Genes Nutr (2007) 2:275–285
DOI 10.1007/s12263-007-0058-x

RESEARCH PAPER

Dietary modulation and structure prediction of rat mucosal pentraxin (Mptx) protein and loss of function in humans

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Received: 12 March 2007 / Accepted: 20 July 2007 / Published online: 16 October 2007

Abstract Mucosal pentraxin (Mptx), identified in rats, is a short pentraxin of unknown function. Other subfamily members are Serum amyloid P component (SAP), C-reactive protein (CRP) and Jeltraxin. Rat Mptx mRNA is predominantly expressed in colon and in vivo is strongly (30-fold) regulated by dietary heme and calcium, modulators of colon cancer risk. This renders Mptx a potential nutrient sensitive biomarker of gut health. To support a role as biomarker, we examined whether the pentraxin protein structure is conserved, whether Mptx protein is nutrient-sensitively expressed and whether Mptx is expressed in mouse and human. Sequence comparison and 3D modelling showed that rat Mptx is highly homologous to the other pentraxins. The calcium-binding site and subunit interaction sites are highly conserved, while a loop deletion and charged residues contribute to a distinctive "top" face of the pentamer. In accordance with mRNA expression, Mptx protein is strongly down-regulated in rat colon mucosa in response to high dietary heme intake. Mptx mRNA is expressed in rat and mouse colon, but not in human colon. A stop codon at the beginning of human exon two indicates loss of function, which may be related to differences in intestinal cell turnover between man and rodents.

Keywords Heme · Calcium · Carcinogenesis · Nutrition · Biomarker · Protein modelling · Absence in human

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Introduction

Mucosal pentraxin (Mptx) has been identified as a gene that is specifically expressed in colon mucosa [37]. Rat in vivo nutrigenomics experiments have shown that *Mptx* is strongly modulated by the dietary components such as heme, a major component of red meat, and calcium, which are both modulators of risk factors of colon carcinogenesis [37, 38]. Furthermore, several other genes, such as carbonic anhydrase or cytokeratin 21, showed differential expression in response to these dietary components, but none of these genes exhibited a 30-fold dynamic range of expression as was observed for Mptx [38]. Based on these results, we have proposed that Mptx might be an appropriate marker for diet-modulated health of colonic mucosa, exhibiting high expression in healthy colonic mucosa, and strongly decreased expression upon intake of dietary components such as heme, inducing luminal cytotoxic and hyperproliferative effects [31, 32, 33], and upon dietary induced oxidative stress [8].

Mptx belongs to the family of pentraxins [13]. This family consists of two subgroups: the classical short

pentraxins (25 kDa) and the long pentraxins (>45kDa). The short pentraxins consist of a signal peptide followed by a pentraxin domain. Two classical short pentraxins are known: serum amyloid P component (SAP, also abbreviated as APCS) and C-reactive protein (CRP). Both are highly conserved in evolution and have been identified in many vertebrates. They are synthesized by hepatocytes and delivered to the blood, where they occur as pentamers. The pentamers are arranged in a discoid structure, with conserved calcium-binding sites at one face that suggest conserved binding to a phospholipids surface [19]. CRP and SAP both have an immune function. They are found to be increased upon inflammation [34] and may have a role in reducing excess complement activation [3, 25]. Reduced levels lead to autoimmune disease [2, 26, 29, 30]. More specifically, they are thought to be involved in the recognition, binding and clearance of dead and apoptotic cells or cellular debris of pathogenic cells and host cells [9, 11, 15, 17, 20].

Mptx is the first short pentraxin that is identified in the colon. One other extra-hepatic short pentraxin has been identified, in frog [24]. This gene, named jeltraxin (Fjtx), is specifically expressed in the oviduct and is supposed to play a role in regulation of the structure of frog egg jelly. Fjtx shows high sequence homology to SAP and CRP has similar structural features and also has calcium-binding properties [24].

We reasoned that comparison of the Mptx protein sequence to that of other pentraxins and across species will allow us to see whether relevant structural and functional sequences are conserved, a prerequisite for its use as a biomarker. For this it is also necessary to establish if Mptx protein is similarly affected by luminal dietary components as its mRNA and whether Mptx is expressed in the human colon. Here, we address these questions.

Materials and methods

Rat feeding experiment

Sixteen Wistar rats (outbred, males, specific pathogen free; Harlan, Horst, The Netherlands) were housed individually and fed a humanized AIN-93 rodent diet, as described previously [37]. The diet of eight rats was supplemented with heme (0.5 mmol/kg diet) (Sigma, Zwijndrecht, The Netherlands) for 2 weeks. Feces were collected daily during days 11–14 and were frozen at –20°C. Fecal water was prepared and its cytotoxicity quantified as described before [31]. Rats were killed after 2 weeks, the colon was excised and rinsed in 154 mM KCl, after which the colon was cut longitudinally and both halves were scraped to recover the mucosa. One half was used for RNA extraction, the other for protein measurements. Scrapings were stored in liquid N_2. The experiments were performed according to national guidelines and were approved by the animal welfare committee of Wageningen University, Wageningen, The Netherlands.

DNA constructs and recombinant proteins

Recombinant Mptx clones were generated by PCR from rat colon cDNA. Primers were designed based on the predicted open reading frame [37] and appropriate restriction sites were introduced at the end of primers. Amplified fragment were ligated into the *Kpn*I–*Not*I sites of the bacterial expression vector PROTet^{TM} 6xHN (Clontech, The Netherlands). In this way, a fusion construct was generated, consisting of Mptx fused to a 6xHisAsn affinity tag (HN-rMptx). The constructs were confirmed by DNA sequencing. Sequence analysis revealed HN-rMptx contained one mismatch that leads to substitution of Ile150 to Val. Recombinant Mptx protein was expressed in *Escherichia coli* DH5αPRO cells according to the protocol of the PROsystem (Clontech). The expression was induced with 80-ng/ml anhydrotetracycline for 4 h.

