Endonuclease activity in lipocalins

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Several lipocalins contain conserved amino acid sequences similar to the phosphodiester bond cleavage domain of sugar non-specific magnesium-dependent nucleases of the *Serratia marcescens* type. His-89 and Glu-127 of the *S. marcescens* endonuclease are believed to have a role in the active catalytic site by the attack of a water molecule at the phosphorus atom of the bridging phosphate. Tear lipocalin contains both amino acids in analogous regions, and is active as a nuclease. Two forms of β-lactoglobulin contain only Glu-134 (analogous to Glu-127 of the *Serratia* nuclease) yet retain nuclease activity equal to or greater than that of tear lipocalin. However, retinol-binding protein lacks both of these motifs and shows no detectable activity. DNA-nicking activity is decreased by 80 % in the mutant of tear lipocalin that replaces Glu-128 but is unchanged by mutations of His-84. The endonuclease activity of tear lipocalin is dependent on the bivalent cations Mg^{2+} or Mn^{2+} but is decreased at high concentrations of NaCl. These findings indicate that some lipocalins have non-specific endonuclease activity similar in characteristics to the Mg^{2+}-dependent nucleases and related to the conserved sequence LEDFXR (where 'X' denotes 'any other residue'), in which the glutamic residue seems to be important for activity.

Key words: β-lactoglobulin, retinol-binding protein, tear lipocalin, von Ebner's gland protein.

INTRODUCTION

The lipocalins comprise a family characterized by sequence homology of specific conserved regions, a β-barrel composed of an eight anti-parallel strands, and the binding of small hydrophobic molecules [1–7]. Most lipocalins have a molecular mass of approx. 18 kDa and function in the transport of lipids and hydrophobic molecules; for example, retinol-binding protein and β-lactoglobulin are believed to transport retinol in blood and milk respectively [1–3]. Tear lipocalin, also known as von Ebner's gland protein, is produced primarily by lacrimal glands and glands in the circumvallate papillae of the tongue [8]. Tear lipocalin has also been found in the prostate and in sweat [9]. Tear lipocalin comprises approx. 33 % of the protein content of tears and is known to carry a broad array of lipid ligands including phospholipids, fatty acids, glycolipids and cholesterol [10,11]. In addition, tear lipocalin can carry retinol in tears [12], inhibit cysteine proteinases [13] and scavenge lipid from the cornea [14]. Tear lipocalin exists as a non-covalently linked homodimer in tears [15].

The *Serratia marcescens* Mg^{2+}-dependent nuclease is a homodimer composed of two polypeptide chains, each 245 residues long and with a molecular mass of 26.7 kDa [16,17]. The enzyme consists of a central six-stranded anti-parallel β-sheet flanked by α-helices on both sides [16]. Each monomer contains one active site. Inspection of the amino acid sequences (see Figure 1) reveals similarity between tear lipocalin and two well-conserved domains in the *S. marcescens* family of nucleases. Both motifs are believed to have a role in the active catalytic site [18]. The first domain, DRG-HQA, has some, albeit tenuous, corresponding sequence in tear lipocalin (see Figure 1) but the evidence presented here suggests that this domain does not contribute to the nuclease activity of tear lipocalin. The second domain, LEDFXR (where 'X' denotes 'any other residue'), is part of an α-helical segment and includes Glu-127. Here we report on and characterize nuclease activity in lipocalins associated with the LEDFXR domain.

EXPERIMENTAL

Zymographic assay for DNA hydrolysis

Zymographic assays were performed on SDS/10 % (w/v) polyacrylamide gels containing 10 μg/ml heat-denatured salmon sperm DNA. After electrophoresis of protein samples, the gels were rinsed in renaturation buffer [40 mM Tris/HCl (pH 7.5)/1 mM $MgCl_2$/0.1 % (v/v) Triton X-100] and incubated in reaction buffer in 40 mM Tris/HCl (pH 7.5)/1 mM $MgCl_2$/0.7 mM 2-mercaptoethanol at 37 °C for 72 h, as described previously [19].

