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Phage display reveals a novel interaction of human tear lipocalin and thioredoxin which is relevant for ligand binding

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Abstract Human tear lipocalin (TL) is an unusual member of the lipocalin protein family, since it is known to bind a large variety of lipophilic ligands in vivo and acts as a cysteine proteinase inhibitor in vitro. It is suggested to function as a physiological protection factor by scavenging lipophilic potentially harmful compounds. Since protein-protein interaction or macromolecular complexation is a common feature of many lipocalins, we applied phage display technology to identify TL interacting proteins. By panning of a human prostate cDNA phagemid library against purified TL we isolated a thioredoxin (Trx) encoding phage clone. Biochemical analysis revealed that TL indeed interacts with Trx and is reduced by this redox protein. Reduction of the TL-specific disulfide bond is of functional relevance, since the reduced protein shows a nine-fold increase in ligand affinity when tested with retinoic acid as ligand.

Key words: Protein-protein interaction; Phage display; Lipocalin; Retinoic acid binding; Von Ebner's gland protein

1. Introduction

The protein superfamily of lipocalins consists of small mainly secretory proteins defined on the basis of conserved amino acid sequence motifs and their common structure. Functionally they share several properties including the ability to bind a remarkable array of small hydrophobic molecules, binding to specific receptors and the formation of macromolecular complexes (reviewed in [1]). However, whereas numerous lipophilic ligands which can bind to lipocalins have been identified, only limited data are available concerning potential interacting protein partners.

Human tear lipocalin (TL, identical with von Ebner's gland protein) is an unusual lipocalin member since it is known to bind a large number of relatively insoluble lipids with binding properties that differ from those of other family members [2,3] and in addition it acts as a cysteine proteinase inhibitor at least in vitro [4]. TL has been found to be expressed in tear glands [5,6], sublingual glands [7], prostate [8], mucosal glands of the tracheobronchial tree [9] and in the human nasal mucosa [10]. Although not all of the biological properties of TL have been determined so far, there is clear evidence that this protein acts as a physiological protection factor of epithelia, probably by scavenging harmful lipophilic molecules, and/or might play a role in the control of inflammatory processes by its protease inhibition [4,9]. Both of these functions would require interaction with other proteins.

One of the most powerful methods to isolate and identify interacting cytoplasmic or secreted proteins is phage display technology [11]. This technique was shown to be, beside its numerous applications in antibody technology [12], especially useful in identification of protease-substrate complexes or in protein-receptor interaction [13,14].

Therefore we investigated the applicability of a phage display shotgun cloning strategy to identify proteins that potentially interact with TL. Here we describe an interaction of TL with the redox mediator human thioredoxin (Trx). This interaction seems to be of physiological relevance for lipophilic ligand binding of TL, since disulfide bond reduction markedly influences the substrate affinity of TL.

2. Materials and methods

2.1. Materials

A polyclonal antiserum against phage protein pVIII was purchased from Clontech (USA) and secondary antibodies were from Sigma (Germany). Human Trx and bovine thioredoxin reductase (TR) were purchased from IMCO Corp. (Sweden). Reduced Trx was prepared according to [15]. Human TL was prepared as described [5] and was routinely delipidated by chloroform/methanol extraction [2]. An enzymatically reduced TL was prepared using the human Trx system as described [16]. Reaction was performed in TE at room temperature for 4 h. Protein aliquots were treated with one volume of 10.8 mM iodoacetamide (Sigma, USA) dissolved in nitrogen-saturated 0.2 M Tris-HCl, pH 8.0, per three volumes of reaction mixture. For chemical reduction protein aliquots were treated with 100 mM 2-mercaptoethanol (2-ME) and iodoacetamide (final concentration 97 μM). The reaction mixtures were purified by ion exchange chromatography as described [5].

