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Original article

Vitamin A excreted in the urine of canines is associated with a Tamm-Horsfall like protein

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Abstract – Under physiological conditions canines transport vitamin A in blood plasma primarily as retinyl esters bound to lipoproteins and excrete substantial amounts of vitamin A as retinol and retinyl esters with urine. In the aqueous environment of urine, the hydrophobic vitamin A has to be associated with a protein. This vitamin A-protein complex was purified to homogeneity, prepared by preparative ultracentrifugation (density 1.21 g/mL), native polyacrylamide gel electrophoresis (PAGE) and size exclusion chromatography. The vitamin A-protein complex has a high molecular mass of > 5 000 kDa under native conditions. SDS PAGE under reduced conditions revealed a single band with a molecular mass of about 100 kDa for the protein moiety. Peptides obtained after limited proteolysis with trypsin from the 100 kDa protein were characterised by MALDI-TOF mass spectrometry and showed amino acid sequence homology to the human Tamm-Horsfall Protein (THP). This was further confirmed by a positive immunoreaction of the isolated protein with crossreacting human THP antibodies. The localisation of THP in dog kidneys was determined by using immunohistology. The reaction was strong along the entire thick ascending limb of the Henle loop and distal convoluted tubule. Our data point to the possibility that THP functions as a novel carrier for vitamin A in the urine of canines.

vitamin A / canine / kidney / urine / Tamm-Horsfall protein

Résumé – La vitamine A excrétée dans l'urine des canidés est associée à une protéine homologue à la Tamm-Horsfall. Dans des conditions physiologiques normales, les canidés transportent la vitamine A dans le plasma sanguin principalement sous forme de rétinyl esters liés aux lipoprotéines et excrètent des taux importants de vitamine A sous forme de rétinol et de rétinyl esters dans l'urine. Dans

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l'environnement aqueux de l'urine, la vitamine A hydrophobe doit être associée à une protéine. Ce complexe protéine-vitamine A a été purifié et homogénéisé, préparé par ultracentrifugation (1,21 g/mL de densité), électrophorèse sur gel de polyacrylamide natif (PAGE) et chromatographie d'exclusion de taille. Le complexe protéine-vitamine A a une masse moléculaire élevée supérieure à 5 000 kDa dans les conditions natives. Le SDS PAGE effectué dans des conditions réduites a révélé une bande unique avec une masse moléculaire d'environ 100 kDa pour la partie protéique. Les peptides obtenus à partir de la protéine de 100 kDa, après protéolyse limitée avec de la trypsine, ont été caractérisés par spectrométrie de masse MALDI-TOF et ont montré une homologie de séquence en acides aminés avec la protéine Tamm-Horsfall (PTH) humaine. Ceci a été confirmé par une immuno-réaction positive entre la protéine isolée et les anticorps présentant une réaction croisée avec la PTH humaine. La localisation de la PTH dans le rein de chien a été déterminée par immunohistologie. La réaction a été forte tout le long de la section ascendante épaisse de l'anse de Henle, et du tubule contourné distal. Nos résultats suggèrent que la PTH fonctionne comme un nouveau transporteur pour la vitamine A dans l'urine des canidés.

vitamine A / canidés / rein / urine / protéine Tamm-Horsfall

1. INTRODUCTION

Vitamin A metabolism in most species in the order *Carnivora* is fundamentally different from that in other animals and in humans. The dog and most other carnivores transport vitamin A under physiological conditions in the blood plasma predominantly as retinyl palmitate and retinyl stearate [23]. In accordance to observations in other species, retinol is transported in the blood plasma bound to the specific carrier protein, the retinol-binding protein (RBP) [18]. The plasma retinyl esters, in contrast, are bound to the different lipoprotein fractions and their levels in plasma are highly variable, depending on the amount of vitamin A in the diet [22].

In addition to the peculiarity of an unspecific transport of retinyl esters in blood plasma, dogs and other canines are different with regard to the excretion of vitamin A in the urine. Contrary to the other species, which do excrete vitamin A in the urine as water-soluble metabolites [13, 21, 29], canines excrete vitamin A in the urine as retinol and retinyl esters [18, 24]. Both retinol and retinyl esters are hydrophilic components, which require a carrier protein in the urine. The first attempts to characterise this carrier protein showed that it behaves like a lipoprotein of the blood plasma [24]. But besides this, no further information is available concerning the possible protein involved in the binding of retinol and retinyl esters in urine, or the possible mechanism underlying this phenomenon of vitamin A excretion in canine species.

