A Cleavage-potentiated Fragment of Tear Lacritin Is Bactericidal*

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Background: The wet visual surface of the eye is essentially a sterile environment.

Results: Proteolytic processing of the prosecretory mitogen lacritin in tears releases a fragment that is required for much of the bactericidal activity of tears.

Conclusion: The protease-released C terminus of lacritin is bactericidal under physiological conditions.

Significance: All known lacritin activities are bundled within the same C-terminal region, although at different dose optimum.

Antimicrobial peptides are important as the first line of innate defense, through their tendency to disrupt bacterial membranes or intracellular pathways and potentially as the next generation of antibiotics. How they protect wet epithelia is not entirely clear, with most individually inactive under physiological conditions and many preferentially targeting Gram-positive bacteria. Tears covering the surface of the eye are bactericidal for Gram-positive and -negative bacteria. Here we narrow much of the bactericidal activity to a latent C-terminal fragment in the prosecretory mitogen lacritin and report that the mechanism combines membrane permeabilization with rapid metabolic changes, including reduced levels of phosphocoenzyme A, spermidine, putrescine, and phosphatidylethanolamines and elevated alanine, leucine, phenylalanine, tryptophan, proline, glycine, lysine, serine, glutamate, cadaverine, and pyrophosphate. Thus, death by metabolic stress occurs within hours by Staphylococcus epidermidis and slowly by another mechanism, in a chymotrypsin- or leupeptin protease-inhibitable manner. Although bactericidal at low micromolar levels, within a biphasic 1–10 nM dose optimum, the same domain is mitogenic and cytotoxic for epithelia via a syndecan-1 targeting mechanism dependent on heparanase. Thus, the C terminus of lacritin is multifunctional by dose and proteolytic processing and appears to play a key role in the innate protection of the eye, with wider potential benefit elsewhere as lacritin flows from exocrine secretory cells.

Antimicrobial peptides protect all classes of life as the first line of innate defense, through the continuity of surface epithelium and activation of subepithelial macrophages (1, 2). Although >1900 antibacterial peptides have been identified to date, most (3–5) are individually inactive under normal physiological conditions (6, 7), with electrostatic binding of the anionic bacterial outer membrane a common characteristic. Yet others are primarily hydrophobic. Although membrane disruption is typical with ensuing lysis or pore formation (8), some pass intracellularly to disrupt function (9). Antimicrobial peptides may represent the future of antibiotics, with sensitivity to proteolysis in the gut being a primary weakness.

Different epithelia have evolved distinct protective mechanisms. The surface epithelium of the eye lacks the enhanced cornified barrier of skin, yet is rarely subject to bacterial penetration (7, 10). This property is largely attributable to the bactericidal tear film that covers the surface of the eye with lipids (11), metabolites (12), salts, and at least 1500 different proteins, some only recently characterized (13). Originally, it was thought that tears only immobilized pathogens by salt agglutination for subsequent removal or that salt levels were responsible for tear bactericidal activity because heat was ineffective (14) or, instead, that a moderate heat-resistant activity could be designated as a lysozyme (15). Since then, a variety of antimicrobial factors have been identified in human tear film, including lactoferrin, immunoglobulin A antibodies (IgA), secretory phospholipase A2, mucins, α- and β-defensins, histatins, and cathelicidin LL-37 (7). Gene knock-out studies support antimicrobial roles for lipocalin 2 (16–18), cathelicidin antimicrobial peptide (LL37 (19)), and defensin β1 (20). However, all except lipocalin 2 are individually inactive under physiological conditions (6).

Lacritin is a pleiotropic tear protein (21) that promotes the survival of stressed human corneal epithelial cells (22), basal tearing (23), and corneal epithelial cell proliferation (24). Lacritin is 21% identical (25) with dermicidin, whose cathespin D-releasable C-terminal domain is processed (26) to the potently bactericidal SSL-25 peptide, the main bactericidal activity in sweat (27). These observations were the rationale for challenging cultures of Escherichia coli, Pseudomonas aeruginosa, and Staphylococcus epidermidis with lacritin...
and lacritin truncation mutants, peptides, and fragments. We report that cleavage of lacritin releases a potent bactericidal fragment that is distinct from SSL-25 and is active on both Gram-negative and -positive bacteria when applied at low micromolar doses. Activity is retained in 280 mosmol/liter buffer and only slightly diminished at 380 mosmol/liter. Thus, a growth factor with a biphasic dose optimum of 1–10 nM is a potent bactericide at low micromolar levels after proteolytic processing.