Endonuclease activity by nick translation

To establish the presence of endonuclease activity, 100 ng of pUC19 DNA (New England Biolabs, Beverly, MA, U.S.A.) was incubated for 10, 20 and 30 min at 37 °C in a 20 μl mixture that contained tear lipocalin (0.25 μg/μl) or bovine DNase I (Sigma, St Louis, MO, U.S.A.) (0.5 ng/μl), 50 mM Tris/HCl, 10 mM $MgSO_4$, 0.1 mM dithiothreitol, 50 μg/ml BSA, 1 mM each of dATP, dGTP, dTTP and 0.5 μCi/μl [α-^{32}P]dCTP (Amersham, Arlington Heights, IL, U.S.A.), pH 7.5. *Escherichia coli* DNA polymerase I (Stratagene, La Jolla, CA, U.S.A.) (5 units) was then added and left at room temperature for 5 min. DNA polymerase I was inactivated by being heated for 5 min at 70 °C. Unincorporated dNTPs were removed with a Centri-Sep column (Princeton Separations, Adelphia, NJ, U.S.A.). The amount of [α-^{32}P]dCTP incorporated was quantified with a Packard 1900 TR scintillation counter. Background counts from a reaction

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mixture that did not contain putative enzyme were subtracted from the radioactive counts incorporated into the plasmid DNA.

Assay for endonuclease activity

Supercoiled pUC19 plasmid DNA (100 ng) was incubated with putative enzymes ranging in concentration from 0.01 to 0.15 μg/μl in 40 mM Tris/HCl/40 mM NaCl/2.5 mM $MgCl_2$ (pH 8.0), in a final volume of 20 μl. At intervals the reactions were quenched with 25 mM EDTA and put on ice. The reaction products were separated on a 1 % (w/v) agarose gel. The relative intensity of bands corresponding to the appearance of the relaxed form of supercoiled plasmid DNA was determined by scanning densitometry (IS-1000 Digital Imaging System; Alpha Innotech Corporation, Leandro, CA, U.S.A.) of the ethidium-bromide-stained gels. To compare the respective activities of DNase I, lipocalins and mutant proteins, the initial rate of appearance of relaxed pUC19 was determined with ORIGIN (Microcal Software, Northampton, MA, U.S.A.) by the method described previously [20]. To compare activities at a variety of salt concentrations, the percentage of relaxed pUC19 was quantified by densitometry and plotted as a function of concentration. The activity was compared after 30 min; during this period the rate of relaxation was linear.

Cloning and mutagenesis: site-directed mutagenesis and plasmid construction

The tear lipocalin cDNA in PCR II (Invitrogen, San Diego, CA, U.S.A.), synthesized previously [21], was used as a template to clone the gene spanning bases 115–592 of the previously published sequence [12] into pET 20b (Novagene, Milwaukee, WI, U.S.A.) with flanking restriction sites for *Nde*I and *Bam*HI as described previously [22]. In addition to the wild type, we prepared three tear lipocalin mutants with substitutions (H84C, H84W and E128W) by the introduction of point mutations by sequential PCR steps [22].

Protein purification

Human retinol-binding protein and bovine β-lactoglobulins A and B were purchased from Sigma. Tear lipocalin was purified from pooled human tear samples by size-exclusion and ion-exchange column chromatographies, as described previously [8,11].

The wild-type and mutant plasmids were transformed in *E. coli* strain BL 21 (DE3). Cells were cultured and protein was expressed in accordance with the manufacturer's protocol (Novagene, Milwaukee, WI, U.S.A.). After cell lysis by sonication as described previously [23], the supernatant was treated with methanol [40 % (v/v) final concentration] at 4 °C for $2\frac{1}{2}$ h. The suspension was centrifuged at 3000 ***g*** for 30 min. The supernatant was dialysed against 50 mM Tris/HCl, pH 8.4. The dialysate was treated with $(NH_4)_2SO_4$ (45–75 % satn). The resulting precipitate was dissolved in 50 mM Tris/HCl, pH 8.4, and applied to a Sephadex G-100 column (2.5 cm × 100 cm) equilibrated with 50 mM Tris/HCl/100 mM NaCl (pH 8.4). The fractions containing the mutant protein were dialysed against 50 mM Tris/HCl, pH 8.4, and applied to a DEAE Sephadex A-25 column. Bound protein was eluted with a 0–0.8 M NaCl gradient. Eluted fractions containing mutant proteins were concentrated centrifugally (Centricon-10; Amicon, Bedford, MA, U.S.A.). Protein concentrations were determined by the biuret method [24]. The purity of the final samples was assessed by a combination of techniques including analytical SDS/tricine/PAGE and MS [11,25].

