Tissue Transglutaminase Is a Negative Regulator of Monomeric Lacritin Bioactivity

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Purpose. Molar accounting of bioactive fluids can expose new regulatory mechanisms in the growing proteomic focus on epithelial biology. Essential for the viability of the surface epithelium of the eye and for normal vision is the thin, but protein-rich, tear film in which the small tear glycoprotein lacritin appears to play a prominent prosecretory, cytoprotective, and mitogenic role. Although optimal bioactive levels in cell culture are 1 to 10 nM over a biphasic dose optimum, ELISA suggests a sustained tear lacritin concentration in the midmicromolar range in healthy adults. Here we identify a reconciling mechanism.

Methods. Monoclonal anti-lacritin 1F5 antibody was generated, and applied together with a new anti-C-terminal polyclonal antibody to tear and tissue Western blotting. In vitro tissue transglutaminase (Tgm2) cross-linking was monitored and characterized by mass spectrometry.

Results. Blotting for lacritin in human tears or saliva surprisingly detected immunoreactive material with a higher molecular weight and prominence equal or exceeding the ~23 to 25 kDa band of monomeric glycosylated lacritin. Exogenous Tgm2 initiated lacritin cross-linking within 1 minute and was complete by 90 minutes—even with as little as 0.1 nM lacritin, and involved the donors lysine 82 and 85 and the acceptor glutamine 106 in the syndecan-1 binding domain. Lacritin spiked into lacritin-depleted tears formed multimers, in keeping with ~0.6 μM TGM2 in tears. Cross-linking was absent when Tgm2 was inactive, and cross-linked lacritin, unlike recombinant monomer, bound syndecan-1 poorly.

Conclusions. Since syndecan-1 binding is necessary for lacritin mitogenic and cytoprotective activities, TGM2 cross-linking negatively regulates lacritin bioactivity. (*Invest Ophthalmol Vis Sci.* 2013;54:2123–2132) DOI:10.1167/iovs.12-11488

The biology of the surface of the eye and optical quality are together dependent on the thin preocular tear film comprising as many as 1543 different proteins,[1] including at least 33 different enzymes.[2] Tissue transglutaminase (TGM2, gene symbol for human tissue transglutaminase; Tgm2, gene symbol for guinea pig tissue transglutaminase employed in cross-linking experiments; EC 2.3.2.13) is a constituent of normal tears[2,3] and is expressed by corneal epithelial cells[4] (NEIBank) where it is UVB inducible. It is also expressed by both conjunctival epithelia[5] and subconjunctival fibroblasts.[6] Enhanced TGM2 expression correlates with reduced cell viability, caspase activation, TNF receptor clustering,[7] and mitochondrial dysfunction[8] associated with hyperosmolar stress in dry eye.[9] Transglutaminases encompass a multifunctional family of enzymes involved in high-fidelity posttranslational modifications that catalyze Ca^{2+}–dependent covalent bond formation between primary amines or ε-amino groups of lysine and γ-carboxamide groups of glutamine. Cross-linking affects function, both negatively and positively. TGM2 cross-linked collagen is resistant to metalloproteinase digestion and less mitogenic[10]; and cross-linked IL-2 (unlike IL-2 monomer) is cytotoxic for oligodendrocytes.[11] TGM2 also positively regulates the activity of midkine, a small heparin binding growth factor,[12] and is required for the activation of latent TGF-β[13] and S100A11.[14] Could TGM2 in tears regulate ocular surface biology?

Lacritin is a 12.3 kDa tear prosecretory mitogen[15] with glutamine and lysine residues suitable for TGM2 catalyzed cross-linking. Lacritin promotes corneal epithelial cell survival (Zimmerman K, et al. *IOVS* 2012;53:ARVO E-Abstract 4231) and proliferation,[16] and basal tear protein secretion by lacrimal acinar cells.[15] When topically applied to rabbit eyes, lacritin acutely increases basal tear flow.[17] Lacritin is largely restricted to tears and to a lesser extent saliva, through its lacrimal acinar cell,[15] meibomian gland,[18] and apparent conjunctival and corneal epithelial cell[19] expression. Some has been detected in the thyroid gland,[15] lactating breast (McKown RL, et al. *IOVS* 2012;53:ARVO E-Abstract 4235), and in lung lavage.[20] Lacritin targets cell surface syndecan-1,[21] which is extensively expressed on ocular surface epithelia,[22] and requires heparanase to expose[21] and in part create[40] the lacritin binding site within its N-terminus. Heparanase is normally expressed by the corneal epithelium.[23] Lacritin–syndecan-1 ligation initiates rapid $G_{\alpha i}$ or $G_{\alpha o}$-PKCα-PLC-Ca^{2+}-calcineurin-NFATC1 and $G_{\alpha i}$ or $G_{\alpha o}$-PLC-PLD-mTOR mitogenic signaling,[16] suggesting involvement of a G-protein coupled receptor. A biphasic dose

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Supported by NIH RO1EY013143, RO1EY018222 (GWL).

Submitted for publication December 13, 2012; revised January 30, 2013; accepted February 15, 2013.

Disclosure: **F. Velez V**, None; **J.A. Romano**, None; **R.L. McKown**, EyeRx Research, Inc. (E), P; **K. Green**, None; **L. Zhang**, None; **R.W. Raab**, P; **D.S. Ryan**, None; **C.M.L. Hutnik**, None; **H.F. Frierson Jr**, None; **G.W. Laurie**, P

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response is typical with a 1 to 10 nM optimum in human cell culture.[16] A similar range of soluble lacritin promoted basal tear secretion by rat lacrimal acinar cells.[15] Approximately 4 μM lacritin stimulated basal tearing in rabbits.[17] However, direct ELISA suggests that lacritin is 18 to 27 μM in normal human basal tears, as implied by an ELISA estimate of 4.2 ng lacritin per 100 ng total tear protein,[24] and an estimated basal tear protein concentration of ~8 mg/mL.[25] If lacritin dosing is biphasic for all forms of epithelial activation in vivo, lacritin in basal tears appears to exceed the beneficial dose range.

The two concentrations might be reconcilable if tear lacritin was partly and actively sequestered in inaccessible complexes. Sequencing of 2-D tear gels detected a higher molecular weight lacritin species (Nichols J, unpublished observations, 2012), together with the expected ~23 to 25 kDa lacritin bands[26]—in keeping with tears replete with both lacritin multimers (or heterocomplexes) and monomers, that would not be distinguishable by ELISA. Twenty-three to 25 kDa lacritin is 50% less, 7-fold less, or at an average ratio of 0.24 of normal, respectively, in blepharitis,[27] contact lens-related dry eye,[26] and aqueous deficient dry eye.[28] One study reported no lacritin change, but the methodology substituted 2-D SDS-PAGE for liquid chromatographic cationic separation followed by reverse phase separation.[29] While characterizing a new generation of monoclonal antilacritin N- (1F5-C9-F4; '1F5') and rabbit C- ('ab C-term') domain-specific polyclonal antibodies, we unexpectedly discovered ~75 kDa lacritin immunoreactive material in human tear and saliva samples that previously had not been subjected to antilacritin immunoblotting. Validation by mass spectrometric analyses, and determination that TGM2 was responsible, led to the realization that only monomeric lacritin was fully active, whereas ~50 and ~75 kDa bands were inactive, and likely represented respective dimers and trimers. TGM2 thus appears to serve as a lacritin-negative regulator.