Sequence homology analysis and modelling of Mptx

Homology relationships were established using BLASTP [1] and Pfam (http://www.sanger.ac.uk/Software/Pfam/) and multiple sequence alignments were made with ClustalW [36]. Alignments are based on the following accession numbers: Rat Mptx AY426671; Mouse Mptx BC024348; Human Mptx XM_060355; Rat SAP NM_017170; Mouse SAP NM_011318; Human SAP NM_001639; Rat CRP NM_017096; Mouse CRP NM_007768; Human CRP NM_000567; Lepidobatrachus laevis egg jelly pentraxin precursor Jtlx AF047712. For chromosomal alignments the following accession codes were used: rat NW_001084686 (start exon1: 2212568, start exon2: 2209770), mouse NW_001030678 (start exon1: 315307, start exon2: 317822) and human NT_004487.18 (start putative exon2: 9736652; stop at: 9736657). Homology modelling of Mptx was performed using the program WHAT IF [41], using the pentameric structure of human serum amyloid P component (Protein data bank code: 1sac; resolution 2.0 Å; 52% amino acid sequence identity) as a template. The 1sac structure consists of five equivalent monomers arranged in a ring, with 10 bound Ca ions and four bound acetate ions. Additionally, we used the highly similar pentameric structure of human C-reactive protein (Protein data bank code: 1b09; resolution 2.5 Å; 58% amino acid sequence identity), which has nine bound

Ca-ions and five bound phosphocholine molecules. The result of the modelling was visualized using Protein Explorer (http://molvis.sdsc.edu/protexpl/frntdoor.htm). This is in agreement with the calculated surface potentials of Mptx and SAP, with the top of Mptx being more negative and the bottom less positive compared to SAP (Fig. 3d) and calcium. Heme is while calcium is a rat that It is unlikely that the absence of expression humans is due to differences in dietary intake, since the expression could be detected in rodents on chow and widely different semi-synthetic diets [37, 38; 8], but not in any of the human subjects, who were not on a specific dietary regimen and therefore represent a variety of dietary intakes. Tharia extent its and physiological Mol. Clin. Biol. [31], [37] at 1.0 [41]. The surface potentials were calculated and drawn with the program Yasara Twinset (http://www.yasara.org).

Antibodies

Two different polyclonal anti-Mptx antibodies were generated by Alpha Diagnostic International (San Antonio, TX, USA) in rabbits against the synthetic peptides DHVIPLEEAHDSCDGGNLIN (Ab1), at the C-terminal region of Mptx, and QSDMDGKAFIFPQES (Ab2), at the N-terminal region of Mptx. The peptides were selected based on (1) their occurrence at the surface of the modelled Mptx protein, and (2) not at interaction sites with other subunits and (3) on displaying a high antigenicity index. Antibodies were affinity purified against the respective peptides that were conjugated to Sepharose.

Western blotting

Mucosal scrapings were homogenized in N_2 and brought into 500-µl buffer A [(200 mM sucrose, 20 mM Tris–HCl pH 7.4, 1 mM DTT and a protease inhibitor cocktail tablet (Roche)]. Total protein concentrations were determined by bicinchoninic acid assays (BCA, Pierce, Perbio, The Netherlands). Homogenate samples (7.5 µg total protein) were denatured at 95°C for 3 min, subjected to SDS-PAGE (10–20% Criterion Precast Gel, Bio-Rad, The Netherlands) and transferred to PVDF membrane. Membranes were blocked overnight at 4°C with 1% serum albumine (BSA) in 50 mM Tris–HCl pH 7.4, containing 0.1% Tween-20. The primary antibodies, affinity purified Mptx antibodies (1:100 dilution), 6xHN polyclonal antibodies (Clontech, 1:4,000 dilution) or actin antibodies (Santa Cruz Biotechnology 1:50) were incubated with the blots in 50 mM Tris–HCl pH 7.4, containing 0.2% BSA and 0.5% Tween-20 at RT for 1 h. The blots were then incubated with appropriate horseradish peroxidase-conjugated anti-IgG antibody (Promega, 1:7,500 dilution). Antibody-bound protein was detected using TMB-stabilized substrate for HRP (Promega, The Netherlands).

PCR

Mucosal scrapings were homogenized in liquid N_2 and total RNA was isolated and quantified as described before [37]. One microgram RNeasy purified and DNase treated total RNA was used for cDNA synthesis (SuperScript Preamplification System for First Strand Synthesis, Life Technologies). Quantitative Real-time PCR (Q-PCR) reactions were performed with the LightCycler (Roche) as described before [38], with the exception that we used intron spanning rat Mptx primers with the following sequence: forward: 5′-TCGGCACCCTGCTTCTCACTG TTC-3′, reverse: 5′-CTCATTGTCCTGAGTTTTTGTGTT GTAG-3′. Amplification of human Mptx mRNA was attempted on cDNA generated from total RNA that was isolated from (1) the human colon cancer cell lines HT-29, its mucin producing derivatives HT-29 MTX and HT-29 18N2 and differentiated (10 day after confluency) and undifferentiated (70 % confluent) CaCo-2 cells, (2) biopsies from the ileum, colon ascendus, colon transversum and rectum of six individuals of 17, 20, 34, 35, 55 and 56 years of age and (3) commercial human colon RNA (Gentaur). For amplification of human Mptx mRNA, primers with the following sequences were used; forward Hu1: 5′-CCTT CACAGACTTCACCTGCCC-3′; forward Hu2 5′-CAAGA GCAGGATTCCTTTGGG-3′; reverse 5′-TTGGGCTTAG TTACCACATAGCC-3′. In all experiments the two forward primers were used with the reverse primer in independent reactions. A control for amplification from genomic DNA, forward primer FHuInt 5′-GGGTCCAC AGTGAGAGCAAATCC-3′ was used with all cDNAs. This primer is located just upstream of the potential second Mptx exon. All primers were tested on human chromosomal DNA for equal amplification efficiency. Intron spanning primers of beta2-microglobulin [28], verified on isolated human chromosomal DNA, were used to establish that the cDNA was of good quality. The following PCR conditions were used: in a total reaction volume of 25 µl, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 400 nM of each primer, and 1.25 U of Taq polymerase (InVitrogen) were used. This mixture was amplified as follows: 3 min at 94°C, followed by 30 cycles of 30 s 94°C, 1 min 58°C, 2 min 72°C and a final step of 10 min at 72°C. All amplifications were done at least two times in independent experiments. In addition to these experiments, a commercial multiple tissue blot containing 1 µg colon poly(A)+ RNA (Clontech) was hybridized according to the protocol of the supplier with a 32P-labeled (Megaprime DNA labelling,

