Characterization of a hemocyte intracellular fatty acid-binding protein from crayfish (*Pacifastacus leniusculus*) and shrimp (*Penaeus monodon*)

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Uptake and translocation of hydrophobic ligands are fundamental for all living cells, and are accomplished by members of the lipid-binding protein superfamily (LBP). This superfamily includes the fatty acid-binding proteins (FABPs), the cellular retinoic acid-binding proteins (CRABPs), the cellular retinol-binding proteins (CRBPs), P2 myelin proteins, adipocyte LBP, and mammary-derived growth inhibitors, all of which are intracellular and extracellular low-molecular-mass proteins that bind a wide range of hydrophobic ligands [1]. The FABPs are intracellular proteins and are present in both vertebrates and invertebrates. Whereas the presence and binding specificity of FABPs, CRABPs and CRBPs are well established in vertebrates, the binding specificity of very few invertebrate intracellular LBPs has been investigated in detail. So far, fatty acid

Intracellular fatty acid-binding proteins (FABPs) are small members of the superfamily of lipid-binding proteins, which occur in invertebrates and vertebrates. Included in this superfamily are the cellular retinoic acid-binding proteins and retinol-binding proteins, which seem to be restricted to vertebrates. Here, we report the cDNA cloning and characterization of two FABPs from hemocytes of the freshwater crayfish *Pacifastacus leniusculus* and the shrimp *Penaeus monodon*. In both these proteins, the binding triad residues involved in interaction with ligand carboxylate groups are present. From the sequence and homology modeling, the proteins are probably FABPs and not retinoic acid-binding proteins. The crayfish transcript (p1FABP) was detected at high level in hemocytes, hepatopancreas, intestine and ovary and at low level in hematopoietic tissue and testis. Its expression in hematopoietic cells varied depending on the state of the crayfish from which it was isolated. Expression was 10–15 times higher in cultures isolated from crayfish with red colored plasma, in which hemocyte synthesis was high, if retinoic acid was added to the culture medium. In normal colored crayfish, with normal levels of hemocytes, no increase in expression of p1FABP was detected. Two other putative p1FABP ligands, stearic acid and oleic acid, did not have any effect on p1FABP expression in hematopoietic cells. These results suggest that retinoic acid-dependent signaling may be present in crustaceans.

Abbreviations

ATRA, all-trans retinoic acid; CPBS, crayfish phosphate buffered saline; CRABP, cellular retinoic acid-binding protein; CRBP, cellular retinol-binding protein; FABP, fatty acid-binding protein; hpt, hematopoietic tissue; LBP, lipid-binding protein; RA, retinoic acid; RARE, retinoic acid-responsive element; RXR, retinoid-X receptor.
binding to LBPs has been demonstrated in the insects Schistocerca gregaria [2] and Locusta migratoria [3]. A recombinant putative CRABP (AAL68638) from the shrimp Metapenaeus ensis has been shown to bind retinoic acid (RA) and retinal [4], and another recombinant putative CRABP from Manduca sexta [5] has been found to bind saturated as well as unsaturated fatty acid, but not RA [6]. The 3D structure of vertebrate LBPs is highly conserved, with a 10-stranded β-barrel structure forming a cavity in which hydrophobic ligands are bound. Binding of RA as well as of many fatty acids requires interaction with the side chains of a characteristic triad of conserved amino-acid residues in the ligand-binding hollow, but it is not possible to predict the binding specificity of the protein in question based on the presence of this triad [6].