Materials and Methods

Cells, Tear Collection, Human Tissues

HEK293-6E cells were grown in suspension culture in F17 medium (Life Technologies, Grand Island, NY) with 4 mM L-glutamine and 0.1% of cell membrane stabilizer (Pluronic F68; Life Technologies). Eyes of normal individuals were anesthetized with 0.5% proparacaine for basal tear collection largely on Schirmer strips. Some tears were collected on wicks.[24] Collected tears were immediately stored at −70°C for later elution with an equal volume of PBS. Saliva (500 μL) was collected into an Eppendorf tube, centrifuged at ×20,000*g* for 10 minutes, and then the supernatant was subjected to precipitation with 1/10 volume trichloroacetic acid on ice. Human procedures and patient consent were approved by the Walter Reed Army Medical Center, the University of Western Ontario, and the University of Virginia Institutional Review Boards.

Lacritin and Antilacritin Antibody Generation, and Blotting

Human recombinant lacritin and lacritin truncation mutants lacking 25 amino acids from the C-terminus,[16] or 24, 45, or 65 amino acids from the N-terminus (Zhang Y, et al. *IOVS* 2010;51:ARVO E-Abstract 4179) (numbering excludes the signal peptide) were generated in *E. coli* as intein fusion proteins. Intein fusion proteins were captured on chitin beads, eluted with β-mercaptoethanol (removes the intein tag[30]), and passed over a diethylaminoethyl (DEAE) cellulose column to remove bacterial impurities (Zimmerman K, et al. *IOVS* 2012;53:ARVO E-Abstract 4231). Four male AJ mice were immunized with keyhole limpet hemocyanin-linked DPAQEAGTSKPNEEIS, corresponding to amino acids 11 to 26 of the mature lacritin N-terminus, and boosted three times. Fusions were screened first against BSA-DPAQEAGTSKP-NEEIS, then to lacritin truncation mutant C-59, and finally to tear lacritin immunocaptured in wells coated with protein A and polyclonal rabbit 'anti-Pep Lac N-term' lacritin antibody.[24] 1F5-C9-F4 ('1F5') showed the highest titer and was expanded in a 500-mL bioreactor (Lampire Biological Products, Piperville, PA), purified (GammaBind Plus Sepharose; GE Healthcare Life Sciences, Piscataway, NJ), and validated with lacritin truncation mutants. 1F5 is an IgG1. Also employed was new rabbit "anti-N-65 Lac C-term" (here designated as "ab C-term"), directed against the lacritin N-65 truncation mutant (amino acids 66–119 of mature lacritin). Rabbit terminal bleeds were collected on day 70 and purified on protein A for subsequent use. Slight background to *E. coli* proteins was depleted by passage over an *E. coli* lysate column prepared by AminoLink (Thermo Fisher Scientific, Inc., Rockford, IL) immobilization of lysate from *E. coli* expressing C-59 lacritin. Similarly, tear background of secondary anti-mouse antibodies was depleted by passage over beads (Thermo Fisher Scientific, Inc.) coupled to normal human tears. Normal human tears were eluted off of flash-frozen Schirmer strips with ice-cold PBS at 1 μL per mm. For blotting, lacritins, tears, or TCA-precipitated saliva were loaded onto 10% or 4% to 20% gradient SDS-PAGE gels using 10 μL per lane of tears (in an equal volume of ×2 sample buffer with β-mercaptoethanol and boiling) and 37.5 μL per lane of TCA precipitated saliva that had been solubilized in 125 μL of ×1 sample buffer with β-mercaptoethanol and boiling. All gels were transferred to nitrocellulose. Blocking with milk was avoided since lacritin appears to be a constituent of milk (McKown RL, et al. *IOVS* 2012;53:ARVO E-Abstract 4235). Instead, blots were blocked with 5% BSA in Tris-buffered saline with a detergent solution (Tween-20; Thermo Fisher Scientific, Inc.); and incubated with 1F5 (0.6 μg/mL) or with anti-N-65 Lac C-term (1/2000), using peroxidase-labeled secondary and ECL (Thermo Fisher Scientific, Inc.). TGM2 was detected with mouse monoclonal antibody CUB 7402 (Abcam, Cambridge, MA). For immunohistochemistry, zinc formalin-fixed, paraffin-embedded human lacrimal gland was sectioned, epitope–exposed, and peroxidase-blocked, as per Sanghi et al.,[15] and incubated with 1F5 (0.03 μg/mL) or ab C-term (1/20,000), followed by detection.

Tgm2 Modification of Lacritin and Inhibition Studies

Active guinea pig liver transglutaminase (1.5 μM, Tgm2; Sigma-Aldrich, St. Louis, MO) was incubated with 0.1 nM to 3 μM purified recombinant lacritin for 0 to 90 minutes at 37°C in HEPES buffer containing 10 mM Ca^{2+}. The reaction was terminated with 50 mM EDTA, mixed with gel loading buffer containing β-mercaptoethanol (0.6 M), boiled, and separated by 10% SDS-PAGE. Gels were transferred to nitrocellulose membranes and blotted for lacritin using 1F5, followed by peroxidase-labeled goat-anti-mouse secondary antibody and ECL detection. Further reactions were performed for 40 minutes (37°C) with 100 nM lacritin to which had been added 1.5 μM Tgm2; 1.5 μM Tgm2 that had been boiled (10 minutes at 99°C), or incubated in the presence of 50 mM EDTA; and finally with recombinant inactive human TGM2 (1.5 μM; Abnova, Walnut, CA). Gels were transferred and blotted as above. Recombinant human TGM2 is tagged with GST at its N-terminus, and generated via an in vitro wheat germ expression system. The reason for its inactivity is not indicated by Abnova. To purify Tgm2 cross-linked lacritin, 1F5 mab (30 μg) was immobilized on 50 μL of AminoLink beads (Thermo Fisher Scientific, Inc.) to which was added 12.3 μg of cross-linked lacritin. After thorough washing, bound lacritin was acid eluted into 1/12.5 volume 1 M Tris, pH 9.5. Bioactivity of Tgm2 modified lacritin was assessed via syndecan-1 affinity precipitation.[21] For affinity precipitation, lacritin-intein on chitin beads was either left untreated, or alternatively was incubated for 40 minutes with 1.5 μM Tgm2, 1.5 μM inactive recombinant TGM2, or 1.5 μM boiled Tgm2. Negative control C-25-intein truncation mutant[21] (incapable of binding syndecan-1) was similarly bacterial-expressed and linked to chitin. After rinsing away Tgm2 in the reaction

mixture, octylglucoside lysates[21] of HEK-2936E cells transiently overexpressing human syndecan-1 were added. Lacritin-intein-chitin and C-25-intein-chitin beads were then washed with octylglucoside buffer, treated with heparitinase/chondroitinase,[21] boiled in gel loading buffer, separated by 10% SDS-PAGE, transferred to nitrocellulose, and blotted for human syndecan-1 using mouse monoclonal antibody BB-4, followed by detection as performed using 1F5. Blots were quantitated using Fiji (http://rsbweb.nih.gov/ij/docs/guide/146-2.html, in the public domain).