Amersham) human sequence (485 bp) corresponding to the potential second exon of Mptx to assess Mptx mRNA expression. Finally, RACE PCR according to the protocol of the supplier (Invitrogen) was done on mRNA as well as on total RNA isolated from a human rectal biopsy, as well as form CaC0-2 using the following primers; first strand primer GSP1: 5′GCTCACATTGACATGG, first amplification primer GSP2 5′AAGCATAAGGAGATCGAGGG CAGGG (+ AAP), nested amplification primer GSP8: GCCATTGAAAGTGACCGCAGCAT or GSP2 (+ UAP) (AAP and UAP were supplied by Invitrogen. PCR of mouse Mptx was done on cDNA generated from RNA isolated from colon scrapings of C57Bl6/J mice using the following intron spanning primers; forward: 5′-CCTGTT TCTCTCTGTTCTTTCAGG-3′; reverse: 5′-GGCCTTCAT ACACAGAGTGAAG-3′ in a total volume of 25 µl, containing 1.5 mM MgCl2, 0.2 mM dNTPs, 400 nM of each primer, and 1.25 U of Taq polymerase (InVitrogen). The following cycling conditions were used; 3 min at 94°C, followed by 35 cycles of 30 s at 94°C, 1 min at 60°C and 2 min 72°C and a final step of 10 min at 72°C.

Results

Effects of heme

The effects of dietary heme on colonic mucosa of Wistar rats ($n = 8$) are shown in Table 1. Dietary heme induces increased cytotoxicity in the colonic lumen. Q-PCR determined mRNA levels shows on average sevenfold lower Mptx levels in the heme-fed group compared to the control group, which confirms strong down regulation of Mptx in response to dietary heme. These results are analogous to our previous reports [37, 38].

Table 1 Effects of dietary heme on Wistar rats ($n = 8$)

	Control	Heme
Gain in body weight (g/day)	2.9 ± 0.4	2.8 ± 0.4
Food intake (g/day)	16.2 ± 0.4	16.8 ± 0.3
Fecal output (g wet weight/day)	1.82 ± 0.2	1.73 ± 0.3
Cytotoxicity of fecal water (% lysis)[a]	0.9 ± 0.2	46.1 ± 4.7
Relative *Mptx* expression (*Mptx/aldoA*)[b]	1.0 ± 0.51	0.14 ± 0.10

Values are given mean ± SEM

[a] Cytotoxicity of 5 µl fecal water was determined against human erythrocytes in a K-release assay [31]

[b] The ratio of Q-PCR measured Mptx and aldolase RNA is indicated. Aldolase A is not modulated by dietary heme [37]. The ratio in the control group was set at 1.0.

Relationship to pentraxins

A search of the protein family database Pfam using the 219 amino acid Mptx sequence resulted in an unambiguous match with the pentraxin gene family (E-value score of 1.8×10^{-100}). Amino acid sequence alignment of rat Mptx with the short pentraxins serum amyloid P component (SAP) and C-reactive protein (CRP) from rat, mouse and human, and the only other extra-hepatic short pentraxin, Fjtx, from frog, is shown in Fig. 1. Mouse Mptx shows 82% amino acid identity to rat Mptx. The human Mptx sequence, derived from the human genome sequence, shows 72% amino acid identity to rat Mptx. As compared to rat and mouse Mptx, the human sequence contains a stop codon at the position of the third amino acid of the second exon (Fig. 2). This was confirmed by sequence analysis (data not shown). Rat Mptx contains a pentraxin signature peptide (H-x-C-x-[S/T]-W-x-[S/T]; Prosite accession number: PS00289) at residues 112–119, although the third conserved residue of this signature differs slightly in Mptx (N instead of S/T). The Mptx mouse homologue shows the same S/T to N replacement and, in addition, the first His is replaced by Arg. Conservative and even non-conservative substitutions in the pentraxin signature peptide are also seen in other pentraxins. Apparently, sequence homology of the entire pentraxin domain is more important than the identity of the consensus signature sequence. The human homologue lacks the conserved Cys-residue in its pentraxin signature peptide, but other Cys-residues, for instance at position 40 or 82, which are unique for the human sequence, might replace the missing Cys. The residues involved in calcium binding are highly conserved in all sequences (Fig. 1). Mptx of rat, mouse and human clearly differ from SAP, CRP and Fjtx by a deletion of 4–5 amino acids around position 190 (numbering of rat Mptx, including signal peptide), and additional Cys-residues upstream of this region. Possibly, this region is specific for Mptx and is related to its function. The genes encoding pentraxins Mptx, SAP and CRP of rat are all located on chromosome 13, while those of human and mouse, are located on chromosome 1. This is plausible, since the rat chromosome 13, mouse chromosome 1 and human chromosome 1 contain highly homologous segments with a high number of orthologs [5].