**EXPERIMENTAL PROCEDURES**

*Tears and Tear Immunodepletion*—Normal human basal tears were collected as described previously (28). The institutional review board at Walter Reed Army Medical Center Department of Clinical Investigation granted approval prior to the initiation of the study. Each participant gave informed consent, and all research adhered to the tenets of the Declaration of Helsinki. Briefly, tears from 0.5% proparacaine-anesthetized eyes were collected onto preweighed wicks and flash-frozen for −70 °C storage. Tears were eluted by immersion of each strip in 30 μl of PBS for 20 min, followed by centrifugation. For immunodepletion, 10-fold diluted tears were incubated overnight (4 °C) or for 1 h at room temperature with protein A beads (0.2 ml, NAb Spin Kit, Peirce/Thermo Scientific) saturated with “anti-N-65 Lac C-term” or preimmune Ig. N-65 is a lacritin truncation mutant lacking 65 N-terminal amino acids (22). The tear flow-through after centrifugation (5000 × g for 1 min) was then assayed for antibacterial activity.

**Lacritin Constructs, Purification, Synthetic Peptides, and Mass Spectrometry**—Lacritin N-terminal truncations N-55, N-65, N-71, and N-75 (29) were generated by PCR from parent cDNA pLAC, as described previously (29). N-terminal deletions of 80 (N-80) and 86 (N-86) amino acids were generated using forward primers 5′-GGTTGTCATATGAAAGCAGGA-AAAGGAATGCAAGG-3′ and 5′-GGTTGTCATATGCAC-GGAGGCCTGCAAGTG-3′, respectively, and common reverse primer 5′-GGTTGTCATATGGTATCTCTCCCTTTAAG-3′. All constructs were verified by sequencing. Bacterial protein expression and purification of recombinant lacritin and lacritin truncations were performed as described previously (24). Briefly, cleared cell (ER2566 or BL21-CP) lysates were loaded on chitin columns (IMPACT-CN System; New England Biolabs Inc., Beverly, MA) equilibrated with 50 mM Tris, 0.5 mM NaCl (pH 8), followed by 20 column volumes of washing, elution with 50 mM 2-mercaptoethanol for 16 h at room temperature in the same buffer, extensive dialysis against PBS (4 °C), and protein quantitation. Further DEAE purification (22) removed a ~9-kDa lacritin proteolytic fragment and bacterial contaminants in which lacritin was collected as the flow-through with 140 mM NaCl in phosphate buffer, pH 7.2. Synthetic peptides N-80-C-25, N-94, N-94/C-6, N-94/C-10, N-94/C-15, N-99, and N-104 were synthesized by Genscript (Piscataway, NJ) with acetylated N termini. Purity was >95%. C termini of all were amidated, with the exception of lacritin C-terminal N-94, N-99, and N-104. N-64/C-31 was neither amidated nor acetylated and was synthesized by the University of Virginia Biomolecular Research Facility.

The nature of the lacritin ~9-kDa fragment was pursued by Western blotting. Briefly, lacritin before and after DEAE separation was separated by SDS-PAGE and then transferred and blotted with anti-Pep Lac N-terminal and anti-N-65 Lac C-terminal antibodies, respectively diluted 1:200 or 1:400 in PBS containing 0.3% Tween 20. Detection was with ECL. For fragment purification, chitin-enriched lacritin was dialyzed against phosphate buffer containing 14 mM NaCl (pH 7.2). Following incubation with DEAE equilibrated in the same buffer, the ~9-kDa fragment was collected in the flow-through, whereas intact (18 kDa) lacritin was eluted with 140 mM NaCl in phosphate buffer, pH 7.2. After determination of protein concentration (BCA assay), both were aliquoted, lyophлизed, and stored at −70 °C. Analysis was by SDS-PAGE on 4–20% gradient gels. The identity of the ~9-kDa fragment was determined by mass spectrometry.

**Bacterial Growth, SYTOX Green Assays, and on Column Cleavage**—*E. coli* (ATCC (Manassas VA) catalog no. 10536), *S. epidermidis* (ATCC catalog no. 12228), and *P. aeruginosa* (ATCC catalog no. 9027) were grown to mid-log phase in 50 ml of Luria-Bertani (LB) medium and washed three times in phosphate buffer containing 10 mM NaCl (pH 7.2; PB-NaCl) with centrifugation. Pellets were resuspended in 1 ml of PB-NaCl.

