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Characterization of the native C-reactive protein (cCRP) and the corresponding liver mRNA in dogs

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ABSTRACT

C-reactive protein (CRP) plays an important role in the acute phase reaction in humans and dogs. For the canine CRP (cCRP) only an *in silico* deduced preliminary transcript and amino acid sequence is available. The objective of this study was to further characterize the native cCRP protein and its corresponding liver mRNA. Furthermore, immunological similarities of serum CRP in related animal species were investigated. Native cCRP protein was isolated from dog-sera by affinity chromatography and further analyzed by immunodetection, protein sequencing (mass spectrometry and N-terminal Edman sequencing), 2D-gel electrophoresis, and glycoprotein analysis. Furthermore, cCRP cDNA sequence was determined from dog liver total RNA by RT-PCR. Gel electrophoresis, immunodetection and glycoprotein detection revealed two cCRP isotypes with different molecular weights (22 and 25 kDa) with the upper band being glycosylated. Selective glycoprotein analysis showed sialic acid terminally linked (2–6) to galactose or N-acetylgalactosamine and subsequent PNGase F treatment identified N-terminal linkage. Mass spectrometry confirmed approximately 45% of the cCRP predicted amino acid sequence and N-terminal amino acid sequencing revealed a shorter native cCRP than expected (204 amino acids). The new canine CRP mRNA sequence confirms 100% of the formerly deduced sequence. Immunological homologies to the canine CRP protein were found in selected dog-related species.

This study contributes major molecular details to the knowledge about canine CRP. Such structural information may assist in developing new diagnostic tools for inflammatory-based diseases in dogs as well as other dog-related species.

1. Introduction

The acute phase reaction is an important part of the defense against pathogens and other inflammatory stimuli in animals. C-reactive protein (CRP) is one of the acute phase proteins (APPs), a group of blood proteins involved in inflammatory processes. In healthy individuals, these proteins are typically produced at low concentrations and based on the proportion of their serum concentration they are classified into minor, moderate and major APPs. CRP is in both human and canine patients a major APP, showing increases of 10 to 10,000 fold within 48 h followed by subsequent declines directly after elimination of the stimulus [1,2].

The human CRP (hCRP), first described in 1930 by Tillett and Francis [3], was characterized as a pentraxin and named after its ability to bind through a calcium-mediated binding site phosphocholine (PCh). PCh is part of biological membranes, bacterial cell walls and lipopolysaccharides of various pathogens and CRP binding activates the classical complement pathway [4]. This unspecific immune-response is an ancient defense mechanism against environmental conditions (infections, injuries) [5,6] and of variable importance in different mammalian species. However, the current knowledge about CRP structure and function in non-domestic animals remains limited. Even if the canine CRP (cCRP) has gained importance in veterinary medicine only incomplete biochemical data are available for cCRP compared to the knowledge regarding the structure of human CRP. Only few older studies have

Abbreviations: APC, *p*-Aminophenyl Phosphoryl Choline; APP(s), acute phase protein(s); CRP, C-reactive protein; cCRP, canine CRP; CKD, chronic kidney insufficiency; GDV, gastric dilatation-volvulus; hCRP, human CRP; IMHA, immune-mediated hemolytic anemia; PCh, phosphorylcholine; SNA, Sambucus nigra agglutinin.

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investigated cCRP describing main features including purification, antiserum preparation, molecular weight, isoelectric point and general glycosylation pattern [7–10]. Currently, detailed information about the cCRP protein and the mRNA sequence are only deduced from genomic data.

In general the pentameric CRP proteins are synthesized and further processed in the liver. The human CRP represents a 23 kDa protein composed of 206 amino acids [11] in contrast to the deduced canine CRP comprising 223 amino acids [9].

The canine CRP have been described with different molecular weights (19.4 kDa and 23.5 kDa) suggesting a partial glycosylation of two out of five subunits [9].