RA is an important modulator of embryonic development, vision, maintenance of epithelial differentiation, immune functions, and reproduction in vertebrates [7]. In vertebrates the parent vitamin A molecule, all-trans-retinol, circulates in blood bound to serum retinol-binding protein. Inside cells, all-trans-retinol, circulates in blood bound to serum retinol-binding protein. Inside cells, all-trans-retinol and its oxidation product (all-trans-retinal) are associated with the cellular retinol-binding proteins (CRBP-I and CRBP-II). All trans-retinoic acid (ATRA) is found intracellularly bound to one of the two isoforms of retinoic acid-binding proteins, CRABP-I and CRABP-II [8]. Normally, micromolar concentrations of RA and nanomolar concentrations of retinol are present in human plasma [9]. RA is transported inside the cell to the nucleus [10]. In the nucleus, it exerts its effect on the target cells by activating the RA receptors. These receptors contain conserved domains that bind to specific DNA sequences termed RA-response elements (RAREs), and function to enhance or reduce gene transcription [7,11]. In invertebrates, only a few CRABPs have been characterized and the first one cloned was cDNA of msCRABP isolated from the tobacco hornworm M. sexta. This protein shows a high degree of similarity in the ligand-binding pocket to bovine and human CRABP [5]. However, recent ligand-binding studies with recombinant msCRABP showed high affinity binding for fatty acids and negligible interaction with RA and other retinoids, and hence this protein may be a FABP rather than a CRABP [6]. Binding studies of another putative arthropod CRABP, from the shrimp Metapenaeus ensis (recombinant meCRABP [4]) revealed binding of ATRA and retinol to this protein. However, as high concentrations of these ligands were needed for binding and no binding studies with other fatty acids were performed, the presence of true CRABPs in invertebrates is still not conclusively demonstrated.

The FABPs are intracellular low-molecular-mass (14–15 kDa) proteins capable of binding long-chain fatty acids. FABPs in vertebrates have been studied in detail for more than three decades, and crystallography and NMR studies have revealed the tertiary structure of a large number of vertebrate FABPs [1]. FABPs in invertebrates were first identified in the desert locust S. gregaria, and now the number identified is ≈30 [12]. However, the physiological role of these proteins and their binding specificities are still largely unknown. Sequence identities between vertebrate and invertebrate FABPs are in general low, although the known crystallographic structure of invertebrate FABPs shows the consensus β-barrel structure found in vertebrates [12]. Vertebrate FABPs are involved in cellular fatty acid transport and utilization and compartmentalization of intracellular fatty acid storage, and also in fatty acid-induced regulation of gene expression (for review, see [1]). However, the biochemical role of these proteins in immunity is not well understood. Expression of epidermal FABPs has been demonstrated in mouse peritoneal macrophages, human macrophages obtained by in vitro differentiation of monocytes, several cell lines derived from monocytes and macrophages [13], alveolar macrophages [14] as well as in dendritic cells of the spleen [15]. Epidermal FABP has been found to be specifically up-regulated in monocytes involved in allograft destruction [16].

In this study, we characterized an arthropod FABP isolated from hemocytes of the freshwater crayfish Pacifastacus leniusculus and cloned the cDNA coding for a similar FABP from hemocytes of the penaeid shrimp Penaeus monodon.

Results

Analysis of sequence and structure

An ≈560-bp hemocyte EST was obtained from the P. leniusculus EST library (clone number HC 246-563; DataBank accession number CF542599). By using a combination of PCR-based techniques, this partial cDNA was amplified from hemocyte RNA. The full cDNA sequence is named Pacifastacus leniusculus FABP or pFBAP. Crayfish pFBAP consists of an ORF encoding a 132-amino-acid protein and one putative polyadenylation signal site (ATTAA) (Fig. 1A). The deduced protein is ≈15 kDa in size, similar to those of other RA-binding protein or FABP family members.