Structure prediction of Mptx

The 3-dimensional structure of mature rat Mptx (without the signal peptide of 19 residues) was modelled based on the pentameric structure of human serum amyloid P component (ID: 1sac). The mature Mptx monomer of 200 residues has 52% amino acid sequence identity with SAP,

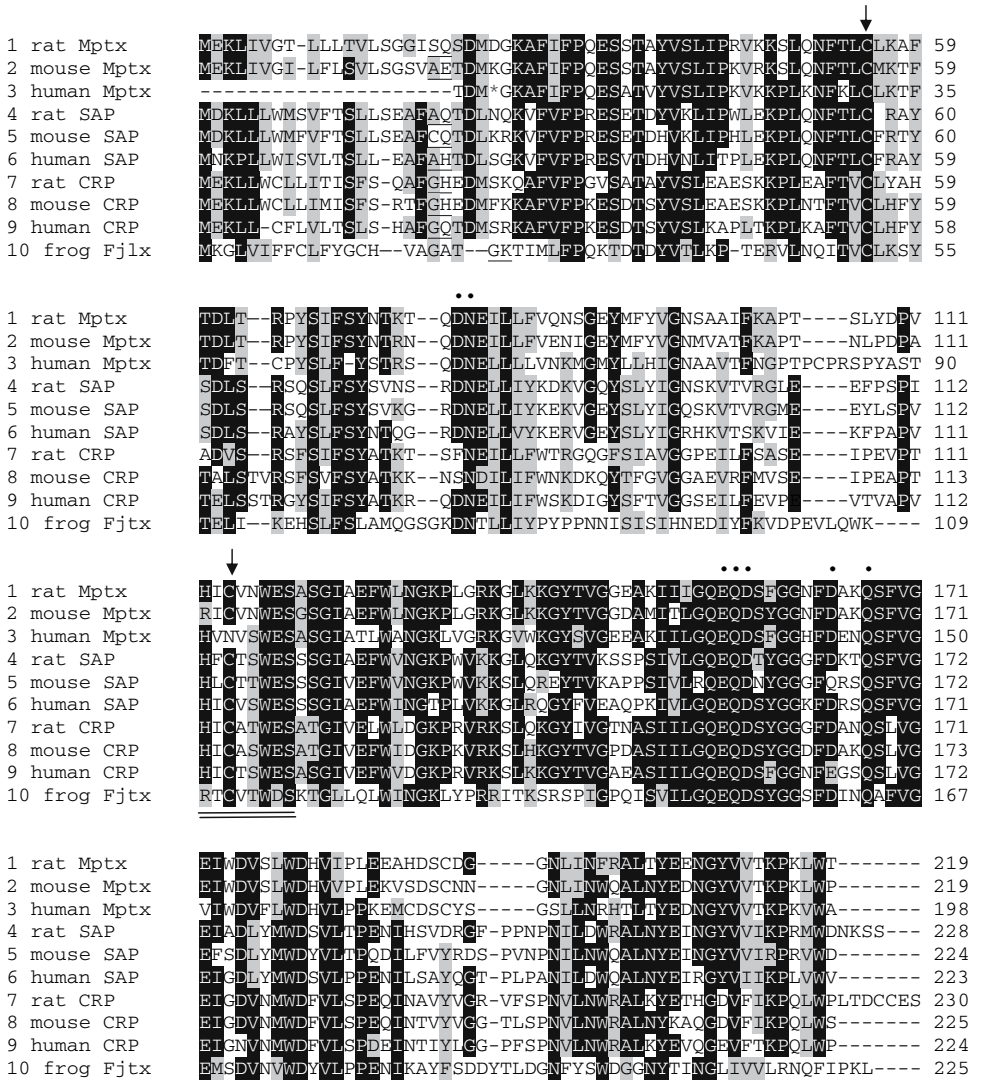

Fig. 1 Alignment of the rat Mptx amino acid sequence with the mouse and human Mptx sequence, with rat, mouse and human C-reactive protein (CRP) and serum amyloid P component (SAP) and with frog jeltraxin (Fjtx). Symbols are as follows: *black* highlighting was used when more than 6 out of 10 aligned amino acids are identical, *grey* highlighting was used likewise for conservative amino acid substitutions. The (putative) cleavage site of the signal peptide is underlined in each sequence. *Arrows* mark conserved cysteine residues. *Double underlined* amino acids indicate the pentraxin consensus sequence. *Dots* indicate the residues known to coordinate calcium ions in human CRP and SAP. The star indicates a stop codon

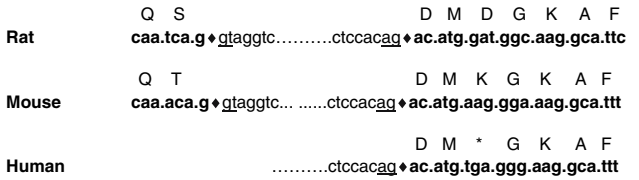

Fig. 2 Alignment of the genome sequence of rat, mouse and human Mptx. (Putative) exon sequences are shown in *bold*, with triplets separated by a *dot*. Intron sequences are in normal type. Splice sites are indicated by *filled diamond*. Consensus splice acceptor and donor sequences are *underlined*