CD spectral measurements

Spectra were recorded (Jasco 600 spectropolarimeter, 0.2 mm path length for far-UV spectra) with protein concentrations of 1.2 mg/ml. Eight scans from 190 to 260 nm were averaged. Results were recorded as mean molar ellipticity.

RESULTS AND DISCUSSION

Lipocalins share partial sequence identity with non-specific nucleases

A comparison of amino acid sequences reveals that several lipocalins share sequence similarity with the non-specific nucleases of the type related to *S. marcescens*. There is a conserved motif LEDFXR (Figure 1) in tear lipocalin, β-lactoglobulin, late lactation protein, odorant-binding protein and rat von Ebner's gland protein. Many other lipocalins show conservation of part of this sequence, especially a glutamic residue (Figure 1). This motif is not preserved in retinol-binding protein. Another conserved nuclease motif from the *Serratia* group, DRG-HQA, seems to be present in tear lipocalin but not in retinol-binding protein or β-lactoglobulin. These motifs contain highly conserved amino acids His-89 and Glu-127, which are considered critical in the mechanism of nuclease-catalysed cleavage of phosphodiester bonds of DNA. We studied the nuclease activity of three lipocalins (tear lipocalin, β-lactoglobulin and retinol-binding protein) to test our hypothesis that lipocalins have nuclease activity associated with these motifs.

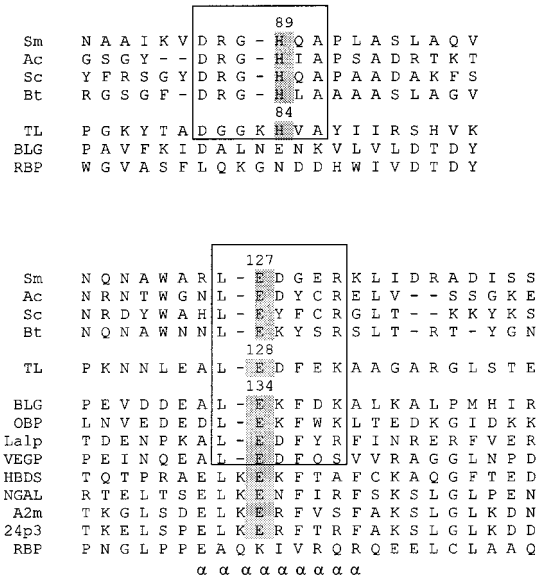

Figure 1 Sequence alignment of *Serratia* class of endonucleases with lipocalins

Representatives of the *Serratia* class of Mg^{2+}-dependent endonucleases and lipocalins include *S. marcescens* (Sm), *Anabaena sp.* (As), *Saccharomyces cerevisiae* (Sc), *Bos taurus* (Bt), human tear lipocalin (TL), bovine β-lactoglobulin (BLG), human retinol-binding protein (RBP), bovine odorant binding protein (OBP), tammar wallaby late lactation protein (Lalp), rat von Ebner's gland protein (VEGP), human brain prostaglandin D synthase (HBDS), human neutrophil gelatinase-associated lipocalin (NGAL), rat α_2-microglobulin (A2m) and mouse oncogene protein 24p3. The two highly conserved sequences are boxed. These residues or those analogous to Glu-127 and His-89 of the *S. marcescens* nuclease are shaded. The notation α designates the secondary structural element α-helix.