Mass Spectrometry

Tgm2 cross-linked lacritin was digested with trypsin prior to capillary-liquid chromatography tandem mass spectrometry (Capillary LC-MS/MS) analysis for the identification of cross-link sites. Briefly, the sample was resuspended in 100 mM ammonium bicarbonate solution and reduced with dithiothreitol (5 μg/μL in 100 mM ammonium bicarbonate). Reduced cysteines were then blocked using iodoacetamide (15 μg/μL in 100 mM ammonium bicarbonate). Trypsin (20 ng/μL) was incubated with the sample at a 1:20 enzyme to substrate ratio for 2 hours (37°C) before adding 0.1% trifluoroacetic acid (TFA) to quench the enzymatic reaction. Digests were then separated on a capillary column (0.2 × 150 mm Magic C18AQ 3 μ 200 A; Michrom Bioresources, Inc., Auburn, CA) using an HPLC system (UltiMate 3000 HPLC; LC-Packings Nederlands B.V., Sunnyvale, CA) before being analyzed on a mass spectrometer (LTQ Orbitrap XL; Thermo Fisher Scientific, Inc.) equipped with a microspray source (Michrom Bioresources, Inc.) operated in positive ion mode. Each sample was injected into short microcolumns (μ-Precolumn Cartridge; Dionex, Sunnyvale, CA) and desalted with 50 mM acetic acid for 5 minutes. The injector port was then switched to inject and the peptides were eluted off of the trap onto the column. Mobile phase A was 50 mM acetic acid in water. Acetonitrile was used as mobile phase B. Flow rate was set at 2 μL/min. Mobile phase B was increased from 2% to 5% in 5 minutes and again from 5% to 30% in 60 minutes, then from 30% to 50% in 20 minutes. The gradient was increased again from 50% to 90% in 5 minutes and then kept at 90% for another 5 minutes before being brought back to 2% in 1 minute. The column was equilibrated at 2% of mobile phase B (or 98% A) for 30 minutes before the next sample injection. MS/MS data was acquired with a spray voltage of 2 KV and a capillary temperature of 175°C. The scan sequence of the mass spectrometer was based on the data-dependent TopFive method in preview mode: the analysis was programmed for a full scan recorded between 400 and 2000 Da and an MS/MS scan to generate product ion spectra to determine amino acid sequence in consecutive scans of the five most abundant peaks in the spectrum. The full scan resolution was set at 60,000 to achieve high mass accuracy MS determination. The CID fragmentation energy was set to 35%. Dynamic exclusion is enabled with a repeat count of 3 within 30 seconds, a mass list size limit of 500, exclusion duration of 350 seconds, and a low-mass width of 0.50 and high-mass width of 1.50 Da.

Sequence information from the MS/MS data was processed by converting the .raw files into a merged file (.mgf) using an inhouse program, (RAW2MZXML_n_MGF_batch; merge.pl, a Perl script). Isotope distributions for the precursor ions of the MS/MS spectra were deconvoluted to obtain the charge states and monoisotopic *m/z* values of the precursor ions during the data conversion. The resulting .mgf files were searched using database software (Mascot Daemon, version 2.3.2; Matrix Science, Boston, MA) against the NCBInr database (version 20120313) for protein identifications. Trypsin was used as the enzyme and four missed cleavages were permitted. Considered variable modifications were oxidation (Met), and carbamidomethylation (Cys). The mass accuracy of the precursor ions were set to 20 ppm and the fragment mass accuracy was set to 0.8 Da. One ^{13}C peak was included in the search in case of the accidental pick of ^{13}C peaks. Cross-link site identification was done by searching the data on database search software (MassMatrix[31]; Hua Xu, Cleveland, OH) against lacritin and TMG2 sequence. The proposed crosslink sites were defined as lysine and glutamine residues and the cross-links between these two residues were formed after the loss of –NH3 group. Therefore, a mass loss of –17.02654Da (–NH3) was used to search for cross-linked peptides. Candidate cross-linked peptides were then manually verified.

Polymerization of Recombinant Lacritin by Depleted Tears

An antibody column was prepared by adding 150 μg of 1F5, 125 μg of ab C-term, and 125 μg of anti-Pep Lac N-term to 300 μL of coupling resin (AminoLink Plus Coupling Resin; Thermo Fisher Scientific, Inc.) for covalent coupling.[24] After washing, 30 μL of normal basal tears was combined with 370 μL of IP/lysis/wash buffer (Thermo Fisher Scientific, Inc.) and incubated with the column overnight at 4°C with end-over-end mixing. Depleted tears were collected, concentrated to <100 μL using a 10 kDa cutoff spin filter (Ultracel YM-10; Millipore, Billerica, MA), and washed with 10 volumes of HEPES buffered saline to dilute out detergent that is incompatible with TGM2 activity. Detergent is incompatible with TGM2 activity. Depleted tears were divided into three aliquots, and then respectively supplemented with: 614 ng recombinant lacritin plus $CaCl_2$ (20 mM final concentration); 614 ng recombinant lacritin and EDTA (50 mM final concentration); or buffer only to an equivalent volume. After overnight incubation at 37°C with shaking, samples were separated by SDS-PAGE, transferred to nitrocellulose, and blotted. Under these conditions, no column antibody leaching was observed. To estimate TGM2 levels in tears, 31.25 to 500 ng of recombinant TGM2 (Abnova) were separated by SDS-PAGE together with 1 to 5 μL of normal basal tears. After transfer, blots were incubated with ab-Tgm2 (CUB 7402; Abcam), followed by detection with a secondary antibody that had been precleared against a column of normal human tears. Bound antibodies were detected by ECL. Blots were analyzed for density and the amount of TGM2 in normal human tears was estimated.

