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cDNA and Genomic Cloning of Lacritin, a Novel Secretion Enhancing Factor from the Human Lacrimal Gland

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Multiple extracellular factors are hypothesized to promote the differentiation of unstimulated and/or stimulated secretory pathways in exocrine secretory cells, but the identity of differentiation factors, particularly those organ-specific, remain largely unknown. Here, we report on the identification of a novel secreted glycoprotein, lacritin, that enhances exocrine secretion in overnight cultures of lacrimal acinar cells which otherwise display loss of secretory function. Lacritin mRNA and protein are highly expressed in human lacrimal gland, moderately in major and minor salivary glands and slightly in thyroid. No lacritin message or protein is detected elsewhere among more than 50 human tissues examined. Lacritin displays partial similarity to the glycosaminoglycan-binding region of brain-specific neuroglycan C (32% identity over 102 amino acid residues) and to the possibly mucin-like amino globular region of fibulin-2 (30% identity over 81 amino acid residues), and localizes primarily to secretory granules and secretory fluid. The lacritin gene consists of five exons, displays no alternative splicing and maps to 12q13. Recombinant lacritin augments unstimulated but not stimulated acinar cell secretion, promotes ductal cell proliferation, and stimulates signaling through tyrosine phosphorylation and release of calcium. It binds collagen IV, laminin-1, entactin/nidogen-1, fibronectin and vitronectin, but not collagen I, heparin or EGF. As an autocrine/paracrine enhancer of the lacrimal constitutive secretory pathway, ductal cell mitogen and stimulator of corneal epithelial cells, lacritin may play a key role in the function of the lacrimal gland-corneal axis.

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Introduction

The polarized exocrine secretory cell is an elegant manifestation of epithelial differentiation, a complex process influenced by mesenchymal interactions[1] and adhesion to elements of the newly formed epithelial basement membrane. A well-studied example is the exocrine pancreas in which basement membrane-coincident[2] acquisition of cell polarity, protein synthetic organelles including subluminal secretory granules, calcium signaling machinery and constitutive secretion is followed by a delayed rab3D-[3,4] and rab4-dependent[5] completion of stimulus-secretion coupling. More complex are salivary glands in which several different parenchymal cells are specified at varying rates in extracellular environments thought to differ subtly.[6]

The regulation of epithelial differentiation in exocrine glands, as examined in gene knockout experiments, appears multifactorial. There is evidence that the TGFβ superfamily has an important role in acinar formation in the exocrine pancreas (type II TGFβ receptor;[7] type II activin receptor[8]) and mammary gland (activins and inhibins[9]). Also involved (mammary gland) are ErbB4,[10] the progesterone receptor,[11] the extracellular matrix glycoprotein osteopontin,[12] and the EGF receptor[13]

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when knocked out with TGFα and amphiregulin.[14] In a similar way, partial or complete knockout of fibroblast growth factor receptor 2 (IIIb) in mammary[15] and salivary gland[16] is associated with impaired acinar formation, or even partial (pancreas), or incomplete (salivary gland) organogenesis.[17] FGF-10 null mice display a complete absence of lacrimal glands, a result in keeping with ectopic lacrimal gland formation in regions of transgenic FGF-10 overexpression.[18,19]

Dissection of the extracellular matrix contribution has taken advantage of primary acinar cell culture in which the delicate and reversible nature of differentiation in polarized secretory cells can become fully apparent. Agonist responsiveness in isolated lacrimal acinar cells is, for example, dependent on adhesion to laminin-1, an effect enhanced by inclusion of a lower molecular mass extracellular matrix fraction enriched in BM180.[20] In a similar way, constitutive casein secretion[21] and insulin-dependent tyrosine phosphorylation[22] by mammary epithelial cells are dependent on contact with a laminin-1 enriched basement membrane. Although a number of exocrine cell lines appear to be fully functional without supplements, several authors have noted a ''differentiative'' morphogenesis by the human submandibular ductal (HSG) cell line on laminin-1,[23] a process reported to be inhibitable with anti-TGFβ3 antibodies.[24] These observations are in keeping with a remarkable tendency of laminin-1 to selectively constitute fetal glandular basement membranes, particularly those of differentiating pancreatic, salivary[25] and mammary[26] glands. Other differentiation factors are expected to exist, particularly those that may confer organ specificity.

We report on the cDNA and genomic cloning, chromosome mapping and initial functional characterization of a new human gene product, lacritin, that has a highly restricted glandular distribution. Lacritin enhances unstimulated but not stimulated secretion, has mitogenic activity and promotes signaling in both lacrimal acinar and corneal epithelial cells. Its highly restricted expression pattern and functional attributes are evidence for its putative autocrine/paracrine differentiative role in the lacrimal gland and neighboring ocular system.

Results

Multiple extracellular factors are hypothesized to modulate the differentiation of stimulated and unstimulated tear secretion pathways in lacrimal acinar cells, a polarized exocrine secretory cell with some mRNAs remarkably under-represented in gene data banks. In a systematic oligonucleotide screen of a human lacrimal gland library, a novel gene product was identified (Figure 1) with a level of lacrimal gland specificity (Figure 2) not previously observed. We refer to this gene product as lacritin. Its 417 bp open reading frame (Figure 1(a)) predicts a 14.3 kDa hydrophilic protein core with a

Figure 1. Lacritin cDNA, deduced protein sequence, and partial similarity to human neuroglycan C and fibulin-2. (a) Lacritin consists of 138 amino acid residues with a putative endoplasmic reticulum signal peptide (underlined, probability score 0.97; SignalP), one N-glycosylation site (PeptideStructure), and six predicted O-glycosylation sites (respective potential 0.62, 0.99, 0.97, 0.88, 0.99 and 0.98; NetOGlyc 2.0), the latter grouped between residues 52 and 64. Lacritin lacks identity with other sequences, even with ESTs (in keeping with an absence of lacrimal-specific ESTs and an expression pattern that is highly restricted). (b) Homology is with the glycosaminoglycan attachment region of neuroglycan C (af059274), and with the cysteine-free N-terminal globular domain (Nb) of fibulin-2 (x82494). BestFit quality of lacritin lacking signal peptide with neuroglycan C or fibulin-2 is 83 (*versus* 37 ± 5 when lacritin sequence is randomized) and 81 (*versus* 38 ± 5), respectively. Shown is lacritin without signal peptide *versus* residues 21 to 130 of neuroglycan C (539 amino acid residues total length) and 217 to 336 of fibulin-2 (1184 amino acid residues total length).