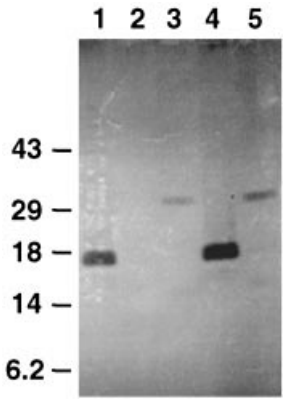

Figure 2 Zymographic assay of lipocalins

Ethidium-bromide-stained SDS/10% (w/v) polyacrylamide gel containing 10 μg/ml salmon sperm DNA. Lane 1, tear lipocalin (15 μg); lane 2, retinol-binding protein (15 μg); lane 3, DNase I (2.0 ng); lane 4, β-lactoglobulin A (15 μg); lane 5, DNase I (5.0 ng). The positions of molecular mass markers are indicated (in kDa) at the left. Tear lipocalin and β-lactoglobulin A show bands corresponding only to their respective monomeric subunit molecular masses at approx. 18 kDa. Retinol-binding protein shows no activity by this assay.

Tear lipocalin and β-lactoglobulin hydrolyse DNA

The zymographic assay in Figure 2 confirms a marked hydrolysis of DNA in the lanes containing tear lipocalin and β-lactoglobulin but not in the lane with retinol-binding protein. A comparison of the respective sequences of these proteins provides circumstantial evidence for the motif responsible for nuclease activity. The LEDFXR motif is present in tear lipocalin and β-lactoglobulin, both of which have nuclease activity. Retinol-binding protein lacks the LEDFXR motif (Figure 1) and is devoid of nuclease activity at the same concentration as tear lipocalin and β-lactoglobulin. β-Lactoglobulin does not contain the DRG-HQA motif yet retains nuclease activity. The zymogram also excludes the possibility of contamination from a ubiquitous endonuclease such as DNase I because the hydrolysis of DNA was observed only at the molecular mass corresponding to the subunit mass of the lipocalin proteins, approx. 17.5–18 kDa.

Tear lipocalin is an endonuclease

To determine whether the nuclease activity observed with lipocalin was an exonuclease or an endonuclease, we performed a modified nick translation reaction with plasmid DNA in the presence of DNA polymerase I. The incorporation of ^{32}P was proportional to the amount of tear lipocalin used (Figure 3A). It is evident from Table 1 that the incorporation of radiolabelled nucleotides followed the nicking of supercoiled DNA in a fashion similar to that of DNase I. Therefore tear lipocalin is an endonuclease. The incorporation efficiency of tear lipocalin is three orders of magnitude lower than that of DNase I. This level of incorporation activity approaches that observed in hydrolysing autoantibodies from the serum of individuals with autoimmune disease [26].

Endonuclease activity was also evaluated by the plasmid relaxation assay, a widely used method for quantification of activity [20,26]. It is evident from Figure 3(B) that the plasmid DNA relaxation activity of tear lipocalin is proportional to the amount of protein used. This relationship permits a comparison of the nuclease activity of tear lipocalin with that of other nucleases and under other conditions.

