Lipophilin, a novel heterodimeric protein of human tears

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Abstract We identified a novel heterodimeric protein, lipophilin AC, in human tears. One of its components, lipophilin A (69 residues; mass, 7575.1; p*I*, 9.47) was homologous to the C1 and C2 components of prostatein ('estramustine-binding protein'), the major secreted protein of rat prostate. Human lipophilin C (77 residues; mass, 8854.1; p*I*, 4.94) was homologous to the rat prostatein C3 component and to human mammaglobin, a protein overexpressed in some mammary carcinomas. Tear lipophilins A and C expand the roster of human uteroglobin superfamily members and provide models for exploring these typically steroid-regulated and steroid-binding molecules.

Key words: Tears; Uteroglobin; Prostatein; Estramustine-binding protein

1. Introduction

Tears play an important role in protecting, nourishing and lubricating the cornea and external eye. Normal tears contain approximately 10 mg/ml of protein, of which 35–45% is comprised of lysozyme, lactoferrin, lipocalin ('tear-specific prealbumin') and IgA [1]. Perhaps because poets have displayed more interest in tears than biochemists, the other proteins of tears have received relatively little attention. While performing preliminary investigations related to antimicrobial components in tears, we noticed a distinctive molecule that had not previously been described. We purified and sequenced this molecule, 'lipophilin', and found it belonged to the uteroglobin superfamily. This report describes our studies.

2. Materials and methods

2.1. Tear collection

Under an approved protocol, healthy adult volunteers were briefly exposed to the vapors of freshly minced onions. Over the next 5–10 min, 100–250 μl of stimulated tears was collected and stored at −20°C until used.

2.2. Purification of lipophilin heterodimers

Tears from different donors were pooled and subjected to RP-HPLC on a 10×250 mm, Vydac 218TP510 C-18 column (Vydac, the Separations Group, Hesperia, CA), using a linear gradient of acetonitrile (ACN) in 0.1% trifluoroacetic acid (TFA), that increased in ACN concentration by 1% min^{-1}. Fractions containing lipophilin heterodimers were identified by performing 16.5% acrylamide SDS-PAGE gels with and without sample reduction by dithiothreitol (DTT). Appropriate fractions were further purified by RP-HPLC on a 4.6×250 mm Vydac 218TP54 C-18 column, using various linear gradients of acetonitrile in 0.1% TFA or in 0.13% heptafluorobutyric acid (HFBA).

2.3. Other methods

Protein was measured by a micro-bicinchoninic acid (BCA) assay (Pierce Chemicals, Rockford, IL), using bovine serum albumin standards. Quantitative amino acid analysis was performed by the PicoTag method. Electrospray mass spectrometry was performed at UCLA in the laboratory of Kym Faull.

2.4. Reduction of lipophilin dimers

HPLC-purified lipophilin (50 μl, 1 mg ml^{-1}) was resuspended in a buffer containing 6 M guanidine.HCl, 20 mM EDTA and 0.5 M Tris, pH 8.07. After the solution had been flushed with nitrogen and incubated in a 52°C water bath for 10 min, freshly prepared DTT was added in approximately 2000-fold molar excess, relative to lipophilin. The samples were again flushed with nitrogen, sealed and incubated for 2.5 h at 52°C. Then, additional DTT was added, followed in 45 min by the addition of glacial acetic acid (5% final concentration) to stabilize the reduced products. These were resolved by performing RP-HPLC on a 4.6×250 mm C-18 column, with a linear ACN gradient in 0.1% TFA.

2.5. Carboxamidomethylation

Rapid reduction of lipophilin heterodimers was achieved by adding a 2000-fold molar excess of DTT at 52°C under the anaerobic conditions described above. After 2.5 h, the mixture was cooled to room temperature, and protected from light. Iodoacetamide was added in a 3-fold molar excess, relative to DTT. After 10 min, DTT (equal in amount to the first addition) was added to quench unreacted iodoacetamide. The carboxamidomethylated (CAM) derivatives of lipophilins A and C were then separated and purified by RP-HPLC.

2.6. Amino acid sequencing

N-terminal sequencing was performed with a Porton Model 2090E sequencer (Beckman Instruments, Fullerton, CA), either directly, or after transferring the CAM-modified peptide to a polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, MA).

2.7. Cyanogen bromide cleavage

CAM-modified components A or C were dissolved at 0.5 mg ml^{-1} in 0.1 N HCl and cyanogen bromide was added in slight excess, by mass, to peptide. The mixture was incubated with occasional shaking for 30 h at room temperature, before performing RP-HPLC to obtain peptide fragments for mass determinations or sequencing.