19 amino acid residue signal peptide giving rise to a mature secreted core protein of 12.3 kDa with an isoelectric point of 5. Lacritin displays a moderately
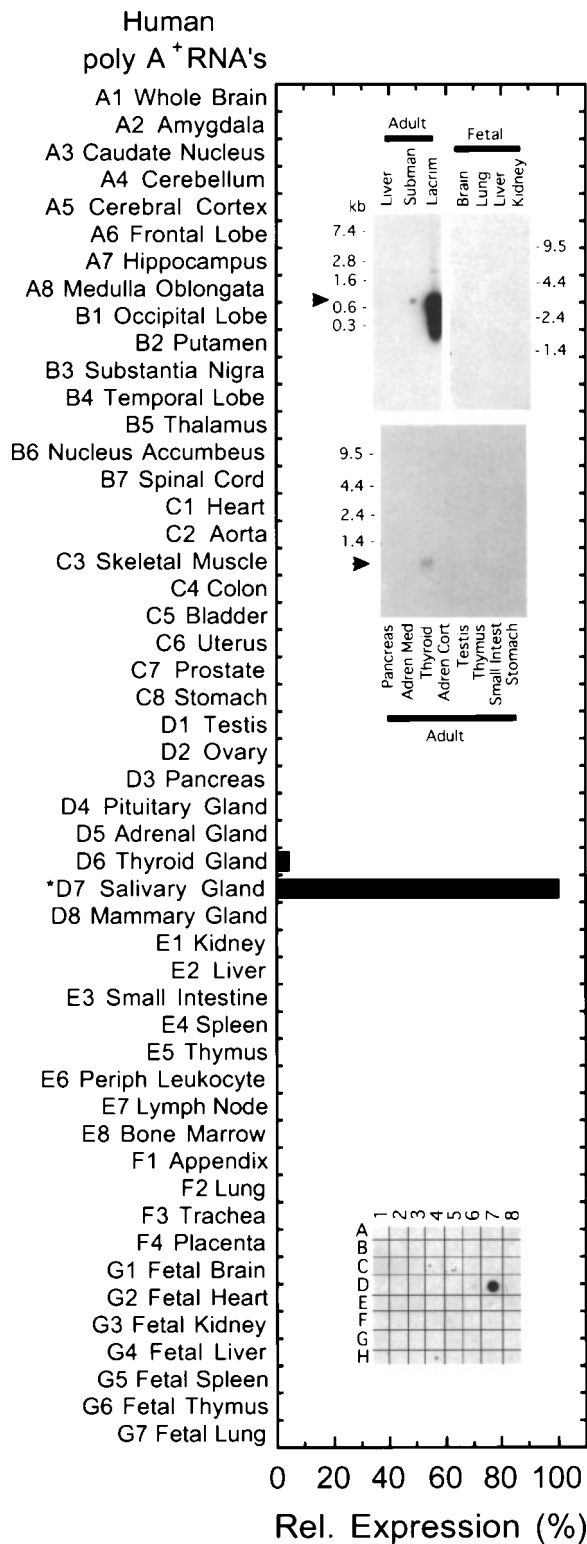
Human poly A$^+$RNA's

A1 Whole Brain
A2 Amygdala
A3 Caudate Nucleus
A4 Cerebellum
A5 Cerebral Cortex
A6 Frontal Lobe
A7 Hippocampus
A8 Medulla Oblongata
B1 Occipital Lobe
B2 Putamen
B3 Substantia Nigra
B4 Temporal Lobe
B5 Thalamus
B6 Nucleus Accumbeus
B7 Spinal Cord
C1 Heart
C2 Aorta
C3 Skeletal Muscle
C4 Colon
C5 Bladder
C6 Uterus
C7 Prostate
C8 Stomach
D1 Testis
D2 Ovary
D3 Pancreas
D4 Pituitary Gland
D5 Adrenal Gland
D6 Thyroid Gland
*D7 Salivary Gland
D8 Mammary Gland
E1 Kidney
E2 Liver
E3 Small Intestine
E4 Spleen
E5 Thymus
E6 Periph Leukocyte
E7 Lymph Node
E8 Bone Marrow
F1 Appendix
F2 Lung
F3 Trachea
F4 Placenta
G1 Fetal Brain
G2 Fetal Heart
G3 Fetal Kidney
G4 Fetal Liver
G5 Fetal Spleen
G6 Fetal Thymus
G7 Fetal Lung

0 20 40 60 80 100

Rel. Expression (%)

Figure 2. Organ expression of lacritin mRNA is highly restricted. Human dot-blot lacking lacrimal RNA but containing 50 other tissue poly(A)$^+$ RNAs reveals lacritin message in salivary (submandibular) gland and thyroid, but not elsewhere. Upper inset, abundant adult human lacrimal gland expression *versus* relatively less submandibular gland and no detectable liver expression. Message size is 760 bp. Top right, no lacritin mRNA is apparent in human fetal brain, lung, liver or kidney. Bottom, slight expression in human adult thyroid, but not in pancreas, adrenal medulla or cortex, testis, thymus, small intestine or stomach. Upper inset load is 5 µg of total RNA/lane (top left); and 2 µg of poly(A)$^+$ RNA/lane (right and bottom). Wash conditions were 0.1 × SSC, 0.1% (w/v) SDS at 55 °C. Lower inset, dot blot from which histogram was developed. No RNA was dotted in B8, F5-F8 and G8. H1-H8 contain control RNAs (yeast total RNA, yeast tRNA, *E. coli* rRNA, *E. coli* DNA, poly r(A), human Cot DNA, human DNA (1×) and human DNA (5×), respectively). Dot blot wash conditions were 2 × SSC, 0.1% (w/v) SDS at 55 °C. Exposure time for both dot blot and Northern was two weeks.

high level of glycosylation with six putative *O*-glycosylation sites between residues 52 and 64, and a single *N*-glycosylation site near the C terminus. In FASTA searches of the primate database, partial homology (Figure 1(b)) is detected with the glycosaminoglycan binding region of human neuroglycan C (32% identity over 102 amino acid residues; BestFit quality = 83 *versus* 37 ± 5 when lacritin sequence was randomized) and with the "cysteine-free", possibly mucin-like, amino globular region of human fibulin-2 (30% identity over 81 amino acid residues; BestFit quality = 81 *versus* 38 ± 5 for random). Although all three are rich in *O*-glycosylation, positioning of serine and threonine residue is not strictly shared (Figure 1(b)); and both lacritin and fibulin-2 lack glycosaminoglycan-binding sites. Neuroglycan C (af059274; 539 amino acid residues) is a component of brain extracellular matrix anchored by a transmembrane domain.[27] Fibulin-2 (x89494; 1184 amino acid residues) is widely dispersed in basement membranes and stroma of embryonic and adult tissues.[28] Searches of non-primate databases pointed to modest homologies with *Trypanosoma cruzi* mucin-like protein (af036464; BestFit quality = 78 *versus* 46 ± 10); *Plasmodium falciparum* merozoite surface antigen 2 (u91656; BestFit quality = 76 *versus* 53 ± 6) and *Pinus taeda* putative arabinogalactan protein (af101791; BestFit quality = 74 *versus* 37 ± 4).