Disease associated glycosylation pattern have been detected for the hCRP [12]. Therefore, a specific knowledge about the distinct molecular cCRP structure and its specific glycosylation will be of great value to improve diagnostic approaches in veterinary medicine.

The aim of this study was to further characterize the molecular structure of native cCRP by use of protein sequencing (mass spectrometry, Edman degradation) as well as liver transcripts sequencing (cDNA). This may add missing information about the native cCRP structure. Furthermore a cCRP-specific antibody was first used in dog-related species to elucidate its suitability as diagnostic tools as well for related wild animals.

The detailed knowledge about the native cCRP protein structure including sugar-side chains will further enhance the development of diagnostic tools analyzing inflammatory diseases in dogs.

2. Materials and methods

2.1. cCRP-purification

Serum used for protein characterization was obtained from dogs presented to the Small Animal Clinic, Freie Universitaet Berlin between March 2011 and March 2012. The dogs were euthanized due to the underlying disease, 50–500 ml blood was collected within 30 min after euthanasia, serum samples were prepared by centrifugation and stored (−20 °C).

Protein measurement was performed by Bradford protein assay (Bio-Rad Protein Assay, Bio-Rad Laboratories GmbH, München, Germany). cCRP protein was quantified using a commercial ELISA (TECOmedical Group, Sissach, Switzerland).

Purification of cCRP was conducted by affinity chromatography based on a previously published protocol [13] using *p*-Aminophenyl Phosphoryl Choline (APC)-agarose (Thermo scientific, Pierce Biotechnology, Rockford, USA). In brief, dog serum was diluted 1:2 with binding buffer (0.1 M Tris, 0.1 M NaCl, 2 mM $CaCl_2$, pH 8) and incubated one hour on the APC column. The column was washed until base-line OD 280 nm absorption was reached using a liquid chromatography system (ÄKTAprime plus, GE Healthcare Bio-Science AB, Uppsala, Sweden; PrimeView 5.0 control software). Bound proteins were eluted (elution buffer 0.1 M Tris, 0.1 M NaCl, 2 mM EDTA, pH 8; flow rate 1 ml/min) and peaks collected in 1 ml fractions.

Selected affinity chromatography-fractions were further investigated for molecular weight by analytical gel filtration (HiPrep 16/60 Sephacryl S-300 HR, GE Healthcare, UK) with running buffer (10 mM Tris, pH 8, 10 mM EDTA, 100 mM NaCl) using ÄKTAprime plus (flow rate 0.7 ml/min, protein detection at OD 280 nm) following calibration with marker proteins (67 kDa BSA, 158 kDa aldolase).

2.2. Protein analysis

Protein fractions were examined by gel electrophoresis (GE) using 15% polyacrylamide gels as previously published [14] under non-reducing conditions (Roti Load, Roth, Karlsruhe, Germany). Proteins were stained with Coomassie brilliant blue (G250, Merck) after fixation (40% isopropanol, 10% acetic acid). For Western blot analysis, proteins were transferred on a 0.2 μm nitrocellulose membrane (Hybond™-ECL, GE Healthcare, UK) using a semidry blotter (TE77 ECL Amersham Bioscience Europe GmbH, Freiburg, Germany; 40 min at 45 W/cm^2). After blocking the membrane (TBST + 0.25% fish gelatin) for 1 h, cCRP-specific proteins were subsequently detected using a biotinylated cCRP-specific antibody (TECOmedical AG, Sissach Switzerland) diluted 1:5000 in blocking solution. Washing steps were performed in TBST. Streptavidin-Horseradish-Peroxidase (Calbiochem, Merck, Darmstadt, Germany) diluted 1:100,000 served as detection system in combination with the ECL Advance Western Blotting Detection Kit (GE Healthcare, UK). Chemiluminescence signals were digitized in a Fusion SL (Vilber Lourmat, France).