2.8. Glycosylation

Lipophilin heterodimer and purified components A and C were tested for glycosylation with the ECL glycoprotein detection kit (Amersham, Arlington Heights, IL), using lactoferrin standards. Neither lipophilin component was glycosylated.

2.9. Western and dot blots

A conventional alkaline phosphatase detection system was used in both blotting procedures. Reagents included affinity-purified goat anti-rabbit IgG conjugated to alkaline phosphatase (BioRad, Hercules, CA), 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tet-

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razolium (Sigma). Dot blots were done on nitrocellulose membranes (MSI, Westboro, MA). Western blots were performed after SDS-PAGE and electro-transfer to Immobilon-P PVDF membranes (Millipore).

2.10. Polyclonal antibodies

Rabbits were immunized with synthetic peptides corresponding to defined regions of lipophilins A and C (at Research Genetics, Inc., Huntsville, AL). One of these peptides, KFKAPLEAVAAKMEVKK, was identical to residues 27–43 in lipophilin A. Another, KLLEDMVEKTINSDIS, corresponded to residues 5–20 of lipophilin C.

When anti-lipophilin A (1:10 000 dilution) or anti-lipophilin C (1:5000 dilution) antibody was tested in dot blots, each easily recognized <100 picogram of the cognate lipophilin component, while requiring about 1000-fold more (>62 nanogram) of the noncognate component. The smallest amounts of lipophilin AC heterodimer detected in dot blots with these dilutions of anti-lipophilin A and anti-lipophilin C were 4–8 ng and 1–2 ng, respectively. However, their sensitivity in detecting heterodimers in both dot blots and Western blots was improved substantially by pre-treating the membranes with DTT (data not shown).

3. Results

3.1. Preliminary experiments

Before undertaking this investigation, we had transferred several tear peptides to PVDF membranes and obtained their N-terminal sequences. One of these peptides had a complex sequence, since most of the initial sequencing 25 cycles yielded two residues, whereas cycles 26–42 yielded a single sequence (AKFKAPLXAVAAKMEVK). We constructed a composite query sequence that contained the more abundant residue in each of the first 25 cycles, followed by the unique sequence shown above and performed a BLAST search. This query sequence lacked any human homologue, but was identical in 15/40 (37.5%) residues to the C1 component of rat prostatein, a known heterodimeric protein. Since its double sequence suggested that the unknown human tear peptide was also heterodimeric, we undertook the additional studies described below.

3.2. Demonstration in tears

When we reduced whole tears with dithiothreitol and ran the samples on SDS-PAGE gels, a prominent band with an apparent mass of $\approx$6 kDa was evident (Fig. 1). This band was absent if the samples were run under nonreducing conditions. This simple procedure – performing SDS-PAGE with and without DTT reduction – provided us with a facile method of identifying the lipophilin heterodimer. Treatment of tears with DTT also caused other changes in the electrophoretic pattern that are evident in Fig. 1. The most prominent of these was the disappearance of an abundant $\approx$35 kDa species and the increased prominence of a protein with about half that mass. We were later able to perform Western blots of tears with antibodies to lipophilin component A (Fig. 2) and component C (data not shown). These studies indicated that almost all of the lipophilin peptides in nonreduced tears had a mass of $\approx$14 kDa, and that only small amounts of any higher molecular weight forms were present. Consequently, the $\approx$35 kDa component of tears that was so affected by DTT treatment was not lipophilin.

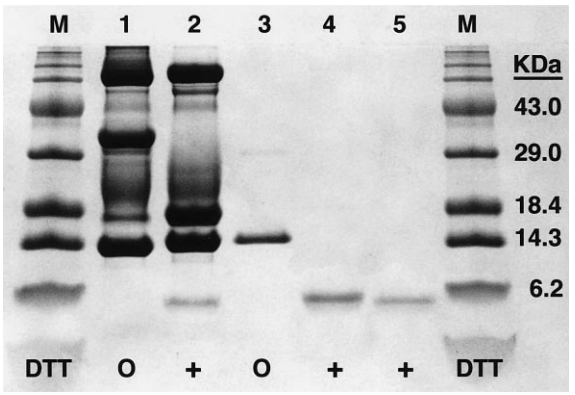

Fig. 1. SDS-PAGE analysis. The lane contents were as follows: M, molecular weight markers, whose sizes are indicated; 1, stimulated tears, 20 μl; 2, DTT-treated stimulated tears, 20 μl; 3, purified lipophilin AC heterodimer, 1 μg; 4, purified lipophilin A monomer, 1 μg; 5, purified lipophilin C monomer, 1 μg. At the bottom of the figure, a + sign indicates dithiothreitol (DTT)-treated samples and ○ denotes samples that were not DTT-treated. The gel was stained with Coomassie blue.