The cDNA encoding a similar protein was isolated from a P. monodon hemocyte cDNA library using degenerated primers. The deduced amino-acid sequence
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Fig. 1. Nucleotide sequence and deduced amino-acid sequence of plFABP and pmFABP. (A) *P. leniusculus* FABP cDNA nucleotide and putative amino-acid sequence. The putative polyadenylation signal site is underlined. The three amino-acid residues (Arg107, Arg127, Tyr129) of the P2 motif characteristic of RA-binding proteins and FABPs are shown in bold and underlined. (B) *P. monodon* FABP cDNA nucleotide and putative amino-acid sequence. The three amino-acid residues (Arg110, Arg130, Tyr132) of the P2 motif characteristic of the RA-binding proteins and FABPs are shown in bold and underlined.
of pmFABP is 136 amino acids long (Fig. 1B). Amino-acid alignment of various FABP sequences shows that crayfish FABP is most closely related to pmFABP (72% amino acid similarity), and this pmFABP sequence is nearly identical (88% identity, 5% similarity) with meCRABP and the *Litopenaeus vannamei* dbEST CK570804 sequence (Fig. 2). plFABP shares high sequence identity with the *Manduca* protein (msCRABP, AAC24317) and also with other arthropod FABPs [6]. The amino-acid sequence of plFABP, as well as that of pmFABP, shows identity with vertebrate CRABPs and FABPs of 35–45% and similarity of 20–25%, some of which are shown in Fig. 2. However, comparison of sequences as such does not give any information about the binding specificity of these two crustacean FABPs. The crayfish and shrimp sequences contain the essential three amino acids R/R/Y (in crayfish Arg107, Arg127 and Tyr129) of the P2 motif considered important for binding ATRA in vertebrates, but they are also important in the binding of some fatty acids [17–20]. Vertebrate CRABPs usually have a tryptophan residue two amino acids before the first R (marked in Fig. 2) in the P2 motif, whereas in plFABP there is a polar tyrosine residue (as in *Manduca*), and in shrimp FABP a leucine is at this position. CRABPs usually have a longer 2nd α-helix compared to FABPs shown in Fig. 2 by the two gaps before Pro38, and we therefore consider the crustacean proteins to be FABPs. A better homology model was obtained by superimposing the plFABP sequence on vertebrate FABPs (Fig. 3A,B) compared with vertebrate CRABPs (Fig. 3C,D). Only one region covering four amino acids (Asn100–Lys103) has low confidence (shown in red in

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**Fig. 2.** Amino-acid sequence alignment of plFABP and pmFABP with various invertebrate and human (hs) FABPs and CRABPs. Alignment shows the following sequences: pmFABP (Barkley accession 800194), pmFABP (BankIt accession 789217), meCRABP (AAL86838), msCRABP (AAC24317), hsFABPb_1FOQb, hsFABPb_1LJXa, hsCRABP1 (NP_000049), hsCRABP2 (NP_0001869). plFABP shows identity/similarity of 51%/21% to pmFABP, 50%/22% to meCRABP, 45%/16% to msCRABP, 38%/23% to hsFABPb_1FOQb, 38%/22% to hsFABPb_1LJXa, 41%/23% to hsCRABP1, and 39%/23% to hsCRABP2. Note the gap in front of Pro38 in all FABPs showing a longer 2nd α-helix in CRABPs.

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**Fig. 3.** Molecular modeling of plFABP using human FABP and CRABP-I as templates. (A) plFABP superimposed on the X-ray crystal structure of human brain FABP (1fdqA, 1fdqB) and colored by secondary structure succession from blue to red. (B) plFABP superimposed as in (A) and colored by confidence, where high confidence is towards blue in the spectrum. (C) plFABP superimposed on human CRABP (lpbd1cblp, lpbd2cbr1) and colored by secondary structure succession from blue to red. (D) plFABP superimposed as in (C) and colored by confidence, where high confidence is towards blue in the spectrum.

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**FABP gene expression and localization**

We isolated plFABP cDNA from hemocyte RNA and analyzed the tissue distribution of plFABP mRNA.
Based on RT-PCR analysis, a strong plFABP signal was detected in hemocytes, hepatopancreas, intestine and ovary, whereas in hpt and testis, FABP expression was low, and in eyestalk and muscle no signal at all could be detected (Fig. 4A). A similar result was achieved by northern blot analysis, which showed that the amount of plFABP transcript was very high in hemocytes, hepatopancreas and intestine, and barely detectable in eyestalk and muscle cells (Fig. 4B). However, in hpt the low expression found by RT-PCR was not confirmed by northern blot (Fig. 4B). This difference may indicate that some mature hemocytes were mixed with the hpt cells, and the more sensitive RT-PCR was able to detect hemocyte plFABP mRNA in the hpt sample. To see if this was the case, we performed in situ hybridization experiments with hpt cells, which were examined under the microscope for the presence of hemocytes before the assay. In situ hybridization experiments were performed using dioxigenin-labeled cDNA probes for plFABP. Fluorescence of the plFABP signal was detected in hemocytes as well as in hpt cells, and the granular cells showed a very strong signal (Fig. 4C). From these experiments, it was obvious that several hpt cells did express plFABP, but at a lower level than the hemocytes. Moreover, the fraction of hpt cells expressing plFABP varied a lot between different animals: in some animals expression was high and in some very low.

