Fatty acid-binding proteins in *Echinococcus* spp.: the family has grown

Jorge L. Pórfido^{1,2} · Michaela Herz³ · Ferenc Kiss³ · Laura Kamenetzky⁴ · Klaus Brehm³ · Mara C. Rosenzvit⁴ · Betina Córscico¹ · Gisela R. Franchini¹

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Abstract

Fatty acid-binding proteins (FABPs) are small intracellular proteins that reversibly bind fatty acids and other hydrophobic ligands. In cestodes, due to their inability to synthesise fatty acids de novo, FABPs have been proposed as essential proteins, and thus, as possible drug targets and/or carriers against these parasites. We performed data mining in *Echinococcus multilocularis* and *Echinococcus granulosus* genomes in order to test whether this family of proteins is more complex than previously reported. By exploring the genomes of *E. multilocularis* and *E. granulosus*, six genes coding for FABPs were found in each organism. In the case of *E. granulosus*, all of them have different coding sequences, whereas in *E. multilocularis*, two of the genes code for the same protein. Remarkably, one of the genes (in both cestodes) encodes a FABP with a C-terminal extension unusual for this family of proteins. The newly described genes present variations in their structure in comparison with previously described FABP genes in *Echinococcus* spp. The coding sequences for *E. multilocularis* were validated by cloning and sequencing. Moreover, differential expression patterns of FABPs were observed at different stages of the life cycle of *E. multilocularis* by exploring transcriptomic data from several sources. In summary, FABP family in cestodes is far more complex than previously thought and includes new members that seem to be only present in flatworms.

Keywords *Echinococcus multilocularis* · FABPs · Fatty acids · Cestodes

Introduction

Echinococcosis is caused by the metacestode larval stages of *Echinococcus* spp., which generate cyst-like structures in the intermediate host's inner organs. Cystic and alveolar

echinococcosis, produced by *Echinococcus granulosus* sensu lato and *Echinococcus multilocularis*, respectively, affect more than one million people around the world. These diseases can significantly reduce life quality and can be life threatening if untreated. The main treatments consist on either surgery or prolonged drug therapy, with varying results (World Health Organization 2019).

Due to the inability of cestodes to synthesise fatty acids de novo (Maule and Marks 2006; Smyth and McManus 2007), fatty acid-binding proteins (FABPs), together with other lipid-binding proteins, have been proposed as essential for these organisms, facilitating lipid uptake from the host. These proteins have also been suggested as possible antiparasitic drug targets and/or carriers (Xu et al. 2011).

FABPs are small intracellular proteins (around 15 kDa) that reversibly bind fatty acids and other hydrophobic ligands. Members of this family have been found throughout the animal kingdom, both in vertebrates and invertebrates, but no counterparts have been found in plants or fungi (Haunerland and Spener 2004). They are usually highly expressed in cells with very active lipid metabolism. The sequence identity

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✉ Gisela R. Franchini
gfranchini@conicet.gov.ar

¹ Instituto de Investigaciones Bioquímicas de La Plata (INIBIOLP), Facultad de Ciencias Médicas, Universidad Nacional de La Plata (UNLP)-Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), La Plata, Argentina

² Present address: Institut Pasteur Montevideo, Montevideo, Uruguay

³ Institut für Hygiene und Mikrobiologie, Universität Würzburg, Würzburg, Germany

⁴ Instituto de Investigaciones en Microbiología y Parasitología Médica (IMPAM), Facultad de Medicina, Universidad de Buenos Aires (UBA)-Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Buenos Aires, Argentina

among the family varies significantly but they share an overall similar 3D protein structure consisting in a solvent accessible β -barrel with an alpha helical cap. In *E. granulosus*, two FABPs have been described, EgFABP1 and EgFABP2 (Esteves et al. 1993, 2003). EgFABP1 has been characterised in more detail, including its 3D structure clearly matching the typical structure of FABPs (Jakobsson et al. 2003), binding properties as well as its interaction with ligands and with artificial lipidic membranes (Alvite et al. 2001; Pórfido et al. 2012).

This study offers a detailed analysis of the FABP family in *E. multilocularis* and *E. granulosus* not only based on genomic and transcriptomic information but also validated by cloning and sequencing of the respective cDNAs from *E. multilocularis*.