No matching or homologous ESTs were detected, in keeping with lacritin's abundance in human lacrimal gland and restricted expression elsewhere (Figure 2). Northern analysis revealed a strong 760 bp lacrimal gland message, and weaker submandibular and thyroid gland messages of the same size (Figure 2, upper inset). No message was detected in human adult adrenal gland, testis, thymus, pancreas, small intestine or stomach; nor in human fetal brain lung, liver or kidney. Similarly, in a commercial dot-blot of 50 different human tissue poly(A)$^+$ RNAs that excluded lacrimal gland (Figure 2), lacritin expression was found only in submandibular gland (salivary gland), and to a lesser degree in thyroid. We subcloned lacritin coding sequence into pET-28b and pcDNA3.1/myc-His(+)C to generate recombinant bacterial (Figure 3(a)) and mammalian (293-T cell;
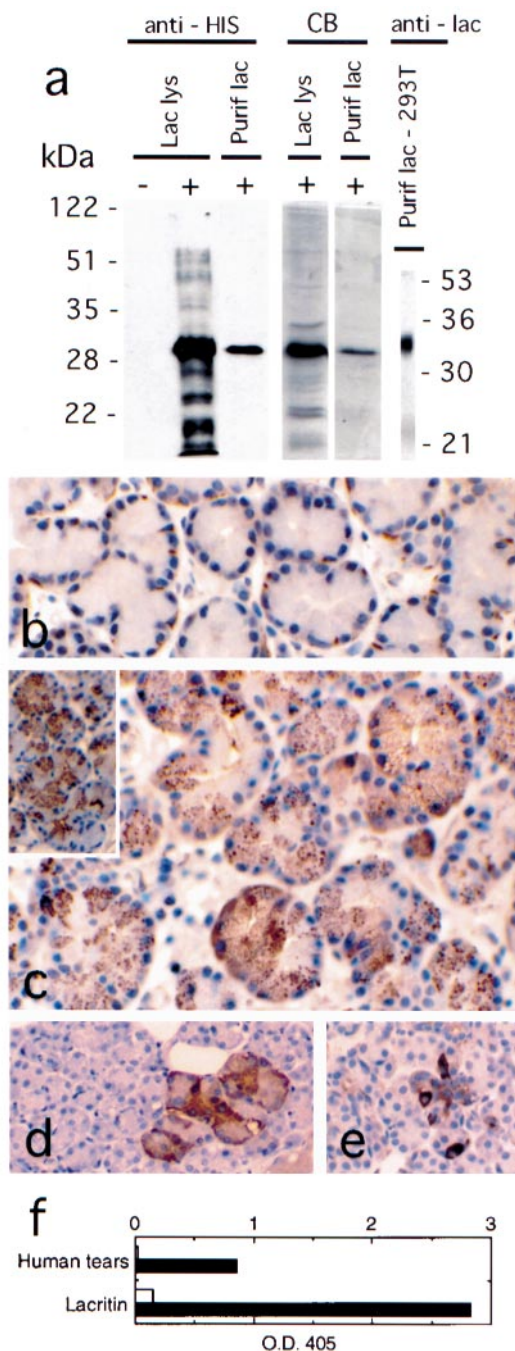
Figure 3. Preparation of recombinant lacritin and immunolocalization. (a) Purification of His-tagged recombinant human lacritin (lac) from induced (+) but not uninduced (−) bacterial lysate (lys), as monitored using anti-His antibodies and after Coomassie blue (CB) staining. Detected with anti-recombinant lacritin (anti-lac) antibodies is purified His-tagged lacritin from the media of transfected 293T cells. Lacritin mobility in SDS-PAGE is anomalous, as confirmed by mass spectroscopic analysis of the excised lacritin band (18.3 kDa mass spectroscopy peak size for His-tagged lacritin with signal peptide from bacterial lysate). Anti-lacritin antibodies were prepared in rabbit. Lack of cross-reactivity is illustrated in Figure 7(a). (b) Preimmune and (c) anti-acritin immunostaining (brown) of human lacrimal gland sections. Lacritin is present in secretory granules. Inset, lower power image. (d) Antilacritin immunostain-

Figure 3(a)) lacritin, respectively. Both forms of lacritin displayed anomalous migration in SDS-PAGE, as confirmed by mass spectroscopic sequencing and molecular mass analysis of the gel-excised band. Antibodies prepared against bacterial lacritin were applied to sections of human lacrimal and salivary glands (Figure 3) and to tissue microarrays containing formalin-fixed, paraffin-embedded sections of 75 different human tissues and organs (Table 1). Immunoreactivity was clearly observed in secretory granules of acinar cells in lacrimal and major and minor salivary glands, but was not apparent in other epithelia or stroma. The presence of lacritin in thyroid was equivocal (Table 1). Frequency of acinar cell staining was high in lacrimal gland (Figure 3(c)), whereas only scattered salivary acinar cells were reactive (Figure 3(d) and (e)). Immunoreactivity was also apparent in secretions within lumens of lacrimal and salivary ducts (not shown). By ELISA, lacritin was detected in human tears (Figure 3(f)) and to a lesser extent in saliva (not shown).

Lacritin's highly restricted expression pattern and strong conservation with mouse (99 % amino acid identity over two-thirds of mouse cDNA on hand; Walton & G.W.L., unpublished data) suggested an important physiological role(s). In the absence of functional clues, advantage was taken of an assortment of well-established assays involving each of the three main cell types in the acinar-corneal axis. Included were experiments asking whether lacritin promoted intracellular signaling through tyrosine phosphorylation and intracellular calcium release, and the consequences of signaling, including cellular secretion and proliferation. Accordingly, we chose to test the effect of lacritin on serum-free cultures of lacrimal acinar, salivary ductal and corneal epithelial cells using secretion (acinar), proliferation (ductal), tyrosine phosphorylation (acinar, ductal) and calcium signaling (corneal epithelial) assays. Freshly isolated rat lacrimal acinar cells were plated on increasing amounts of lacritin (with a constant small amount of laminin-1 to ensure adherence), or on laminin-1-coated wells in which lacritin was added to the medium. Both coated and soluble lacritin enhanced unstimulated secretion in a dose-dependent manner (Figure 4(a)), but no effect was observed on the stimulated secretory pathways activated by the agonists carbachol and VIP (Figure 4(b)). These results suggest an autocrine or paracrine role, possibly *via* receptors on the luminal acinar cell surface. We next cultured quiescent HSG cells in serum-

ing of human submandibular gland, as compared to (e) human parotid. (f) ELISA detection of lacritin (filled bars) in human tears *versus* a recombinant lacritin positive control. Open bars, preimmune. Original magnification 400× ((b) and (c)) and 200× (inset, (d) and (e)). Sections have been counterstained with hematoxylin (blue) and eosin (red).

Table 1. Restricted immunolocalization of lacritin in human organs

Adrenal medulla	–	Esophagus	–	Parathyroid	–	Small intestine	–
Adrenal cortex	–	Gall bladder	–	Parotid gland	+	Spinal cord	–
Appendix	–	Ganglia	–	Periph. nerve	–	Spleen	–
Bladder	–	Heart	–	Pituitary gland	–	Stomach	–
Bone/marrow	–	Kidney	–	Placenta	–	Subman gland	++
Brain	–	Lacrimal gland	++++	Prostate	–	Testis	–
Breast	–	Liver	–	Testes	–	Thymus	–
Bronchus	–	Lung	–	Minor salivary	+	Thyroid gland	?
Cerebellum	–	Lymphatics	–	Sem. vesicle	–	Uterus/vagina	–
Colon	–	Ovary	–	Skel. muscle	–		
Epididymis	–	Pancreas	–	Skin	–		

Relative intensity; not all tissues shown.