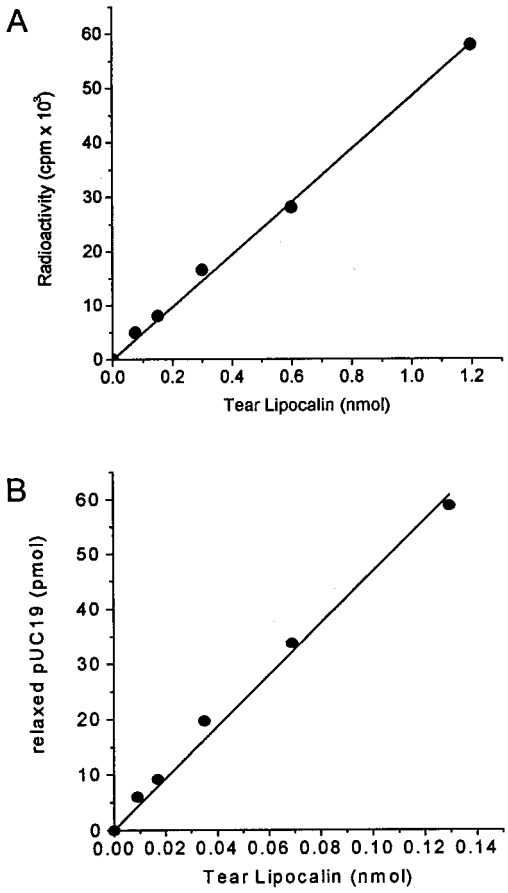

Figure 3 Endonuclease activity as a function of the amount of tear lipocalin

(**A**) Nicking of DNA determined by the coupled DNA polymerase I reaction (as shown in the Experimental section). DNA pUC19 (100 ng) was incubated for 1 h with the indicated amount of tear lipocalin. Incorporated ^{32}P was measured. (**B**) Supercoiled pUC19 plasmid DNA (100 ng) was incubated with the indicated amounts of tear lipocalin for 30 min. The reaction products were separated by 1% (w/v) agarose-gel electrophoresis, stained with ethidium bromide at 5 μg/ml and then photographed. The films were scanned with a densitometer and the amount of relaxed pUC19 was calculated.

Table 1 Incorporation of radioactivity into pUC19 by tear lipocalin and DNase I

Quantitative analysis of [α-^{32}P]dCTP incorporation into pUC19 incubated with tear lipocalin (5 μg; 0.3 nmol) and with DNase I (10 ng; 0.3 pmol), for various durations and supplemented with DNA polymerase I as described in the Experimental section.

	10^{-3} × Radioactivity (c.p.m./nmol of protein)	
Time (min)	Tear lipocalin	DNase I
10	7.3	15900
20	14.1	29400
30	26.4	54600

Endonuclease activity of tear lipocalin is dependent on bivalent cations

The effect of metal ions and salt concentration on DNA digestion of tear lipocalins is evident from Figure 4. The activity was

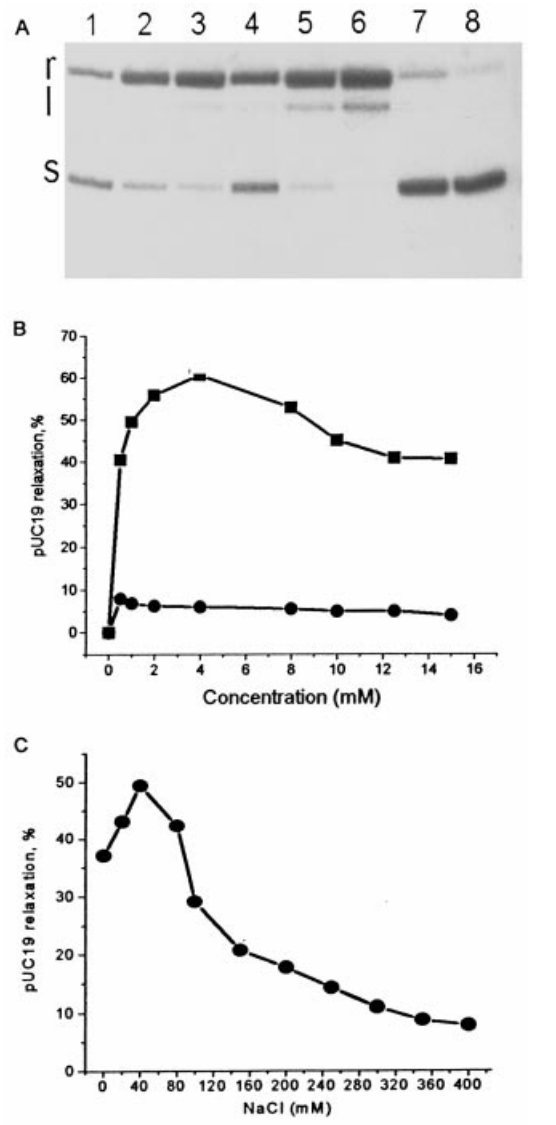

Figure 4 Nuclease activity of tear lipocalin as a function of cation concentration