For lacritin inhibition assays, 50 μl of bacterial pellets each diluted 1:100 in PB-NaCl were incubated for 1.5 h (37 °C) with 100 μl of lacritin, lacritin truncations, or synthetic peptides at a final concentration of 0.1–6 μM. Mixtures were diluted 1:10 in PB-NaCl before plating 100 μl in quadruplicate on LB agar plates for overnight growth at 37 °C. Colonies were manually counted. In other experiments, mid-log *E. coli* was treated at 37 °C for 0, 1, 2, or 3 h with 2 μM lacritin or lacritin truncations or with ampicillin (5 μM) or tetracycline (2 μM). After each treatment, 100 μl was centrifuged, resuspended in 1 ml of PB-NaCl, and plated (100 μl) onto LB agar for overnight growth (37°C) and colony counting.

For salt sensitivity studies, pelleted and washed mid-log phase *E. coli, S. epidermidis, or P. aeruginosa* were resuspended in 1 ml of PB-NaCl and then treated as above with PB-NaCl or with 3 μM N-65 in 130, 280, or 380 mosmol/liter PB-NaCl for 1.5 h (37 °C). Mixtures were diluted 1:10 in PB-NaCl before plating 100 μl of each in quadruplicate on LB agar plates for overnight growth at 37 °C. Colonies were manually counted.

For bacterial permeability assays, pelleted and washed mid-log phase *E. coli* were resuspended in 1 ml of PB-NaCl and then treated as above with 3 μM lacritin, N-65, or C-25 or with 10% Triton X-100. Similarly, washed mid-log phase *S. epidermidis* were resuspended in 1 ml of PB-NaCl and then treated with lacritin or C-25 or a ~9-kDa purified lacritin fragment. Later, 1 μl of 0.5 mM SYTOX Green was added to each well of 96-well fluorescent microtiter plates. Readings were taken at 5-min intervals at respective excitation and emission wavelengths of 485 and 538 nm using a Fluoroskan Ascent FL fluorometer (Thermo Fisher Scientific). In parallel, SYTOX Green internalization was visualized by confocal microscopy after 1 h of 10% Triton X-100, PB-NaCl, or 3 μM N-65 treatment of washed mid-log phase *E. coli*. 

**Lacritin Bactericidal Activity**
For cell-free synthesis without glycosylation, full-length lacritin cDNA in pLacSL was PCR-amplified and subcloned into pTXB1 supplied by the manufacturer (New England Biolabs, Ipswich, MA). Cell-free synthesis and subsequent removal of ribosomes, followed by metal affinity resin adsorption of His-tagged factors, was performed as per the manufacturer’s instructions (New England Biolabs; PURExpress). Immediately following expression, an aliquot was stored at −60 °C. Other aliquots were incubated at 37 °C for 24 and 48 h. Each was separated by SDS-PAGE, transferred, and blotted with anti-N-65 Lac C-terminal antibodies.

For lacritin cleavage assays, supernatants from saturated 50-ml overnight cultures of S. epidermidis were collected by centrifugation (10 min; 11,000 rpm). Each supernatant was then incubated for 4, 16, and 20 h (37 °C) in PB-NaCl with chitin beads containing lacritin intein immobilized via N termini. C-terminal cleavage products were collected by PB-NaCl washing, separated by SDS-PAGE, transferred, and blotted with anti-N-65 Lac C-terminal antibodies. In some experiments, supernatants and lysates from overnight cultures of S. epidermidis, Staphylococcus aureus, P. aeruginosa, and E. coli were incubated overnight (37 °C) with lacritin in solution in PB-NaCl. Mixtures were then separated by SDS-PAGE, transferred, and blotted with anti-N-65 Lac C-terminal antibodies. Parallel studies monitored the integrity of chitin-intein-immobilized lacritin in PB-NaCl at 37 °C for 0, 24, 48, and 72 h or for 24 h (37 °C) with 1 μM pepstatin, 10 μM bestatin, 100 μM antipain, 1 mM 4-benzensulfonyl fluoride hydrochloride, 100 μM chymostatin, 10 μM E64, 100 μM leupeptin, or 10 mM phosphoramidon or for 24 h after boiling for 5 min at 100 °C.