Results

Detection of Higher Molecular Weight Lacritin

An ocular surface or secretory mechanism that restricts the availability of epithelial bioactive lacritin was implied by the ~18 to 27 μM level of lacritin in normal human basal tears[24] versus the 1 to 10 nM biphasic dose optimum on cultured human cells.[15] New antibodies 1F5 mab and ab C-term were generated and characterized. Both detect ~18 kDa recombinant lacritin (Figs. 1A, 1B) and acinar cells in human lacrimal glands (Figs. 1C, 1D), but differ in their respective N- (Fig. 1A) or C- (Fig. 1B) terminal specificity. Thus, 1F5 mab detects lacritin lacking 25 amino acids from the C-terminus (C-25) but not N-terminal truncations of 24 through 80 amino acids—in keeping with a peptide antigen corresponding to amino acids 11 through 26 (numbering excludes the signal peptide that is absent in all recombinant constructs). In contrast, the immunizing antigen for ab C-term is lacritin's 54 amino acid C-terminus. This is in accordance with no reactivity for lacritin C-59. Ab C-term binds lacritin N-65, and also C-terminal truncations of 10 through 25 amino acids (Fig. 1B), in keeping with the polyclonal nature of the antibody. We then blotted for lacritin in human tears or saliva (Figs. 1E, 1F), taking care to control for possible cross-reactivity of secondary antibodies with Ig's prevalent in both fluids. Only the ab C-term detected the ~25 kDa band of glycosylated monomeric lacritin. Instead, under standard denaturing conditions, a ~75 kDa band was prominent with both antibodies (Figs. 1E, 1F), as well as a ~50 kDa band with ab C-term, and a lower band of ~13 kDa as a possible fragment. Moderate detection of the ~24 to 25 kDa and ~13 kDa bands had previously been demonstrated with ab C-term[32] in human tears and with new anti-Pep Lac-N-Term

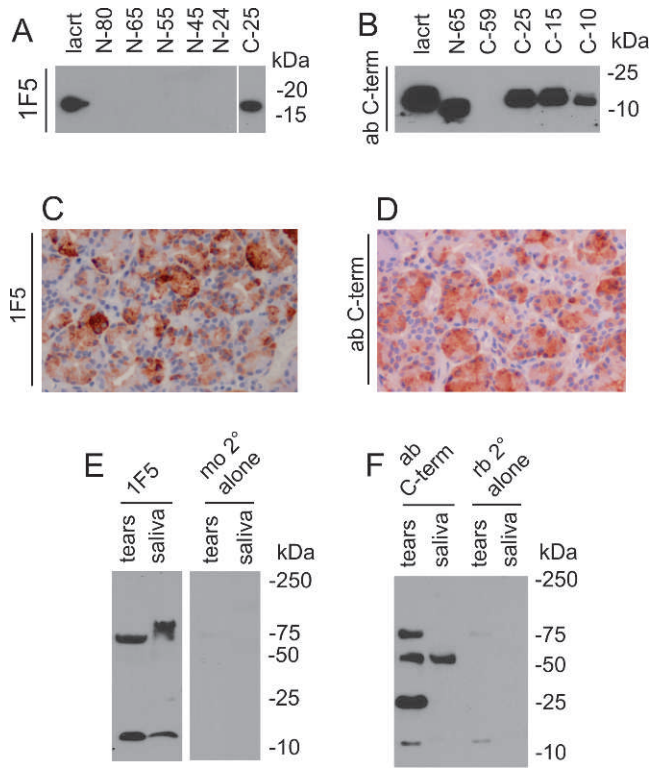

Figure 1. Respective N- and C-terminal specificity of new anti-lacritin antibodies 1F5 mab and ab C-term, and primary detection of a ~75 kDa and lower lacritin band human tears. (**A**) 1F5 mab blot of equal loads of recombinant lacritin, lacritin N-truncations, and a lacritin C truncation. (**B**) Ab C-term polyclonal antibody blot of equal loads of recombinant lacritin, lacritin C-truncations, and a lacritin N truncation. (**C**) 1F5 mab localization of lacritin in human lacrimal gland. Primary staining is over acini. Some background is associated with endothelial cells. Original magnification ×200. (**D**) Ab C-term localization of lacritin in human lacrimal gland. (**E**) 1F5 mab blot of human tears and saliva. (**F**) Ab C-term blot of human tears and saliva.

antibodies,[24,32] but we had previously not paid serious attention to the higher molecular weight bands whose potential significance was validated with 1F5. Since protein separation was denaturing, the implication is that much tear lacritin may be covalently cross-linked with itself (as dimers and trimers), or with another tear protein.

Oligomerization of Lacritin

How might lacritin become covalently cross-linked? Although absence of cysteines and inclusion of β-mercaptoethanol or dithiothreitol in gel-loading buffers ruled out disulfide bonding, lacritin is relatively rich in lysine and glutamine residues (Fig. 2A). TGM2 catalyzes the covalent interaction of glutamine γ-carboxamide groups with lysine ε-amino groups (Fig. 2B). TGM2 is a 77.3 kDa member of the normal tear proteome.[2,3] Could cross-linking be TGM2 dependent?

We purchased active guinea pig liver transglutaminase (Tgm2),[12] and performed incubations with 1 μM recombinant lacritin at 37°C. Tgm2 rapidly and progressively converted lacritin monomer into oligomers with detectable monomer exhausted by 10 or 20 minutes (Figs. 3A, 3B), although some monomer occasionally remained when mixing was not optimal (Figs. 3C, 3D). By 90 minutes, cross-linked lacritin below and greater than ~75 kDa form had formed (Figs. 3A, 3B) in preparations containing 3 μM lacritin, although oligomerization was apparent over a broad lacritin dose range and initially formed very high molecular species (Fig. 3A). Boiled Tgm2 or inactive human recombinant TGM2 all failed to cross-link lacritin monomer (Fig. 4A). Interestingly, the amount of immunodetected material increased with time of Tgm2 incubation (Figs. 3A, 3C). We considered whether Tgm2 may have incorporated into the lacritin complex and/or have some affinity for 1F5 mab. The sequence 'P_QEAGT' of the 1F5 antigen DPAQEAGTSKPNEEIS is shared with human TGM2 amino acids 66 to 73, the latter with a below threshold antigenic score of 0.992,[33] and is conserved in guinea pig Tgm2 as DPAseEAGT. To address these questions, Tgm2 cross-linked lacritin (Fig. 4B) was purified on a 1F5 mab column and then blotted with ab Tgm2. No Tgm2 was detected (Fig. 4C). We also tested blots of 1.5 μM Tgm2 incubated with lacritin for 0 or 90 minutes (Fig. 4D) or with itself (Fig. 4E). No 1F5 cross-reactivity was detected in the absence of lacritin (Fig. 4D), and introduction of lacritin had no effect on the size distribution of Tgm2 (Fig. 4E).

Donor and Acceptor Sites in Lacritin Cross-Linking

Deprotonated lysines couple with acceptor glutamines via ε-(γ-glutamyl)-lysl linkages. To ask which lysines and glutamines are affected, we analyzed Tgm2-modified lacritin by mass spectrometry (Figs. 5, 6), and discovered that glutamine 106 was bridged to lysines 82 and 85. Interestingly, glutamine 106 resides within the syndecan-1 binding domain,[16,21] as recently confirmed and further refined by Zhang et al. (Zhang Y, et al. *IOVS* 2010;51:ARVO E-Abstract 4179).