2.2. RNA isolation, reverse transcription and construction of a human prostate cDNA library in phagemid pEZM3

Total RNA from human prostate was extracted by RNazol B (Cinna/Biotecx) which is based on the method developed by Chomczynski and Sacchi [17]. Poly(A)$^+$ RNA was prepared using Dynabeads Oligo(dT)$_{25}$ (Dynal, Norway) exactly as described by the supplier. A portion of prostate mRNA was reverse transcribed using random primers and Superscript II reverse transcriptase (Gibco BRL, UK), according to the manufacturer's specifications. The cDNA was blunt ended with Klenow fragment, subjected to agarose gel electrophoresis and the region between 3000 and 200 base pairs was electroeluted and cloned into the *Eco*RV site of phagemid pEZM3 (Clontech, USA). Another portion of the prostate mRNA was reverse transcribed using oligo(dT)-*Bgl*II primers or oligo(dT)-*Apa*I primers and then ligating the dsDNA to an adapter containing a *Sal*I site. After digestion with an excess amount of the appropriate restriction enzyme (50–100 U/μg DNA) the cDNA was size selected by agarose gel electrophoresis and directionally cloned into the *Sal*I and *Bgl*II or *Apa*I sites of phagemid pEZM3 (Clontech, USA).

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Abbreviations: CD, circular dichroism; 2-ME, 2-mercaptoethanol; TL, tear lipocalin; Trx, thioredoxin; TR, thioredoxin reductase

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2.3. Phage production and panning procedure

Phagemids from both batches were mixed and transformed into the amber suppressor *Escherichia coli* strain TG1 by electroporation and grown in 2×YT/glucose medium. Cultures were grown to mid-log phase and infected with M13K07 helper phage (5×10^{10} plaque-forming units/ml) and grown for an additional 2 h. After centrifugation the supernatant was discarded to remove glucose. The pellet was resuspended in 2×YT supplemented with ampicillin (50 μg/ml) and kanamycin (50 μg/ml) and the culture was grown overnight. Phages were isolated by polyethylene glycol 8000. Enrichment of phages was performed in microtiter plates (MaxiSorp, Nunc, Denmark) under the following conditions: wells were precoated with 2 μg purified TL for 2 h at room temperature, washed and blocked with 1% bovine serum albumin (BSA). Wells were incubated (1 h, 37°C) with 50 μl of mixed phages (10^{10}–10^{11} colony-forming units). Following incubation the wells were washed twice (first round of panning), five times (second to fifth round of panning) with TBS, 0.05% Tween-20. Trapped phages were rescued by addition of 0.1 ml of log-phase TG1 cells and incubation for 30 min at 37°C. The enriched TG1 library was plated out on 2×YT/amp/glucose/$MgCl_2$ plates for titer determination and estimation of recovery of trapped phages.

2.4. Confirmation of TL-Trx interaction by ELISA

Wells were precoated with human Trx (1 μg/well) and incubated with purified TL (0.5–2 μg/well) at 4°C for 12 h. After blocking (PBS with 0.2% gelatin, 0.1% Tween 20) and washing, the wells were incubated with anti-TL antiserum for 2 h at room temperature. After removing primary antibody and washing, the wells were incubated with horseradish peroxidase-conjugated secondary antibody for 2 h. Afterwards 50 μl of substrate solution (containing H_2O_2 and 3,3′,5,5′-tetramethylbenzidine) was added and incubated for 30 min. The reaction was stopped by adding 10 μl of 1 N H_2SO_4 and the A_{405} was determined. BSA-coated wells were used as a blank. The Trx-precoated wells without TL showed the same background as the blank.

2.5. Trx reduction assay and gel electrophoresis

The reduction of TL by the Trx/TR system was assayed spectrophotometrically essentially as described previously [15,16] in a Milton-Roy Spectronic 3000 spectrophotometer by monitoring the oxidation of NADPH at 340 nm using 1 cm semi-microcuvettes. A change in A_{340} of 0.0062 between the reaction and reference cuvettes represents the reduction of 1 μM disulfide. Gel electrophoresis under non-reducing and reducing conditions was performed using 14% polyacrylamide Tris-glycine gels (Novex, USA) without or with addition of 2-ME.