**Treatment of hpt cells with putative ligands in vitro**

As there was large variation in plFABP expression in hpt cells, we assessed the effect of different putative ligands for the plFABP protein on hpt cells in vitro. Hpt cell cultures were initiated and then cultured for 48 h. Different concentrations of ATRA, oleic acid or stearic acid were then added to the medium, and the morphology of the cells was observed every day. After 7 days, plFABP expression was compared. As shown in Fig. 5A–D, cell spreading was affected slightly after culture in the presence of ATRA at a concentration of 10 nM or higher, whereas no obvious change was observed with the other fatty acids. Interestingly, lipid droplet formation, as judged by oil red staining, was observed in hpt cells cultured with an unsaturated fatty acid (oleic acid), but not in those cultured with stearic acid (a saturated fatty acid) or ATRA (Fig. 5E–G). Lipid droplet formation was always observed in the presence of oleic acid at concentrations of 0.1–1.0 mM, whereas the changes in spreading in the presence of ATRA were more variable. Because of variable results, we investigated some characteristics of the individual crayfish from which the hpt cells were isolated. We divided the crayfish into two different groups according to the color of the hemolymph and called these groups CN (for normal color) and CR (for red color) (Fig. 6, absorbance peak between 450 and 520 nm). In normal colored crayfish, the total number of hemocytes was 0.6 (±0.2) · 10⁶ (n = 5), whereas in the red colored crayfish, it was 3.1 (±0.9) · 10⁶ (n = 5), per ml hemolymph, indicating that hemocyte synthesis is higher in crayfish with red colored plasma. Furthermore, the clotting reaction was much more rapid in crayfish with red plasma, clotting occurring within 5 min compared with several hours in normal colored crayfish. The crayfish with red plasma usually had a thick hpt, which again implies a higher hemocyte count and thus higher hemocyte synthesis than normal colored crayfish. Therefore, we speculated that the
hematopoietic process may be more active in these crayfish. We, therefore, tested whether exogenous fatty acids or ATRA could affect plFABP mRNA expression in cultured hpt cells from the CN and CR groups. Whereas no difference was observed on the addition of the two fatty acids (Fig. 7), a dramatic effect was achieved by the addition of ATRA at 100 nM. As shown in Fig. 7, there was a 13-fold increase in plFABP expression in hpt cells isolated from the CR group after ATRA treatment, whereas in the control group (CN) no change in expression was found (Fig. 7).

**Discussion**

FABPs belong to a large superfamily of low-molecular-mass, small cytosolic lipid-binding proteins responsible for the binding of RA and/or fatty acid molecules. The biological roles of these proteins span over a wide range of processes such as transport, cellular uptake and cytoplasmic trafficking of fatty acids, and modulation of the amount of RA available to nuclear receptors [1,20]. FABPs are found intracellularly in vertebrates as well as in invertebrates, but the
presence of CRABPs in invertebrates has not been conclusively demonstrated [6].

We report for the first time the presence of a FABP in invertebrate hemocytes and hpt, and that the expression of this protein can be modulated by external addition of ATRA. However, our data do not support the existence of CRABPs in invertebrates, as our sequence analysis and 3D structural modeling clearly show that the crayfish and shrimp FABPs do not possess the characteristics of vertebrate CRABPs, although they express the conserved R/R/Y of the P2 motif. FABPs isolated from the same tissue, from different vertebrate species, consistently display sequence identities higher than 70%, whereas FABPs from different tissues of a given species share identities as low as 20%, and pIFABP shows ~30-45% identity with vertebrate FABPs [1].

