

Secretoglobins: Lacrimal Gland-Specific Rabbit Lipophilin mRNAs

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PURPOSE. RNAs encoding secretoglobulin subunits, also known as lipophilins, are found in rabbit lacrimal glands. Some secretoglobins in other species are tissue- and/or sex-specific, whereas other secretoglobins are expressed in a variety of tissues of both sexes. In this study the tissue-specific and sexual dimorphic expression patterns of rabbit lacrimal gland lipophilins AL, AL2, BL, CL, and CL2 were determined.

METHODS. RNAs from male and female rabbit lacrimal glands were compared in a differential display analysis, and a new rabbit lacrimal gland secretoglobulin, lipophilin AL2, was identified. Next, RNAs from male and female rabbit harderian, lacrimal, mandibular, sublingual, and parotid glands and from liver, kidney, pancreas, testis (male), ovary, and mammary gland (female) were isolated, electrophoresed in agarose gels, and transferred to nylon membranes. cDNA probes encoding the lipophilins AL, AL2, BL, and CL/CL2 were hybridized to the RNA in the blots.

RESULTS. Rabbit mRNAs encoding the lipophilins AL, AL2, BL, CL, and CL2 were detected only in the lacrimal gland. Lipophilin AL2 mRNA was detected only in male rabbit lacrimal gland.

CONCLUSIONS. In the rabbit, several lipophilins were expressed only in the lacrimal gland. (*Invest Ophthalmol Vis Sci.* 2008;49:2856–2862) DOI:10.1167/iovs.07-1496

The ocular tear film coats the corneal and conjunctival epithelia, providing lubrication and protection for the eye surface. The tear film is an aqueous-mucin gel, covered by a thin lipid layer at the air-fluid interface. Lacrimal glands secrete the bulk of the fluid phase, consisting primarily of water, ions and a variety of dissolved proteins. Goblet cells in the conjunctival epithelium supply many dispersible mucins, proteins with large carbohydrate moieties, whereas corneal and conjunctival epithelial cells express membrane-bound mucins. Meibomian glands embedded in the eyelid margin secrete tear film lipids. The intraocular harderian glands of nonprimate vertebrates also contribute tear proteins and lipids.

Among the proteins present in the aqueous component of tears are members of the secretoglobulin family.¹ Secretoglobins are small, multimeric proteins secreted in the mucosa of mammalian epithelial tissues.² Uteroglobulin represents the founding member of this family,³ designated SCGB 1A1 (see the phylogenetic tree available through the Human Genome Organisa-

tion Gene Nomenclature Committee [HGNC] at http://www.genenames.org/genefamily/scgb_tree.pdf). Homodimers of uteroglobulin are present in the uterus, the lung (known as CCSP), and several other tissues.⁴ Earlier studies have shown that uteroglobulin binds progesterone, inhibits phospholipase A2 and exhibits anti-inflammatory or immunomodulatory properties.^{5–7}

Several investigators have shown that the secretoglobins in saliva and tears are composed of multiple, nonidentical subunits in the form of heterodimers and heterotetramers.^{1,8–10} In mouse salivary glands, the secretoglobins, known as androgen-binding proteins (ABPs), are composed of three subunits (α , β , and γ), and form α - β and α - γ heterodimers.¹¹ The corresponding genes are designated *abpa*, *abpb*, and *abpg*. Mouse lacrimal glands express a distinct set of ABP subunits encoded by *abpd*, *abpe*, *abpz*, and *abph*.¹² The secretoglobulin genes of mouse salivary and lacrimal glands, along with cat salivary gland Fel dI,¹³ belong to two branches of the secretoglobulin family tree: SCGB 1B and its heterodimer partner designated 1C (see the phylogenetic tree available through HGNC at http://www.genenames.org/genefamily/scgb_tree.pdf). Mouse salivary ABP multimers bind steroid hormones^{14,15} and have been proposed to function as pheromones.^{16,17}

In human lacrimal glands and tears, two different secretoglobulin subunits have been identified belonging to the 1D and 2A SCGB branches: lipophilins A and C, also known as SCGB1D1 and SCGB2A1, respectively.¹ In rabbit lacrimal glands, the mRNAs for five lipophilin subunits have been identified (GenBank accession numbers AF308614, AF308617, AF308618, AY063770, and AY303698; <http://www.ncbi.nlm.nih.gov/Genbank>; provided in the public domain by the National Center for Biotechnology Information, Bethesda, MD), belonging to the same 1D and 2A branches of the secretoglobulin tree. (Other lipophilin sequences of the 1D and 2A branches have been found in rabbit salivary glands and prostate; GenBank accession numbers AF308615, AF308619 and AF308620.) Rabbit lipophilin proteins are secreted in tears as 17- to 20-kDa covalent-linked heterodimers, which associate as noncovalent heterotetramers of approximately 36.5 kDa.^{18,19}

Several secretoglobulin subunits exhibit sexually dimorphic expression in salivary or ocular glands; examples include cat Fel dI,^{20,21} hamster heteroglobulin,^{10,22} the C3 component of rat prostatein,⁸ and mRNAs encoding the mouse ABP- δ and ABP- η .¹² Individual secretoglobulin subunits also differ widely in their tissue distribution. Some, such as SCGB1A1 (uteroglobulin) and SCGB2A1 (also known as mammaglobin B) in humans, are expressed in a variety of tissues throughout the body; whereas human SCGB1D1 is found primarily in the eye (based on GenBank human EST [expressed sequence tag] database entries, 2007). Likewise in the mouse, secretoglobins ABP- δ and ABP- ζ are expressed in multiple tissues, whereas lacrimal gland ABP- ϵ and ABP- η , show tissue expression limited to the lacrimal gland.¹²

In this study, we characterized the mRNA expression patterns of five rabbit lacrimal gland secretoglobins in RNA blots of male and female rabbit tissues. The lipophilins AL, AL2, and BL (SCGB1D branch) and CL and CL2 (SCGB2A branch) exhibited lacrimal gland-specific expression in the rabbit. In addi-

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tion, lipophilin AL2 mRNA was expressed only in male rabbit lacrimal glands.