Cross-Linking Suppresses Lacritin Cell Surface Targeting

Lacritin targeting of cells is dependent on local heparanase deglycanation of the ubiquitous cell surface proteoglycan syndecan-1[21] (Fig. 7A), with coupling between lacritin's C-terminal amphiphathic α-helix and a domain within syndecan-1's heparan sulfate rich N-terminus.[40] When syndecan-1 or heparanase are depleted by siRNA, no lacritin-dependent mitogenesis is observed.[21] To ask whether or not cross-linking interferes with coupling, we treated lacritin with Tgm2 while still an intein fusion protein immobilized on chitin beads. In controls, immobilized lacritin was similarly treated with inactive recombinant TGM2, or boiled Tgm2. Tgm2 cross-linking substantially diminished the affinity of lacritin for syndecan-1. Ligation was unaffected by incubation under the same conditions and same duration with inactive TGM2, or with boiled Tgm2 (Fig. 7B).

Native TGM2 Activity in Tears

If a significant population of tear lacritin is covalently cross-linked in normal human tears and Tgm2 readily cross-links lacritin in vitro, can native tear TGM2 do the same? We immunodepleted all lacritin monomers, multimers, and fragments from normal human basal tears, and then spiked in 1.1 μM recombinant lacritin for overnight incubation at 37°C (Fig. 8). TGM2 requires Ca^{++} for activity. Incubations that included Ca^{++} displayed lacritin species with mobilities appropriate of a lacritin monomer, dimer, trimer, and tetramer, at relative levels (Fig. 8A) similar to that seen for endogenous lacritin (Figs. 1E, 1F). However, without Ca^{++} and instead with EDTA, lacritin remained monomeric, with trace amounts of apparent tetramer (Fig. 8A). Without tears, no multimerization occurred although there was some dimer with Ca^{++} (Fig. 8B). We next compared blottable levels of human recombinant and tear TGM2 over several concentrations or loading volumes. As a GST fusion protein generated in a wheat germ translation

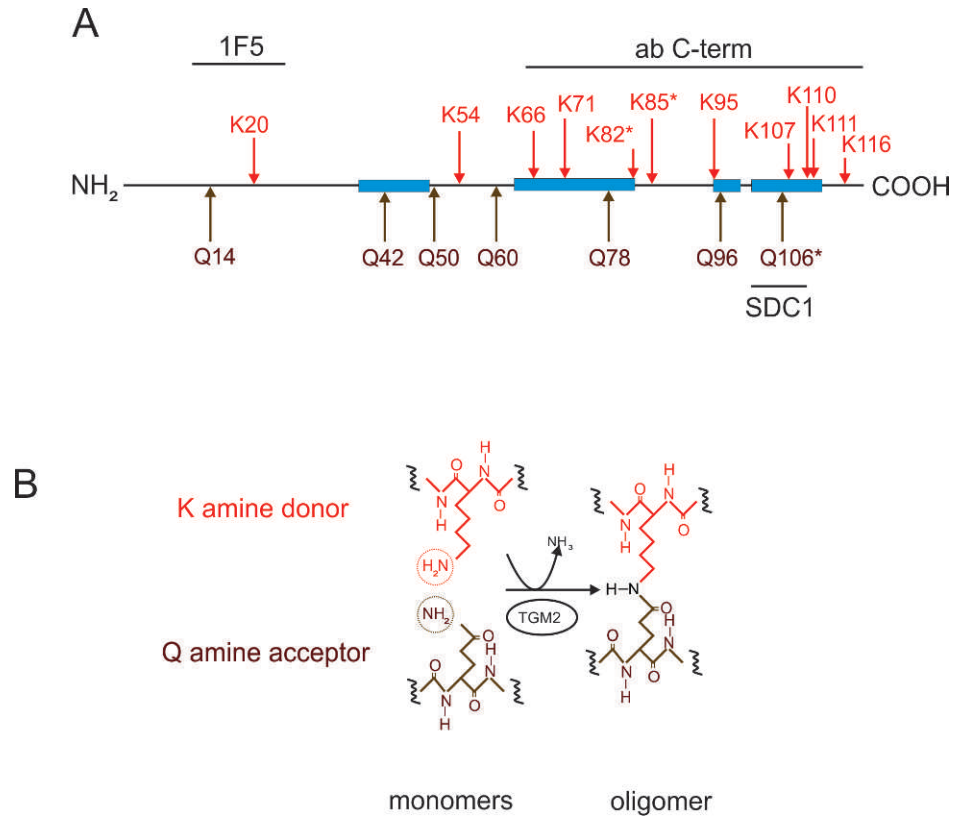

Figure 2. Lacritin is rich in lysines and glutamines, and is thus a potential TGM2 substrate. **(A)** Schematic diagram of lacritin indicating the location of lysines and glutamines with respect to the syndecan-1 binding site, and also the 1F5 mab and ab C-term antigens. Numbering excludes the signal peptide. **(B)** TGM2 transamidation reaction.

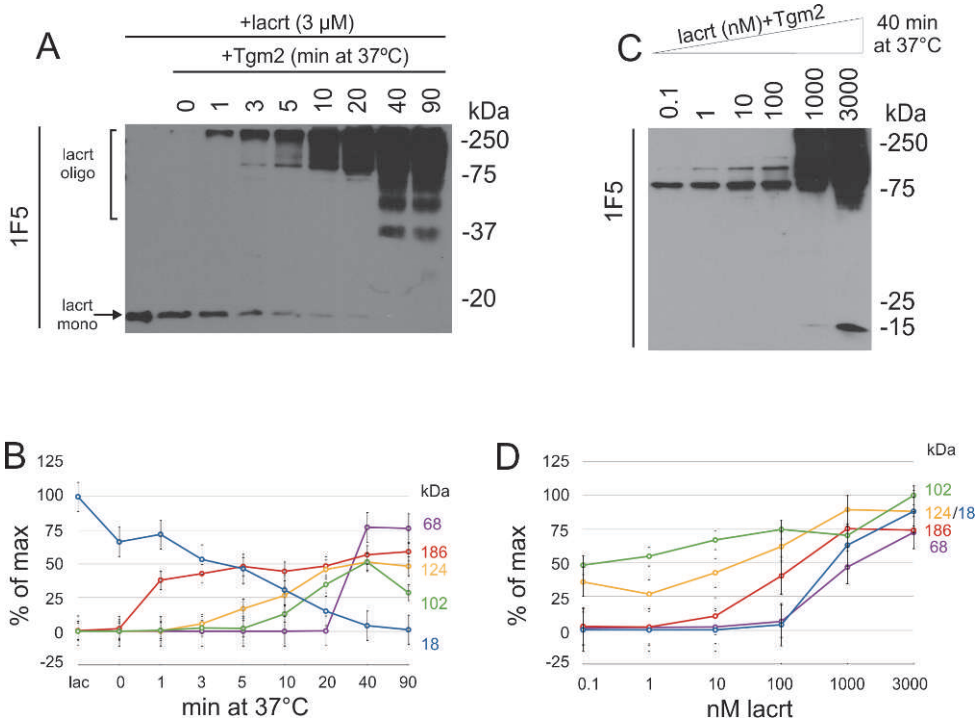

Figure 3. Tgm2 rapidly polymerizes lacritin, and is effective over a broad lacritin dose range. **(A)** 1F5 mab blot of recombinant lacritin (3 μM) incubated with 1.5 μM Tgm2 for different times at 37°C. **(B)** Quantitation of selected bands from **(A)**. **(C)** 1F5 mab blot of increasing amounts of recombinant lacritin incubated with 1.5 μM Tgm2 for 40 minutes at 37°C. **(D)** Quantitation of selected bands from **(C)**.