Our new crustacean FABPs show high similarity to other invertebrate FABPs, although the tissue distribution is different from that of the shrimp _M. ensis_ CRABP transcript, which according to the sequence is probably a FABP. pIFABP was highly expressed in the hepatopancreas and hemocytes, whereas in _M. ensis_ no expression was found in the hepatopancreas [4]. The opposite result to that reported in this paper was obtained for the eyestalk: expression in shrimp eyestalk was fairly high, whereas expression in crayfish eyestalk was absent [4].

In _M. ensis_, the ovaries at the early and late stages of vitellogenesis display similar high levels of mRNA transcripts, and we also found high pIFABP expression in the ovaries of crayfish, indicating a role for these FABPs in gonadal maturation. The presence of FABP in the early larval stages of shrimp (_M. ensis_) also suggests that this protein may be involved in early larval development [4].

So far no role has been indicated for FABPs in invertebrate immunity or hematopoiesis. In vertebrate immunity, the function of different FABPs is not clearly understood, although several FABPs are expressed in immune active cells [14]. Long-chain fatty acids also regulate gene transcription via FABPs by activating nuclear peroxisome proliferator-activated receptors, which are essential for induction of differentiation in some cell types [21].

RA, the biologically active metabolite of vitamin A, regulates the patterning and development of many vertebrate organs [22–24], and also exerts a wide range of effects on both normal and malignant hematopoietic cells [25,26]. However, in invertebrates (or nonchordates), no clear evidence exists for the presence of a RA signaling pathway [23], although in our work an effect of externally added ATRA was found, clearly indicating that some sort of RA signaling exists. In another crustacean, the fiddler crab _Uga pulgator_, endogenous retinoids have been isolated from the limb blastema [27], and external addition of RA to the crab during limb regeneration was also shown to induce malformation and slow growth [28]. Moreover, expression of retinoid-X receptor (UpRXR) was increased after treatment with RA in the fiddler crab during limb regeneration [29]. RXR is a heterodimeric receptor that can bind RA receptor but also other nuclear receptor proteins. Although RA receptor can bind ATRA and 9-cis RA, after dimerization with RXR, RXR in vertebrates can only bind 9-cis RA. RXR homologs have been described in insects, and the _Drosophila_ homolog ultraspiracle forms heterodimers with the ecdysone receptor and is unable to bind RA [23]. However, although the fiddler crab _UpRXR_ shares high similarity in its DNA binding domain to insect ultraspiracles, its ligand-binding domain is more closely related to vertebrate RXRs [29]. Recent results also show that _UpRXR_ occurs in several different splicing variants, indicating that more has to be learned about these receptor proteins before a lack of RA signaling in invertebrates can be ruled out [30]. In _Ciona intestinalis_, more than 20 genes have been shown to be up-regulated in the embryo by RA treatment, and in _M. sexta_ a RARE-like motif has been found in the 5' regulatory region of the msCRABP (AAC24317) gene [5]. Whether similar RA-dependent regulatory sequences are present in the promoter region of pIFABP and whether the effect of external RA mimics some endogenous substance still has to be investigated in crayfish. Externally added ATRA was only effective in hpt cells isolated from crayfish with red hemolymph. Crayfish with red plasma had both a high number of hemocytes and rapid clotting ability compared with crayfish with normal colored plasma, indicating that they have higher hemocyte synthesis and a more rapid clotting system. We have previously shown that, in a high-density lipoprotein, the β1,3-glucan-binding protein present in plasma, ~1.6% of its total lipids is carotenoids, and a low density lipoprotein, the clotting protein, is pink–orange in color [31,32]. Therefore these proteins may contribute to the increase in red color of the plasma. However, in the pacific white shrimp _Litopenaeus vannamei_, red hemolymph in combination with decreased clotting ability was detected after infection with Taura syndrome virus [33]. In kuruma prawn, _Metapenaeus japonicus_, the hemolymph turned red after injection with a toxic serine proteinase isolated from the pathogen _Vibrio alginolyticus_ [34]. If crayfish with red colored plasma respond to ATRA by greatly increased expression of pIFABP, this may indicate that this FABP is involved in
hemocyte synthesis, as hemocyte numbers are higher in these crayfish. However, more experiments are needed to conclusively explain the physiological relevance of this ATRA-induced plFABP expression.