(**A**) Supercoiled pUC19 plasmid DNA and tear lipocalin, at final concentrations of 3 nM and 1.2 μM respectively, were incubated with 2.5 mM $MgCl_2$ or 2.5 mM $MnCl_2$ as described below. The 1% (w/v) agarose gel shows relaxed (r), linear (l), and supercoiled (s) plasmid products. Lanes 1–3, $MgCl_2$ at 30, 60 and 90 min respectively; lanes 4–6, $MnCl_2$ at 30, 60 and 90 minutes respectively; lane 7, 10 mM EDTA added to the reaction mixture containing 2.5 mM $MgCl_2$; lane 8, reaction mixture containing 2.5 mM $MgCl_2$ in the absence of tear lipocalin. (**B**) The percentage of relaxed pUC19 as a function of $MgCl_2$ (■) and $CaCl_2$ (●) concentrations. (**C**) The percentage of relaxed pUC19 is shown as a function of NaCl concentration.

enhanced in the presence of either $MgCl_2$ or $MnCl_2$ (Figure 4A) but was not affected by $CaCl_2$ (Figure 4B). Optimal activity occurred at 4 mM $MgCl_2$. For the *Serratia* endonuclease there was a strict dependence on bivalent cations for catalytic activity that included Mg^{2+} and Mn^{2+} but not Ca^{2+} [27]. Our results indicate a similar pattern for tear lipocalin.

There was optimal activity with increasing concentration up to 40 mM NaCl (Figure 4C) but further increases in salt concentration resulted in a rapid decline in activity. A decrease in activity with increasing NaCl concentration has been observed with DNase I and has been attributed to a lower substrate DNA-binding affinity [20].

Table 2 DNA digestion activity of the lipocalins

Plasmid relaxation assays were performed as described in the Experimental section. Activities are shown relative to the activity of native tear lipocalin. Results are means ± S.D. for five independent experiments. Mutants are identified by one-letter amino acid codes. Abbreviations: TL, tear lipocalin; BLGA and BLGB, bovine β-lactoglobulins A and B; RBP, human retinol-binding protein; n.d., not detectable. The initial rate of appearance of relaxed pUC19 is shown.

Protein	Rate of relaxed pUC19 formation (pmol/min per nmol)	Relative activity
TL native	19.1 ± 0.6	1
TL wild-type	18.2 ± 0.8	1.0
BLGA	33.5 ± 2.2	1.8
BLGB	17.5 ± 0.4	0.9
RBP	n.d.	–
E128W	4.1 ± 0.2	0.2
H84C	23.5 ± 0.8	1.2
H84W	24.7 ± 0.2	1.3
DNase I	25740 ± 2002	1355

Glu-128 is important for endonuclease activity in tear lipocalin

In the *Serratia* class of Mg^{2+}-dependent nucleases, Glu and His residues are hydrogen-bonded to water molecules and are believed to form a conserved Mg^{2+}/water cluster that participates in the active catalysis of DNA [28]. The quantitative studies of DNA digestion activity with four available lipocalins and three mutants of tear lipocalin implicate Glu-128 in conferring endonuclease activity on the lipocalin family (Table 2). Retinol-binding protein does not possess amino acids analogous to either Glu-128 or His-84 of tear lipocalin and features no detectable activity. β-Lactoglobulin A, which contains only Glu-134 (analogous to Glu-127 of the *Serratia* nuclease), shows the highest relative activity of the lipocalins. The sequence of β-lactoglobulin A does not contain His-84, yet the activity is greater than that of tear lipocalin (Table 2).

To verify the contribution of these two pivotal amino acids we constructed lipocalins with point mutations at these sites. DNA-nicking was decreased by 80% in the tear lipocalin point mutant E128W (Table 2). The decrease in endonuclease activity was not explained by changes in the secondary structure of this mutant. The superimposed CD spectra of all the mutants were extremely similar and showed nearly identical β-sheet and α-helical structures (Figure 5). The difference CD spectra obtained by subtracting the spectrum of native tear lipocalin from those of E128W and H84W show spectral minima at 226 nm and 230 nm for E128W and 230 nm for H84W respectively, (Figure 5, inset). Most proteins show a contribution to the CD band at 220–250 nm conferred by the rotational strength associated with aromatic side chains and between-nearest-neighbour interactions [29,30]. Differences in CD spectra between E128W and H84W are therefore probably related to mutation-associated changes in the aromatic contribution rather than a distorsion of secondary-structure elements. The loss of DNA-hydrolysing activity with point mutations at E128W is remarkably similar to what has been described for the *Serratia* class of endonucleases [31]. Nuclease activity was unchanged in point mutants H84C and H84W (Table 2). Taken together, these results suggest that Glu-128, but not His-84, contributes to endonuclease activity in the lipocalins. Because the activity of tear lipocalin is dependent on