Results and discussion

We identified six FABP encoding genes on the available *E. multilocularis* genome. Two of them encode proteins with identical primary sequence and 93% identity to EgFABP1; thus, they were named *emfabp1.1* [EmuJ_002165500] and *emfabp1.2* [EmuJ_000550000] (accession codes correspond to WormBase ParaSite). Of the other four genes, one is orthologous to *egfabp2* [EmuJ_000549800] (100% identity between EmFABP2 and EgFABP2), whereas the other three represent novel genes (named *emfabp3* [EmuJ_000551000], *emfabp4* [EmuJ_000417200] and *emfabp5* [manually annotated by us], each with distinctive features. In particular, although the coding sequence of *emfabp3* is slightly longer than previously described for *egfabp1* and *egfabp2*, the gene contains a considerable longer intron (4443 versus 80 bp) reminiscent of those from mammalian FABPs (Esteves and Ehrlich 2006; Alvite et al. 2008; Smathers and Petersen 2011) (Fig. 1). *emfabp4*, on the other hand, codes for a longer protein compared with all other FABPs (176 versus 130 aminoacids), and also has an unusually long intron compared with what had been mostly described for cestodes FABPs (Esteves et al. 2003; Esteves and Ehrlich 2006; Alvite et al. 2008). In this work, the coding sequences abovementioned have been corroborated by cloning and sequencing of *E. multilocularis* cDNAs. Obtained sequences have been deposited in Gene Bank under the following accession codes: MN809107 (*emfabp1.2*); MN809108 (*emfabp1.1*); MN809109 (*emfabp2*); MN809110 (*emfabp3*); MN809111 (*emfabp4*); MN809112 (*emfabp5*). It is important to note that Zheng and co-workers (2013b) have proposed the presence of more than two FABP genes in *Echinococcus* spp. In particular, based on in silico data, the authors stated the existence of five genes each in *E. granulosus* and *E. multilocularis*. Importantly, our study revealed several differences to those predictions (Zheng et al. 2013b). On the one hand, these authors proposed intronless genes that would correspond to

Fig. 1 Schematic representation of the length and position of the introns in EmFABPs' protein sequences. The figure displays the length of the introns (in base pairs) at the upper-right corner of the triangles as well as the relative position of the splicing sites in the translated protein sequence for the five EmFABPs (under the triangles). EmFABP1 accounts for EmFABP1.1 and EmFABP1.2 since they are identical

emfabp3 and *emfabp4*, but that does not agree with our experimental evidence (cloning and sequencing of *E. multilocularis* cDNAs). We found, instead, that both these genes have unusually long introns compared with what had been described for cestodes FABPs (Esteves et al. 2003; Esteves and Ehrlich 2006; Alvite et al. 2008). In addition to that, the protein sequences proposed by them for EmFABP3 and EmFABP4 (called Emul_FABP1 and Emul_FABP4, respectively, in their article) are identical to our proposed EmFABP3 and EmFABP4 up to the single intron splice site in each gene. After that site, their Emul_FABP1 continues with amino acids that do not share similarities with equivalent FABPs' positions, and Emul_FABP4 ends at amino acid 124. Finally, we found that *emfabp5* was similar to *emfabp1* and *emfabp2*, although carrying two introns instead of the only one previously described in cestodes FABP genes (Esteves and Ehrlich 2006; Alvite et al. 2008). More than one intron is not uncommon for FABP genes of different species, but it has not been described in cestodes yet (Zimmerman and Veerkamp 2002; Esteves and Ehrlich 2006; Smathers and Petersen 2011). Moreover, in intron 1 of *emfabp5*, an alternative GC splice donor sequence was found which, although being less common than GT, appears quite frequently in species such as *Homo sapiens*, *Caenorhabditis elegans* and *Arabidopsis thaliana* (Sheth et al. 2006; Parada et al. 2014).

With respect to genomic organization, five of the genes (*emfabp1.1*, *emfabp1.2*, *emfabp2*, *emfabp3* and *emfabp4*) are present on the same chromosome (chromosome 7), and all of them but *emfabp3* are coded by the same strand. The other

gene, *emfabp5*, lies on chromosome 4. In addition, three of the genes encoded by chromosome 7 (*emfabp1.1*, *emfabp1.2* and *emfabp2*) are clustered in *E. multilocularis* within a 13.7-kb region, with intergenic regions of 5.6 kb (between *emfabp2* and *emfabp1.1*) and 6.7 kb (between *emfabp1.1* and *emfabp1.2*).