Two-dimensional (2D) GE was performed according to a published protocol [15] using 20 μg protein per strip. Samples were applied to isoelectric focusing (IEF) strips (3–10 pH range, nonlinear, 17 cm; Bio-Rad), covered with 3 ml Plus One Dry Strip Cover Fluid (Amersham Bioscience) and equilibrated for 14 h at 50 V followed by isoelectric focusing at 1 h 200 V, 1 h 500 V, and finally at 10,000 V for 7 h by using a Protean IEF cell (Bio-Rad). For the second dimension, IEF strips were equilibrated, placed on 12% SDS–polyacrylamide vertical gels, covered with 0.5% agarose, and electrophoresis was carried out in an ETTAN DALT six electrophoresis unit (GE Healthcare) using appropriate conditions: 0.2 W/gel for 1 h, then at 2 W/gel for 18 h. Subsequently, the gels were either Coomassie stained or further analyzed (Western blot, glycoprotein analysis).

2.3. Glycoprotein analysis

Two different glycoprotein characterizations were performed: detection of glycosylated cCRP subunits and identification of sugar-side chains by lectins. Selectivity of the glyco-detection was further proven by enzymatic O-/N-specific deglycosylation. General glycosylation of CRP was performed using the Pierce Glykoprotein Staining kit (Pierce Biotechnology, Rockford, USA) based on in-gel oxidation of hydroxyl groups followed by a specific staining of the generated aldehydes.

After western-blotting sugar-specific glycosylation pattern were analyzed introducing five different lectins (Galanthus nivalis agglutinin (GNA), Sambucus nigra agglutinin (SNA), Maackia amurensis agglutinin (MAA), Peanut agglutinin (PNA), Datura stramonium agglutinin (DSA)) (DIG Glykan Differentiation Kit, Roche Diagnostics, Mannheim, Germany). Ponceau stains (0.5% Ponceau) ascertain for equal amounts of blotted proteins.

To elucidate the linkage-type of the glycans (N-type vs. O-type) an enzymatic N-glycan-specific cleavage was performed by incubation of 1 μg of cCRP with PNGase F (New England Biolabs Inc.). The PNGase F treated cCRP was subsequently identified by lectin Western blot (SNA).

2.4. Protein sequencing

Protein identification was performed with highly enriched cCRP (>95%) further purified by 15% SDS–PAGE. The protein bands were excised, trypsin digested and protein identification was carried out using MALDI TOF MS as described earlier [16].

For N-terminal sequencing (Edman degradation) 7 μg purified cCRP protein was separated by SDS–PAGE, blotted on a nitrocellulose membrane and after Ponceau staining submitted for commercial N-terminal sequencing (Proteome Factory Berlin, Germany).

2.5. mRNA sequencing

A fresh liver sample was taken from an euthanized female, eight year old dog suffering from a vaginal sarcoma, snap-frozen in liquid nitrogen and stored (−80 °C). Approx. 10 mg dog liver was homogenized (SpeedMill P12, AnalytikJena GmbH, Jena, Germany) and total RNA extracted using the InviTrap® Spin Cell RNA Mini Kit (Invitek GmbH, Berlin, Germany), RNA content as well as purity was measured spectroscopically (NanoDrop 1000, peqlab Biotechnologie GmbH, Erlangen, Germany). One μg total dog liver RNA was introduced for cDNA synthesis with random hexamers as described previously [17]. A primer pair deduced from the dog genome (NCBI NW 0037261241) flanking the 5′ and 3′ regions of the open reading frame of the cCRP gene (cCRP-for: 3′-GGAGTCATGGAGGAGCTGTGGC-5′; cCRP-rev: 3′-ACGGGCCTCAGGACCAC-5′) served to PCR-amplify the complete canine CRP-specific mRNA coding region. The proofreading Platinum Taq-polymerase (Invitrogen) was used for amplification using a thermocycler (Biometra) applying the following cycling conditions: 30 cycles 94 °C 30 s and 60 °C 30 s, final step 72 °C 1 min. The resulting 680 bp long amplicon was gel-purified and commercially sequenced (GATC biotech AG Konstanz, Germany).