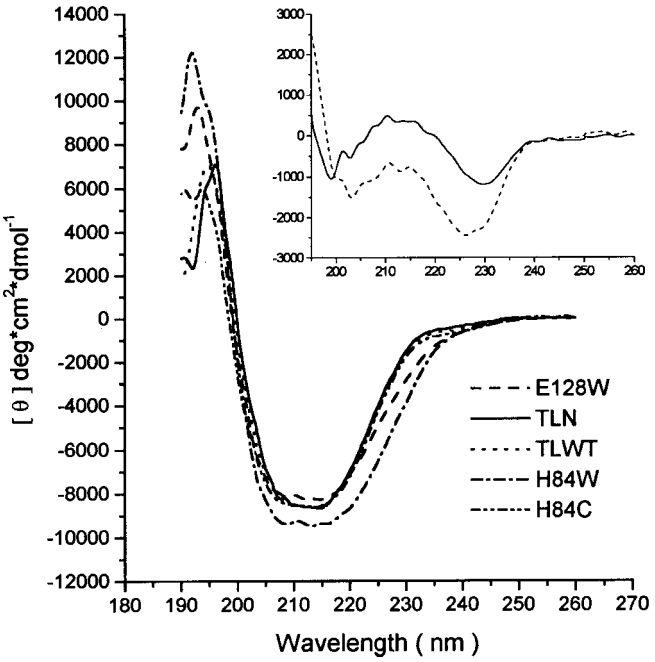

Figure 5 CD spectroscopy of tear lipocalin and mutant proteins

CD spectroscopy was performed as described in the the Experimental section. Abbreviations: TLN, native tear lipocalin; TLWT, expressed wild-type tear lipocalin. Mutant proteins are designated by the numbered amino acid substitution. Inset: difference CD spectra of E128W — TLWT (solid line) and H84W — TLWT (broken line).

the presence of Mg^{2+}, a mechanism employing a Mg^{2+}/water cluster is plausible. Other than the presence of anti-parallel β-sheets, the structures of lipocalins are not particularly similar to those of the *Serratia* class of nucleases. In some nucleases the anti-parallel β-sheet facilitates DNA binding by forming a structure complementary to the major groove of the B form of DNA [32]. Lipocalins feature anti-parallel β-sheets, which might facilitate DNA binding but lack one of the two domains responsible for cleavage of the phosphodiester bonds. Other amino acids that are important for the suggested Mg^{2+}-dependent mechanism of *S. marcescens*, such as Asn-119, are conspicuously absent from β-lactoglobulin, which exhibits nuclease activity. These differences suggest that the mechanism of endonuclease activity in lipocalins is quite different from that in the enzymes of the *S. marcescens* class.

To our knowledge this is the first biochemical demonstration of endonuclease activity in lipocalins. The presence of endonuclease activity in some lipocalins permits speculation on the role of endonuclease activity in tear lipocalin and β-lactoglobulin. Both are present in extracorporeal fluids that are exposed to a variety of microbes including bacteria and viruses, as well as epithelial cells shed from mucosal surfaces. DNA is known to bind and decrease the bacteriocidal activity of lysozyme [33]. Nucleases can act as effective anti-viral agents and inhibit the replication of both DNA and RNA viruses [34]. The clearance of extracellular DNA from tears, saliva, milk, sweat and prostatic secretions could neutralize viruses and help to prevent infection. In addition, the clearance of DNA from the tear film could promote optical clarity. Further studies into the function, extent and mechanism of endonuclease activity in lipocalins are warranted.

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