free media containing increasing amounts of lacritin and studied cell proliferation. The lacritin cultures looked healthier; after four days, a dose-dependent increase in ductal cell number was apparent (Figure 5(a)) that reached a level more than twofold that of the BSA (10 ng/ml) negative control (Figure 5(b)). The same level of lacritin promoted the transient tyrosine phosphorylation of a 48 kDa band in both HSG and rat lacrimal cells (Figure 5(c)). Next, we examined calcium transients in human corneal epithelial cells. Whereas the basal level of signaling was negligible, the addition of lacritin resulted in rapid and sustained calcium waves that propagated throughout the cells (Figure 6). Wave onset preceded that of the usual response to epidermal growth factor (20-40 seconds), and the amplitude of the response depended on the concentration of lacritin. To ensure that bacterial lipopolysaccharide (a possible contaminant of recombinant protein preps) was not involved, samples were tested in the limulus amebocyte lysate assay, and no lipopolysaccharide was detected (<0.05 EU/ml). Finally, we examined the ability of lacritin to bind (Figure 7) the tear film components fibronectin or vitronectin; as well as constituents of the periacinar basement membrane that might harbor small amounts of lacritin not detectable by our immunohistochemical procedure. Also tested was binding to EGF, a common glandular growth factor that might synergize with lacritin to enhance acinar cell secretion. Lack of anti-lacritin antibody cross-reactivity with all ligands (Figure 7(a)) made feasible an ELISA-based binding approach in which lacritin displayed a remarkable avidity for all constituents tested with the exception of collagen I, EGF or heparin (Figure 7(b)). Binding to the tear film constituents fibronectin and vitronectin, and to the basement membrane component laminin-1, was exceeded somewhat by collagen IV and nidogen/entactin. Lacritin is therefore capable of multiple different binding partners. Partially analogous is the affinity of fibulin-2 for collagen IV, entactin/nidogen and laminin-1.[29]

The rather broad lacritin lacrimal gland message (Figure 2; upper inset) was suggestive of alterna-tively spliced forms, or RNA degradation. The same was not true for submandibular gland in which a discrete, but much less intense signal was apparent (Figure 2; upper inset). To address this issue and to gain information on how the lacritin gene is arranged, we sequenced a 12.4 kb genomic fragment, the largest lacritin-positive fragment readily obtainable from lacritin genomic clones. The gene consists of five exons (Figure 8) preceded by a predicted promoter sequence 109 to 59 bp upstream of the translation start site (promoter score = 1.0; NNPP/Eukaryotic). Exon 1 (Figure 8(a)) encodes the complete signal peptide and includes 38 bp of 5′ untranslated sequence. Exon 3 contains sequence for all putative *O*-glyco-sylation sites. The predicted *N*-glycosylation site is formed at the exon 4/exon 5 splice junction. Exon 5 includes 53 bp of 3′ untranslated sequence. Three potential polyadenylation sites are detected 367, 474 and 534 bp downstream of exon 5, the first of which would be in keeping with a 760 bp tran-script. Sequences at exon-intron boundaries all con-form to predicted splice donors or acceptors (Table 2), with the exception of the exon 4 splice acceptor. Intronic sequences revealed common intronic repeat elements. Also independently dis-covered on a separate genomic fragment was a lacritin pseudogene lacking 38 bp of 5′ exon 1 sequence. To examine possible alternative splicing, we used RT-PCR with submandibular or lacrimal gland cDNA as template (Figure 8(b)) and forward and reverse primers from exons 1 and 5, respect-ively, each including untranslated flanking sequence. A single PCR product was detected in both organs whose size (449 bp) was in keeping with transcription from all five exons without alternative splicing. Fluorescent *in situ* hybridiz-ation (FISH) revealed that the lacritin gene is located on chromosome 12 (Figure 9), a result con-firmed by double labeling with a probe for 12q15. Measurement of ten specifically labeled chromo-somes located the lacritin gene approximately 16 % of the distance from the centromere to the telomere of 12q, an area that corresponds to 12q13. Also found on 12q13 is a rare genetic alacrimia known as triple A syndrome.[30] Attempted PCR using
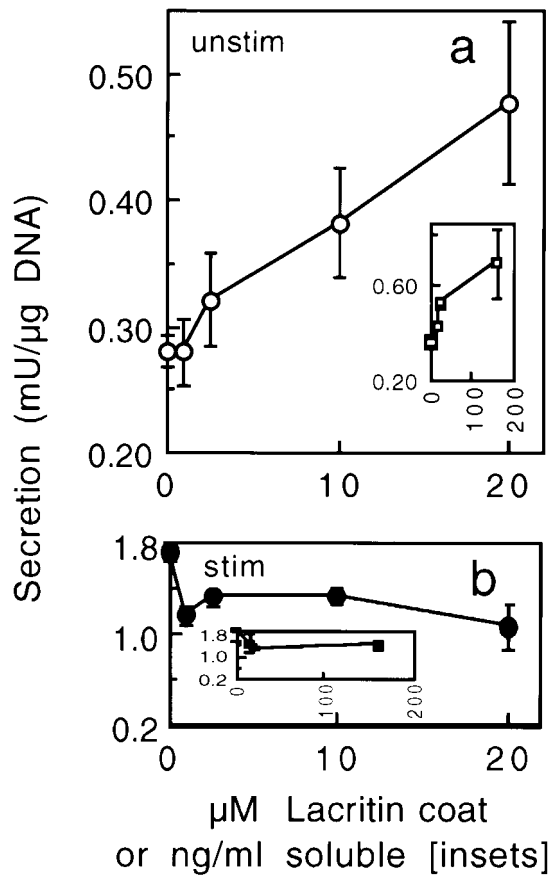
Figure 4. Recombinant lacritin enhances unstimulated secretion by isolated rat lacrimal acinar cells. (a) Enhancement of unstimulated secretion in the presence of increasing amounts of coated lacritin. Inset, soluble lacritin (0 to 162 ng/ml) similarly augments unstimulated secretion. (b) No effect on carbachol-stimulated (10^{-4} M) and VIP-stimulated (10^{-8} M) secretion either on lacritin-coated wells, or in the presence of soluble lacritin (inset). All experiments were performed in serum-free medium on laminin-1 (0.05 μM) co-coated wells (or coated; insets) to ensure cell adhesion. Secretion is assessed as mU of peroxidase secreted per μg of cellular DNA. In this and all subsequent functional experiments (except where noted), purified lacritin without His-tag was utilized.
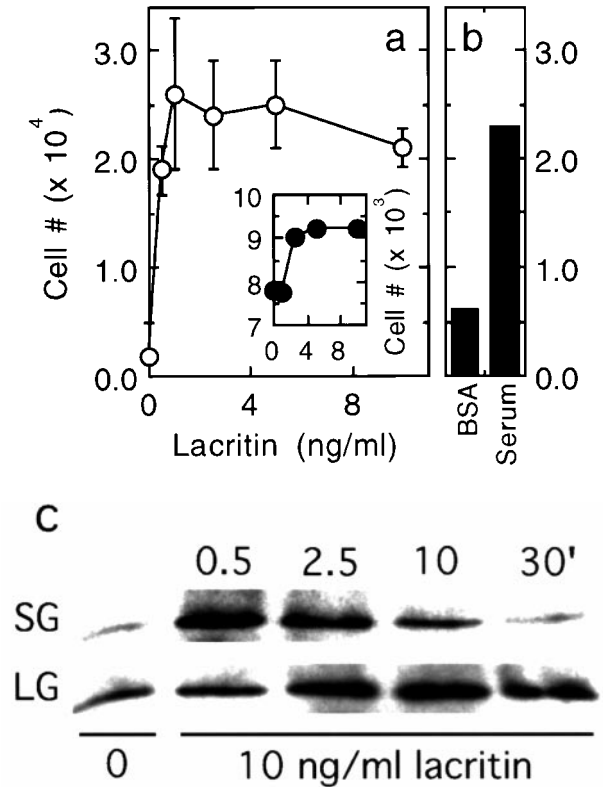