2. MATERIALS AND METHODS

2.1. Urine acquisition

Urine samples (generally 15–50 mL) were collected from healthy dogs (*Canis familiaris*) of both sexes and were placed in tubes, which were wrapped in aluminium foil to protect their contents from light. Sodium azide (2 mg/L) was added in order to prevent bacterial growth. Protease activity was inhibited by the addition of 1 mM EDTA, 0.1 μg/mL phenylmethylsulfonyl fluoride, 5 μg/mL leupeptin and 5 μg/mL aprotinin (Boehringer Mannheim, Germany). Cells and other non-soluble material were cleared from the sample by brief centrifugation ($300 \times g$, 2 min). Centrifuged urine was kept at 4 °C and processed as soon as possible.

2.2. Ultracentrifugation

Urine samples were adjusted to a density of 1.21 g/mL with solid potassium bromide, and aliquots of 11 mL were pipetted into centrifuge tubes (Ultra-Clear, Beckmann Instruments, Inc., Palo Alto, USA). Ultracentrifugation was carried out in a SW-41 rotor at 100 000 × *g* for 18 h at 10 °C using a Beckmann Optima LE-70 preparative ultracentrifuge [6]. Aliquots of the floating fraction and infranatant were collected and stored at 4 °C until further analysis by high performance liquid chromatography (HPLC) and SDS polyacrylamide gel electrophoresis (PAGE).

2.3. Size exclusion chromatography

Urine samples were dialysed 18 h against the elution buffer (0.15 M NaCl, 0.05 M $NaH_2PO_4 \times H_2O$, 1 mM EDTA, 0.02% NaN_3, pH 7.0). Dialysed urine (800 µL; containing approximately 500 µg of total protein) was applied to a Superose-6 HR 10/30 fast protein liquid chromatography column (Pharmacia, Uppsala, Sweden), equilibrated with the elution buffer [28]. Isocratic elution was performed at a flow rate of 30 mL/h. The absorbance was monitored at 280 and 325 nm. Fractions of 2.5 mL were collected, and further analysed by HPLC and SDS PAGE.

2.4. Native PAGE and vitamin A autofluorescence

Urine samples were initially separated on a 3–15% linear gradient native PAGE [12]. The autofluorescence of vitamin A under UV excitation at 312 nm was then used to localise the vitamin A-protein complex [26]. To quantify the amount of vitamin A in the native gel, individual lanes were cut into 5 mm-strips. These strips were either extracted by n-hexane for HPLC analysis to quantify the amount of vitamin A present in the individual fractions of the native gel, or strips were subjected to a 12% SDS PAGE run to determine the molecular mass of the protein and to purify the protein to homogeneity. In the next step, the single band of the purified protein was cut from the SDS gel for in gel digestion. The digested fragments were subjected to mass spectrometry (MS) analysis.

2.5. Determination of vitamin A

Samples containing vitamin A were incubated with an equal volume of ethanol, followed by two extractions with five volumes of n-hexane containing 0.05% (w/v) butylated hydroxy toluene (BHT). The samples were centrifuged at 1800 × *g* for 10 min to facilitate phase separation, and the organic extracts were pooled, evaporated under nitrogen and dissolved in 200 µL of acetonitrile/methanol (85/15, v/v; 0.01% ammonium acetate). Vitamin A (retinol and retinyl esters) was analysed using rp-HPLC in which 150 µL were injected onto an Inertsil-ODS column (250 × 4 mm, 5 µm; Grom, Germany). The solvent system consisted of a step-gradient of acetonitrile/methanol (85/15, v/v; 0.01% ammonium acetate) and isopropanol as described previously [25]. Retinol and retinyl esters were quantified by measuring the absorption at 325 nm. Concentration of retinyl esters were calculated as retinol equivalents using retinol as an external standard [20].

2.6. Analyses of total protein

Protein concentrations were determined using the Bradford method with bovine serum albumin (BSA, Serva, Heidelberg, Germany) as the standard [2]. Apparent molecular mass was determined using a linear 12% SDS PAGE [12]. All gels were stained with silver nitrate [8]. Protein bands were densitometrically scanned using a Fluor-S™ Multimager (BioRad, Munich, Germany).

2.7. Immunoblot analysis

For Western blotting, samples of urine and fractions obtained from ultracentrifugation, native PAGE and size exclusion chromatography were separated through 12% SDS PAGE, and transferred to an Immobilon polyvinylidene difluoride (PVDF) membrane (Millipore Corp., Bedford, USA). The blots were fixed with 20% methanol, blocked with Tris-buffered saline (TBS), 0.1% Tween 20, (TBST, pH 7.6) containing 5% defatted milk, for 1 h, washed 3 times with TBST, and then probed with sheep anti-human THP IgG (Chemicon, Temecula, USA; 1:1000 diluted in 5% defatted milk in 0.05% TBST) at 4 °C overnight. Following incubation, peroxidase conjugated rabbit anti-sheep IgG (Dako Diagnostica, Hamburg, Germany; 1:1000 dilution in 5% defatted milk in 0.05% TBST) was applied. Finally, the immunoreactive bands were visualised by the enhanced chemiluminescence kit (Boehringer Mannheim).