MATERIALS AND METHODS

Animals

New Zealand (NZ) rabbits (2–3 kg; Bakkom Rabbitry, Viroqua, WI) and Dutch Belted (DB) rabbits (1–2 kg; Birchwood Farms, Red Wing, MN) were treated in accordance with the ARVO Statement for the Care and Use of Animals in Ophthalmic and Vision Research. Male and female rabbits were euthanatized and the following tissues were collected: lacrimal, harderian, mandibular, sublingual, and parotid glands and liver, kidney, pancreas, testis, mammary gland, and ovary. The tissues were frozen in liquid nitrogen and stored at -70°C until needed.

RNA Isolation

Poly(A)⁺ RNA was isolated from 125 mg of male and female NZ lacrimal glands in a single-step procedure (PolyAtract System 1000; Promega, Madison, WI). Poly(A)⁺ RNA from lacrimal glands was reverse transcribed into cDNA, as previously reported.²³

Total RNA was isolated from approximately 100 mg of each DB and NZ tissue (TRIzol Reagent; Invitrogen Life Technologies, Carlsbad, CA, Phase Lock Gel-Heavy; Brinkmann Instruments, Inc., Westbury, NY).

Differential Display-PCR and Gel Analysis

We compared male and female NZ lacrimal gland cDNAs using differential display-polymerase chain reaction (DDRT-PCR) with three downstream primers and 24 upstream primers (72 combinations; National Biosciences, Plymouth, MN).²⁴ Experimental details have been published.²³ Briefly, male and female rabbit cDNAs were amplified with each primer combination, PCR products were electrophoresed in nondenaturing polyacrylamide gels, and the DNA was silver stained (Promega). Differential fragments were excised and eluted from hydrated gels, and reamplified using the original primer set. The resultant PCR products were subcloned into a plasmid vector (pCR2.1; Invitrogen) and sequenced (Silver Sequence DNA Sequencing System; Promega).

RACE-PCR

The complete cDNA sequence of lipophilins AL2 and BL were obtained using the rapid amplification of cDNA ends (RACE) technique.²⁵ cDNA was synthesized with AMV reverse transcriptase (Promega) after priming poly(A)⁺ RNA from male and female NZ rabbit lacrimal glands with a sequence-specific oligonucleotide (Sigma Genosys; The Woodlands, TX) for 5' RACE (lipophilin AL2 and BL), or with oligo-d(T)₁₇-adapter primer for 3' RACE (lipophilin BL). Gene-specific PCR primers are listed in Table 1; the adapter primer was 5'-GACTCGAGGATC-CAAGC-3' and the oligo-d(T)₁₇-adapter primer was 5'-GACTCGAG-GATCCAAGC(T)₁₇-3'.

DNA polymerase (AmpliTag; Applied Biosystems, Foster City, CA) was used for all amplifications. The PCR reaction mixtures were denatured at 94°C for 2 minutes, then cycled 30 times in a DNA thermal cycler (model 480; Perkin-Elmer, Wellesley, MA) as follows: 94°C for 45 seconds, 52°C for 1 minute, and 72°C for 2 minutes. The last cycle

was incubated at 72°C an additional 10 minutes. The PCR products were subcloned into plasmid vector pCR2.1.

Sequence-Specific PCR

Sequence-specific primers were designed by visual inspection of an alignment of the rabbit lipophilin CL and CL2 sequences. For CL, the sense oligonucleotide was 5'-AGTACATCCTCACTGATGCCG-3' and the antisense oligonucleotide was 5'-CTGCAGTTGATTGGATGCC-3'; for CL2, the sense oligonucleotide was 5'-TCATCGACACTGAG-CAAAC-3' and the antisense oligonucleotide was 5'-TTCTGCCAGTC-CACAGTCG-3'. The PCR reaction mixtures were denatured at 95°C for 1 minute, then cycled 30 times in the DNA thermal cycler as follows: 95°C for 30 seconds, 60°C for 60 seconds, and 72°C for 90 seconds. The last cycle was incubated at 72°C an additional 5 minutes. The products were electrophoresed in an 8% nondenaturing polyacrylamide gel and silver stained.

Cloning and Sequencing

The AL2 RACE-PCR product was electrophoresed in an agarose gel, extracted, and cloned into the plasmid vector pCR2.1 (Invitrogen). Double-stranded plasmid inserts were sequenced (Silver Sequence DNA Sequencing System; Promega).