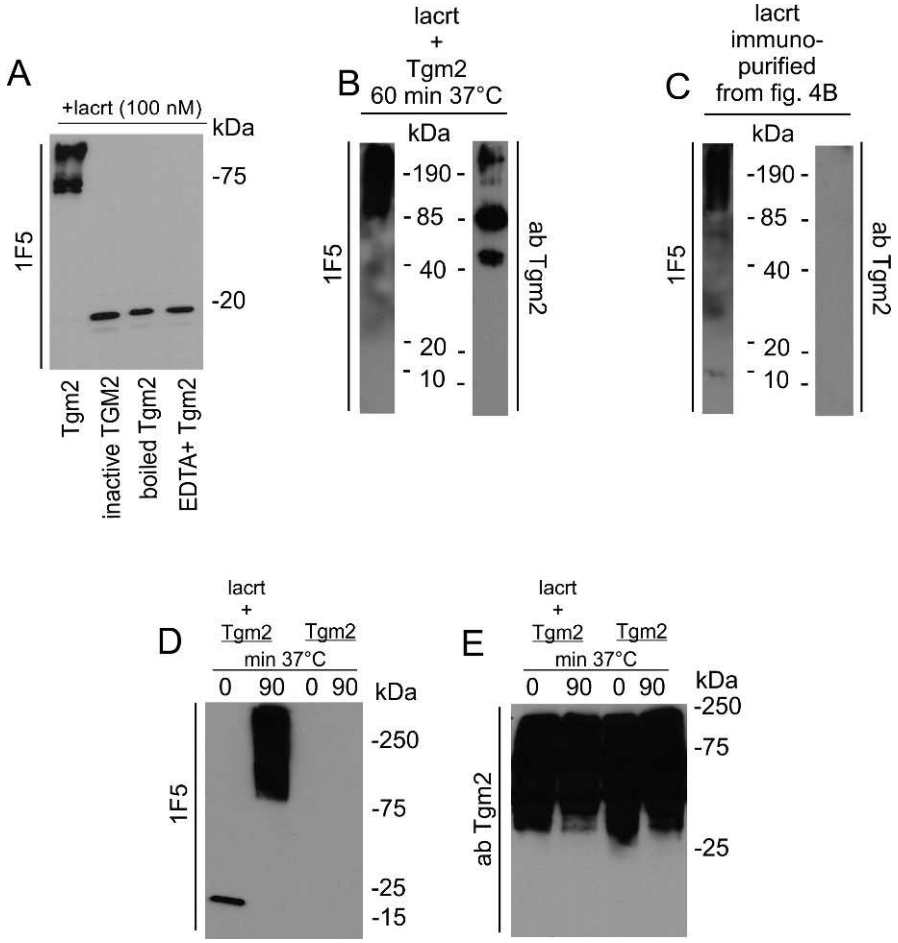

Figure 4. Tgm2 polymerization of lacritin is specific. (**A**) 1F5 mab blot of 100 nM recombinant lacritin incubated with 1.5 μM each of Tgm2, inactive recombinant human TGM2, boiled Tgm2, or Tgm2 in the presence of 50 mM EDTA for 40 minutes at 37°C. (**B**) 1F5 mab and anti-Tgm2 blot of 3 μM lacritin incubated for 60 minutes at 37°C with 1.5 μM Tgm2. (**C**) 1F5 mab and anti-Tgm2 blot of the incubation from (**B**) immunopurifed on a 1F5 column. (**D**) 1F5 blot of 3 μM lacritin incubated with 1.5 μM Tgm2, or 1.5 μM Tgm2 alone. Incubation was for 0 or 90 minutes. (**E**) Anti-Tgm2 blot of the incubation mixture from (**D**).

system (therefore not glycosylated; one N-linked site predicted), recombinant TGM2 (~80 kDa) migrates only slightly higher than tear TGM2 (~75 kDa). Comparative densities suggest a tear level of 0.6 μM TGM2 (Fig. 8C), in line with 1.5 μM Tgm2 employed in Figures 3 and 4, but with kinetics presumably slowed by competing tear proteins. The nature of a ~10.5 kDa band in tears is unknown. Thus, normal tear TGM2 appears to reconcile the discrepancy between total tear lacritin levels, and the much lower level necessary for lacritin bioactivity on ocular surface epithelia. Corneal TGM2 increases in dry eye stress,[9] and might thereby differentially downregulate levels of epithelial bioactive lacritin.

Discussion

We sought to reconcile lacritin's 1 to 10 nM biphasic dose optimum in epithelial cell culture with the ~18 to 27 μM level in normal human tears, as implied by an ELISA estimate of 4.2 ng lacritin per 100 ng total tear protein,[24] and a suggested basal tear protein concentration of ~8 mg/mL.[25] We observed by immunoblotting that some tear lacritin appeared to be sequestered in larger complexes—likely dimers and trimers—and demonstrated that recombinant lacritin is a substrate for exogenous and native tear Tgm2. Since Tgm2 complexed lacritin binds syndecan-1 poorly and syndecan-1 both mediates lacritin dependent survival (Zimmerman K, et al. *IOVS* 2012;53:ARVO E-Abstract 4231) and mitogenic[16] signaling, TGM2 in tears appears to be a negative regulator of lacritin activity.

Lacritin cross-linking by TGM2 was rapid. Only 1 minute was required to assemble the largest detected complex. Smaller complexes followed, including ones at, below, and above the ~75 kDa form observed in tears. The implication is that donor and acceptor residues are readily available, and that massive cross-linking is immediate. Midkine cross-linking in vitro was similar, although dimer was also detected at 5 minutes.[12] However, when recombinant lacritin was spiked into lacritin-depleted tears, much slower kinetics were observed even though the estimated level of TGM2 in tears (~0.6 μM) did not differ substantially from exogenous (1.5 μM). Slower kinetics and smaller lacritin oligomers in tears may reflect competition of other tear substrates for TGM2, and perhaps in vivo an endocytic or tear clearance mechanism for large complexes. Although we detected covalent binding of glutamine 106 with lysines 82 and 85 (numbering excludes the signal peptide), other glutamines and lysines may contribute. Lacritin truncation mutant C-25 lacks the above residues, but also oligomerized in the presence of Tgm2 (not shown). Thus, the kinetics are in keeping with immediate or delayed cross-linking upon lacritin secretion by extracellular or plasma membrane–associated TGM2, dependent likely on the proxim-