2.6. Ligand binding studies by fluorescence quenching experiments

For binding studies an assay which is based on the decrease in tryptophan fluorescence after ligand binding according to [18] was used. Fluorescence measurements were made with a Hitachi F4500 fluorescence spectrophotometer using 5 nm band width at a constant temperature (22°C). Excitation was at 295 nm and emission was recorded between 330 and 350 nm. For each measurement 200 μl protein solution (40 nM–1 μM) in 10 mM sodium phosphate, 100 mM NaCl (pH 7.3) was mixed with 2 μl of appropriately diluted retinoic acid solution. The mixtures were stirred for 5 min at room temperature and the tryptophan fluorescence was measured in three independent experiments. A stock solution of all-*trans* retinoic acid (Sigma, USA) was gravimetrically prepared in DMF and further diluted in DMF as required.

3. Results

3.1. Characterization of the human prostate cDNA phagemid library and panning against TL

TL was found to be expressed in a variety of human glands or tissues [5–10]. For experimental reasons we decided to use human prostate as a source of mRNA for constructing a phagemid cDNA library. As cloning vector we used a gene pIII fusion-based phagemid system, since it is known that relatively large fusion moieties can be added onto pIII without resulting in steric interference with protein-ligand interaction [19]. To maximize the complexity of the phagemid cDNA library two different cDNA synthesis strategies were performed as described above. Each final library consisted of more than 3×10^6 individual clones. Before panning both libraries were pooled. Analysis of the insert size of the pooled phagemids showed that although we used a size-selected cDNA some of the phages contained inserts smaller than 200 bp.

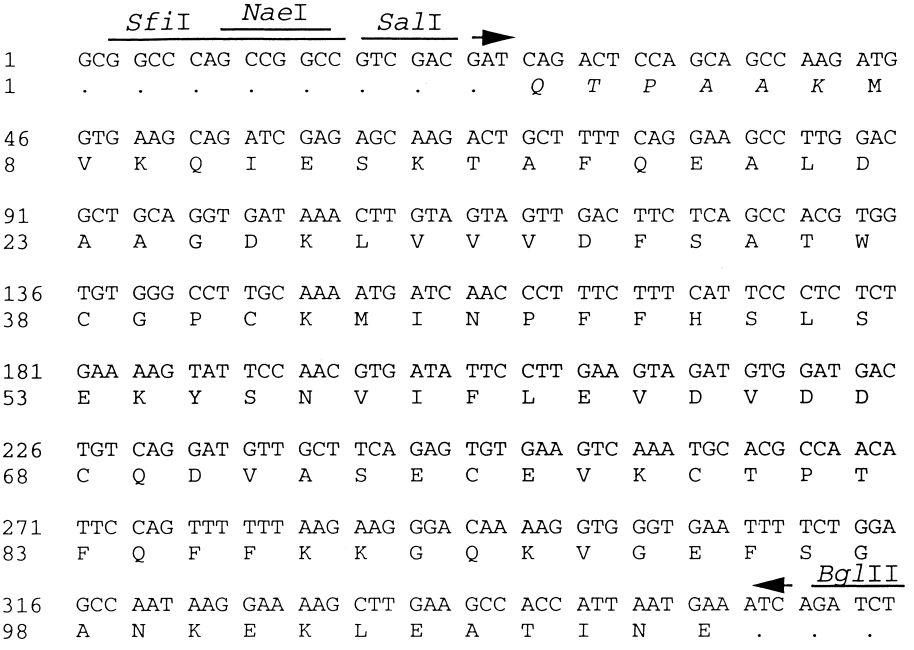

Fig. 1. Nucleotide sequence and deduced amino acid sequence of the phagemid insert encoding human Trx. Following the gene III leader sequence the multiple cloning site is indicated. The *Eco*RV half sites enclosing the insert are marked by arrows. The first six amino acids of the insert (in italics) are encoded by the 5′-untranslated region of the Trx mRNA.