In conclusion, there is still no evidence for true RA-regulated gene expression in invertebrate animals. Although no substrate-binding experiments were performed on purified native or recombinant plFABP in this study, our data give some support to the idea that a RA signaling pathway is likely to be present in crustaceans, and this pathway may be more active during stress.

**Experimental procedures**

**Experimental animals**

Freshwater crayfish, *P. leniusculus*, were purchased from Nils Fors, Torsång at Lake Vättern, Sweden and were maintained in tanks with aerated running water at 10 °C. Only intermolt crayfish were used in this study.

**Cloning of crayfish and shrimp FABP cDNA**

An EST sequence of crayfish FABP was found in a *P. leniusculus* hemocyte EST library. Several sets of gene-specific primers based on the crayfish EST sequence were designed for 5'-RACE and 3'-RACE. First-strand cDNA was synthesized from 4 µg total RNA-3' RACE System for Rapid Amplification of cDNA Ends kit (Invitrogen, Carlsbad, CA, USA), using an oligo-dT-adapter primer. An initial amplification by PCR was carried out with specific F7-FABP (5'-AGGGACAAGCTTATCCAGACGCAG-3') as sense primer and AUAP as antisense primer, under the following conditions: a first step of 3 min at 94 °C was followed by 35 cycles of 30 s at 92 °C, 30 s at 55 °C and 1 min at 72 °C, and 72 °C for 7 min. The nested PCR was performed with specific F106-FABP (5'-GATGGCGTG GTGTCCAAGCGTATC-3') as sense primer and abridged universal amplification primer (AUAP) as antisense primer with a 10-fold dilution of the 1st PCR as the template, under the same conditions.

For 5'-RACE, first-strand cDNA (4 µg total RNA) was synthesized by reverse transcription. Thermoscript™ reverse transcriptase and 0.5 µM oligo(dT)20 or 5'-AAAGTAGCA ATGGCAGCAACA-3' (reverse, R191-FABP) primer were allowed to react for 1 h at 50 °C in 20 µL reaction mixture containing 1 mM dNTP and 5 mM dithiothreitol in first-strand buffer. The cDNA was purified using a QIAquick PCR purification kit (Qiagen, Hilden, Germany). To anchor the PCR product at the 5' end, cDNA was tailed for 10 min at 37 °C using 15 units of terminal deoxynucleotidyl transferase (Promega, San Diego, CA, USA) and 200 µM dCTP. To amplify the 5'-mRNA ends of crayfish FABP, we used a series of FABP-specific primers as follows: 5'-CTCAGAGGACTCGAGGTTGTT-3' (reverse, R2-CRPB), 5'-CTCAGAGGACTCGAGGTTGTT-3' (reverse, R1-FABP), R191-FABP and 5'-GGCCACCGCTGAC TAGTACGGGGIGGGGGIIG-3' (forward, anchor primer, AP). A first amplification by PCR was carried out with specific AP as sense primer and R191-FABP as antisense primer, using 1 µL reverse transcription reaction mixture in a total volume of 25 µL: a first step of 3 min at 94 °C was followed by 35 cycles of 30 s at 92 °C, 30 s at 53 °C and 1 min at 72 °C, and 72 °C for 7 min. The first round of PCR was carried out with AP-specific and TG-specific primer under the conditions described above. Nested PCR was performed with the AP and reverse FAPB-specific primer using 1 µL of the first round PCR product. The RACE PCR products were visually examined on a 1.2% agarose gel after electrophoresis. PCR products were cut out of the gel slice and purified using the GFX PCR DNA and Gel Band Purification kit (Amersham Bioscience, Uppsala, Sweden) and then cloned into a PCR 2.1-TOPO TA cloning vector (Invitrogen) following the manufacturer’s instructions.