2.6. CRP homology search in other species

For immunological homology analysis of CRP in other species, four Caniformia species were used, including a racoon (*Procyon lotor*) and a stone marten (*Martes foina*) with unknown disease, a brown bear (*Ursus arctos*) with arthritis and a seal (*Phoca vitulina*) with a necrotizing inflammation in the lung. Additionally, five not directly related species including cat (*Felis silvestris catus*), horse (*Equus caballus*), swine (*Sus scrofa domestica*), cow (*Bos taurus*) and rabbit (*Oryctolagus cuniculus*) with different inflammatory processes were taken as control. All animals had different clinical status and were collected either *post mortem* or for diagnostic purposes. For immunological investigation of sera Western blot with a specific anti-dog cCRP-antibody (TECOmedical) was conducted as described.

3. Results

3.1. cCRP protein purification

Purification of cCRP was performed from pooled blood serum samples (approx. 700 ml from 7 dogs). Total protein amount in individual serum samples was 60–90 mg/ml, and the cCRP concentration varied between 25 and 200 mg/l. After the one-step APC-affinity chromatography about 16 mg native cCRP protein with 93% purity was isolated.

The molecular weight as well as potential impurities of this native cCRP protein preparation were subsequently analyzed through gel filtration comprising a major peak around 110 kDa (Fig. 1A). Corresponding to the estimated molecular weight this peak represents the native pentameric 3D structure of cCRP. Under denaturing conditions two different cCRP protein monomers of approximately 22 and 24 kDa were detected by SDS–PAGE (Fig. 1B).

3.2. Protein sequencing

Mass spectrometry analysis of both gel-purified native cCRP protein isotypes (22 and 24 kDa) reveals identical proteins comprising peptide pattern perfectly matching to the predicted formerly published sequence XP_545746. Matching peptide fragments resulting from the MALDI–TOF–MS analysis of both cCRP isotypes are depicted as bold and underlined letters in the predicted protein sequence. Furthermore, when analyzing the N-terminus of native cCRP protein by Edman degradation, both isotypes comprise the same amino acid sequence: QIDLDE (bold italics letters). The reassessed protein-structure of native canine CRP protein represents a 204 amino acids sequence starting at amino acid 20 (N-terminus) with a hypothetical molecular weight of 23.21 Da and a pI 5.75 (internet resource Expasy). Finally, we were able to verify 92 out of the 204 (45%) earlier predicted amino acid sequence of the native cCRP:

```
1    MEKLWPCLLV LMNLPGAFL***Q IDLDE***KAFVF PR**ESENSYVI**
     **LFPQLQKPMK**
51   AFTVCLQVYT DLTRPHSLFS YATK**SQSNEI LLFKERPGLF**
     **SVSVGGSDAF**
101  **INFPQK**FYAP QHFCVTWESV TGLTELWVDG KPMVRASLR**R**
     **GYTVGSGASI**
151  **VLGQEQDSFG GGFDK**NQSLV GDIEDVNMWD FVLSPSQILT
     LYTTR**ALSPN**
201  **VLNWRN**LRYE TRGEVFLKKE LWS
```

When searching data bases (EMBL, NCBI) for most homologous proteins to this new native canine CRP sequence only low similarity with published pig (65%), human (60%) or rat (59%) CRP proteins were found.

3.3. RNA sequencing

To further validate the findings of the MS peptide mapping the coding region of the cCRP mRNA has been sequenced. A cDNA sequence generated from canine total liver RNA revealed a 678 bases long nucleotide sequence encoding for a 223 amino acid long protein perfectly matching with the prior predicted protein sequencing data (XP_545746). This new native canine cDNA sequence has been submitted to and validated by the EMBL data base (Acc. No. HG 003655) and matched 100% with the formerly reported cCRP sequence (Acc. No. XM_545746) deduced *in silico* from a dog genome annotation.