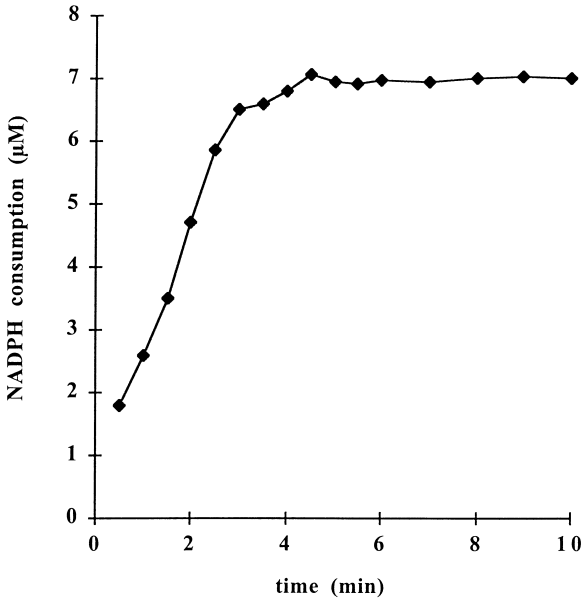

Fig. 2. Disulfide reduction of TL by the human Trx system. The reactions were performed in TE buffer containing 400 μM NADPH. TL was added to both reaction and reference cuvettes, to a final concentration of 10 μM. Human Trx and bovine TR were added to a final concentration of 3 μM to the reaction cuvette. Reference cuvettes contained either Trx (3 μM) or TR (3 μM). Reaction and reference cuvettes were measured simultaneously in an 8-position cell holder and the background was subtracted automatically.

3.2. Nucleotide sequence analysis of affinity-selected phage clones

After five rounds of panning against purified TL as a bait, the enriched phagemid library was screened for interacting clones by ELISA using an anti-pVIII polyclonal antibody and horseradish peroxidase as secondary antibody. From 88 clones used per ELISA plate about 15% reacted with the anti-pVIII antibody. Positive reacting phagemid clones were subjected to further analysis. We selected 10 positive phages, six of them harbored a 330 bp insert. Sequence analysis showed that these inserts encode human Trx (Fig. 1). All of the six phages contained the same insert. The Trx insert starts at the gene III leader sequence-insert fusion with CAG encoding an in frame Gln which is located six amino acids upstream of the original Trx-specific start Met and stops with GAA encoding a Glu. In the original Trx sequence this Glu is located in front of the last two C-terminal amino acids [20].

It should be mentioned that a set of other positive phages contained an insert which showed significant homology (75% and 65% identity) with uncharacterized genes from *Mus musculus* and *Fugu rubripes*. This insert is currently under further investigation.

3.3. Confirmation of TL-Trx interaction by ELISA and enzymatic analysis

To confirm the TL-Trx interaction revealed by phage display, in the next step we performed a sandwich ELISA assay using microtiter wells coated with purified human Trx and incubated with different amounts of purified human TL. Bound TL was detected with an anti-TL-specific antibody [5]. A linear increase of A_{405} values up to 0.26 (corrected by subtraction of the BSA-coated blank) was found with increasing amounts of TL added, thus indicating significant binding of TL to Trx and confirmation of the results from phage display.

Enzymatic cleavage of disulfide bonds by Trx can be followed by a Trx/TR-coupled spectrophotometric assay [15]. Since it is known that TL has one characteristic disulfide bond formed by Cys^{79} and Cys^{171}, we used this assay to test whether TL serves as a substrate for Trx (Fig. 2). The significant NADPH consumption and a time-dependent kinetics show that the disulfide bond of TL is indeed cleaved by the Trx system.