The six predicted protein sequences of FABPs were assigned to the FABP family according to different patterns found in their primary structure: PROSITE pattern *Cytosolic fatty-acid binding proteins signature* (accession number PS00214), Pfam pattern *Lipocalin/Cytosolic fatty acid binding protein family* (accession number PF00061) and PRINTS pattern *Fatty acid-binding protein signature* (accession number PR00178). The PRINTS pattern is defined by 3 elements. The first one includes the first β -strand (β A) that forms part of the barrel and the first α -helix (α I). Additionally, on β A, it has a characteristic Gly-X-Tip triplet (where X represents any amino acid). The second element goes from the C-terminal end of β D across β E, and the third represents the last two strands of the barrel, β I and β J. Within these three elements, all six FABPs are very well conserved (Fig. 2). What is more, they all invariantly contain the P2 ligand-binding motif (an Arg... Arg-x-Tyr motif involved in ligand binding) (Jones et al. 1988; Jakobsson et al. 2003), with the sole exception of EmFABP4 that has a Tyr instead of an Arg in the first position.

All the analysed sequences showed a predicted secondary structure of ten β -strands with two α -helices between the first two strands, in good accordance with the typical arrangement of secondary structure elements in FABPs. In the case of EmFABP3, the prediction includes longer turns between strands β B and β C, and between β F and β G. Nevertheless, whether the insertions affect these turns or other regions of the protein remains to be determined. Finally, in EmFABP4, the most striking feature is that the predicted sequence is much longer (176 amino acids) than what is expected for a FABP (around 130 amino acids). In this case, a typical FABP fold is predicted but no specific structure is assigned to the C terminus of the protein. The coding sequence has been amplified by RT-PCR, which gives evidence for its correct transcription and expression within the organism so it is expected to constitute a new FABP variant in which the C terminus' fold and function should be addressed. It is noticeable that, recently, FABPs with C-terminal extensions from *Fasciola* spp. have been also reported (Morphew et al. 2016).

In order to define the relatedness of the proteins across the species, a phylogenetic analysis was performed using FABPs from cestodes and model organisms (Fig. 3). As observed, all cestodes FABPs are clustered together with the sole exception of EmFABP5 and EgFABP5 which represent a different node. This protein sequence divergence is in accordance with its

Fig. 2 Multiple alignment of protein sequences of EmFABPs. This figure shows the alignment of the EmFABPs. Three regions are highlighted with dotted lines, representing the three elements that constitute PRINTS pattern PR00178 (fatty acid-binding protein signature) which represent a fingerprint of the FABP family. The grey-shaded amino acids represent conserved positions whereas light blue-shaded ones represent positions with similar amino acids in the different proteins. (*) indicates the P2-binding motif. The yellow bars represent approximate location of β -sheets (β A- β J), while red bars symbolise α -helices (α I and α II), both according to EgFABP1 structure (PDB, 1o8v) and secondary structure predictions performed employing PSIPRED tool

FABPs were found in *E. granulosus* as well. Two of them correspond to the previously reported *egfabp1* (from now on named *egfabp1.1*) [*EgrG_000549850 commented on database*] and *egfabp2* [*EgrG_000549800*] (Esteves et al. 1993, 2003). Three other genes are, clearly, orthologues of *emfabp3*, *emfabp4* and *emfabp5*, and thus named *egfabp3* [*EgrG_000551000*], *egfabp4* [*EgrG_000417200*] and *egfabp5* [*EGR_05655, commented on database*]. The sixth gene turned out to be located in a cluster equivalent to that described above for *E. multilocularis*. It is found immediately after *egfabp2* and *egfabp1.1*, but in this case, the CDS (or the ORF) is not identical to that of *egfabp1.1*. As it is placed in an equivalent position to where *emfabp1.2* is found in the *E. multilocularis* genome, relative to other FABP genes, it was named *egfabp1.2* [*EgrG_000550000*].

Noticeable is the fact that in EgFABP1.2, two of the three amino acids that shape the P2-binding motif are not conserved. This could imply that this protein may bind its ligands employing a different mechanism (as certain mammal FABPs that lack this motif do), bind other kind of ligands or be non-functional.