which implies that this homology model should be quite accurate. The model shows a conserved intra-chain disulfide bridge between Cys 55 and Cys114 (numbering based on full sequence, including signal peptide). Mptx has a deletion of four amino acids around position 190 compared to 1sac. Based on the SAP 3D-structure, the most likely structural position for this deletion is between Ser 172 and Cys 173, in a loop on the outer surface of the monomer. Five monomers together form a flat, donut-shaped pentamer with a large hole of about 20 Å diameter in the middle (Fig. 3a). The overall folding of the backbone in each monomer is very similar in SAP and Mptx, with the only differences found at residue 104 (Ile in 1sac is replaced by Pro in Mptx) and at the position of the introduced deletion of four residues. In SAP there are many non-covalent interactions between the monomers forming the pentamer, consisting both of ionic and hydrophobic interactions. Although not all the residues involved in these interactions are conserved in Mptx, the amino acid substitutions are conservative and hence the monomer–monomer interactions in Mptx are predicted to be highly comparable to those in SAP. Each monomer has binding sites for two calcium ions, located on one side of the pentamer, which is designated as the "bottom" side (Fig. 3b). The residues involved in binding of calcium-ions in SAP and human C-reactive protein (ID: 1b09) as well as the residues involved in binding of acetate and phosphatidyl choline, that

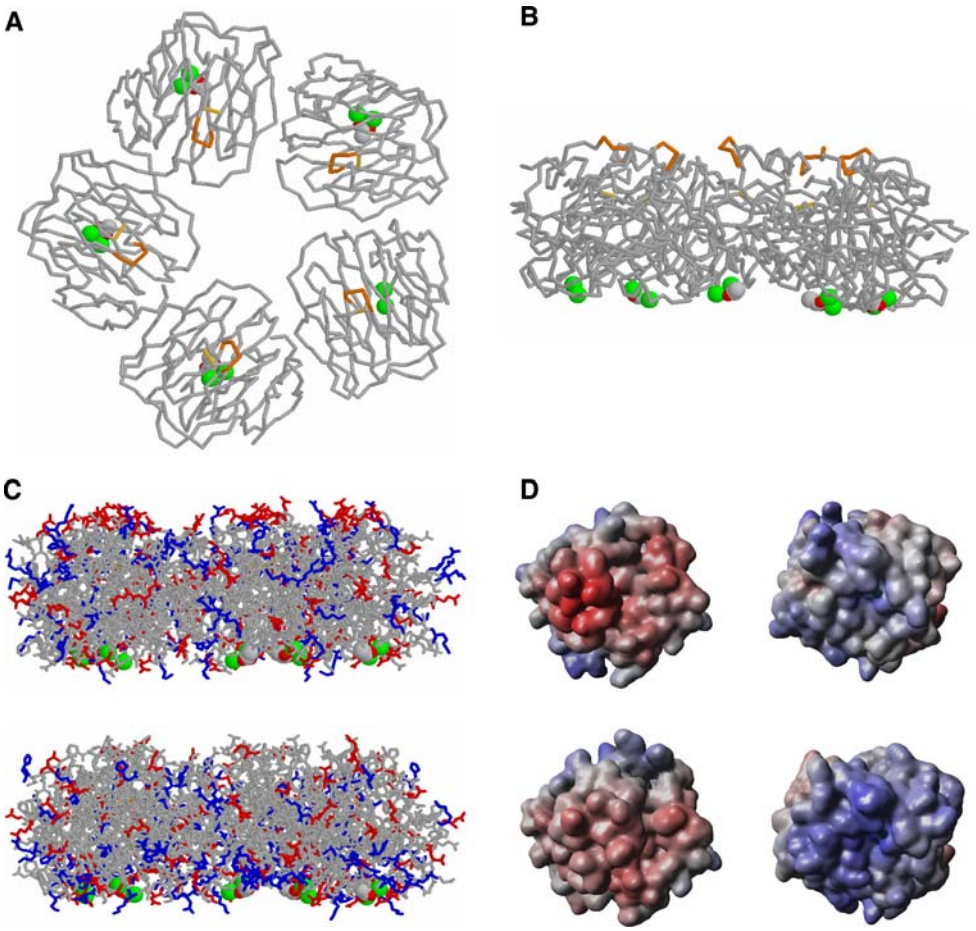

Fig. 3 Structural model of rat Mptx pentamer. The program 'WHAT IF' [41] was used to predict the 3D-structure of Mptx using the pentameric structure of human serum amyloid P component (PDB code: 1sac) as a template. **a** "top" view, **b**, **c** side view. Only the alpha-carbon backbone is shown (*in grey*). **a**, **b**: *orange*: loop where four residues have been deleted relative to 1sac; *yellow*: single disulfide bond; *green* spheres: calcium ions; *red* and *grey spheres*: acetate ion. **c**: basic (*blue*) and acidic (*red*) side chains of Mptx (*upper*) and SAP (*lower*). **d**: shows the surface electrostatic potential of the Mptx (*upper*) and SAP (*lower*) monomers. The top is shown on the *left* and the bottom on the *right*. *Red* is negative and *blue* is positive; calcium ions (just visible) in *green*

neutralize the calcium in the SAP and CRP structure, respectively, are highly conserved in the model of Mptx (Fig. 3). It can, therefore, be concluded that the 3D structure of these binding sites will essentially be the same in Mptx, SAP and CRP. The loop with the 4-residue deletion in each monomer is located on the opposite "top" face of the pentamer (Fig. 3b). Neither this loop nor the calcium-binding site is near the monomer–monomer interface. Differences between the structures of Mptx and SAP are mainly in the type of side chains on the surface of the pentamer. Both structures have a number of positively charged side chains (Lys residues) on the "bottom" face of the pentamer, which have the potential to bind to phospholipids. These side chains are in equivalent positions in the 3D structure CRP, complexed to phosphatidylcholine [43]. The Mptx model has significantly more acidic residues on the "top" face than SAP (and CRP) (Fig. 3c). This is in agreement with the calculated surface potentials of Mptx and SAP, with the top of Mptx being more negative and the bottom less positive compared to SAP (Fig. 3d). Together with the shortened loop on this face, this suggests that the "top" face of the pentamer could display quite different functional properties.