Sequence Analyses

Sequence information was organized by computer (GeneWorks suite; IntelliGenetics, Campbell, CA). The translation start sites and signal peptide cleavage site were predicted by using NetStart 1.0²⁶ and SignalP 3.0,^{27,28} respectively. (NetStart and SignalP are web-based programs provided in the public domain by the Center for Biological Sequence Analysis, Technical University of Denmark, Lyngby, Denmark and are available at <http://www.cbs.dtu.dk/services/>.) Sequences were compared with those in the GenBank databases using the BLAST programs²⁹ (provided in the public domain by the National Center for Biotechnology Information (NCBI), Bethesda MD, available at <http://www.ncbi.nlm.nih.gov/blast/>). ClustalW was used to align nucleic acid and amino acid sequences.³⁰ (ClustalW is provided in the public domain by the European Bioinformatics Institute at EMBL, Hinxton, UK, available at <http://www.ebi.ac.uk/tools/>.)

RNA Blots

Poly(A)⁺ RNA (2 μg per lane) or total RNA samples (10 μg per lane) were denatured in 50% formamide and 6% formaldehyde in low ionic strength buffer (20 mM phosphate, pH 7.7) at 65°C for 5 minutes. The samples were electrophoresed through a 1.8% agarose gel containing 6% formaldehyde in 20 mM phosphate buffer (pH 7.7). RNA standards (Life Technologies, Rockville, MD) were visualized by staining a portion of the gel with 0.5 $\mu\text{g}/\text{mL}$ ethidium bromide. The RNA in the remainder of the gels was capillary transferred to GeneScreen (NEN Life Science Products, Boston, MA) using $10\times$ SSC (1.5 M NaCl, 0.15 M sodium citrate, pH 7.0). Prehybridizations and overnight hybridizations were in 50% formamide at 42°C (39°C for lipophilin BL) according to the manufacturer's protocol.

The rabbit sequences represented in the lipophilin probes are listed in Table 2. Lipophilin clones were digested with appropriate

TABLE 1. RACE-PCR Primers

Clone/Accession No.	Reverse Transcription Primer*	Bases	PCR Primer*	Bases
5' RACE				
Lipophilin AL2/AY063770	5'-AAGCAAGATGTTGAAACC-3'	417–434	5'-AGATAGTAGAAAGCAAGGC-3'	363–381
Lipophilin BL/AY303698	5'-GAAAGCAATTATTTATTAATC-3'	507–528	5'-CATCTCAAATCATGTGTTGTG-3'	486–506
3' RACE				
Lipophilin BL/AY303698			5'-TCACTTTCCTTGCAATTCCTC-3'	440–459

* For 5' RACE, the primer sequences for the respective clones represent the complementary strands.

TABLE 2. Northern Blot Probes

Lipophilin Probe	Bases	GenBank Accession No.
AL (SCGB1D)	115–407	AF308614
AL2(SCGB1D)	315–454	AY063770
BL (SCGB1D)	459–532	AY303698
CL2(SCGB2A)	296–503	AF308618

restriction enzymes, the desired fragments were purified in agarose gels, and each fragment was resubcloned before isolation and labeling.

The actin probe was a 1.5-kb mouse α -actin fragment (Stratagene, La Jolla, CA). The G3PDH probe was 1-kb fragment of human glycerol-3-phosphate dehydrogenase (BD-Clontech, Palo Alto, CA). DNA fragments were random prime-labeled with [α - ^{32} P]dCTP (NEN Life Science Products) using the a gene labeling system (Prime-a-Gene; Promega). The labeled probes were denatured in a boiling water bath for 5 minutes and added to fresh hybridization solution at 1 to 6×10^6 cpm/mL for overnight incubations.

The blots were washed several times in $2\times$ SSC, 1% SDS at 60°C (55°C for mouse actin; 52°C for lipophilin BL) and finally in $0.2\times$ SSC at room temperature. The blots were exposed to XAR film (Eastman Kodak Co., Rochester, NY) for 2 to 4 hours (lipophilins AL and CL2) or to XAR film with an intensifying screen at -70°C for approximately 1 week (lipophilins AL2 and BL). After each hybridization, the blots were stripped in boiling $0.1\times$ SSC with 1% SDS.

RESULTS

New Rabbit Lacrimal Gland Secretoglobulin: Lipophilin AL2

We compared mRNA sequences of male and female rabbit lacrimal glands using DDRT-PCR. From male lacrimal glands, we isolated a sequence fragment encoding a previously unidentified rabbit lipophilin mRNA, predicted to encode a SCGB1D protein subunit. We obtained the full-length sequence of the new lipophilin, which we named lipophilin AL2 (GenBank accession no. AY063770). Two other members of the SCGB1D branch had already been identified from rabbit lacrimal glands: lipophilins AL and BL. We also obtained a full-length sequence for lipophilin BL, which included the 5' untranslated region (GenBank accession no. AY303698).

We compared the cDNA sequence of lipophilin AL2 with other rabbit lipophilin sequences of the SCGB1D branch. The 3' untranslated region of lipophilin AL2 contains a 40-base sequence element, bases 410 to 450, conserved among SCGB1D mRNAs of human, rabbit and rat (Fig. 1).

The deduced amino acid sequence of lipophilin AL2, bases 73 to 363, encodes a 96 residue preprotein. SignalP predicts cleavage of a signal peptide after serine 21, yielding a 75-amino-acid mature protein subunit with a calculated pI of 4.8.

We aligned the deduced amino acid sequence of lipophilin AL2 with those of other rabbit and human members of the SCGB1D branch (Fig. 2). For reference, we included an alignment of rabbit and human members of the SCGB2A branch. Rabbit lacrimal gland lipophilins CL and CL2 were available in sequence databases. Note that each predicted mature subunit contains three conserved cysteine residues, available to participate in disulfide bond formation in secretoglobin heterodimers or heterotetramers.