2.8. In gel digestion and MALDI-TOF mass spectrometry

The protein bands were excised from the gel, cut into small pieces and washed for 2 h in a mixture of 40% (v/v) acetonitrile and 60% (v/v) 50 mM NH_4HCO_3. The gel pieces were dried by vacuum centrifugation. An amount of sequencing grade trypsin (Boehringer Mannheim) corresponding to 10% in weight of the estimated protein content of the gel slice was dissolved in 50 µL of 50 mM NH_4HCO_3, added to the dried gel pieces and soaked. Subsequently, 60 µL of 50 mM NH_4HCO_3 was added and digestion was allowed to proceed overnight at 37 °C. Proteolytic peptides were extracted by incubation with 30 µL of 5% (v/v) formic acid for 10 min followed by the addition of 50 µL of acetonitrile. The supernatant was removed and the 5% formic acid/acetonitrile treatment was repeated once. The combined supernatants were lyophilised, dissolved in 40 µL of 0.1% (v/v) trifluoroacetic acid (TFA) and were then used for MALDI-TOF MS analysis or HPLC separation. The purification of the peptides obtained by proteolytic cleavage was performed by reverse phase HPLC on a C18 column using a SMART system from Pharmacia (Uppsala, Sweden). Mass spectra of peptides (before and after HPLC) as well as post source decay (PSD) spectra were obtained using a Reflex II MALDI-TOF MS instrument (Bruker-Daltonik, Bremen, Germany). Alpha-cyano-4-hydroxy cinnamic acid (Fluka, Buchs, Switzerland) at a concentration of 15 mg/mL in 7 volumes acetonitrile and 3 volumes 0.1% (v/v) TFA, served as the matrix. Samples of in gel digested proteins were prepared by depositing equal volumes of sample, 2% (v/v) TFA and matrix solution on the target. Spectra were recorded in the reflector mode and calibrated using the monoisotopic peaks from a known autoproteolysis product of bovine trypsin (residue 50 to 69, M+H+ = 2163.06 Da) and from the matrix trimer ion (3M+H+ = 568.14 Da). Mass accuracy was better than ±0.2 Da up to a mass of 3000 Da. Mass spectra of peptides in HPLC fractions were obtained by directly applying 1 or 2 µL of the eluate fraction to the target followed by 0.3 µL of the matrix solution. The spectra were externally calibrated on Angiotensin II and matrix ions. PSD spectra were obtained from this sample as well [5].

2.9. Database search

The database search using the proteolytic peptide masses was performed with the PeptideSearch program developed at the EMBL Protein & Peptide Group, available on the internet (http://www.mann.embl-heidelberg.de/). The searches were performed at a mass accuracy of ±0.2 Da and the protein masses were restricted to the range between 20 and 150 kDa. Database searches using the

amino acid sequence stretches derived from PSD spectra were performed with the bic_sw protein database search program from the European Bioinformatics Institute (EBI) available over the internet (http://www2.ebi.ac.uk/bic_sw/).

2.10. Immunohistology

The immunohistological localisation of the Tamm-Horsfall protein was performed in tissue sections from the dog kidney fixed in a 4% formaldehyde solution. The samples were embedded in paraffin, sectioned at 4 μm and mounted on glass slides. The sections were deparaffinised, rehydrated, and incubated for 30 min in 0.5% hydrogen peroxide in methanol in order to deactivate endogenous peroxidases. Non-specific antibody binding was blocked for 10 min in TBS, pH 7.6, containing 5% BSA (Serva). The slides were incubated with polyclonal sheep anti-human THP IgG (Chemicon, 1:1000 in 1% BSA in TBS) at 4 °C overnight. The sections were then incubated with peroxidase labelled rabbit anti-sheep IgG (Dako, 1:100 in 1% BSA in TBS) for 30 min. A solution of diaminobenzidine tetrahydrochloride (DAB, Fluka) and 0.01% hydrogen peroxide were used in 0.1 M imidazole buffer (pH 7.1) as substrates for the peroxidase reaction. Finally, the sections were counterstained with hematoxylin, dehydrated and covered. In control sections 1% BSA in TBS was used instead of the primary antibody. All incubations except for the primary antibody were performed at room temperature.