Generation of anti-Mptx antibodies

Based on the 3-dimensional structure model of rat Mptx, potential antigenic regions were predicted by selecting linear, hydrophilic peptide segments located on the surface of the pentamer. Two antigenic peptides (Mptx-1 and Mptx-2) were synthesized and used for immunization of rabbits, to obtain anti-Mptx polyclonal antibodies. Affinity purified Mptx antibodies (Ab1 and Ab2) tested in the standard ELISA protocol showed antigen-specific positive signals up to a dilution of $1{:}10^5$. The antibodies were tested for reactivity with recombinant Mptx. To this end, the full-length Mptx sequence (lacking the putative signal peptide sequence) was expressed as recombinant fusion protein in *E. coli*. For this purpose, rat Mptx was amplified by RT-PCR and sub-cloned into the PROTet expression vector (Clontech), downstream of the 6xHis–Arg affinity tag (HN-tag). The recombinant Mptx (HN-Mptx) contains at the N-terminus the HN-tag leading to an expected molecular mass of 25.5 kDa. Figure 4 shows the protein pattern of the total cell extracts of *E.coli* transformed with the HN-Mptx plasmid, after immunostaining with custom anti-HN-tag antibodies, or the anti-Mptx antibodies Ab1 and Ab2.

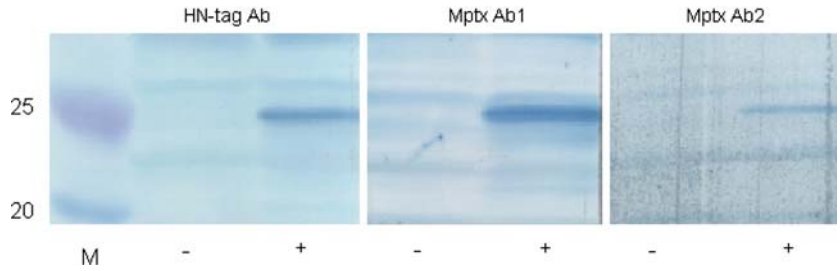

Fig. 4 SDS-PAGE analysis of HN-Mptx expressed in *E. coli*, immunostained with anti-HN-tag and anti-Mptx antibodies. Each *lane* was loaded with 7.5 µg of total protein extracted from a 4 h (mid-exponential phase) cell culture of *E.coli*. *M*: marker protein with known molecular weight. Lanes indicated with *minus* are uninduced controls, lanes indicated with *plus* are loaded with protein extract from a cell culture induced with 80 ng/ml anhydrotetracycline

Anhydrotetracycline induction clearly generates a protein band that is absent in non-induced cells, consistent with the expected molecular weight (25.5 kDa) of the recombinant protein. Anti-Mptx antibodies Ab1 and Ab2 stain the same band as HN-tag antibodies. Staining with Ab1 is most intense.

Modulation of Mptx protein levels by dietary heme

Anti-Mptx antibodies were used for detection of Mptx in mucosal scrapings obtained from the rat feeding experiment to study the effects of dietary heme. Cell homogenates of eight rats from each group were pooled and normalised for total protein content. As Fig. 5 shows, the level of Mptx in colonic mucosa of heme-fed rats is much lower than in that of control rats. Similar results were obtained for Ab2 (data not shown). The intensity of the housekeeping gene β-actin is equal, confirming equal total protein amounts of both samples. These results demonstrate that Mptx protein levels are strongly down-regulated in response to dietary heme, which is in accordance to its mRNA levels.

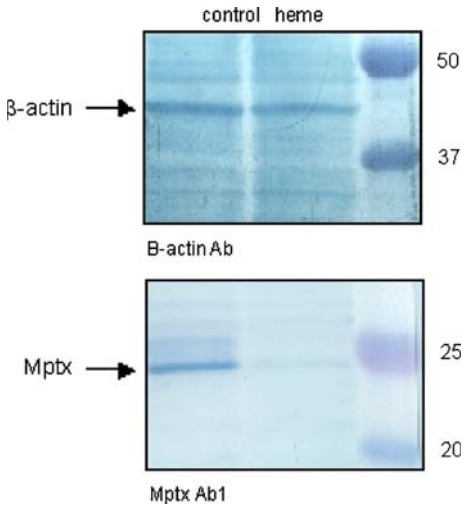

Fig. 5 SDS-PAGE analysis of rat Mptx from colonic scrapings of rats from the feeding experiment. In each lane, 7.5 µg of total protein extracted from colonic scrapings from three control rats (*left lane*) or heme-fed rats (*middle lane*) was loaded. At the right, marker proteins are loaded. β-actin antibodies shows equal amounts of β-actin in both extracts. Mptx levels are strongly reduced in heme-fed rats

Expression of human and mouse Mptx

The rat Mptx gene has two exons. The first exon predominantly encodes the signal peptide sequence and the second exon consists largely of the pentraxin domain [37]. In the human genome, the second exon of Mptx can readily be identified. However, this sequence contains a stop