Male-Specific Expression of Lipophilin AL2

We identified rabbit lipophilin AL2 in a sex-based differential expression screen of lacrimal gland mRNAs. To confirm the male-specific expression of lipophilin AL2, we hybridized a labeled 3' lipophilin AL2 cDNA fragment (Table 2) to blots of RNA from lacrimal glands of male and female NZ and DB rabbits (Fig. 3). 3'-Lipophilin AL2 hybridized to RNA from male rabbit lacrimal glands, but not to RNA from female lacrimal glands. In contrast, the cDNA fragments of lipophilins AL and BL hybridized to RNA from male and female lacrimal glands.

In addition to lipophilins AL, AL2, and BL of the SCGB1D branch, we also subcloned fragments of rabbit lipophilin CL2 of the SCGB2A branch. A labeled cDNA fragment of lipophilin CL2 hybridized to RNA from both male and female lacrimal glands (Fig. 3). Note that the lipophilin CL2 probe probably cross-hybridized with the lipophilin CL sequence.

Lacrimal Gland-Specific Expression of Lipophilins AL, AL2, BL, CL, and CL2

To address sex and tissue specificity of the rabbit lacrimal gland lipophilins, we hybridized labeled cDNA fragments of lipophilins AL, BL, and CL2 to RNA blots of NZ rabbit male and female tissues (Fig. 4). For this experiment, we specifically collected several salivary glands from one male and one female NZ rabbit, since other lipophilins (AS and CS) have been identified in rabbit salivary glands (see Fig. 2 legend). Lipophilins AL, BL, and CL2 hybridized to RNA from male and female lacrimal glands as found previously for both DB and NZ rabbits (Figs. 3, 4). None of the lacrimal gland lipophilin sequences hybridized with RNA from any of the other rabbit tissues tested (Fig. 4). Unexpectedly, lipophilin AL2 did not hybridize (data not shown) to the lacrimal gland RNA of the adult male NZ rabbit, from which we isolated the tissues for the RNA blot depicted in Figure 4. Actin and G3PDH control hybridizations demonstrated that each lane of the RNA blots contained intact RNA (data not shown).

The lipophilin CL2 cDNA fragment likely cross-hybridized with lipophilin CL. To address whether lipophilins CL and CL2 were each expressed in male and female lacrimal glands, we amplified male and female rabbit lacrimal gland cDNA with sequence-specific primers. Lipophilin CL primers yielded a 197-base product and lipophilin CL2 primers a 204-base prod-

3' UTR

rb lip AL2	AATGTAAAGGTTTCAACATCTTGCTTTAATAAATTACTT	(409–447; AY063770)
hs SCGB1D2	AATGTAAAGGTTTCAACGCTCTTGCTTTAATAAATCACTT	(392–430; NM_006551)
hs SCGB1D1	AATGTAAAGGTTTCAACGCTCTTGCTCTAATAAATCACTT	(385–423; NM_006552)
hs SCGB1D4	--TGTAAGGTTTCAACACGTTGCTTTAATAAATCACTT	(392–427; NM_206998)
rb lip AL	--TGTAAGGTTTCAACGCTCTTGCT--TAATAAATTACTT	(366–401; AF308614)
rb lip BL	-ATATAAGGTTTCAACGCTCTT-CTCTAATAAATCACTT	(409–445; AY303698)
rat C2	AATGTGAAGGTTTCAATATCTTGCACTAATAAATTACT-	(379–416; BC099748)

FIGURE 1. Alignment of 3' untranslated region (UTR) nucleic acid sequences from rabbit lipophilins, human SCGBs, and the C2 component of rat prostatein, all members of the SCGB1D branch. Underscored bases indicate the conserved polyadenylation signal. Nucleic acids are shaded when identical in at least five of the seven sequences.