Figure 5. Lacritin-induced proliferation and tyrosine phosphorylation. (a) Number of human salivary gland (HSG) cells four days after adding 0 to 10 ng/ml of His-tagged lacritin to HSG cells in serum-free medium. Inset, similar mitogenic activity of lacritin with His tag removed by thrombin cleavage. (b) Instead of lacritin, BSA (10 ng/ml) or serum (10%) was added for the same period. The corresponding 10% serum value for the inset is 10,469. All experiments were performed on laminin-1 (0.05 μM) coated wells. (a) and (b) Cell number was determined using DNA dye, and (inset) MTS assays. (c) Addition of 10 ng/ml lacritin promotes the phosphorylation of a 48 kDa band that is optimal at 0.5 and 10 minutes in HSG (SG) and rat lacrimal acinar (LG) cells, respectively.

lacritin genomic primers and BAC templates spanning the triple A syndrome region failed to produce PCR product,[31] in keeping with recent attribution of triple A syndrome to the AAAS gene.[32,33] Human genome sequence analysis[31] suggests a 1.4-1.6 Mb separation of the AAAS and lacritin genes, and a lacritin gene location approximately 61.86-61.87 Mb from the centromere within 12q13 contig assembly NT009563.

Table 2. Donor and acceptor splice sites of the human lacritin gene

Exon	Splice acceptor	Exon length (bp)	Splice donor	Intron length (bp)
1	agaccttctGCAG[a]	96	CTGgtgagt	1550
2	ccattgcagAAGA	54	CCTgtgagt	798
3	cattcacagCTAA	141	TGAgtaagt	401
4	caaaattagAATC[b]	102	AAAgtgagt	784
5	tccttctagATGG	115	AGCccagag[c]	

Exonic sequence is capitalized. Intronic sequence is lowercase.
[a] The 5′ terminus of exon 1.
[b] Not predicted from SpliceView.
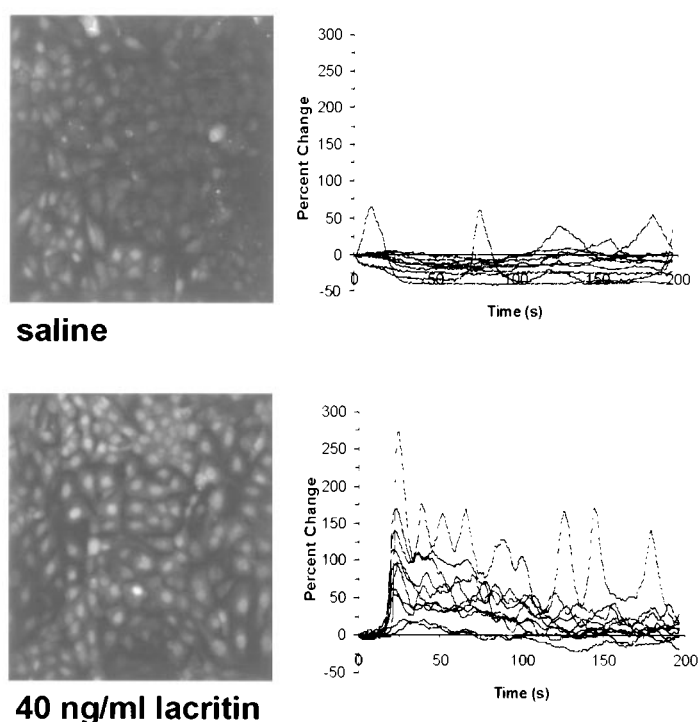[c] The 3′ terminus of exon 5.

Figure 6. Lacritin-induced calcium signaling. Human corneal epithelial cell calcium response to Hepes-buffered saline in the presence or absence of 40 ng/ml lacritin. Images are of human corneal epithelial cells eight seconds after addition. Graphs indicate individual cell tracings of percentage change in fluorescence for 200 seconds after addition. Ten randomly sampled tracings per condition are presented. Most lacritin cells display an elevated oscillatory pattern, whereas saline controls remain mostly flat at or below the baseline.

Discussion

We report on the molecular cloning, chromosomal mapping, expression and initial functional analysis of lacritin, a novel secreted glycoprotein with partial homology to the N-terminal domains of human neuroglycan C and fibulin-2. Lacritin enhances unstimulated tear secretion by lacrimal acinar cells, promotes ductal cell proliferation and tyrosine phosphorylation, stimulates corneal epithelial cell calcium signaling, and like fibulin-2 binds multiple components of the extracellular matrix.

Discovery of lacritin developed from the hypothesis that multiple extracellular factors trigger glandular differentiation, particularly growth factors and components of the surrounding extracellular matrix. Indeed, partial or failed acinar formation has been reported in mice lacking the TGFβ superfamily members or receptors,[7−9] ErbB4,[10] the progesterone receptor,[11] the extracellular matrix glycoprotein osteopontin,[12] EGF receptor (with TGFα and amphiregulin),[13,14] fibroblast growth factor receptor 2 (IIIb),[15−17] or the growth factor FGF-10.[18,19] Linking such factors to the secretory function of acinar cells in culture has proven more complex. Nonetheless, it is clear that the periacinar mesenchymal[18,19] and hormonal[34] environment affect glandular development and function, and that both autocrine and paracrine regulation play important roles.[20, 35−38]

Most delicate are primary cultures of freshly isolated exocrine cells,[39−42] particularly lacrimal acinar cells,[43−46] that functionally dedifferentiate in the absence of laminin-1 and lower molecular mass

factors derived from the extracellular matrix[20,47] and elsewhere.[44] Remarkably under-represented in gene databanks, human lacrimal gland mRNAs may code for a rich array of differentiation factors, a presumption underlying the paired oligonucleotide screening of a little used human lacrimal gland cDNA library. Among the clones identified by this approach was a novel cDNA sequence represented by several independent clones and corresponding to a 760 bp transcript. The secreted gene product of this lacrimal gland-specific transcript was designated lacritin. Lacritin's predicted six *O* and one *N*-glycosylation sites, reflected in whole or part by periodic acid-Schiff staining (not shown) of mammalian recombinant lacritin, point to a moderately well-glycosylated core protein much like the neuroglycan C glycosaminoglycan-binding domain[48] and fibulin-2 amino globular domain to which lacritin bears partial homology.