3. RESULTS

3.1. Detection, purification, and characterisation of the 100 kDa urinary protein

One of the major urinary proteins that migrated as a 100 kDa protein on SDS PAGE was purified initially by preparative ultracentrifugation (Tab. I). The resulting protein preparation was analysed by SDS PAGE. A major protein band migrating as a 100 kDa species was detected by staining the gels with silver nitrate and by immunoblot detection of chemiluminescence in the gel (Figs. 1A and 1B). The native urinary protein was migrated as a high molecular weight species (> 5000 kDa) in the void volume from a calibrated gel filtration column, and revealed the presence of a 100 kDa protein band (Fig. 1, lane 5 and lane 9). It was not determined whether the native protein is composed of multiple identical 100 kDa protein containing subunits, or is a hetero- oligomeric complex. The absorption spectrum of the protein complex eluted in the void volume is characterised by two absorbance maxima in the UV region at 280 and 325 nm (Fig. 2A). The retinoids extracted from the purified protein were examined by HPLC analysis. The absorption spectra of the extracted retinoids was identical to that of

Table I. Summary of purification.

Fraction	Retinol and retinyl esters	Protein	Ratio of vitamin A and protein	Purification
	(ng/mL)	(μg/mL)	(ng/μg)	(-fold)
Urine	580	126	4.6	1
Supernatant	463	12	38.6	8.4

Each value is the average of six urine determinations after ultracentrifugation (density = 1.21 g/mL) of dog urine.

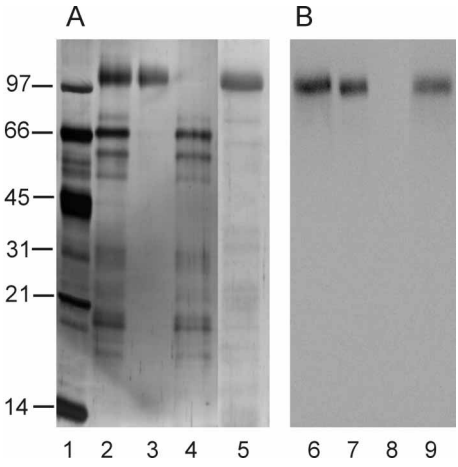

Figure 1. Characterisation of the canine urinary 100 kDa protein. A 100 kDa urinary protein was purified by using preparative ultracentrifugation, size exclusion chromatography and SDS PAGE as described under "Materials and Methods". Gel A was stained with silver nitrate; Gel B was transferred to PVDF and immunoblotted using sheep anti-human Tamm- Horsfall protein antibody. *Lane 1*, standard proteins; *lane 2*, SDS PAGE analysis of dog urine; *lane 3*, SDS PAGE of the floated 100 kDa protein after the ultracentrifugation purification step, *lane 4*, infranatant fraction; *lane 5*, SDS PAGE of the 100 kDa protein obtained from the void volume of the gelfiltration column; *lane 6*, immunoblot analysis of THP (total urine); *lane 7*, floated fraction; *lane 8*, infranatant fraction; *lane 9*, immunoblot detection of THP purified by size exclusion chromatography.

retinol and retinyl ester (palmitate and stearate) standards (Fig. 2B, inset). Under conditions of native gradient PAGE the protein complex had no electrophoretic mobility. Vitamin A could be detected by its typically yellowish-greenish fluorescence under exposure to UV light (Figs. 3A and 3B). The excised fluorescent band migrated under reducing SDS conditions as a 100 kDa protein as depicted in Figure 3C.

3.2. Sequences of tryptic peptides

The 100 kDa protein band was cut out from the SDS-PAGE and incubated with trypsin. The peptide mixture extracted from the gel band was analysed by MALDI-TOF MS; the resulting spectrum can be seen in Figure 4A. Database searches based on the observed peptide masses did not result in the identification of a known protein. Thus the peptide mixture was separated by HPLC and a number of the purified peptides were sequenced by post source decay (PSD) measurements using MALDI-TOF MS. A typical PSD spectrum is shown in Figure 4B. The sequence information obtained from the individual peptides was used to perform database searches using the bic_sw protein database search program from the European Bioinformatics Institute (EBI). The results obtained from six sequenced peptides allowed to identify a clear homology to the human THP (accession number P07911). The homology to bovine, rat and mouse THP (P48733, P27590) was almost as good. Some sequence homology was also found to the human, dog and rat pancreatic secretory granule membrane major glycoprotein GP2 precursor (P55259, P25291, P19218). The determined peptide sequences as well as the homology to human THP is depicted in Figure 5. Sequence identity is indicated by bold face letters.