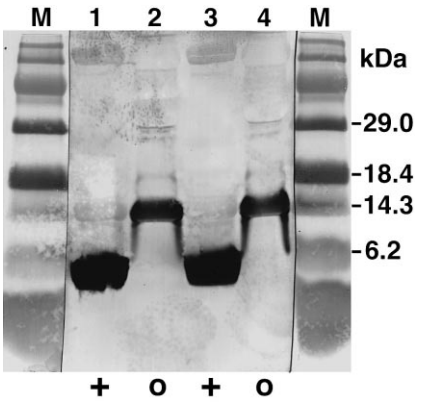

Fig. 2. Western blot. Lanes 1–4 each contained 6 μl of pooled normal tears. The samples in lanes 1 and 3 were reduced with dithiothreitol (+), samples in lanes 2 and 4 were unreduced (0). The lanes marked M contained molecular weight standards, as indicated. Samples 3 and 4 had been lyophilized prior to loading; samples 1 and 2 were nonlyophilized. The polyclonal rabbit antibody was raised against a synthetic peptide corresponding to residues 27–43 of lipophilin A. A similar result was obtained with antibody to lipophilin component C (not shown).

3.3. Purification of lipophilin

We applied 1 ml aliquots of whole tears to a large C-18

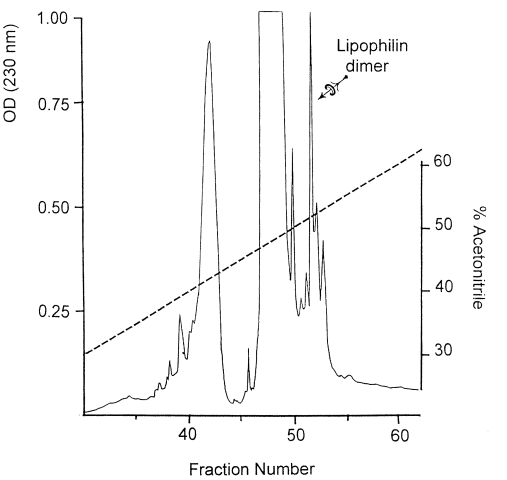

Fig. 3. RP-HPLC fractionation of whole tears on a 10×250 mm C-18 column. Lipophilin was present in the peak indicated by the arrow.

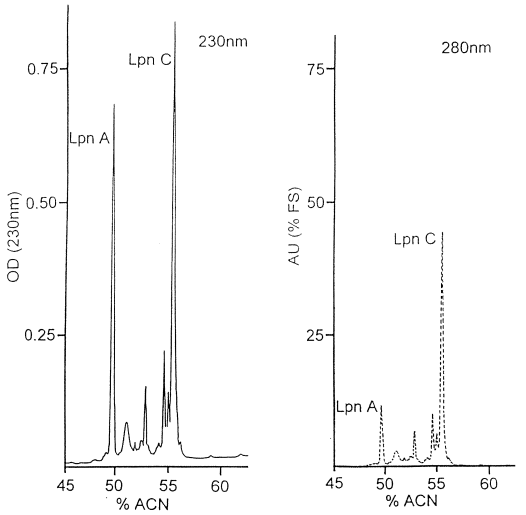

Fig. 4. Separation of lipophilins A and C. Purified lipophilin dimer was reduced with dithiothreitol and chromatographed on a small C-18 column with a 1% min^{-1} gradient of acetonitrile in 0.1% trifluoroacetic acid. Elution was monitored at 230 nm (left panel) and at 280 nm (right panel).

column that was eluted with a gradient of ACN in 0.1% TFA, and identified the lipophilin peak by performing SDS-PAGE ± DTT reduction. Fig. 3 shows the late emerging peak that contained the lipophilin heterodimers. These dimers were further purified to apparent homogeneity on a small C-18 column, using HFBA as the ion-pairing agent. The mass of these purified lipophilin dimers was 16424.01 ± 1.17 by ESI-MS (data not shown). Since we recovered approximately 414 μg of highly purified lipophilin dimer from 9 ml of pooled human tears, its concentration in tears necessarily exceeds 45 μg/ml.

3.4. Separation of the lipophilin monomers

When lipophilin heterodimers were reduced prior to RP-HPLC, their monomeric components eluted separately. Component A emerged before component C (Fig. 4) and showed slightly less absorbance than component C at 230 nm, and considerably less absorbance at 280 nm. This suggested that component A was slightly smaller and contained fewer tryptophan and/or tyrosine residues than component C.