**Hemolysis Assay**—The method of Cerovský et al. (30) was followed with some modifications. Washed sheep red blood cell pellets (MP Biomedicals, Santa Ana, CA) were suspended for 1 h at 37 °C in 565 μl of PBS plus 100 μl of lacritin, N-55, N-65, N-71, N-75, N-80, or C-25 at a final concentration of 2 μM or with N-65, N-64/C-31, N-80/C-25, N-94, N-94/C-6, N-94/C-10, N-94/C-15, N-99, or N-104 at a final concentration of 6 μM. As respective positive and negative controls, Triton X-100 (final concentration of 5%) or PBS was included in place of lacritin or lacritin fragments. After centrifugation (250 × g; 5 min), the absorbances of supernatants at 540 nm were monitored.

**Metabolome Analysis**—Washed mid-log E. coli were incubated with 6 μM N-65 or PB-NaCl for 15 min at 37 °C in replicates of six, each at 1 × 10⁸ cells/replicate. Cells were then washed once, and pellets were flash-frozen for storage at −70 °C. Unbiased metabolite analysis was performed by Metabolon Inc. (Durham, NC) using GC/MS and LC/MS/MS. 78 metabolites were identified.

**Statistical Analyses**—With the exception of the single metabolomic analysis, all experiments were performed at least
Statistical analysis of metabolite data was as described previously (22), where raw data values were first log transformed to be closely distributed as a normal distribution and then assessed by a non-parametric Wilcoxon test and two-sample t test. For both tests with \( p < 0.05 \), metabolites were considered significantly different and further analyzed by hierarchical clustering for their association patterns. Data are reported as the mean ± S.E.
Lacritin Bactericidal Activity

RESULTS

Lacritin Bactericidal Activity in Tears—Tears protect the surface of the eye against environmental pathogens and are enriched in the prosecretory mitogen lacritin (Fig. 1A), which flows onto the eye during basal and reflex tearing (21, 31). Lacritin is 21% identical to dermcidin, whose proteolytically processed C terminus contributes to the bactericidal activity of human sweat (26, 27, 32). We first sought to validate whether basal human tears (33–35) (Fig. 1B), like reflex tears (36, 37), are bactericidal and, if so, whether lacritin or a lacritin fragment(s) is in part responsible. Indeed, half-diluted basal tears completely blocked E. coli growth (Fig. 1C). E. coli is a significant contributor to bacterial conjunctivitis in the developing world, as is P. aeruginosa (38, 39). We next tested tears that had been passed over immobilized anti-N-65 Lac C-terminal antibodies (ab C-term) to immunodeplete both lacritin and C-terminal lacritin fragments (Fig. 1D, lane 2), or over preimmune Ig (mock-depleted; Fig. 1D, lane 4). Both were diluted 10-fold for dose-dependent challenge of E. coli and P. aeruginosa. Mock-depleted tears suppressed E. coli and P. aeruginosa colonies in a tear volume-dependent manner (Fig. 1, E and F). This contrasted with C-terminal antibody-immunodepleted tears, which were as ineffective as the phosphate buffer negative control (Fig. 1, E and F).

Lacritin’s C Terminus Contains a Bactericidal Domain—Lacritin’s C terminus contains three predicted α-helices (Fig. 1A), each validated by circular dichroism (24, 29) (Fig. 2G). The most C-terminal α-helix is amphipathic and targets syndecan-1 as an initiator of corneal epithelial cell proliferation (24) and survival (22), largely via hydrophobic face residues (29). Association of amphipathic α-helices with bacterial membranes can be destabilizing (40). To explore whether these or other lacritin domains are bactericidal, we generated recombinant lacritin and lacritin truncations (24) (Fig. 2A). Each was generated as an intein fusion protein, purified on chitin to also remove the intein tag and then on DEAE to exclude bacterial contaminants. Lacritin and truncations were then assayed in equimolar (2 μM) amounts in the presence of mid-log E. coli, P. aeruginosa, or S. epidermidis. P. aeruginosa is an eye pathogen often responsible for keratitis in contact lens wear (41). S. epidermidis is a common cause of conjunctivitis and keratitis and is abundant in blepharitis (42, 43), an eyelid inflammation associated with slightly altered tear composition, including selectively less lacritin (44). Lacritin without truncation had no effect on the appearance of colonies, with numbers equivalent to the phosphate buffer negative control (Fig. 1, E and F). This contrasted with C-terminal antibody-immunodepleted tears, which were as ineffective as the phosphate buffer negative control (Fig. 1, E and F).