3.4. 2D protein analysis

In Coomassie-stained SDS–PAGE gels as well as in Western blotting analysis using a cCRP specific antibody denatured cCRP was always found as a double band (22 kDa and 24 kDa; Figs. 1B, 2 and 3). To elucidate the nature of these protein isotypes a 2D analysis was conducted. Isoelectric focusing and 2D analysis of the purified denatured protein resulted in specific protein spots showing isoelectric points (pI) around 6 and two different molecular weights (22 kDa and 24 kDa). In detail, six individual protein spots were found: three differently charged spots each at a molecular weight of 22 kDa and 24 kDa, respectively. The verification of these six cCRP isotypes was ascertained by immune-detection of all six protein spots using a cCRP-specific antibody (Fig. 2A).

3.5. Glycoprotein detection

Initial experiments introducing purified cCRP to a general glycoprotein staining experiment showed that only the upper 24 kDa band is glycosylated (Fig. 2C). A further sugar-characterization introducing five different glyco-selective lectins showed that the larger cCRP protein binds only the SNA lectin. No interactions could be detected with the other tested lectins. Therefore, the glycosylated 24 kDa isoform of the cCRP protein contains alpha(2,6)-linked sialic acid residues terminally linked to either galactose or N-acetylgalactosamine (SAα(2-6)-Gal resp. SAα(2-6)-GalNac).

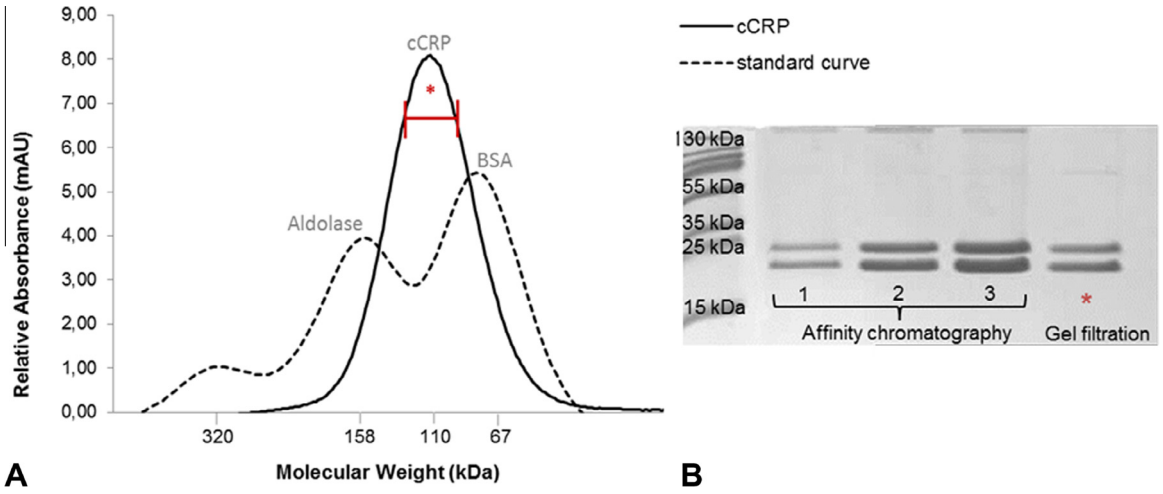

Fig. 1. Gel filtration analysis of affinity-purified cCRP: A typical gel filtration curve is shown (black line reflects the cCRP protein) together with the calibration curve (dashed line showing standard proteins aldolase and BSA) (A). SDS–PAGE gel depicting peak-fractions from affinity chromatography elution (1–3) as well as peak samples derived from gel filtration (*) (B).