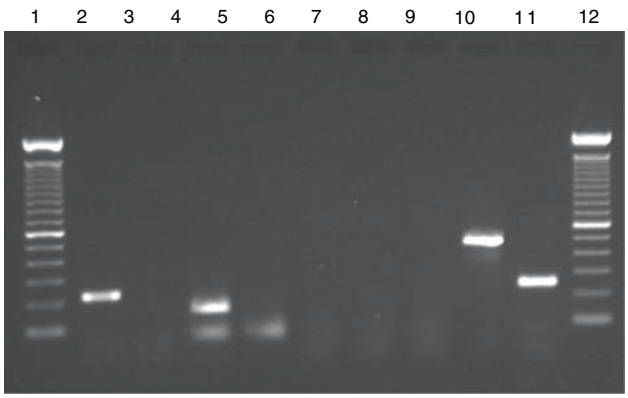

Fig. 6 Expression of rat and mouse Mptx mRNA and absence of human MPtx expression, using RT-PCR. Lane (L)2: rat cDNA; L3: rat CDNA without reverse transcriptase (RT) step; L4 mouse cDNA; L5 mouse cDNA without RT step; lanes L6–L10: human cDNA from ileum (L6), colon transversum (L7, L9) and rectum (L8); cloned human chromosomal DNA containing Mptx (positive control for PCR) (L10). PCRs were performed with primers for rat Mptx (L2, L3; calculated 220 bp), mouse Mptx (L4, L5; calculated 145 bp), human Mptx (L6-L9; calculated 484 bp) and, to control human cDNA quality, human ß2-microglobulin (L11, re-amplification of L7; calculated 249 bp), as described in the Materials and methods section. L1, L12: molecular weight marker (100bp ladder)

codon, which replaces the third amino acid of the second exon (Fig. 2). This implies that in humans the Mptx gene has lost its function or that it is spliced at a different position as compared to rat and mouse. To ascertain the expression of mRNA in humans, the presence of Mptx mRNA was assessed in the human colon cancer cell lines HT-29 and CaCo-2, in biopsies of the human intestinal tract, including colon *ascendens*, colon *descendens* and the rectum, of different people, and a commercial colon RNA preparation, using PCR based methods. In all cases no mRNA could be detected. Figure 6 shows a summarizing example of mRNA expression in rat and mouse and its absence in humans. Also Northern blotting (multiple tissue blot and intestinal tract blot) and RACE-PCR did not reveal expression of Mptx in human tissue (data not shown). Together, this indicates that Mptx is not expressed in humans and implicates that Mptx is a pseudogene in humans. In mouse, as in rat, colon, Mptx expression was readily detected using intron-spanning primers, with no detectable signal in the absence of the reverse transcriptase step (data not shown).

Discussion

Here, we describe the predicted 3D structure of the rat Mptx protein and the dietary regulation of its biosynthesis. Mptx belongs to the family of short pentraxins. Mptx is expressed in the colon mucosa, while the related pentraxins SAP and CRP are synthesized in the liver. The only one other extra-hepatic short pentraxin, Fjtx, was identified in frog egg jelly. The sequence alignment shows that Mptx is most closely related to SAP and CRP and is a more distant homologue of Fjtx. Comparison of the amino acid sequence of rat Mptx with the pentraxins SAP and CRP shows that more than 60% of the aligned amino acids are either identical or are conservative substitutions. Rat Mptx contains a pentraxin signature sequence, a putative signal peptide cleavage site at residue 19–20, conservative Cys residues at positions 55 and 114, which are all common features of pentraxins. A putative glycosylation site at residue Asp-51 is shared with SAP. Remarkably, Mptx lacks a stretch of four amino acids in its C-terminal end, compared to the serum pentraxins.

By homology modelling, Mptx is predicted to be a globular protein with an intra-chain disulfide bridge between Cys55 and Cys114. The overall structure of the monomers and the donut-shaped pentamer is equivalent to that of human SAP. Generally, the residues involved in monomer interactions and pentamer assembly are also conserved in Mptx, which indicates a very similar pentameric organisation of the proteins. The calcium coordinating residues are strictly conserved between Mptx, SAP and CRP. Therefore, it is very likely that Mptx exhibits calcium-dependent ligand binding, a property that is conserved for nearly all pentraxins. Calcium has an important role mediating phospholipid binding. In the 3D-structure of CRP, the "bottom face" of the pentamer, containing the Ca-binding sites, is complexed to phosphocholine. This suggests that the bottom face of pentraxins is involved in binding to phospholipids present in cell membranes. This is supported by the presence of conserved positively charged lysine residues on the "bottom" face of the pentamer. In this configuration, the "bottom" face may interact with cell surfaces, while the "top" face of the pentamer is available for specific interactions with other components. Interestingly, the "top" face of the Mptx pentamer is more negatively charged than SAP and CRP. Furthermore, the Mptx sequence from rat, mouse and human is characterized by a four amino acid deletion in the C-terminal loop region. This region, which is not involved in pentamer interactions, is also positioned on "top" face of the monomer, opposite to the calcium-binding residues. Possibly, this region, being most different from SAP and CRP, determines Mptx-specific functions.

Previously, we have reported that Mptx may serve as a marker for gut health since it is significant differentially regulated by heme and calcium. Heme is a dietary component that has detrimental effects on colonic mucosa, while calcium is a model dietary component that has beneficial effects on colon health. In rat-feeding experiments we observed that intake of dietary heme resulted in increase of cytotoxicity of the colonic content, hyperproliferation of the epithelium and concomitant tenfold decrease of Mptx mRNA levels in response to heme [37, 38]. Intake of calcium has opposite effects and was accompanied by a threefold increase of Mptx mRNA levels. Moreover, calcium appeared to counteract the negative effects of heme, and heme-induced physiological and genetic effects were significantly reduced [38]. In addition, mRNA levels in the colon were decreased in the absence of the antioxidants vitamin C or vitamin E in the diet. MRNA levels are not necessarily reliable indicators of the corresponding protein abundances. Analyses in yeast and mammalian cells have demonstrated discrepancies between mRNA and protein levels, which emphasize the relevance of additional experiments besides mRNA profiling [27, 44]. Here, we demonstrate that protein levels of Mptx are high in healthy rat colonic mucosa, while Mptx is hardly detected in mucosa from heme-fed rats, which corresponds directly to its transcriptional regulation.