To ask whether the LAKAGKG region was responsible, we generated AKAGKGMHGGVPGG (amino acids 81–94; N-80/C-25), comprising the truncation-narrowed portion of the SSL-25 homologous region. Also generated were partially overlapping LKSIVEKSILTEQALAKAGKGMH (amino acids 65–88; N-64/C-31) and C-terminal KQFIENGSEFAKLLKFSSLKPKWA (amino acids 95–119; N-94). Unexpectedly, colonies were abundant with N-80/C-25 and N-64/C-31, whereas few or no colonies were apparent with N-94, a region only 12.5% identical with the C terminus of dermcidin. To narrow this site, we generated synthetic peptides N-94/C-6, N-94/C-10, N-94/C-15, N-99, and N-104 (Fig. 2F). N-94 and N-104 were fully active but

FIGURE 3. N-65 permeabilizes bacterial membranes without hemolysis and is effective at 380 mosmol/liter. A, washed mid-log E. coli were incubated (37 °C) with PB- NaCl (phos) without or with 3 μM lacritin, N-65, or C-25 or with 10% Triton X-100 in the presence of 5 μM SYTOX Green in microtiter plates. Fluorescent readings were monitored at 485 nm (excitation) and 538 nm (emission). B, fluorescent confocal microscopic visualization of washed mid-log E. coli 1 h after treatment (37 °C) with 5 μM SYTOX Green in the presence of PB- NaCl (phos) without or with 10% Triton X-100 or 3 μM N-65. Double staining with DAPI is shown. C and D, washed sheep red blood cells were incubated (37 °C) for 1 h with PB- NaCl (phos) without or with 1 μM lacritin or truncations (C) or with 6 μM N-65 or synthetic peptides (D). Others were similarly incubated with 5% Triton X-100. The A540 nm of supernatants after centrifugation was measured. E–G, pelleted and washed mid-log phase E. coli (E), P. aeruginosa (F), or S. epidermidis (G) were resuspended in 1 ml of PB- NaCl and then treated as above with PB- NaCl or with 3 μM N-65 in 130, 280, or 380 mosmol/liter PB- NaCl for 1.5 h (37 °C). The mixtures were then diluted and transferred to agar plates for overnight growth (37 °C) and cfu counting. Colonies were manually counted. Error bars, S.E.
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N-65 is bactericidal and equipotent to ampicillin (Fig. 2I). In dose-response studies, N-104 was almost as effective as N-65 (Fig. 2H and J), with a half-maximal inhibition of $<1 \mu M$ for E. coli (Fig. 2H) and $\sim 1–1.5 \mu M$ for P. aeruginosa (Fig. 2J), a dose range common to antimicrobial peptides.

**Antimicrobial Mechanism**—To ask whether N-65 was destabilizing the outer bacterial membrane such that small extracellular molecules were gaining entry, we challenged mid-log E. coli with it in the presence of the membrane-impermeable dye SYTOX Green (45). After entry, SYTOX Green binds nucleic acids. We also monitored the release of hemoglobin from sheep red blood cells to control for lysis of mammalian cells under identical incubation conditions. N-65 (Fig. 3, A and B), but not C-25 or lacritin (Fig. 3A), with a half-maximal inhibition of $<1 \mu M$ for E. coli (Fig. 2H) and $\sim 1–1.5 \mu M$ for P. aeruginosa (Fig. 2J), a dose range common to antimicrobial peptides.

**FIGURE 4.** Metabolomic heat map of mid-log phase E. coli revealing N-65-triggered changes at 15 min versus no treatment control. Washed mid-log E. coli were incubated with PB-NaCl (untreated) without or with 6 $\mu M$ N-65 for 15 min at 37 °C. Cells were washed, flash-frozen, and subjected to metabolite analysis using GC/MS and LC/MS/MS. Raw data values were first log-transformed to be closely distributed as a normal distribution and then assessed by non-parametric Wilcoxon test and two-sample t test. For both tests with $p < 0.05$, metabolites were considered significantly different and further analyzed by hierarchical clustering for their association patterns.

