) 133–138.
- [48] A. Payne, The Harderian gland: a tercentennial review, *J. Anat.* 185 (1994) 1–49.
- [49] M. Kohn, H. Kehrner-Sawatzki, W. Vogel, J.A. Graves, H. Hameister, Wide genome comparisons reveal the origins of the human X chromosome, *Trends Genet.* 20 (2004) 598–603.
- [50] M.D. Ganformina, D. Sanchez, M.J. Bastiani, Lazarillo, a new GPI-linked surface lipocalin, is restricted to a subset of neurons in the grasshopper embryo, *Development* 121 (1995) 123–134.