3.3. Immunohistology

An immunoreactive staining of THP in the dog kidneys was observed in the epithelial cells lining the thick ascending limb of the Henle loop and the distal convoluted tubule (Fig. 6A). The cells of the glomeruli as well as those of the proximal convoluted tubules, the thin parts of the Henle loop and collecting ducts were always negative. All cells of the thick ascending limb of the Henle loop had an extensive staining of the

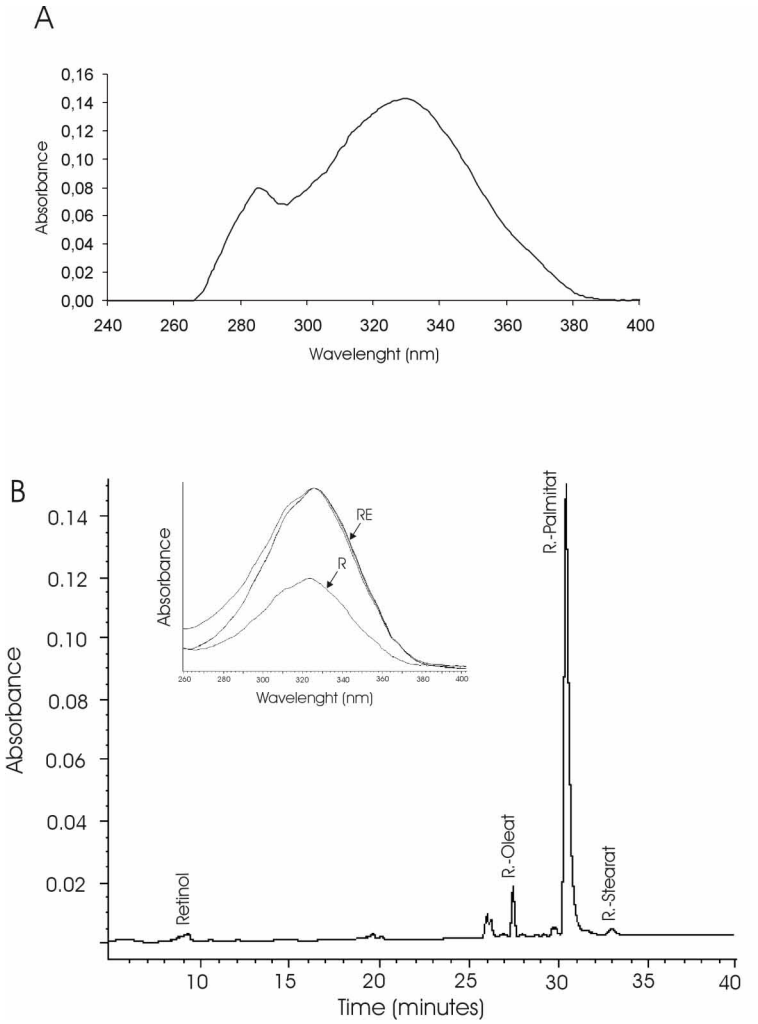

Figure 2. Ultraviolet-visible absorption spectrum of the Tamm-Horsfall vitamin A-protein complex. (A) Purified vitamin A-protein complex isolated from the void volume by gelfiltration. The absorption spectrum of the complex (diluted in elution buffer) was monitored in a Shimadzu UV-visible spectrophotometer between 240 and 600 nm. (B) Gradient HPLC analysis of the retinoids extracted from the purified Tamm-Horsfall protein were analysed on an Intersil C18 column. Retention times and peak areas are recorded. *Inset*, chromatogram of the extracted retinyl esters (RE) and the retinol standard (R) using the photodiode array detector.

luminal membrane, while the cytoplasm and the basolateral membrane were less strongly stained. Kidney sections incubated without primary antibody showed no significant labelling (Fig. 6B).

4. DISCUSSION

As early as 1938, it was reported that dogs excrete substantial amounts of vitamin A in the urine [3]. We were later able to