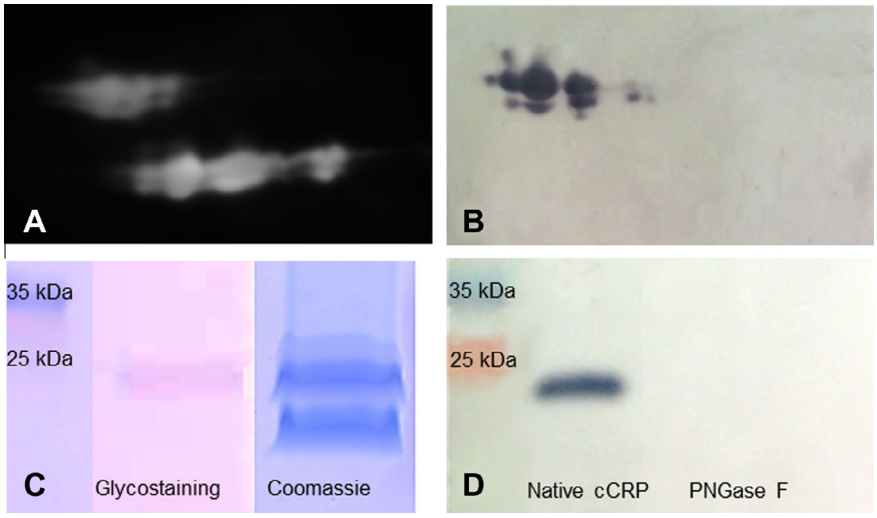

Fig. 2. Characterization of purified cCRP protein by 2D-GE: immunostain using anti cCRP antibody (A), SNA lectin-specific detection of sugar residues (B), general glycoprotein staining (C) and validation of N-linked sugar chains by enzymatic treatment with PNGase F (D) and subsequent glycostaining.

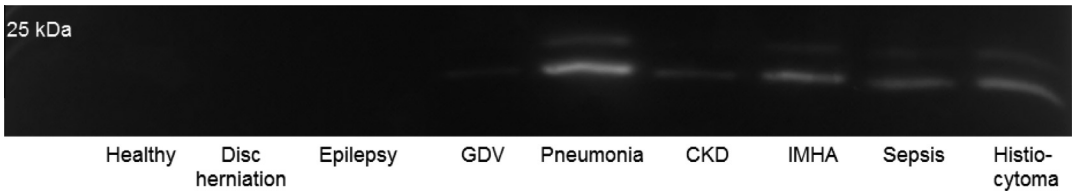

Fig. 3. Comparison of sera from healthy or diseased dogs. Sera from dogs with different non-inflammatory (disc herniation, epilepsy, chronic kidney disease (CKD), histiocytoma) and inflammatory (gastric dilatation-volvulus (GDV), pneumonia, immune-mediated hemolytic anemia (IMHA), sepsis) diseases after cCRP-specific Western blotting.

By introducing cCRP protein to 2D-GE followed by a SNA lectin staining it was shown that the three upper 24 kDa protein spots were stained positive for alpha(2,6)-linked sialic acid residues within the glycan-sides chains of all three differently charged isoforms (Fig. 2B).

After N-selective enzymatic hydrolysis of the cCRP glycan side chains by PNGase F, the specific SNA lectin signals disappeared (Fig. 2D). Therefore, it can be suggested that the glycan side chains of cCRP contain GlcNAc N-linked to asparagine as well as sialic acid residues terminally alpha(2,6)-linked to either galactose or N-acetylgalactosamine. Derived from the protein sequence data two asparagine residues (N17, N181) out of 8 within the cCRP protein may be predicted possessing the highest prevalence being glycosylated.

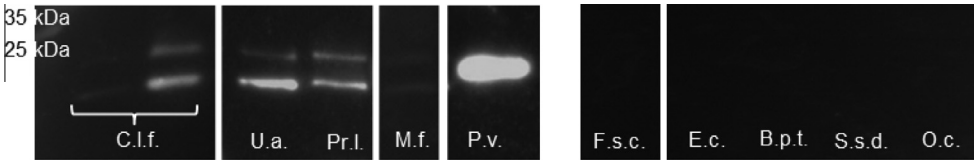

Fig. 4. Western blot analysis of different Canifornia species in comparison to other domestic animals: dog (C.l.f.), brown bear (U.a.), racoon (Pr.l.), stone marten (M.f.), seal (P.v.) cat (F.s.c.), horse (E.c.), cow (B.p.t.), swine (S.s.d.), rabbit (O.c.).