Mptx is expressed in rat and mouse. In both species the Mptx genes has two exons, the first encoding a putative signal peptide and the second the pentraxin domain. In the human genome a 600-bp region that is 72% identical to the second exon of rat Mptx was identified on chromosome 1.

However, the open reading frame contains a stop codon replacing the third amino acid of the (putative) second exon (Fig. 2). This indicates that that the human Mptx gene is not functional. If the human Mptx gene would be a functional gene, the chromosomal organisation of human Mptx must be somewhat different from that of rat and mouse, in such a way that the intron-exon boundary would be more downstream. Alternatively, expression might be dependent on incorporation of a selenocysteine, but no clear SECIS element [14] is present, which renders this unlikely. Our inability to detect Mptx mRNA expression in human colon biopsies and cell lines confirms that Mptx is indeed a pseudogene in humans. It is unlikely that the absence of expression humans is due to differences in dietary intake, since the expression could be detected in rodents on chow and widely different semi-synthetic diets [37, 38; 8], but not in any of the human subjects, who were not on a specific dietary regimen and therefore represent a variety of dietary intakes. The Mptx sequences have high homology to the pentraxins CRP and SAP. It has been suggested that CRP and SAP arose from a duplication event of an ancestral pentraxin gene early in evolution [35]. The high-sequence homology and gene organization of Mptx suggests that it arose from a similar gene-duplication event. This is supported by location on the same chromosome as SAP and CRP. Functionality of human Mptx may subsequently be lost in the human lineage, as has been described for vitamin C synthesis [6]. It was shown that in humans, other primates and guinea pigs, L-gulono-γ-lactone oxidase, which catalyzes the terminal step in L-ascobic acid biosynthesis, is lacking [22]. Other examples of genes that are present in rodents, but functionally absent in man are α-galactosyltransferase 1 [18] and ADP-ribosyltransferase (ART1 = RT6) [16]. Interestingly, while in humans the L-gulono-γ-lactone oxidase lacks at least two exons and contains a large number of functional mutations [23], the α-galactosyltransferase 1 gene and, in particular, ART1 gene are conserved to a similar extent as Mptx. As in Mptx, the open reading frame in human ART1 is maintained, despite three in frame stop codons (1 in Mptx) [16]. The very high degree of conservation of the Mptx sequence suggests a recent evolutionary event, as has been suggested for α-galactosyltransferase 1 [12].

Rats are widely used as a model for humans in nutritional and toxicological studies. However, anatomical and physiological differences of the gastrointestinal tract exist, e.g., related to absorption [7] and permeability [21] and microsomal metabolism [42]. Despite such differences, a high predictive value of rats of absorption of pharmaceutical compounds for human has been established [45]. Nevertheless, important differences between rodents and humans exist, especially related to cell turnover. For example, deregulation of Wnt signalling in the intestinal epithelium of mouse does not result in over-expression of the tumorogenesis related tyrosine kinase MET, while it does in humans [4]. Furthermore, rats display considerable luminal shedding of senescent colonocytes, whereas in humans mucosal phagocytosis is the main route of colonocyte disposal [39]. Especially, the latter observation may be of high relevance if Mptx indeed has a role in recognition, binding and clearance of apoptotic (dead) cells, similar to SAP and CRP [9, 11, 15, 17, 20]. The question is how this difference between man and rodents will affect extrapolation of dietary exposure and other studies in these animals. Elucidation of the physiological function of Mptx in rat and mouse, for example by gene inactivation, will allow insight in the underlying differences in physiology between rodents and man and facilitate improved translation of results obtained in these model species.

Since Mptx is highly expressed in healthy mucosa, its biological function is most likely part of normal biological processes in colonic mucosa. Mptx is synthesized at distinct locations near the bottom of the colonic crypts [8]. Its signal sequence suggests that Mptx is subsequently secreted. Although we could not confirm this using immunochemistry due to high unspecific background signals of our antibodies (data not shown), we speculate that Mptx non-covalently attaches to the surface of intestinal epithelial cells, based on its potential calcium-mediated cell surface binding properties. There, similar to SAP and CRP [17, 20], it may be involved in binding and clearance of dying cells. Possibly, Mptx binds specifically to apoptotic epithelial cells, similar to SAP [10], and mediates their clearance as part of the normal cell turnover processes in healthy colonic mucosa. Alternatively, Mptx may be involved in the normal regulation of apoptosis. Recently, CRP has been shown to increase apoptosis of endothelial progenitor cells [40]. In analogy, after diet-induced damage a strong down-regulation of Mptx might be induced to prevent cells from going into apoptosis. Inhibition of apoptosis, in addition to increase of the proliferation rate, could be an efficient strategy to maintain the epithelial barrier in the colon.

We conclude that the structural pentraxin features, including the pentameric shape and the calcium-binding sites, are predicted to be highly conserved. Mptx is found to be co-ordinately regulated at the mRNA and protein level in a diet-sensitive manner. The genomic loss of function of Mptx in humans is most likely related to differences in cell turnover between man and rodents.

Acknowledgments We thank D. Jonker-Termont (NIZO food research, Ede, The Netherlands) and B. Weijers (Small Animal Research Centre, Wageningen, The Netherlands) for expert technical assistance, Prof. Dr. J. Kleibeuker (University Groningen, The Netherlands) for assistance in provision of human colon biopsy

cDNA, Prof. Dr. M. Katan (WCFS) for helpful discussions and Dr. B. Renckens and Dr. C. van Gelder (CMBI) for assistance with homology modelling and for useful discussions.

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