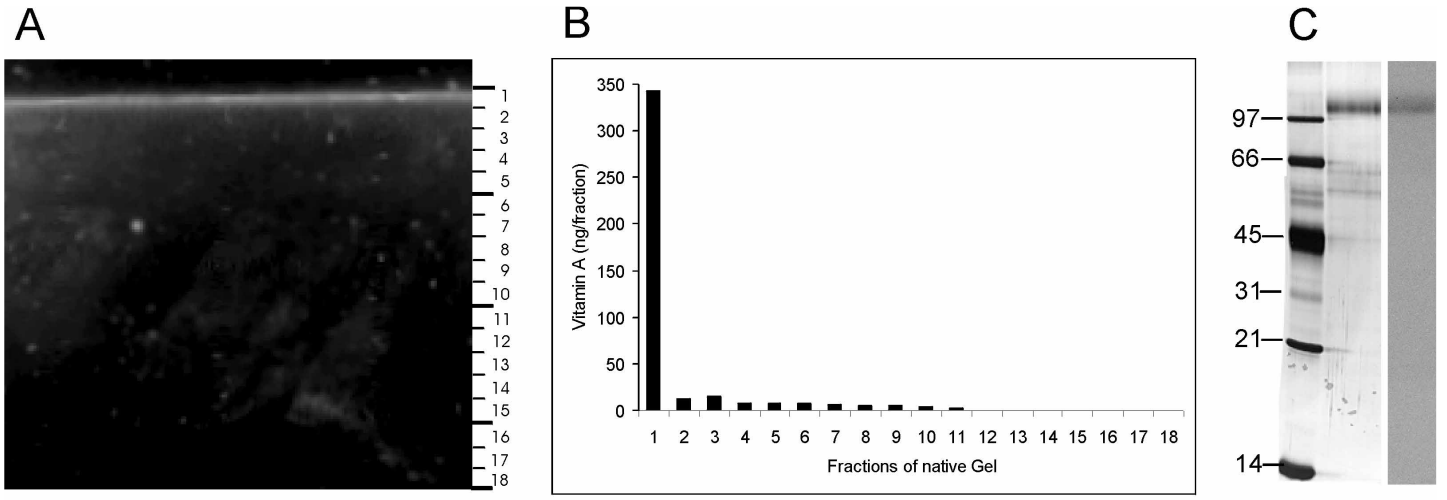

Figure 3. Vitamin A autofluorescence in the native gel under UV exposition. (A) Urine was subjected to a native 3–15% PAGE gradient. Under UV exposure (excitation 312 nm) one fluorescent band was observed only on the cathode. (B) Individual lanes (1–18) were excised from the gel and its retinoid content analysed by HPLC. The retinoid content in the fluorescent band was more than 20-fold higher than those in the other lanes. (C) The native urinary protein migrated as a 100 kDa species on SDS PAGE (*middle lane*) and immunoblot detection showing Tamm-Horsfall protein (*right lane*); *left lane*, standards.