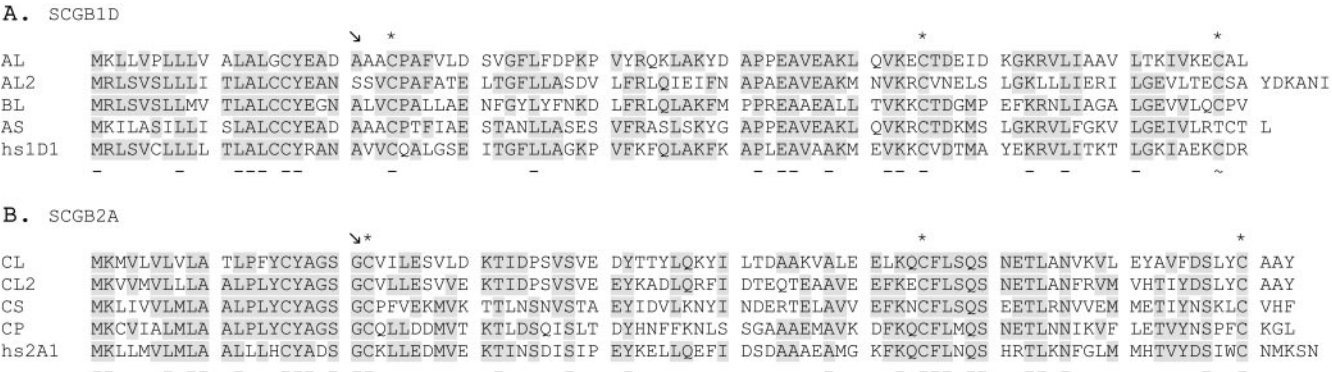


FIGURE 2. Clustal W alignments of the deduced amino acid sequences of rabbit lipophilins of the (A) SCGB1D and (B) SCGB2A branches, aligned with their respective human lacrimal gland counterparts. Identical amino acids are shaded when common to three or more sequences. *Underscored* columns denote positions in which all five amino acids are identical. *Arrows*: predicted signal peptide cleavage sites. *Asterisks*: cysteine residues present in the mature peptides. (NCBI database accession numbers: (A) AL: AAG42802; AL2: AY063770; BL: AAG42804; AS: AAG42803; hs1D1: NP_006543; (B) CL: AAG42805; CL2: AAG42806; CS: AAG42808; CP: AAG42807; hs2A1: NP_002398.)

uct, each from cDNAs generated from male and female NZ rabbit lacrimal glands (data not shown). Mixed primer pairs did not yield amplified products.

We also tested the tissue specificity of rabbit lacrimal gland lipophilins in an RNA blot of DB harderian, lacrimal, and salivary glands; liver; and pancreas (data not shown). The lacrimal gland lipophilins AL, AL2, BL, CL, and CL2 were

expressed only in rabbit lacrimal gland tissues in both DB and NZ rabbits.

DISCUSSION

Lacrimal Gland-Specific Expression

Rabbit lacrimal glands expressed mRNAs for five secretoglobins belonging to the 1D and 2A branches: lipophilins AL, AL2, and BL (1D) and lipophilins CL and CL2 (2A). Individual rabbit secretoglobins exhibited sequence conservation across species in the protein coding regions. In addition, a 40-base motif in the 3' untranslated region of SCGB1Ds was 84% identical among rabbit, human, and rat sequences. Remarkable conservation of the untranslated regions of secretoglobins has been noted previously for members of the SCGB1D branch in rat³¹ and hamster¹⁰ and for mouse secretoglobins known as ABPs.^{12,32}

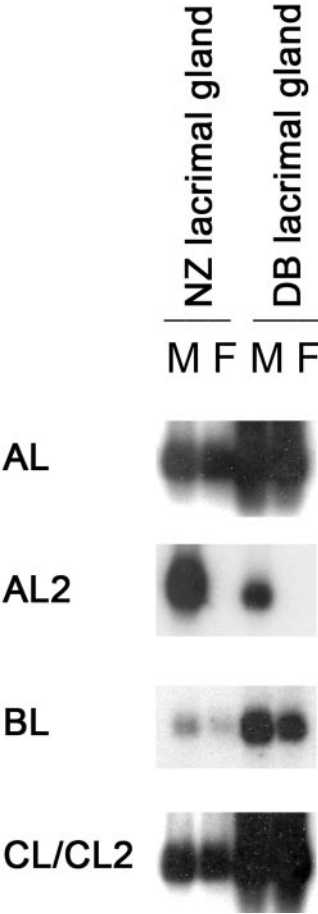


FIGURE 3. Northern blot of lacrimal gland poly(A)⁺ RNA from juvenile male and female NZ and DB rabbits hybridized to probes from lipophilins AL, AL2, BL, and CL2 (Table 2). Each labeled RNA band is approximately 500 bases in length.

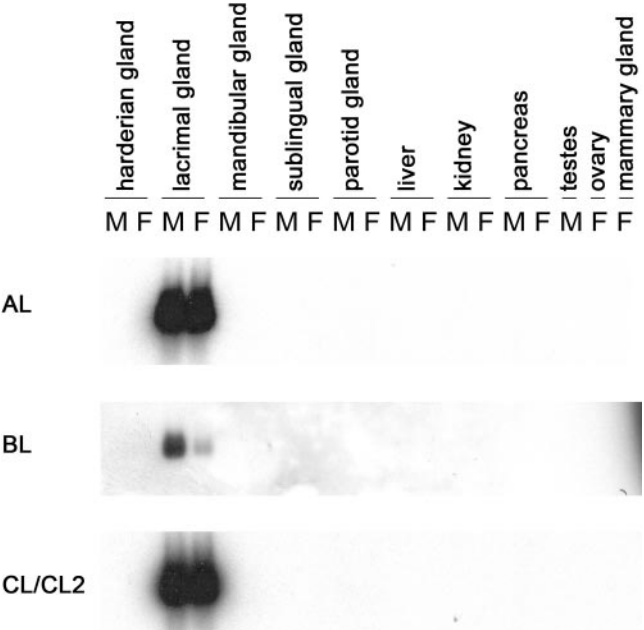


FIGURE 4. Northern blot of total RNA from adult male and female NZ rabbit tissues hybridized to probes from lipophilins AL, BL, and CL2 (Table 2). Each labeled RNA band is approximately 500 bases in length.

In RNA blots of several different rabbit tissues, we found expression of each of the rabbit lipophilins AL, AL2, BL, CL, and CL2 only in lacrimal glands. We did not find evidence for the expression of these lipophilins in rabbit harderian, mandibular, sublingual, parotid, or mammary glands or in heart, liver, kidney, pancreas, ovary, or testis.