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played less dephospho coenzyme A, spermidine, putrescine, and phosphatidylethanolamines. Dephospho coenzyme A is the immediate precursor of widely employed cofactor coenzyme A (50). Spermidine and putrescine counter damage from reactive oxygen species (51), and phosphatidylethanolamine is the primary bacterial membrane phospholipid (52) that proportionally decreases in osmotic stress and is necessary for transport protein function (53). Thus, N-65 appears to widely compromise cellular metabolic capacity, protection against reactive oxygen species that are elevated by antibiotics, and perhaps integral transmembrane transport processes. We also observed an accumulation of alanine, leucine, phenylalanine, tryptophan, proline, glycine, lysine, serine, and glutamate (Fig. 4) and a decrease of valine, as also occurs in cold- or heat-stressed E. coli (54). Other changes included more cadaverine and pyrophosphate. Generation
of cadaverine from lysine decarboxylation is a mechanism by which *E. coli* counters acid stress (55), and pyrophosphate is beneficial to bacterial growth (56). Thus, stress from N-65 disruption of the bacterial membrane and the availability of dephospho-enzymes A, spermidine, putrescine, and phosphatidylethanolamines appears to go hand in hand with mechanisms attempting to counteract it.

**Proteolysis of Lacritin**—If this bactericidal mechanism is available in basal tears, as suggested by lacritin immunodepletion experiments (Fig. 1, E and F), C-terminal fragments must be available at a sufficiently high micromolar level. Levels may be enhanced by bacteria-dependent cleavage, for which there is plenty of lacritin available. Basal human tears appear to contain \(~27\,\mu\text{M}\) lacritin antigen, as implied by an ELISA estimate of 4.2 ng of lacritin per 100 ng of total tear protein (28) and a suggested basal tear protein concentration of \(~8\,\mu\text{g}\/\text{ml}\) (57). We therefore searched for evidence of lacritin C-terminal processing in normal tears and in manipulations of recombinant lacritin without or with bacterial supernatant. Monomer is \(~25\,\text{kDa}\), with dimer and trimer at 50 and 75 kDa, respectively. Blotting of normal basal tears with anti-C-terminal lacritin antibodies detected all three, as well as immunoreactive bands of \(\sim12, 10,\) and \(9\,\text{kDa}\), suggesting that C-terminal fragments are natural constituents of basal tears (Fig. 5A). Quantities of these fragments appear to be relatively low. To ask whether lacritin is subject to bacteria-dependent cleavage, we incubated recombinant lacritin (\(\sim18\,\text{kDa}\)) with *S. epidermidis* supernatant. This promoted the appearance of \(\sim17, 14, 9, 8.5,\) and \(6\,\text{kDa}\) bands within \(4\,\text{h}\) at \(37^\circ\text{C}\) that resolved to a single \(~6\,\text{kDa}\) (Fig. 5B) or \(8.7\,\text{kDa}\) (Fig. 5C) anti-C-terminal detectable band by \(18-20\,\text{h}\). Supernatants from other species were tested. *S. aureus* supernatant also gave rise to a \(~8.7\,\text{kDa}\) anti-C-terminal detectable band (Fig. 5C). *P. aeruginosa* and *E. coli* supernatants were less effective over \(18\,\text{h}\), with monomer largely left intact but with some \(8.7\,-\text{kDa}\) fragment detectable (not shown). Purification of recombinant lacritin has often yielded a second \(~9\,\text{kDa}\) band after DEAE purification (Fig. 5D) that promotes entry of SYTOX Green (Fig. 5E); it is detectable with anti-C-terminal, but not anti-N-terminal, specific antibodies (Fig. 5F); and was validated as a lacritin C-terminal fragment by mass spectrometry (Fig. 5G). Its migration in SDS-PAGE is similar to that of N-80 (Fig. 5H). The same band also slowly developed with time of lacritin incubation alone at \(37^\circ\text{C}\) (Fig. 6A), suggesting that an *E. coli* proteolytic enzyme co-purifies with lacritin, as is not uncommon with recombinant proteins. It is unlikely but possible that lacritin has self-cleavage activity (58). To consider this possibility, we generated lacritin using a cell-free translation system. Lacritin was detectable initially as a doublet (Fig. 6B), which decreased substantially by \(24\,\text{h}\) at \(37^\circ\text{C}\) and was barely apparent at \(48\,\text{h}\). No C-terminal fragment was detected (Fig. 6B). To address the nature of the C-terminal fragment generating proteolytic activity, we subjected *E. coli* recombinant lacritin to a panel of proteolytic inhibitors and discovered that processing was inhibitable with chymostatin, with leupeptin, or by boiling but not with 4-(2-aminoethyl) benzene-sulfonfyl fluoride hydrochloride, antipain, bestatin, E64, pepstatin, or phosphoramidon (Fig. 6C). Thus, a serine protease-like activity may be responsible.