Particularly notable is lacritin's preferential and abundant expression in the lacrimal gland, a quality apparent at both mRNA and protein levels. Lesser amounts were observed in salivary (mRNA, protein) and thyroid (mRNA) glands, but none elsewhere. Several other proteins have been described as enriched in the lacrimal gland, but none appears to be as restricted nor highly expressed as lacritin. Two proline-rich proteins are somewhat similar in expression. One is detectable at high levels in the lacrimal gland, and at lower levels in the submandibular, parotid, sublingual, and von Ebners gland.[49] Another is a basic proline-rich protein expressed in the lacrimal and submandibular glands, but not parotid or sublingual glands.[50] Tear lipocalins are moderately expressed
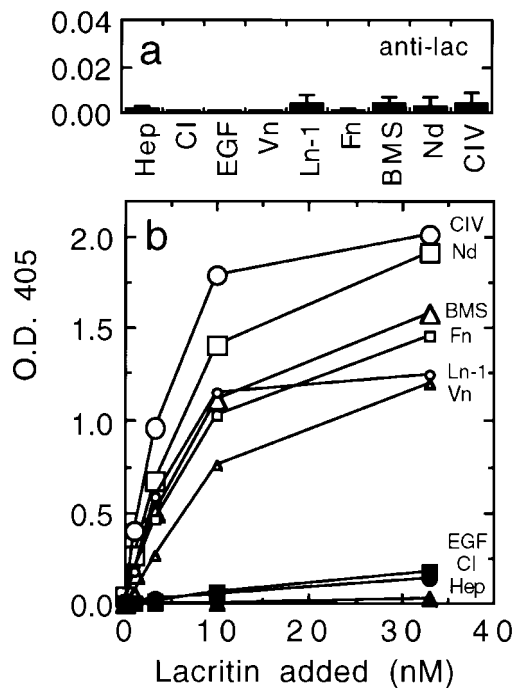
Figure 7. Lacritin binds ECM molecules. (a) Lack of cross-reactivity of anti-lacritin antibodies with heparin (Hep), collagen I (CI), EGF, vitronectin (Vn), laminin-1 (Ln-1), fibronectin (Fn), basement membrane substrate (BMS), nidogen/entactin (Nd) and collagen IV (CIV). (b) Dose-dependent binding of lacritin to collagen IV, nidogen/entactin, basement membrane substrate, fibronectin, laminin-1 and vitronectin. No binding is observed to equivalent amounts of EGF, collagen I or heparin. Lacritin was incubated with coated molecules for one hour. After washing, bound lacritin was detected with anti-lacritin antibody.
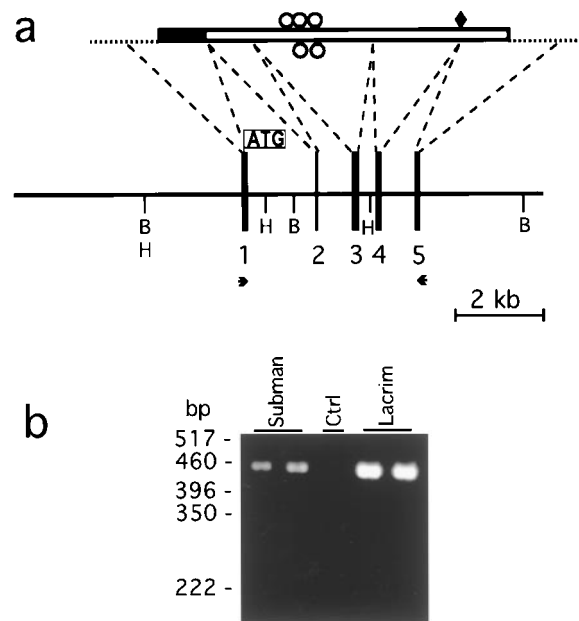

Figure 8. Lacritin gene structure and lack of alternative splicing. (a) Lacritin's coding sequence is distributed among five exons each spanning 54-141 bp. Region of potential *O*-glycosylation (Os; all six grouped together; one of six not diagrammed due to lack of space) is completely coded by exon 3. Signal peptide is shaded; diamond is the predicted *N*-glycosylation site. The lacritin gene is contained within a 12.4-kb *Eco*RI genomic fragment. Restriction sites for *Bam*HI (B) and *Hind*III (H) are indicated. Arrows indicate PCR primers used for examination of alternative splicing. (b) RT-PCR using primers from 5' portion (coding nucleotides 32 to 51) of first exon and 3' portion of fifth exon (coding nucleotides 480 to 462) generate a 449 bp product from human submandibular and lacrimal cDNAs. cDNAs were generated from a lacritin-specific reverse primer (lacritin cDNA nucleotides 523 to 503) or from oligo (dT) (first and second band, respectively of paired samples). Control (Ctrl) is without reverse transcriptase.

in lacrimal gland and von Ebner's glands[51] and have been reported by some,[52] but not others[51] in saliva, sweat, and nasal mucus. Alpha-1 microglobulin, a lipocalin, is widely distributed.[53] Mammaglobin is expressed at low levels in lacrimal gland; and at similar levels in salivary and mammary glands.[54] Small amounts of prolactin-inducible protein are detected in lacrimal gland compared to high levels in parotid, apocrine secretory cells, and human breast cancer cells.[55] Parotid secretory protein expression is moderate in lacrimal gland, pancreas, submandibular gland, parotid, and heart,[56] much like aquaporin-5 that displays similar levels in corneal epithelium,[57] lung, trachea and high levels in parotid, submandibular and sublingual glands.[58] Finally, cystatins are moderate to high in lacrimal gland, but are also detected in prostate, brain (high), kidney, spleen, muscle (moderate) and liver (low).[59]

Introduction of recombinant lacritin to cultures of lacrimal acinar, salivary ductal and corneal epithelial cells provided interesting functional insights. Lacrimal acinar cells displayed enhanced unstimulated (but not stimulated) secretion and

rapid tyrosine phosphorylation of a 48 kDa protein. Ductal cells phosphorylated the same 48 kDa band and were proliferative. A rapid and sustained calcium transient was noted in corneal epithelial cells. Thus all cell types contributing to or benefitting from lacritin outflow appear to be lacritin-inducible, whereas controls were negative and there was no evidence of contaminating bacterial lipopolysaccharide (known to be proliferative in immune cell cultures).[60] How lacritin acts remains to be elucidated. Possibly a common receptor is mediatory, ligation of which may be jointly linked to tyrosine phosphorylation and calcium release as in neural retina where tyrosine kinases have been associated with capacitative calcium entry and inositol-3-phosphate-induced release of intracellular calcium stores.[61,62] Alternatively, lacritin signaling in the three cell types may differ. Lacrimal acinar, ductal and corneal epithelial cells perform strikingly different functions. Although some intracellu-
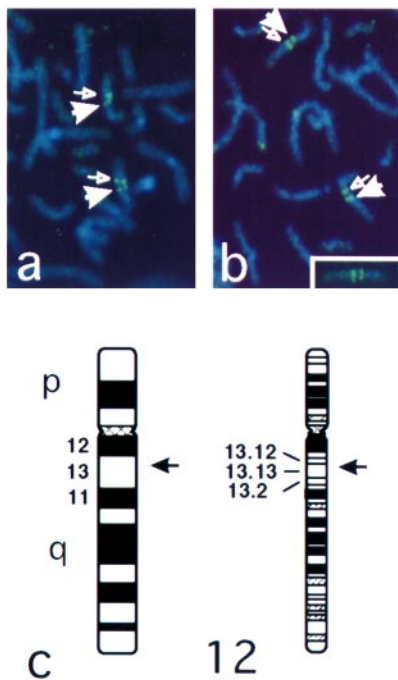
Figure 9. The lacritin gene is located on 12q13. Human FISH mapping of the lacritin gene to 12q13. (a) and (b) Hybridization of lacritin genomic DNA (large arrows) together with a marker genomic DNA (short arrows) from 12q15. (c) Schematic representation indicating sub-band location on 13.13. Triple A syndrome, a rare genetic alacrimia has been linked to 12q13[30]. 12q13 is a gene-rich band containing over 47 gene loci including aquaporin-5, two Rabs, eight keratins, several transcription factors and integrin α7.