We have observed a similarly restricted secretoglobin expression pattern in the mouse. In previous work, we found lacrimal gland-specific expression of mRNAs encoding several mouse secretoglobins, known as ABP- δ , - ϵ , - ζ , and - η .¹² In RNA blots of mouse eye and salivary glands, we documented the expression of the corresponding genes *abpd*, *abpe*, *abpz*, and *apbb* only in lacrimal glands. Although RNA blotting data suggested restricted expression of the mouse lacrimal gland secretoglobins, EST data implied broader tissue distribution of two of these lacrimal gland secretoglobins: the genes *abpd* and *abpz*.

Expression patterns for individual secretoglobin subunits differ widely. In contrast to the apparent restricted expression pattern we observed for rabbit lacrimal gland lipophilins and some mouse ABPs, there is evidence that several secretoglobins in various species are expressed in both ocular glands and other tissues. In the rat, lacrimal gland and prostate express the mRNA and protein product for the C3 component of prostectin.^{8,33} Hamster heteroglobin oligonucleotide probes hybridize to RNA from lacrimal, parotid, and submaxillary glands.¹⁰ In the cat, monoclonal antibodies to Fel dI indicate that this allergen is secreted in cat tears as well as saliva.³⁴ In the NOD mouse, PCR amplification studies have shown that both lacrimal and salivary glands express LGP10 (77% identical with ABP- η), identified as a Sjögren's syndrome autoantigen.³⁵ (Note that mouse salivary glands also express a distinct set of secretoglobin subunit sequences, which include *abpa*, *abpb*, and *abpg*.¹¹)

In human lacrimal glands, Zhao et al.³⁶ identified two secretoglobin subunits, SCGB1D1 and SCGB2A1, and reported broad tissue expression for the genes encoding both subunits. Human ESTs for SCGB2A1 were found in the eye and in a variety of other tissues, including brain, pancreas, thyroid, kidney, muscle, mammary gland, ovary, uterus, prostate, and testis. In contrast, human ESTs for SCGB1D1 have been found only in the eye, probably corresponding to lacrimal gland mRNA expression (based on GenBank human EST database entries, 2007). Different detection methods may be responsible for some of the contradictory findings.

There is precedent in the literature for either broad or highly restricted tissue expression patterns of individual secretoglobins. Although our rabbit RNA blots may have missed some low-level expression, we found limited expression, confined to the lacrimal glands, of each of five rabbit secretoglobins: lipophilins AL, AL2, BL, CL, and CL2.

Sexual Dimorphism

Of the five secretoglobins expressed in rabbit lacrimal glands, we detected the mRNA for one of them, lipophilin AL2, only in male rabbit lacrimal glands from juvenile NZ and DB rabbits. However, we were unable to detect lipophilin AL2 expression in the lacrimal glands of one adult male NZ rabbit. The ages of individual animals (juvenile versus adult) may have affected the observed differences in secretogloblin expression. Our data also indicated that male NZ and DB lacrimal glands expressed higher levels of lipophilin BL mRNA than female lacrimal glands (Figs. 3, 4).

Several secretoglobins in other species exhibit sex differences in ocular and/or salivary gland expression. In many cases, sex steroid hormones have been shown to regulate the differential expression. Androgens influence the expression of

the C3 component of prostectin in the rat lacrimal gland. This secretogloblin subunit, expressed in lacrimal glands of male rats, is not found in the lacrimal glands of females or castrated males. Administration of androgens induces mRNA and protein expression of the C3 component of rat prostectin in the lacrimal glands of both females and castrated males.⁸ The developmental ages of animals have also been shown to affect prostectin expression. For example, the levels of RNA encoding the C3 component are age dependent in male rat lacrimal gland (and prostate), reaching a peak at 60 days and declining thereafter.³³

In RNA blots of mouse glands, male lacrimal glands express more RNA encoding two ABP subunits (*scgbd* and *scgbz*) than female glands.¹² In another laboratory, differential display analyses showed that female mouse lacrimal glands express more mRNA for ABP subunit α (*abpa*) than male mouse lacrimal glands.³⁷

Female hamster harderian glands express mRNAs encoding secretogloblin subunits known as heteroglobin, which are undetected in male harderian glands. This female pattern in harderian gland is induced transiently in castrated males and could be stimulated by estradiol administration.³⁸ (Heteroglobin mRNA and protein were detected in parotid and submandibular glands of both sexes.¹⁰)

In cat salivary glands and skin, the major allergen Fel dI is present in both sexes, but is more abundant in male cats.²⁰ Fel dI production is regulated by androgens.^{21,39}

In humans, sex-based differential expression in the lacrimal gland has not been reported for either SCGB1D1 or SCGB2A1.

Secretogloblin Multimer Composition

Human EST database entries indicated that SCGB1D1 expression is limited to the eye. In contrast, the mRNA for SCGB2A1 is found in the eye and a wide variety of tissues throughout the body. The protein subunits corresponding to human SCGB1D1 and SCGB2A1 are both expressed in lacrimal gland and secreted in the tears.^{1,36} Several other tissues within the human eye also react with an antibody to SCGB2A1,⁴⁰ the ocular secretogloblin subunit exhibiting broad EST tissue distribution.

The two protein subunits expressed in human lacrimal glands, SCGB1D1 and SCGB2A1, form disulfide-bonded heterodimers and associate as heterotetramers secreted in tears.¹ In rabbit lacrimal glands, we found mRNAs encoding multiple subunits of the secretogloblin 1D and 2A branches. If these are translated into protein subunits, there is potential for several distinct heterodimer and heterotetramer combinations to exist in rabbit tears. Rabbit tear lipophilin dimers migrate as relatively broad 17- to 20-kDa heterodimers,¹⁹ possibly reflecting heterogeneity in subunit composition. N-glycosylation of rabbit secretogloblin 2A subunits may also contribute to the broad band observed in gel electrophoresis.