**Lacritin Bactericidal Activity**

![FIGURE 6. Lacritin C-terminal bactericidal activity is exposed at slower kinetics in a serine protease-inhibitable manner when recombinant lacritin is incubated alone. A, DEAE-purified lacritin (18 kDa) after incubation for different times in PB-NaCl, stained with Coomassie Blue. B, cell-free translated and purified lacritin retained at \(-60^\circ\text{C}\) (lane 1) or incubated at \(37^\circ\text{C}\) for \(24\,\text{h}\) (lane 2) or \(48\,\text{h}\) (lane 3). C, DEAE-purified lacritin (18 kDa) before (4) or after \(37^\circ\text{C}\) incubation for \(24\,\text{h}\) in PB-NaCl in the presence of proteolytic inhibitors or no inhibitor. Some lacritin was also preboiled at \(100^\circ\text{C}\) (lane 1). Mixtures were separated by SDS-PAGE, transferred, and blotted with C-terminal antibodies. Inhibitors were as follows: 1 mM pepstatin, 100 mM bestatin, 1 mM antipain, 1 mM 4-benzenesulfonfyl fluoride hydrochloride (AEBSF), 100 mM chymostatin, 10 mM E64, 100 mM leupeptin, 10 mM phosphoramidon.](image-url)
Lacritin Bactericidal Activity

DISCUSSION

Antimicrobial peptides protect all classes of life as the first line of innate defense. The surface epithelium of the eye lacks the enhanced cornified barrier of skin and yet is rarely subject to bacterial penetration (7, 10), a property largely attributed to the bactericidal tear film. Tears are enriched in the prosecretory mitogen lacritin (21) that flows onto the eye during basal and reflex tearing. Here we discover that lacritin is subject to C-terminal proteolytic processing and that the amphipathic α-helix-containing fragment appears to account for much of the bactericidal activity of normal basal tears by creating pores without hemolysis and a rapid form of bacterial death that may be regulated.

Our rationale for exploring whether lacritin might be bactericidal was its 21% identity with dermcidin (Figs. 1A and 2E), whose proteolytically processed C terminus contributes to the bactericidal activity of human sweat (26, 27, 31) and is in tears (13). Tear bactericidal activity has been the subject of much curiosity for over a century (7), including the original discovery (13). Tear bactericidal activity has been the subject of much curiosity for over a century (7), including the original discovery (13). Tear bactericidal activity has been the subject of much curiosity for over a century (7), including the original discovery (13). Tear bactericidal activity has been the subject of much curiosity for over a century (7), including the original discovery (13).

Surprisingly, dermcidin primary sequence homology was not the source of lacritin activity. Only 40.7% identity is shared between dermcidin’s bactericidal SSL-25 peptide and the homologous lacritin region that as a synthetic peptide was inactive. Instead, lacritin N-104 fragment with 7% dermcidin identity embodies the core activity, a hybrid domain consisting of an N-terminal amphipathic α-helix and hydrophobic C-terminal coiled coil tail, together appropriate for bacterial membrane contact and insertion, as was apparent by rapid entry of membrane-impermeable SYTOX Green in N-65-treated cells. Yet, dermcidin’s SSL-25 peptide also forms an amphipathic α-helix (60) and, together with an adjoining C-terminal α-helix (60), binds artificial 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1-rac-glycerol)-rich membranes to form Zn2+-dependent toroidal pores (61). If N-104 or -65 create pores, as per SYTOX Green entry and metabolomic changes incompatible with lysis, how is pore formation linked to death? The answer is not clear, although levels of some cellular elements fell, such as phosphatidylethanolamine, the primary bacterial membrane phospholipid (52) necessary for transport protein function (53). Also lower were spermidine and putrescine, the primary bacterial membrane phospholipid (52) necessary for transport protein function (53). Also lower were spermidine and putrescine.

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