In addition, the enzymatic reduction of TL can also be followed by SDS-PAGE, since the oxidized TL has a more enhanced electrophoretic mobility than the fully reduced form when analyzed without 2-ME treatment (Fig. 3).

3.4. Influence of disulfide reduction on lipophilic ligand binding of TL

Recently, it was demonstrated that the disulfide bridge in TL is important for conferring protein rigidity and there was some evidence that it might influence ligand affinity [21]. Since the latter finding would be of physiological relevance, we investigated the retinoic acid binding activities of a TL reduced by the Trx system compared to a TL which was chemically reduced and an oxidized TL. These investigations were done using fluorescence titration analysis by taking advantage of the intrinsic fluorescence of the conserved Trp in TL. It is known for several lipocalins that binding of lipophilic ligands results in a drastic decrease of the intrinsic protein fluorescence due to energy transfer from excited Trp [18]. We found that retinoic acid possesses essentially the same affinity for TL as retinol [5], therefore retinoic acid was used as a ligand, since it is more soluble in water and chemically more stable than retinol. Fig. 4 shows the fluorescence data from these experiments. Addition of increasing amounts of retinoic acid resulted in a decrease of fluorescence in both oxidized and reduced TL. However, the decrease obtained with reduced TL was sharper than with oxidized TL. The binding constant derived from mathematical analysis was calculated to be 0.96 μM for the oxidized protein and 0.11 μM for the reduced

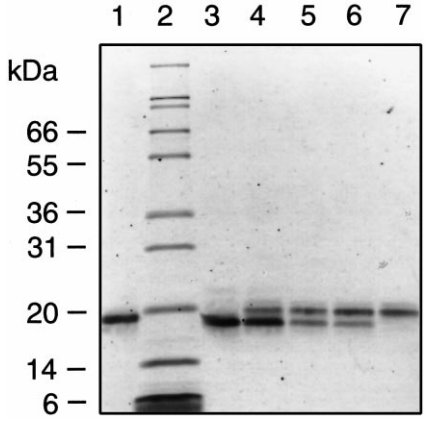

Fig. 3. SDS-PAGE of human TL following incubation with the human Trx system and subsequent alkylation. Lane 1: fully 2-ME-reduced TL; lane 2: molecular weight standards; lanes 3–7: TL treated with Trx system for 15 s (lane 3), 5 min (lane 4), 10 min (lane 5), 15 min (lane 6) and 1 h (lane 7) followed by alkylation with iodoacetamide. Samples in lanes 3–7 were treated with SDS buffer without 2-ME prior to electrophoresis.