lar signaling machinery may be common, others are unique, and some common machinery may be put to different use. Calcium signaling in lacrimal acinar cells is most frequently a downstream effect of muscarinic receptor ligation that mediates the release of tear proteins by the stimulated secretory pathway, a pathway apparently unaffected by lacritin. However, subtleties in calcium amplitude, frequency and localization, dependent on the nature and dose of the agonist, can have dramatically different effects. Contrasting lacritin is BM180, a periacinar basement membrane constituent that appears to act only on the stimulated secretory pathway.[20] Balancing the amounts of available lacritin and BM180 may offer a simple mechanism by which secretory capacity in adult and developing glands may be controlled.

Immunolocalization of lacritin in secretory granules, in secretory content of ducts and in tears was extended by binding studies revealing a strong affinity for tear constituents fibronectin and vitronectin. Though not immunodetected elsewhere, lacritin also displayed equal or greater affinity for the common periacinar basement membrane components nidogen/entactin, collagen IV, and laminin-1; but not collagen I, EGF or heparin. Similar

binding properties have been reported for fibulin-2.[29] Although the significance and precise nature of these interactions remains to be determined, basement membrane binding is perhaps analogous to growth factors whose extracellular matrix accumulation, although functionally potent, is often too low for reliable immunodetection. Alternatively, basement membrane binding (if any) could possibly occur secondary to tissue damage.

Future study of lacritin gene regulatory units could prove particularly rewarding. We isolated the complete lacritin gene together with 5.2 kb of 5′ upstream and 2.8 kb of 3′ downstream genomic sequence. Two promoters were predicted, the most likely with a transcription start site 55 bp upstream of the ATG start site. All six predicted *O*-glycosylation sites occur within exon 3, much like the proteoglycan perlecan in which all three heparan sulfate attachment sites derive directly or indirectly from a single exon.[63] Unlike perlecan in which alternative splicing can give rise to core protein without glycosaminoglycan, no evidence of spliced removal of exon 3 was detected. Lacritin's location on gene-rich 12q13 is coincident with a rare lacrimal deficiency syndrome known as triple A syndrome.[30] Lack of PCR product in reactions combining triple A syndrome BAC clones with lacritin primers,[31] recent attribution of triple A syndrome to the AAAS gene;[32,33] and human genome sequence analysis suggesting a lacritin/AAAS separation of 1.4-1.6 Mb, argue against involvement of the lacritin gene.

In summary, a novel human glycoprotein with remarkably restricted expression and unique functional characteristics has been identified. Elucidation of mechanisms of controlled gene regulation, how lacritin rapidly elicits cellular responses and under what circumstances, opens up a broad area of detailed investigation that may provide long-term insights into our understanding of exocrine secretion.

Materials and Methods

cDNA and genomic cloning

Duplicate filters containing plaques (5×10^4 per filter) from each of ten sublibraries of a human lacrimal gland cDNA library[49] were prehybridized at 42 °C for four hours in $5 \times$ Denhardt's, $6.76 \times$ SSC, 10 mM sodium phosphate, 1 mM EDTA, 0.5 % (w/v) SDS and 182 μg/ml salmon sperm DNA, and then hybridized overnight at 42 °C with one of two overlapping 23-mer oligonucleotides (S1 (AGCTGGGGCACAGGCACCCGCAC) and S2 (GGGGTTCTGGGGCTGCAGCTGGG)) that had been end-labeled with [γ-^{32}P]ATP 7000 Ci/mmol (ICN, Irvine, CA) and purified. Final wash conditions were $2 \times$ SSC (45 °C), corresponding to 29.5 deg.C less than the S1 or S2 t_m (74.5 °C in $2 \times$ SSC for both). Plaques positive in both filters were picked and rescreened three times in duplicate with each oligonucleotide, giving rise to 47 clones. Each was subsequently reanalyzed at increasing wash stringency (−29.5, −24.5, −19.5, and −14.5 °C t_m). Inserts were excised into pBluescript and both strands sequenced *via* a Prizm 377 DNA Sequencer (Perkin-

Elmer, Branchburg, NJ; University of Virginia Biomolecular Research Facility). Of identical clones, most common was a novel sequence lacking homology to BM180 (BestFit quality = 16, *versus* random quality of 17 ± 2) from which the poly(G)-rich S1 and S2 oligonucleotides were derived.[64] Predicted was a 417 bp open reading frame, whose expected protein product was designated lacritin, in keeping with its lacrimal gland expression. Lacritin insert was subsequently used to screen a human P1 genomic library (carried out by Genome Systems Inc; St. Louis, MO) and three identical clones were obtained, as determined by restriction digestion and Southern analysis. The largest lacritin-positive fragment (12.4 kb) was subcloned intact into pBluescript and both strands were completely sequenced. Alignment and analyses[65] of cDNA and genomic sequence was primarily with Unix-based (Gelstart, Gap) and web-based (FASTA, BestFit, Gap) Genetics Computer Group (Madison WI) software using default settings and *E* values (FASTA) restricted to 5 or less. Genomic exon searching and identification of splice sites was facilitated by the Baylor College of Medicine Human Genome Sequencing Center web-site.

Northern analysis

Human lacrimal and submandibular glands were obtained during autopsy through the Southern division of the Cooperative Human Tissue Network within 18 hours of death and most within eight hours to minimize autolytic degradation. The tenets of the Declaration of Helsinki were followed and informed consent and full IRB approval were obtained. Donors were without known systemic bacterial or viral infections, and tissues were normal as determined from cause of death, pathology reports and in most cases histological examination. Tissues were snap frozen in liquid nitrogen after removal and stored at $-85\,°C$ until used for RNA preparation. Total RNA was extracted from 100-300 mg of tissue using a commercial version of the acidified guanidine thiocyanate/phenol method (RNazol B, Tel-Test, The Woodlands, TX). Purified RNA was dissolved in diethylpyrocarbonate-treated water, and the concentration and purity determined from the $A_{260/280}$ absorption values. A ratio close to 2.0 was considered acceptable. RNA integrity was initially determined by electrophoresis of ethidium bromide-complexed RNA samples in a gel containing 0.22 M formaldehyde. Samples that did not show prominent 28 S and 18 S rRNA bands in a 1:1-2:1 ratio under UV light were rejected. For blotting, RNA (5 μg/lane) was separated on a 0.8 % agarose gel under denaturing conditions[66] and transferred to nitrocellulose. Also assayed were two purchased (cat # 7756-1 and 7751-1; Clontech Labs, Palo Alto, CA) Northern blots with multiple human fetal and adult poly(A)$^+$ RNAs and a dot blot (cat # 7770-1; Clontech Labs) containing 50 different human poly(A)$^+$ RNAs together with control RNAs and DNAs. Blots were hybridized with ^{32}P-labeled lacritin insert, washed in $0.1 \times$ SSC, 0.1 % (w/v) SDS (Northern) or $2 \times$ SSC, 0.1 % (w/v) SDS (dot blot) at $55\,°C$, and exposed to X-ray film. Dot blots were then quantified using NIH Image by measurement of pixel gray values of individual dots.