Secretogloblin Functions

What functions might these diverse sets of small proteins fulfill? Molecular recognition could provide a plausible explanation for the variability observed in individual secretogloblin subunit sequences and protein multimer composition. Many secretoglobins are expressed in the mucosa at the interface of the organism with its environment. For example, lacrimal gland secretoglobins in tears cover the exposed ocular surface; salivary gland secretoglobins reside in the saliva that coats the oral cavity and constitutes the first contact with ingested substances; lung epithelial cells secrete secretoglobins that inhabit the bronchial mucosa, the interface between inhaled substances and the respiratory system.

Several possible roles for secretogloblin proteins are consistent with their individual compositional variability and tissue

distribution. Putative roles include communication with the external world, either in a form of defense as a constituent of immune recognition systems,^{41,42} and/or in signaling with other organisms.⁴³ In support of these conjectures, uteroglobin/CCSP exhibited anti-inflammatory properties,⁴ and rodent salivary secretoglobins were suggested to function as pheromones or in odorant recognition.^{16,17} The propensity of secretoglobins to bind small hydrophobic molecules, such as steroid hormones, may constitute an integral aspect of their function.^{5,44,45}

There are tissue-specific and sex-specific gene expression differences among members of the secretoglobin family, within species as well as across species. In this study, we characterized five rabbit lacrimal gland-specific lipophilins. These secretoglobins exhibited sequence homology and subunit structure similarities to the secretoglobins found in human lacrimal glands.