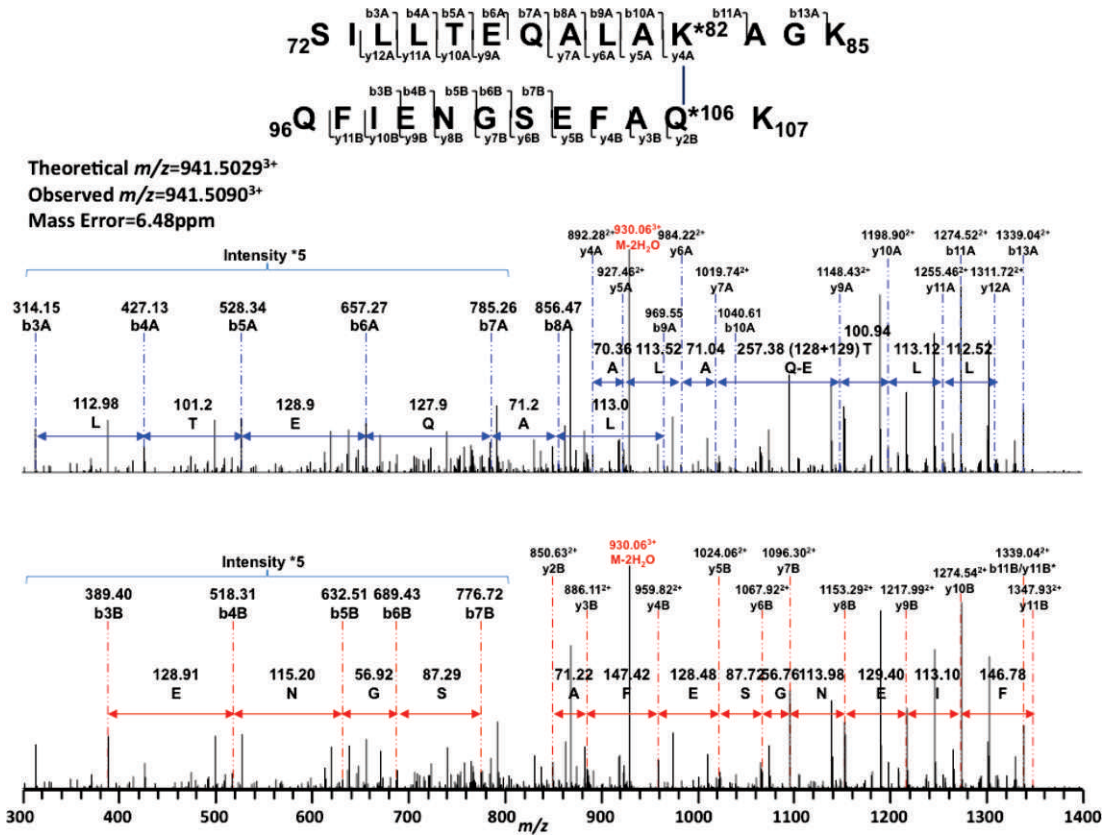

Figure 5. Mass spectrometric identification of glutamine 106 cross-linked to lysine 82 from 1 μM lacritin incubated with 1.5 μM Tgm2 for 40 minutes at 37°C.

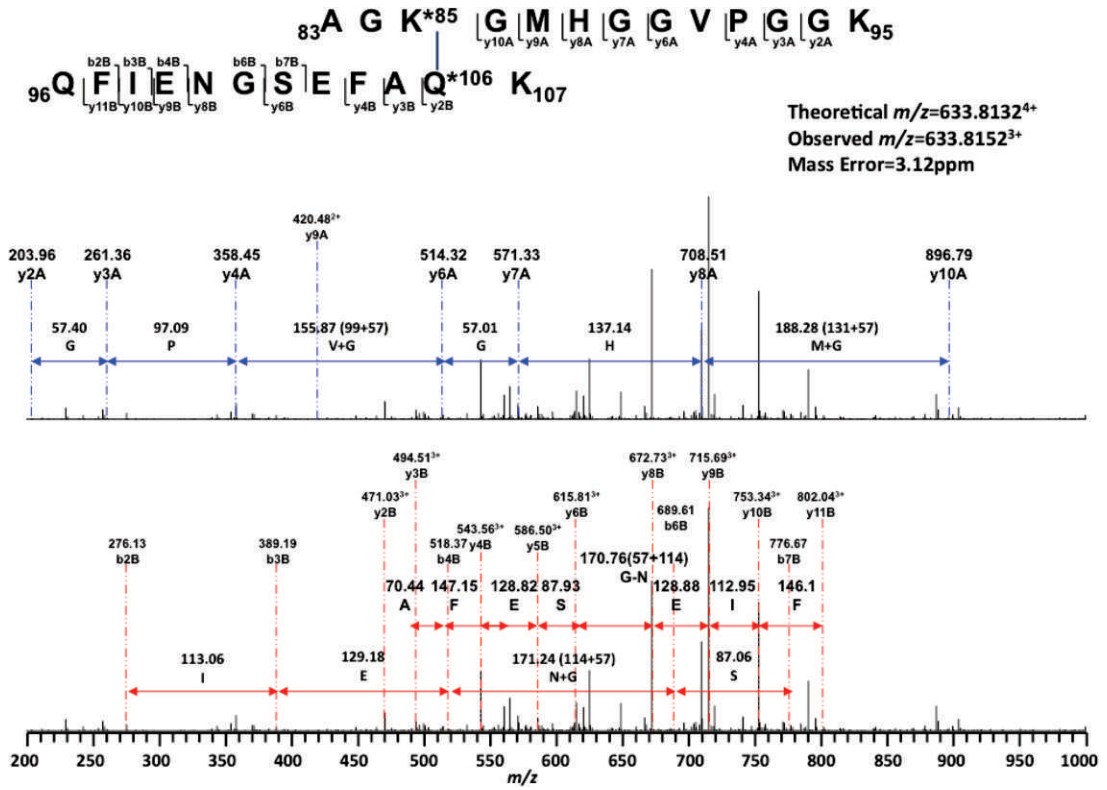

Figure 6. Mass spectrometric identification of glutamine 106 cross-linked to lysine 85 from 1 μM lacritin incubated with 1.5 μM Tgm2 for 40 minutes at 37°C.

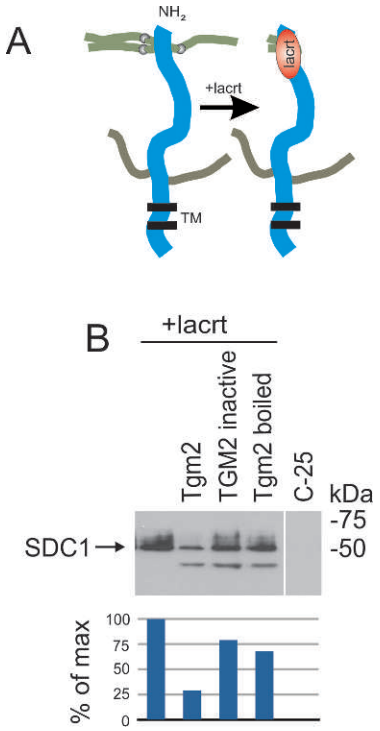

Figure 7. Tgm2 cross-linked lacritin binds syndecan-1 poorly. (**A**) Schematic diagram illustrating how lacritin targets the heparan sulfate-rich N-terminus of syndecan-1, but only after heparanase digestion. (**B**) Lacritin-intein on chitin beads was either left untreated, or alternatively was incubated with 1.5 μM Tgm2, 1.5 μM inactive recombinant TGM2, or 1.5 μM boiled Tgm2. Washed beads were incubated with lysates of HEK-2936E cells transiently overexpressing human syndecan-1, and then treated with heparitinase/chondroitinase, separated by SDS-PAGE, and blotted for human syndecan-1 using mouse monoclonal antibody BB-4.