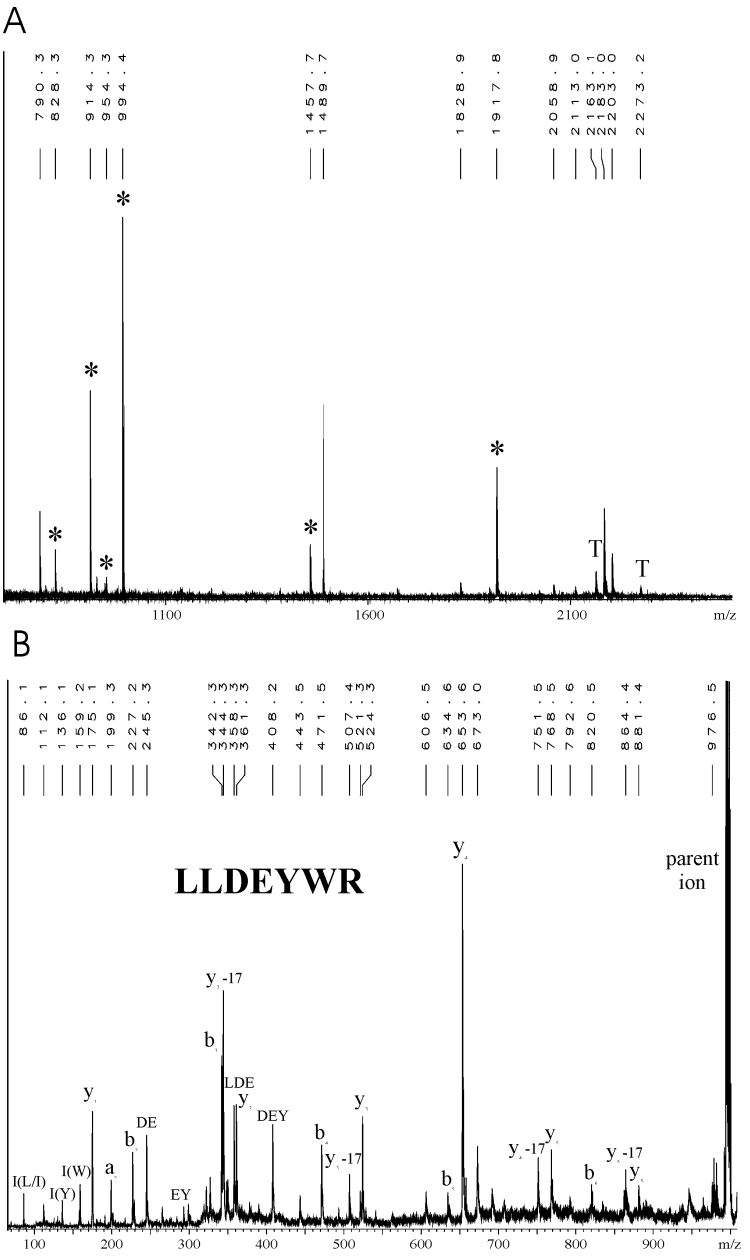

Figure 4. (A) Peptide map of the 100 kDa protein after in-gel tryptic digestion. Peptides marked with a T result from trypsin autoproteolysis. Peptides marked with an * were submitted to PSD analysis, which enabled the determination of their amino acid sequences. (B) PSD spectrum of the peptide at m/z = 994.4. The observed fragments enabled the determination of the amino acid sequence LLDEYWR. L and I cannot be distinguished, however, because of their identical masses.

Figure 5. Homology of the sequenced tryptic peptides to the human Tamm-Horsfall urinary glycoprotein (THP, accession number P07911).

show that vitamin A in the urine of dogs is present in the form of retinol and retinyl esters, primarily as retinyl palmitate. In the same study we showed that other canines such as the fox and the raccoon dog excrete vitamin A as retinol and retinyl esters as well [18, 24].

From early studies onward, it has been speculated that vitamin A might be associated with a protein when excreted in the urine of canines. First experiments showed that the vitamin A-complex in the urine of dogs is of protein origin and stable to heating [14]. Much later, we were the first to extend this limited information and showed that the vitamin A-protein complex in urine could float under ultracentrifugation at a density of 1.21 g/mL and precipitated when the urine was incubated with dextran sulfate in the presence of divalent cations [24]. The results of this study showed for the first time that vitamin A in canine urine is associated with a protein complex that can be isolated from the urine matrix as a single protein band with a molecular mass of 100 kDa. This protein has been purified to homogeneity by three different techniques of protein separation, namely preparative ultracentrifugation, native PAGE and size exclusion gel chromatography, with a subsequent SDS PAGE run in all three approaches. Based on sequence information derived from MALDI-TOF MS and recognition by a crossreacting polyclonal human THP antibody, this protein has been characterised to be closely related or identical to human THP and/or uromodulin [17].

In accordance to humans and rats, the THP in the urine of dogs exists in a polymeric form. Based on the results deduced from the native PAGE and on the behaviour of the native protein complex during size exclusion gel chromatography, the native vitamin A-protein complex has a molecular mass above 5000 kDa which dissociates into monomeric molecules (molecular mass 100 kDa) when subject to SDS PAGE [4]. Similar to human THP, canine THP has an extremely high content of cystine residues relative to other glycoproteins. The reduction of these disulfide bonds with mercaptoethanol results in a reduced