On the basis of this sequence, degenerated and specific primers were designed from both plus and minus strands: forward-Sh1, 5'-GGAAYTTCGAYGARTTYATGAA-3'; reverse-Sh2, 5'-GTAGGTATCGCCGTCCTTGGTG-3'; reverse-Sh3, 5'-GCCATACGCTGGTGTTCTTCA-3'. The UNI-ZAP XR cDNA library from hemocyte of *P. monodon* was used as a template to amplify by PCR combination with T3 and T7, and the resulting PCR products were cloned and sequenced as above.

**Preparation of hpt**

The hpt was dissected from the dorsal side of the stomach, as described in [35]. It was separated into single cells by incubation in 650 µL 0.1% collagenase (Type I + Type IV) solution at room temperature for 40 min. After collagenase treatment the tissue was gently passed 10–20 times through a pipette and centrifuged at 380 g for 5 min to remove the collagenase solution. The pellet was washed twice with 500 µL CPBS by centrifugation at 380 g for 5 min and then resuspended in CPBS and used in subsequent experiments [35].

**Separation of hemocytes**

The different hemocyte populations of *P. leniusculus* were separated and harvested by a sucrose density gradient centrifugation method [36].

**In situ hybridization**

Circulating hemocytes and isolated hpt cells were analyzed by in situ hybridization using cDNA probes for crayfish
FABP. The cells were attached to slides as previously described [37] and fixed with 95% ethanol for 5 min at room temperature and stored in 70% ethanol at −20 °C until used. Sequences corresponding to 332 bp of the FABP region of the crayfish were labeled with dioxigenin using Klenow enzyme according to the manufacturer’s protocol (Boehringer-Mannheim, Mannheim, Germany). The fixed slides were pretreated with 0.1–4 μg·mL −1 proteinase K (PCR grade; Roche, Mannheim, Germany) for 15 min at 37 °C and then hybridized with dioxigenin-labeled probe in 1.1× Denhart’s solution, 5.5% dextran sulfate, 0.2 mg·mL −1 sonicated salmon sperm DNA, and 4.4× NaCl/Cit overnight at 42 °C. The samples were washed in 2× NaCl/Cit for 2× 5 min at 20 °C and 0.1× NaCl/Cit for 10 min at 42 °C. Hybrids were detected using anti-dioxigenin-fluorescein (Fab fragments) and two secondary fluorescein-labeled antibodies. Hybridization of RNase-treated cells and nonprobe was used as negative controls.

RT-PCR
Expression of FABP mRNA in tissues was demonstrated by RT-PCR. Total RNA was extracted from a variety of crayfish tissues including hemocyte, hpt, hepatopancreas, muscle, eyestalk, intestine, testis and ovary using Trizol. One microgram total RNA was reverse-transcribed using Thermoscript™ reverse transcriptase with oligo(dT) as primer. Two specific primers, forward 5′-GGCAAGTACACCCTCGAGTCC-3′ and reverse 5′-AAGGATGATGGAAGAGTGGGA TTTTGGTGGTGGT-3′, were used in PCR as described above. Crayfish ribosomal 40S gene was used as a housekeeping gene; the primer sequences were as follows: forward 5′-CCAGGACCCCCAATCTTTCA-3′ and reverse 5′-GAAAATGCCACGAGCGTTCG-3′.

Northern blot analysis
Total RNA of hemocytes, hpt, hepatopancreas, muscle, eyestalk, intestine, testis and ovary of crayfish were extracted with Trizol (Invitrogen), by capillary blotting overnight. The membrane was hybridized at 42 °C in a 1% formaldehyde–agarose gel at 70 V for 2.5 h and transferred to a nylon Hybond N membrane (Amer sham Pharmacia Biotech) by capillary blotting overnight. The blotted membrane was crosslinked by UV-linker (Stratagene, La Jolla, CA, USA). The FABP DNA fragment (residue 300–609) was used as a probe. The DNA probe was labeled with [32P]dCTP[32P] using the Megas prime DNA Labeling System (Amersham Pharmacia Biotech). The membrane was hybridized at 42 °C and washed with high stringency according to the manufacturer’s instruction for Hybond N membrane. The membranes were then subjected to image plates for the phosphorimager BAS-2040 (Fuji, Tokyo, Japan). The radioactivity was quantified using the IMAGE GAUGE program, version 3.4 (Fuji). Crayfish actin was used as an internal control for quantification of total RNA.