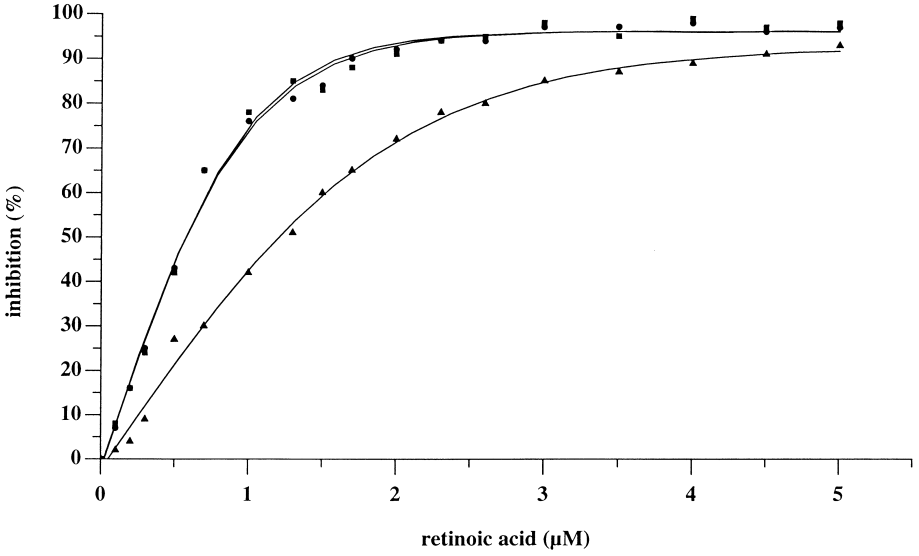

Fig. 4. Determination of the ligand affinity for retinoic acid of oxidized and reduced TL by fluorescence titration experiments. Purified samples of oxidized (▲), enzymatically (■) or chemically (●) reduced TL were each applied at a concentration of 1 μM and mixed with increasing amounts of retinoic acid. Ligand concentration was plotted against the relative decrease in fluorescence. The set of data points was analyzed by standard mathematical methods and a curve was fitted using non-linear hyperbolic regression. The association constant for retinoic acid as calculated was 0.96 μM for the oxidized protein and 0.11 μM for the reduced protein.

protein. Thus the reduced protein has a nine-fold higher affinity for the ligand. There was no significant difference between an TL enzymatically reduced by the Trx system or a chemically reduced protein (Fig. 4).

4. Discussion

In the present study we demonstrated that human TL physically interacts with Trx and found that this interaction significantly influenced the ligand binding properties of TL. Since all Trx proteins can serve as general physiological protein disulfide oxidoreductases that in combination with NADPH and Trx reductase are able to catalyze protein reduction [22], our data strongly suggest that this activity is of relevance for lipocalins in vivo. Almost all of the lipocalin members investigated so far are presumed to have at least one intramolecular disulfide bond. In nearly all of them there is a conserved disulfide bond involving the carboxy-terminal end, indicating functional importance of this structural feature. Although there have been few investigations on the significance of these intracellular disulfide bonds in general, it was clearly demonstrated recently [21] that reduction of the disulfide bond in TL has no influence on the predominant β-sheet structure of the protein but is important in conferring protein rigidity. In addition, when comparing CD spectra from oxidized and chemically reduced protein which was incubated with retinol, enhanced optical activity was generated by bound retinol with the reduced protein, which was interpreted as a more avid ligand binding in the reduced state [21]. Our fluorescence titration experiments with retinoic acid as ligand clearly support this interpretation. The reduced protein showed a nine-fold increase in affinity to retinoic acid compared to the oxidized form. Since Trx is a physiological redox protein our studies strongly suggest that reduction of TL by this system might be of relevance in vivo.

There is increasing evidence that human TL is involved in tissue protection during inflammation and infection [4,9]. On the other hand, it is well known that expression of Trx is inducible by inflammatory cytokines and elevated levels of Trx have been found in chronic infection and inflammation [23,24]. Moreover, activated lymphocytes secrete Trx [25], potentially elevating local concentrations. Higher rates of reduction could also be expected under less aerobic conditions, a situation which is likely to be found in bacterial infections. One might therefore speculate that modification of TL by Trx resulting in enhanced ligand affinity could be a very fast and efficient mechanism to increase the protective potential of this lipid scavenger at very local areas.

The modulation of ligand binding affinity by disulfide bond reduction may have implications for the entire lipocalin family. Therefore a Trx-based reduction mechanism could be of relevance also for other lipocalin members. Indeed, a first preliminary experiment indicated that apoD, another lipocalin member, can also be enzymatically reduced by the Trx system (data not shown). However, more extensive investigations will be necessary to prove the functional consequence of this reaction and to see whether this mechanism is of general relevance for the lipocalins.

Finally, our results demonstrate that a phage display shotgun cloning strategy using complex cDNA libraries is a reliable method to find novel protein interactions with lipocalins. This approach might also be useful for isolation and characterization of lipocalin receptors, since with conventional biochemical methods only limited results have been obtained so far.

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