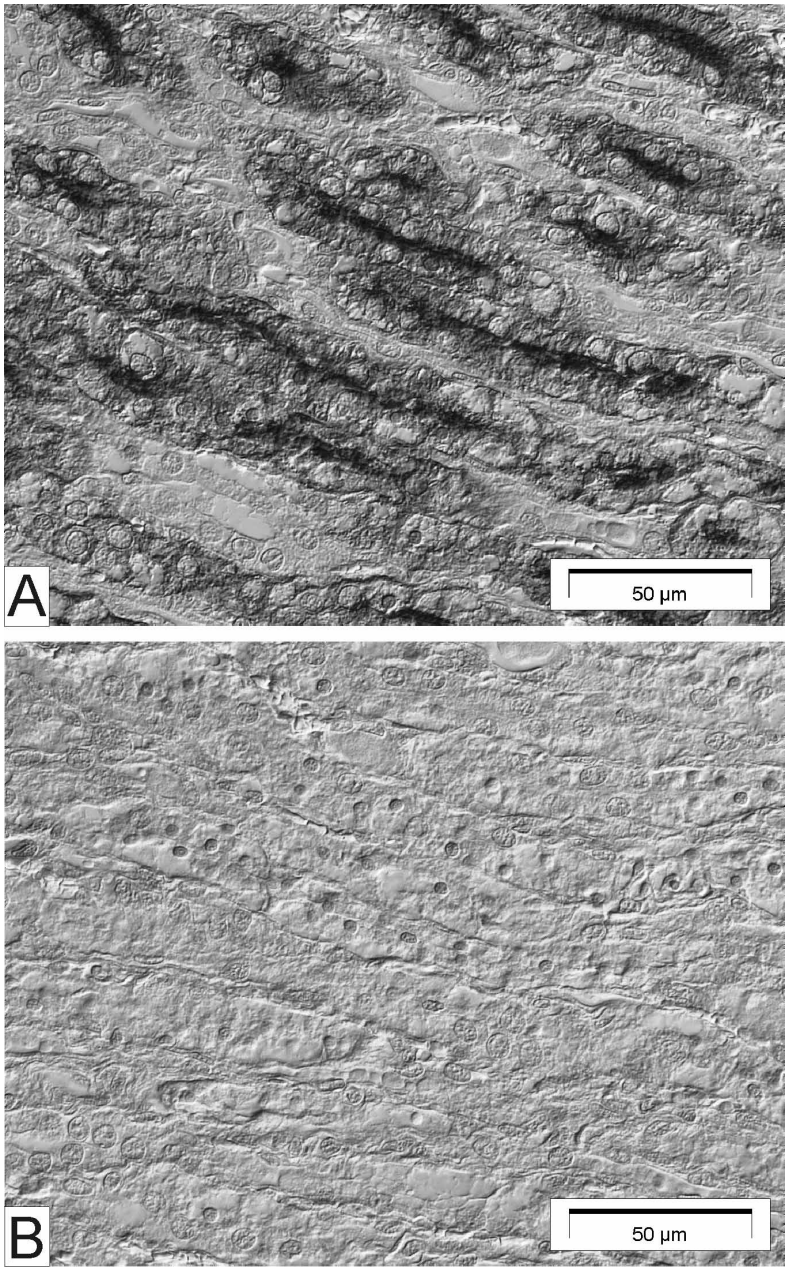

Figure 6. (A) Localisation of the Tamm-Horsfall protein in the adult dog kidney. Specific immunoreactive staining in the epithelial cells lining the thick ascending limb of the Henle loop. The luminal plasma membrane were intensely labelled for THP, while the cytoplasm and the basolateral membrane were less strongly stained. (B) Section incubated without primary antibody shows no significant labelling (Immunohistology, Nomarsky interference contrast).

electrophoretic mobility [4, 15]. The high moiety of carbohydrate has been identified by staining the canine THP after blotting with periodic acid Schiff reagent (data not shown). As in humans and certain other mammals, the THP of dogs was found to be located primarily at the apical membrane of the renal tubular cells of the thick ascending limb of the Henle loop and in the early distal convoluted tubule segments of the nephron [1].

Differences between human and canine THP might be due to the high proportion of the lipophilic vitamin A, which reduces the specific weight. Thus, in canines this complex floats during centrifugation, rather than precipitating [27]. Since both retinol and retinyl esters are associated with the protein, a specific binding as observed in other binding proteins of retinoid metabolism is less probable [16]. However, it remains elusive how retinoids are associated with THP and whether the native polymeric form, the helical organisation or the carbohydrate content of THP are responsible for the binding or association of vitamin A with canine THP.

Although THP has previously been identified in the urine of many mammalian species including the dog, the physiological function of this protein is at present still enigmatic. Since it has been shown that THP is present in the kidney as well as in the superficial layers of the epidermis [9], it has been suggested that this protein may be an important component of epithelia, which absorb sodium and chloride ions but are impermeable to water [11]. Additionally, THP might play a protective role against urinary tract infections [10]. Another function attributed to THP is a possible role in the inhibition or promotion of renal stone formation [7]. In this study we are the first to show that THP might be involved in vitamin A metabolism as well. It has previously been speculated that the excretion of vitamin A in the urine of canines is regulated, instead of being a simple process of glomerular filtration because of the differences in the retinyl ester pattern between plasma (primarily retinyl stearate) and urine (nearly exclusively retinyl palmitate), and because of the size of the lipoproteins, which are carriers of retinyl esters in the blood plasma [19, 24, 30]. Since the excretion of vitamin A in the urine is not as dependent on the concentration of vitamin A in the plasma as on the amount of vitamin A in the diet [22], the knowledge of the protein carrier will greatly help to elucidate those signals and mechanisms on the cellular and molecular levels that are responsible for the regulated excretion of vitamin A in the urine of canines. This might include the basolateral uptake of vitamin A by distal tubule cells, the hydrolysis and reesterification of retinyl esters and retinol within the cells respectively, and finally, the release of vitamin A associated with its carrier into the urine.

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