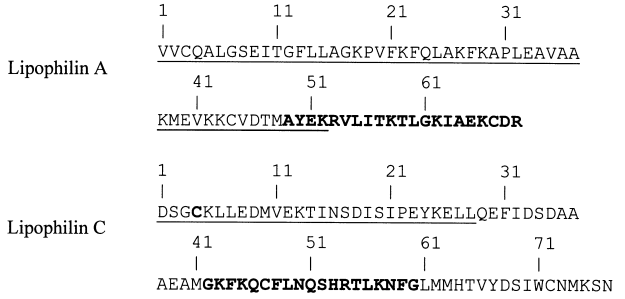

Fig. 5. Primary amino acid sequences of lipophilins A and C. The singly underlined residues were obtained by directly sequencing CAM-modified purified lipophilins A and C. Residues shown in bold type were obtained by sequencing HPLC-purified, cyanogen bromide fragments of carboxamidomethylated lipophilin components A and C.

3.5. Mass measurements

The masses of the reduced and CAM-modified lipophilin A were 7574.69 and 7745.36, respectively. Since alkylating a single half-cystine residue with iodoacetamide increases its mass by 57 AMU, the mass difference (170.67 AMU) between reduced and CAM-modified component A revealed that it contained three half-cystine residues. We studied component C in a similar manner and found the masses of reduced and CAM-modified lipophilin C to be 8854.94 and 9025.74, respectively. The mass difference of 170.80 AMU indicated that component C also contained three half-cystine residues. The measured mass of the parent lipophilin heterodimer (16424.01) corresponded to the calculated mass (16423.63) of a heterodimer that contained one molecule of component A (7574.69 AMU) linked to one molecule of component C (8854.94 AMU) by three intramolecular disulfide bonds. This calculation takes into account that formation of three disulfides will cause the loss of 6 AMU.

3.6. Primary sequences of components A and C

The complete primary sequence of lipophilin A was determined by peptide sequencing and is shown in Fig. 5. Lipo-

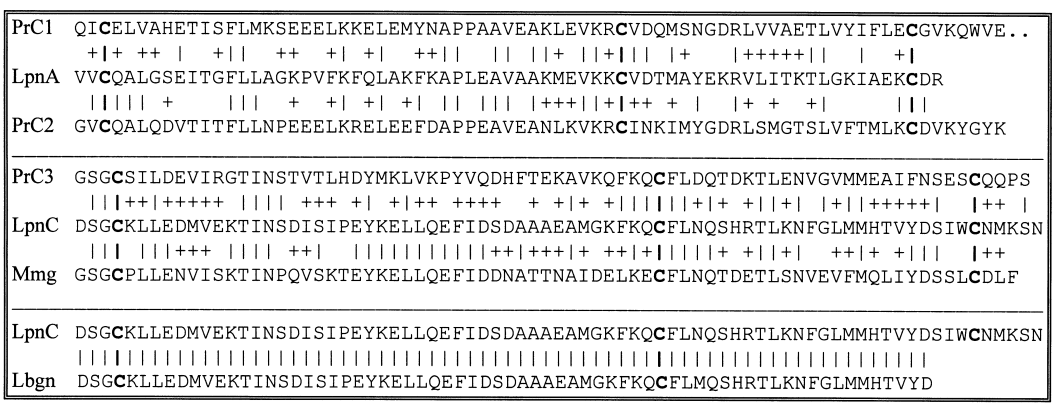

Fig. 6. Lipophilin homology. In the top panel, human lipophilin component A (LpnA) is aligned with rat prostatein components C1 (PrC1) and C2 (PrC2). In the middle panel, human lipophilin component C (LpnC) is aligned with rat prostatein component C3 (PrC3) and human mammaglobin (Mmg). In the bottom panel, lipophilin C is aligned with lacryglobin, a human tear peptide whose partial sequence was recently reported [2]. Identical residues are connected by vertical lines (ı) and similar residues are denoted by a +. The conserved cysteines are bolded.

philin A contained 69 residues and had a calculated p*I* of 9.47. The calculated mass of its reduced form, 7575.1, agreed almost perfectly with the measured mass of the lipophilin A we had purified from tears (7574.69), indicating that the tear molecule was not glycosylated or otherwise post-translationally modified.