Preparation of recombinant lacritin and anti-lacritin antisera

Full-length lacritin cDNA was subcloned in-frame into pET-28b (Novagen, Madison, WI), with orientation confirmed by completely sequencing through the insert. Recombinant His-tagged lacritin was then generated by IPTG-induction of BL-21 transformed cells, and purified from cell lysate on Talon (Clontech; Palo Alto, CA) resin using standard denaturing procedures. After elution, lacritin was extensively dialyzed *versus* PBS, and the His tag was removed by thrombin cleavage. Protein quality was assessed by SDS-PAGE and Western blotting with anti-His antibody (Santa Cruz Biotechnology; Santa Cruz, CA). Lacritin displays anomalous mobility in SDS-PAGE. Lack of contaminating bacterial lipopolysaccharide was confirmed by the limulus amebocyte lysate assay (MRL Reference Lab; Cypress, CA). For analytical comparison, small amounts of mammalian lacritin were expressed in 293T cells using pcDNA3.1/myc-His(+) (Invitrogen, Carlsbad, CA) containing lacritin insert, and then purified under native conditions. Preimmune serum and anti-bacterial lacritin antiserum were subsequently prepared in rabbits (Covance Research Products, Denver, PA), partially purified by precipitation in 4 % caprylic acid[67] and treatment with 293T cell acetone extract (to eliminate background), and assessed by ELISA (1/1000 dilution) using recombinant bacterial lacritin (4 μg/ml) as coat. For immunohistochemistry, sections of zinc formalin-fixed, paraffin-embedded human tissues and a human tissue microarray were deparaffinized and rehydrated, and microwave heated (20 minutes in 10 mM citrate buffer (pH 6.0)) to expose antigen. Endogenous peroxidase was blocked, and then immunodetection was performed using the avidin-biotin-peroxidase complex method (Vectastain Elite kit, Vector Laboratories, Burlingame, CA) after incubation with anti-lacritin I$_g$ or preimmune I$_g$ (1/1000; prepared as above) for one hour at room temperature. Sections were counterstained with hematoxylin, placed in cupric sulfate, and then immersed in lithium carbonate.

Cell function analysis

Freshly isolated rat lacrimal acinar cells[20,47,67] and HSG (human salivary gland) ductal[23] and HCE (human corneal epithelial)[68] cell lines were used to study lacritin function. For secretion studies, rat acinar cells were plated serum-free overnight on wells co-coated with 0.05 μM laminin-1 (to ensure adhesion) and 0 to 20 μM lacritin, or alternatively with laminin-1 (0.05 μM) and treated the next day with serum-free medium containing 0 to 162 ng/ml of soluble lacritin for four hours. Unstimulated and stimulated (carbachol 10^{-4} M/VIP 10^{-8} M) secretions were then collected, assessed (peroxidase assay) and normalized to μg cellular DNA.[20,47] Cell proliferation was examined in serum-free HSG (human salivary gland ductal cell line) cultures grown for four days in 0 to 10 ng/ml of soluble lacritin. Cell number was assessed using DNA dye (Quantos kit; Stratagene, La Jolla, CA) and MTS (Promega, Madison, WI) assays in parallel with standard curves of increasing cell quantity. In controls, lacritin was replaced with BSA (10 ng/ml) or 10 % (w/v) serum. To study tyrosine phosphorylation, overnight serum-free cultures of both rat lacrimal acinar and HSG cells were washed and treated with 10 ng/ml of soluble lacritin for 0.5, 2.5, 10 and 30 minutes. Py(20) anti-phosphotyrosine antibody immunoprecipitation of cell lysates was then examined in Western blots of SDS-7 % PAGE gels using Py(20) and ECL for detection. Calcium signaling in human corneal epithelial cells was similarly carried out in serum-free culture[69] (Klepeis & V.T.-R., unpublished data). HCE cells were grown to confluency on glass coverslips in keratinocyte media

(Life Technologies, Rockville, MD) containing bovine pituitary extract (30 μg/ml), EGF (0.1 ng/ml) and peni-cillin/streptomycin, and rendered quiescent 18 hours before loading with Fluo-3AM (2 μM; Molecular Probes, Eugene, OR) at 37 °C for 30 minutes. Using an inverted Zeiss 510 LSM for visualization, 50 seconds baseline images were first recorded. While the laser was running, lacritin was added (final concentration 4 and 40 ng/ml) and the response continually monitored every 786 ms for a minimum of 200 seconds.

ECM-binding studies

Binding studies were carried out in 96-well plates coated with 10 μg/well of collagen IV, laminin-1, entac-tin/nidogen-1, collagen I, fibronectin, vitronectin, EGF, heparin or BMS.[70] Wells were washed, blocked (PBS-T), incubated with 0-30 nM lacritin (in PBS-T containing 1% (w/v) BSA) for one hour at 4 °C, washed and detected with anti-lacritin antibody (1/1000) by ELISA.

PCR analysis and chromosome mapping

Alternative splicing was examined by RT-PCR using human submandibular or lacrimal total RNA and initial priming with oligo(dT), or in a gene-specific manner with lacritin reverse primer CGCTACAAGGGTATT-TAAGGC (corresponding to nucleotides 523 to 503 from lacritin cDNA). Subsequent amplification with lacritin forward primer ACTCACTCCTCATCCCAAAG (from exon 1; lacritin cDNA nucleotides 32 to 51) and reverse primer TTTTCAGCTTCTCATGCCC (from exon 5; lacri-tin cDNA nucleotides 480 to 462) involved denaturation for two minutes at 94 °C, 30 cycles of amplification (94 °C for 30 seconds, 52 °C for 30 seconds and 72 °C for one minute), and a final cycle for five minutes at 72 °C. PCR product was analyzed in agarose gels.

For FISH mapping (Genome Systems; St. Louis, MO), lacritin genomic DNA was labeled with digoxigenin dUTP by nick translation and hybridized (50 % (v/v) for-mamide, 10 % (w/v) dextran, 2 × SSC) to metaphase chromosomes from PHA-stimulated human peripheral blood lymphocytes. Following washes, specific labeling was detected with fluoresceinated antidigoxigenin anti-bodies and DAPI, and examined in a Nikon Labophot microscope. A total of 80 metaphase cells were analyzed with 60 exhibiting specific labeling. Confirmation was achieved by double labeling using a 12q15 marker, and by comparison with human genome sequence. Photo-graphs were taken on a Nikon AFX at a final magnifi-cation of 1435×.

Statistical analysis

All values are expressed as the mean ± SD.

Data Bank accession numbers

All nucleotide sequences have been submitted to the GenBank/EBI Data Bank with accession numbers af238867 (cDNA) and ay005150 (genomic).

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