3.6. Case reports: disease correlation and species comparison

When examining differently diseased dogs a variation of immuno-reactive cCRP was found between the individuals when comparing the upper (glycosylated) versus the lower (non-glycosylated) isotype of the cCRP protein band staining intensities (Fig. 3). There was no correlation to any specific disease as cCRP was detected in some of the dogs suffering from non-inflammatory diseases and only slightly visible protein bands were present in the dog with a gastric dilatation-volvulus (GDV).

Immune-reactive CRP protein could be detected by Western blot only in sera of dog-related species (Canifornia), in other domestic animals no signal was generated. Specific signals were detected at molecular weights of 22 kDa resp. 24 kDa, with a higher intensity of the lower band in all Canifornia. But in the seal only one very pronounced protein isotype between 22 and 24 kDa was found (Fig. 4). In the cat, horses, cow, pig, rabbit sera no cCRP-specific immune-reactions were detected.

4. Discussion

In human as well as in veterinary medicine CRP represents a useful nonspecific inflammatory marker for disease screening and monitoring [1,2,18]. As CRP synthesis appears unaffected by e.g. glucocorticoids its analysis has advantages over the traditional inflammatory markers [19]. Currently, it is not possible to diagnose a specific disease based on the CRP protein itself, but in human medicine its different glycosylation pattern has found to correlate with specific diseases [20]. In comparison similar glycosylation patterns are described for canine haptoglobin of patients with liver failure and anemia [21].

Current knowledge about the cCRP protein structure is mainly based on a genome-deduced protein sequence predicting a protein containing 223 amino acids (Acc. No. XP_545746). Successful cDNA sequencing of the CRP transcript from dog liver confirms now both the liver as organ of CRP expression as well as the transcript sequence of the formerly genome-deduced data set.

The peptide fragments examined by mass spectrometry covered and affirms 41% of the previously reported full *in silico* cCRP protein sequence. For the first time the N-terminus of the serum cCRP could be sequenced providing a truncated, 19 amino acids shorter sequence. This indicates a posttranslational modification of the protein structure like it is described for the human counterpart [20], resulting in native serum proteins comprising glutamine at their N-termini.

Furthermore, the specific glycosylation pattern of the larger protein subunit shows similarities in human and canine CRP. Both glycosylated isoforms could be detected with the lectin SNA [20]. In 2D GE analysis the mean pI was found to be approx. pH 6 which fits to the newly calculated pI of 5.75. In the 2D GE the pI of the upper subunit was shifted to a more acidic pH compared to the lower isoform, which could be explained by posttranslational protein modification with additional sialic acid or potential phosphorylation sites e.g. thyrosins present in the protein structure.

All posttranslational modifications found in our study could influence the endogenous regulation characteristics of the cCRP protein. It is therefore likely that correlations between the truncation of the protein and its release may exist in dogs as well as described for humans [22]. In this study different cCRP glycosylation patterns could not be directly correlated to distinct diseases in dogs. However, more research on this topic needs to be undertaken before the association between protein modification and their influence on pathophysiological situations is more clearly understood. In conclusion, both the native cCRP protein sequence and its corresponding mRNA sequence are now made available either from canine blood sera (protein) or dog liver.

The detection of CRP in different species of the Canifornia super family by a specific anti-cCRP-antibody makes this biomarker also attractive for use in veterinary zoo and wildlife practice. However, the fact that the antibody did not bind any of the other domestic animals is an indication for obvious immunological differences between the species for members of the CRP proteins.

In conclusion, the increased knowledge about the detailed molecular cCRP structure and the specific changes of glycosylation pattern may achieve importance for deeper diagnostic purposes in veterinary medicine. This might open new approaches using an analogous dog model on the route investigating human inflammatory diseases as well.

Conflict of interest

The authors declare no conflict of interest concerning financial, personal or other relationships with other people or organizations.

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