Treatment with fatty acids and ATRA
ATRA (Sigma-Aldrich, Steinheim, Germany) was dissolved in dimethyl sulfoxide at 10 mM and stored at −80 °C in amber (light-protected) Eppendorf safe lock tubes as a stock solution. Oleic acid and stearic acid solutions were prepared as described by Stremmel & Berk [38]. Briefly, the fatty acids (Sigma) were dissolved at 50 μM in 0.1 M NaOH at 37 °C, and a solution of 2.5 mM fatty acid-free BSA (Sigma) in NaCl/Pi, was added to a fatty acid/albumin molar ratio of 5; the pH was adjusted to 7.4 with NaOH. The stearic acid solution was incubated at 60 °C for 15 min to allow solubilization of the fatty acid complex. The solutions were then filter sterilized and diluted with culture medium to its final working concentration. The hpt cells were cultured in the presence or absence of 0.1–1.0 mM fatty acids or ATRA from 1 nm to 1 μM. The fatty acid-ATRA were introduced 3–5 days after initiation of the culture, and the medium was changed (to one with fatty acid/ATRA maintained) every 2 days until the 7th of culture. The morphology was observed every day. Expression of FABP in hpt cells was determined by real-time RT-PCR at different time intervals after the treatment.

Comparative quantitation of plFABP mRNA expression using real-time RT-PCR
Hpt cell cDNA was synthesized using oligo(dT) as described above and diluted to 1:10 with MilliQ filter sterilized water. Real-time RT-PCR and data analysis were performed in a Roter-gene3000 (Corbett Robotics, Australia) using QuantiTect SYBR® Green PCR kit (Qiagen). Primers (458+ GGCACAGCAGCTGACAGTC GCAATAG and 596–ACGAGAAAGCAGGATTGATG TGATGG) were chosen to amplify a 139-base pair fragment of the plFABP. Primers specific to the crayfish ribosomal 40S gene (156+ GACGAATGGCATACACTGAG AGG and 280–CAGACTCTGCATTCAAGCTGATG) were used to quantify cellular RNA input of each preparation with a 125-bp PCR product. The PCR mixture contained 12.5 μL 2× QuantiTect™ SYBR Green PCR master mix, 0.4 μM each primer, and 5 μL diluted cDNA template. RNase-free distilled water was added to reach a total volume of 25 μL per reaction. All runs used a negative control without target DNA. Thermal cycling conditions were as follows: 95 °C for 15 min, followed by 45 cycles of 94 °C for 15 s, 62 °C for 30 s, and 72 °C for 30 s. All PCRs were performed in triplicate.
Assay of hemocyte number and clotting

Total hemocyte number was determined as previously described [35]. The absorption spectra of plasma from different crayfish were analyzed after removal of the hemocytes from the hemolymph by centrifugation (800 g, 10 min at 4 °C). Clotting ability was analyzed as described previously [39].

Oil red O staining

After being cultured for 2 days in the presence of fatty acids or ATRA, the cells were washed twice with ice-cold NaCl/P₈, fixed in 10% formalin for 10 min, rinsed in distilled water, infiltrated into 100% propylene glycol for 5 min, and then stained with oil red O (Wako, Dusseldorf, Germany) for 8 min at 60 °C. The cells were counterstained with 0.5% methyl green (Sigma, St Louis, MO, USA) in 0.1 m sodium acetate, pH 4.2, for 5 min at 37 °C, followed by rinsing in distilled water (3 × 3 min each) and mounting in aqueous mounting medium.

Protein homology modeling

Structural models of the crayfish FABP were created using Deep View analysis software (http://www.expasy.org/spdbv/). One model was based on the published X-ray crystal structure of human brain FABP (1fdqA, 1fdqB) and NMR structure of human brain FABP. In another model, pIFABP was superimposed on human CRABP (pdb/1cbp, pdb/2cbcr).

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