Fig. 5 also shows the primary sequence of lipophilin C. The first 28 residues of this peptide had been established by sequencing the holopeptide, and we obtained 19 additional residues by sequencing a cyanogen bromide fragment. The sequence was completed by cloning experiments, to be described elsewhere. Component C was a 77-residue peptide with a calculated p*I* of 4.94. The calculated mass of its reduced form was 8854.07, which matched its measured mass (8854.94) almost perfectly. This measurement confirmed the results of our ECL glycosylation studies, which also indicated that human tear lipophilin C was not glycosylated.

3.7. Orientation in heterodimers

Having determined the primary sequences of the lipophilin A and C monomers, we could decipher the double sequence we had obtained for residues 1–25 in the lipophilin fragment encountered at the outset of this work. It corresponded to the N-terminal residues of both lipophilin components, A and C. We concluded from this that lipophilin A and C monomers associate head-to-head when they form AC heterodimers.

4. Discussion

This report describes the two peptides that comprise lipophilin AC, a heterodimeric molecule present in human tears. Although lipophilin AC is relatively abundant, its existence in tears has largely escaped prior detection. Only a single prior report provides partial sequence data for any lipophilin component [2], the peptide we have designated lipophilin C. These investigators mapped human tear proteins by doing two-dimensional polyacrylamide gel electrophoresis, and ascertaining the p*I*, approximate mass, amino acid composition and N-terminal sequence of the separated proteins. Of the thirty tear proteins mentioned in their report, only six (lysozyme, lipocalin, and lactoferrin, cystatin S, cystatin N and zinc 2A-glycoprotein) matched molecules described in the SWISS-PROT database [2]. A molecule that they called 'lacryglobin' was identical in 68/68 residues to component C of lipophilin. These investigators also noted a second 'lacryglobin spot' on their gels, and speculated that it might represent either C-terminally processed lacryglobin (lipophilin C) or a highly homologous but different gene product. Our data show that lipophilin A (calculated p*I* 9.47) and lipophilin C (calculated p*I* 4.94) differ markedly with respect to cationicity, making it quite unlikely that their second lacryglobin spot corresponds to lipophilin A.

The primary sequence data and heterodimeric structure of human tear lipophilin shows remarkable similarity to that of prostatein, a secreted molecule of the rat prostate gland (Fig. 6). Prostatein has also been called alpha protein [3], prostatic steroid-binding protein [4] and EMBP or estramustine-binding protein [5]. Prostatein/EMBP is a tetrameric molecule, composed of three peptide components named C1, C2 and C3. When we chromatographed human tears on a Sephadex G-100 column, the elution profile of lipophilin was consistent with a tetrameric molecule composed of two AC heterodimers (data not shown). Human lipophilin A is homologous to the C1 and C2 components of rat prostatein, and human lipophilin C is homologous to rat prostatein component C3 (Fig. 6). Whereas the C3 component of rat prostatein was reported to be glycosylated [6], we found that human tear lipophilin C is not. It was recently shown that the C3 component of prostatein is also expressed in the rat lachrymal gland [7] and that primary cultures of rat lachrymal gland cells respond to androgens by secreting a prostatein-like molecule in vitro [8].

The rat ventral prostate has been used extensively as a model system to study the effects of androgens. Prostatein/EMBP constitutes 30–50% of the protein secreted by rat prostate [7,9], and approximately 18–30% of total prostatic cytosolic protein [5]. As the synthesis of prostatein/EMBP is also regulated by androgens [10], these features have made prostatein a valuable experimental marker. Although there is immunochemical evidence that prostatein-like molecules exist in humans [11,12], the sequences of these molecules have not been reported.

We have shown that the molecules, lipophilins A and C, described in this report are homologous to two of the three peptides that constitute rat prostatein/EMBP. In studies that will be described elsewhere, we identified an additional human peptide – lipophilin B – that is a homologue of human lipophilin A and the C1 and C2 components of rat prostatein/EMBP. Lipophilin component B was absent from tears, but its mRNA was prominently expressed by human prostate and other endocrine-responsive organs.

Lipophilin C is also homologous to mammaglobin, a recently described human peptide frequently overexpressed in breast cancer cells [13,14]. To date, mammaglobin has been described only at the nucleotide level, and it remains to be learned if it forms prostatein-like heterodimers or higher order molecular assemblies with lipophilins A and B.

Several immunochemical studies have indicated that the tumor cell expression of prostatein/EMBP can provide useful prognostic information and perhaps predict responsiveness to estramustine (Estracyt) treatment in patients with prostate cancer [15–18] and other malignancies [19,20]. The precise structural characterization of these human lipophilins will facilitate studies to examine their effects on the responses of normal and malignant cells to steroids. The nucleotide and peptide sequences will also permit the design of more precise probes to examine their tissue expression in health and disease.

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