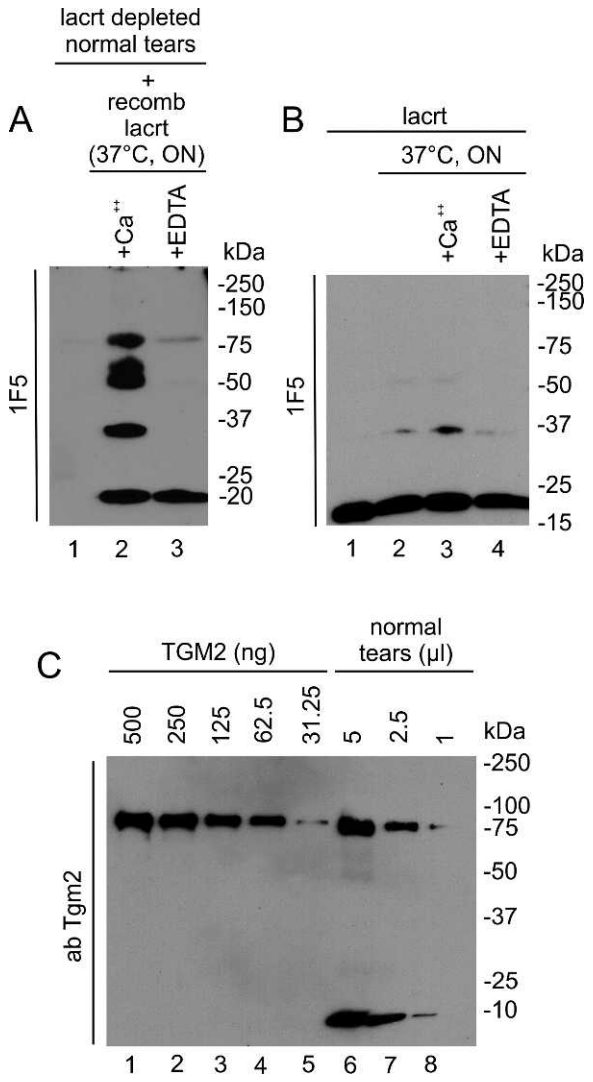

Figure 8. Normal basal tears contain cross-linking activity and ~0.6 μM TGM2. (**A**) 1F5 mab blot of lacritin depleted tears with or without 1.1 μM spiked recombinant lacritin, the latter after overnight incubation at 37°C with 20 mM $CaCl_2$ or with 50 mM EDTA. (**B**) 1F5 mab blot of recombinant lacritin incubated in a similar manner, but without tears. (**C**) Anti-Tgm2 blot of recombinant human TGM2 in increasing amounts run in parallel with increasing tear volumes.

ity of competing substrates. Since cross-linking is covalent, monomer is irreversibly coupled and activity diminished.

Cross-linking increased the affinity of 1F5 mab for lacritin. We ruled out cross-reactivity for Tgm2, although six to seven amino acids of the 1F5 lacritin antigen were identical to a nonantigenic region, respectively, in human TGM2 and pig Tgm2. The 1F5 antigen contains glutamine 14 and lysine 20. Antigen injection into a Tgm2-rich subcutaneous or intramuscular environment might lead to rapid deamidation of glutamine 14 and possible intrachain covalent binding with lysine 20, as would be replicated by combination of Tgm2 with lacritin in vitro. The capacity of deamidation to generate new epitopes underlies celiac disease where deamidation of wheat gliadin (and possibly endogenous antigens) creates unique epitopes for T cell receptors that initiate inflammation.[34]

If 1F5 does indeed prefer TGM2 modified lacritin, it might prove a useful marker for dry eye disease, and ocular surface stress, assuming that our observations are correct that increased tear or plasma membrane-associated TGM2 negatively regulates the level of monomeric (epithelial) bioactive lacritin. Human corneal epithelial cells stressed with hyperosmolar culture medium display increased TGM2 mRNA expression and reduced viability.[9] Also, UVB radiation of human corneal epithelial cells increases TGM2 and reduces viability, and TGM2 siRNA depletion improves viability.[7] TGM2 is a well-known regulator of molecular function, both negative and positive. Excess TGM2 is thought to contribute to glaucoma where accumulation of poorly degradable extracellular matrix hinders aqueous humor outflow,[35] and to development of lens cataracts, where TGM2 activation increases with UVC stress.[36] Collagen mitogenic activity and stimulated αvβ3 integrin expression by perisinusoidal stellate cells of the liver are diminished by TGM2 crosslinking.[10] A nerve-derived tissue transglutaminase (apparently Tgm2) generates an IL-2-dimer that is cytotoxic for oligodendrocytes, and yet promotes optic nerve regeneration in rats.[37] Midkine-dependent stimulation of plasminogen activator activity in bovine aortic endothelial cells is dependent on prior Tgm2 cross-linking of midkine, a heparin binding growth factor.[12] Similarly, Tgm2 triggers the activation of latent TGF-β in bovine aortic endothelial cells,[38,39] and by dimerizing the calgranulin S100A11 generates an agonist for the catabolism of cartilage matrix and hypertrophy of chondrocytes in osteoarthritis.[14] Association of increased TGM2 with dry eye suggests a mechanism whereby less monomeric lacritin may be available to promote basal tearing[17] or protect the ocular surface from stress (Zimmerman K, et al. *IOVS* 2012;53:ARVO E-Abstract 4231). Thus, increased TGM2

through less lacritin monomer could reduce tear volume, thereby generating or further exacerbating dry eye.

We chose to monitor the effect of Tgm2 on lacritin activity by immobilizing lacritin via its intein fusion tag to chitin-coated beads in a lacritin-rich environment that facilitated both the exposure and thorough removal of added Tgm2, or negative controls. Only Tgm2-modified lacritin displayed diminished affinity for syndecan-1. Lacritin also displays a cleavage dependent bactericidal activity (McKown RL, et al. *IOVS* 2009;50:ARVO E-Abstract 4264). Unlike the nanomolar doses optimal for epithelial mitogenesis and survival, the C-terminal bactericidal fragment works at micromolar levels over a broad sigmoidal dose response range. Could this activity also be sensitive to cross-linking? Possibly so, if proteolysis is hindered. If not affected, cross-linked lacritin could serve as a pool of latent bactericidal activity releasable by bacterial proteases. Thus tear TGM2 may both negatively regulate the level of bioactive monomer and perhaps accumulate a micromolar level pool of cross-linked lacritin for innate defense.

Acknowledgments

We thank the UVa Lymphocyte Culture Center for their preparation of 1F5 mab.

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