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References

- Lehrer RI, Xu G, Abduragimov A, et al. Lipophilin, a novel heterodimeric protein of human tears. *FEBS Lett.* 1998;432:163–167.
- Ni J, Kalf-Suske M, Gentz R, Schageman J, Beato M, Klug J. All human genes of the uteroglobin family are localized on chromosome 11q12.2 and form a dense cluster. *Ann NY Acad Sci.* 2000;923:25–42.
- Nieto A, Ponstingl H, Beato M. Purification and quaternary structure of the hormonally induced protein uteroglobin. *Arch Biochem Biophys.* 1977;180:82–92.
- Mukherjee AB, Zhang Z, Chilton BS. Uteroglobin: a steroid-inducible immunomodulatory protein that founded the secretoglobin superfamily (published correction appears in *Endocr Rev.* 2008;29(1):131). *Endocr Rev.* 2007;28(7):707–725.
- Beato M. Binding of steroids to uteroglobin. *J Steroid Biochem.* 1976;7:327–334.
- Levin SW, Butler JD, Schumacher UK, Wightman PD, Mukherjee AB. Uteroglobin inhibits phospholipase A₂ activity. *Life Sci.* 1986;38:1813–1819.
- Mukherjee AB, Cordella-Miele E, Kikukawa T, Miele L. Modulation of cellular response to antigens by uteroglobin and transglutaminase. *Adv Exp Med Biol.* 1988;231:135–152.
- Vercaeren I, Vanaken H, Devos A, Peeters B, Verhoeven G, Heyns W. Androgens transcriptionally regulate the expression of cystatin-related protein and the C3 component of prostatic binding protein in rat ventral prostate and lacrimal gland. *Endocrinology.* 1996;137:4713–4720.
- Kristensen AK, Schou C, Roepstorff P. Determination of isoforms, N-linked glycan structure and disulfide bond linkages of the major cat allergen Fel d 1 by a mass spectrometric approach. *Biol Chem.* 1997;378:899–908.
- Alvarez J, Viñas J, Alonso JMM, Albar JP, Ashman K, Domínguez P. 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.* 2002;277:233–242.
- Dlouhy SR, Taylor BA, Karn RC. The genes for mouse salivary androgen-binding protein (ABP) subunits alpha and gamma are located on chromosome 7. *Genetics.* 1987;115:535–543.
- Remington SG, Nelson JD. Secretoglobins: sexually dimorphic expression of androgen-binding protein mRNA in mouse lacrimal glands. *Invest Ophthalmol Vis Sci.* 2005;46:31–38.
- Morgenstern JP, Griffith JJ, Brauer AW, et al. Amino acid sequence of Fel dI, the major allergen of the domestic cat: Protein sequence analysis and cDNA cloning. *Proc Natl Acad Sci USA.* 1991;88:9690–9694.
- Dlouhy SR, Karn RC. The tissue source and cellular control of the apparent size of androgen binding protein (Abp), a mouse salivary protein whose electrophoretic mobility is under the control of sex-limited saliva pattern (*Ssp*). *Biochem Genet.* 1983;21:1057–1070.
- Karn RC, Russell R. The amino acid sequence of the alpha subunit of mouse salivary androgen-binding protein (ABP), with a comparison to the partial sequence of the beta subunit and to other ligand-binding proteins. *Biochem Genet.* 1993;31:307–319.
- Laukaitis CM, Critser ES, Karn RC. Salivary androgen-binding protein (ABP) mediates sexual isolation in *Mus musculus*. *Evolution.* 1997;51:2000–2005.
- Emes RD, Riley MC, Laukaitis CM, Goodstadt L, Karn RC, Ponting CP. Comparative evolutionary genomics of androgen-binding protein genes. *Genome Res.* 2004;14:1516–1529.
- Lehrer RI, Nguyen T, Zhao C, Ha CX, Glasgow BJ. Secretory lipophilins: a tale of two species. *Ann N Y Acad Sci.* 2000;923:59–67.
- Glasgow BJ, Abduragimov AR, Gassymov OK, Faull KF, Yusifov TN, Lehrer RI. Characterization of a lipophilin in rabbit tears. *Adv Exp Med Biol.* 2002;506:573–580.
- Wentz PE, Swanson MC, Reed CE. Variability of cat-allergen shedding. *J Allergy Clin Immunol.* 1990;85:94–98.
- Ramadour M, Birnbaum J, Magalon C, Lanteaume A, Charpin D, Vervloet D. Cat sex differences in major allergen production (Fel d 1). *J Allergy Clin Immunol.* 1998;101:282–284.
- Domínguez P. Cloning of a Syrian hamster cDNA related to sexual dimorphism: establishment of a new family of proteins. *FEBS Lett.* 1995;376:257–261.
- Remington SG, Lima PH, Nelson JD. Pancreatic lipase-related protein 1 mRNA in female mouse lacrimal gland. *Invest Ophthalmol Vis Sci.* 1999;40:1081–1090.
- Bauer D, Warthoe P, Rohde M, Strauss M. Detection and differential display of expressed genes by DDRT-PCR. *PCR Methods Appl.* 1994;4:S97–S108.
- Frohman MA. RACE: rapid amplification of cDNA ends. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ, eds. *PCR Protocols: A Guide to Methods and Applications*. San Diego: Academic Press, Inc.; 1990:28–38.
- Pedersen AG, Nielsen H. Neural network prediction of translation initiation sites in eukaryotes: perspectives for EST and genome analysis. *Proc Int Conf Intell Syst Mol Biol.* 1997;5:226–233.
- Nielsen H, Engelbrecht J, Brunak S, von Heijne G. Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. *Protein Eng.* 1997;10:1–6.
- Bendtsen JD, Nielsen H, von Heijne G, Brunak S. Improved prediction of signal peptides - SignalP 3.0. *J Mol Biol.* 2004;340(4):783–795.
- Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. *J Mol Biol.* 1990;215:403–410.
- Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* 1994;22:4673–4680.
- Parker M, Needham M, White R. Prostatic steroid binding protein: gene duplication and steroid binding. *Nature.* 1982;298:92–94.
- Laukaitis CM, Dlouhy SR, Karn RC. The mouse salivary androgen-binding protein (ABP) gene cluster on chromosome 7: characterization and evolutionary relationships. *Mamm Genome.* 2003;14:679–691.
- Vercaeren I, Vanaken H, Van Dorpe J, Verhoeven G, Heyns W. Expression of cystatin-related protein and of the C3-component of prostatic-binding protein during postnatal development in the rat ventral prostate and lacrimal gland. *Cell Tissue Res.* 1998;292:115–128.
- van Milligen FJ, Vroom TM, Aalberse RC. Presence of *Felis domesticus* allergen I in the cat's salivary and lacrimal glands. *Int Arch Allergy Appl Immunol.* 1990;92:375–378.
- Esch TR, Poveromo JD, Aikins MC, Levanos VA. A novel lacrimal gland autoantigen in the NOD mouse model of Sjögren's syndrome. *Scand J Immunol.* 2002;55:304–310.
- Zhao C, Nguyen T, Yusifov T, Glasgow BJ, Lehrer RI. Lipophilins: human peptides homologous to rat prostetatin. *Biochem Biophys Res Commun.* 1999;256:147–155.

37. Richards SM, Jensen RV, Liu M, et al. Influence of sex on gene expression in the mouse lacrimal gland. *Exp Eye Res.* 2006;82:13-23.
38. Varriale B, Alvarez J, Prieto F, Domínguez P. Hormonal regulation of FHG22 mRNA in Syrian hamster harderian glands: role of estradiol. *Mol Cell Endocrinol.* 1996;124:87-96.
39. Zielonka TM, Charpin D, Berbis P, Luciani P, Casanova D, Vervloet D. Effects of castration and testosterone on *Fel d 1* production by sebaceous glands of male cats: I. immunological assessment. *Clin Exp Allergy.* 1994;24:1169-1173.
40. Stoeckelhuber M, Messmer EM, Schmidt C, Xiao F, Schubert C, Klug J. Immunohistochemical analysis of secretoglobin SCGB 2A1 expression in human ocular glands and tissues. *Histochem Cell Biol.* 2005;126:103-109.
41. Hughes AL, Nei M. Pattern of nucleotide substitution at major histocompatibility complex class I loci reveals overdominant selection. *Nature.* 1988;335:167-170.
42. Maxwell AI, Morrison GM, Dorin JR. Rapid sequence divergence in mammalian β -defensins by adaptive evolution. *Mol Immunol.* 2003;40:413-421.
43. Swanson WJ, Vacquier VD. The rapid evolution of reproductive proteins. *Nat Rev Genet.* 2002;3:137-144.
44. Heyns W, DeMoor P. Prostatic binding protein: a steroid-binding protein secreted by rat prostate. *Eur J Biochem.* 1977;78:221-230.
45. Karn RC. Steroid binding by mouse salivary proteins. *Biochem Genet.* 1998;36:105-117.