Recently published transcriptomic information regarding *E. multilocularis* oncospheres together with transcriptomic data published by Tsai and co-workers (2013) suggest that all the genes analysed in the present work (with the exception of *emfabp5* that was not annotated) are transcribed in different stages of *E. multilocularis* (Zheng et al. 2013a; Huang et al. 2016).

In addition to that, the transcriptomic data published later, together with the genome of *E. granulosus* (Zheng et al. 2013a), indicate that all the FABP genes of *E. granulosus* are transcribed in, at least, one stage of the parasite (*egfabp1.1* and *egfabp5* are not well annotated in that genome).

According to the different sets of data, *emfabp1.1* and *emfabp1.2* are the most highly expressed FABP genes in *E. multilocularis* (data not shown). Given that *emfabp1.1* and *emfabp1.2* are mostly identical in CDS sequence, it is hard to distinguish which reads correspond to each of the two genes. However, the flanking regions of both genes present several differences that allowed us to map transcripts unequivocally to the genome, reinforcing the existence of two genes (*emfabp1.1* and *emfabp1.2*) and giving evidence that both are transcribed and show differences in expression profile (Fig. 4).

Although expressed in many stages, *emfabp2* is reported to show higher expression levels in metacestodes and adult stage, as well as in primary cells (i.e., cells isolated from metacestode tissues of *Echinococcus multilocularis* which are able to regenerate vesicles in vitro, part of them being germinal cells, the only proliferative ones (Kozioł et al. 2014)). *emfabp3* seems to be poorly expressed, being higher at the adult stage. Moreover, reported data indicates that it would be expressed at the oncosphere stage (non-activated)

of *E. multilocularis*, which are indeed included in the gravid adult samples employed for the analysis (Huang et al. 2016). In the case of *emfabp4*, it is more expressed in adult stages, both gravid and pre-gravid (Tsai et al. 2013). A reanalysis of the data obtained by Tsai et al. showed that *emfabp5* is moderately expressed with its expression higher in the late metacestode stage, which contains brood capsules with protoscoleces. In the adult stage, it would be expressed in levels equivalent to *emfabp3* (Fig. 4).

In the case of *E. granulosus*, according to Zheng et al. (2013a), *egfabp1.2* is more highly transcribed in adult stage than in oncosphere, protoscoleces or cyst, suggesting a possible expression in adult tissues absent in the other stages. *egfabp1.1* is very highly expressed in all stages, *egfabp2* mainly in adult and cyst, *egfabp3* in oncospheres and cyst, and *egfabp4* primarily in adults (Zheng et al. 2013a). *egfabp5*, which is not properly annotated in Zheng et al. (2013a) but, however, is present in transcriptomic data, could be confined to oncospheres and adult stage (which could be due to the expression in oncospheres contained in the adult, as previously mentioned).

It is worth noticing that, in every case, each stage is considered as a whole, not having into account (for technical reasons) the different tissues that compose them. It is thus important, in the future, to deepen the study of those aspects of FABPs expression.

This study shows that FABP family in cestodes is far more complex than previously thought and includes new members, some of them with features that could be unique to flatworms.

Materials and methods

Bioinformatic analysis

The high-quality *E. multilocularis* genome assembly and *E. granulosus* sensu stricto draft genome assembly (Tsai et al., 2013) were retrieved from the WormBase Parasite database (<http://parasite.wormbase.org/>). The reference sequences EgFABP1 (GenBank AF321119.1) and EgFABP2 (GenBank AF321117.1) from *E. granulosus* were used to perform BLASTN, BLASTP, TBLASTN and BLASTX (hosted in the sequence alignment editor BioEdit, <http://www.mbio.ncsu.edu/bioedit/bioedit.html>) against *E. multilocularis* and *E. granulosus* genomes. The predicted protein sequences were analysed employing InterPro (<http://www.ebi.ac.uk/interpro/>), for domain identification, and PSIPRED (<http://bioinf.cs.ucl.ac.uk/psipred/>), for secondary structure prediction.

In order to detect possible expression of all *E. multilocularis* FABPs, including *emfabp1.1*, *emfabp1.2* and *emfabp5*, two approaches were used. In the first approach, RNAseq data from (Tsai et al. 2013) was remapped to the

Fig. 4 Transcripts' level for the different EmFABP genes. The figure shows the transcription levels (in fragments per kilobase of exon model per million reads mapped, FPKM) for the different FABP genes in *E. multilocularis* in different stages (or samples) of the parasite. All transcriptomic data were obtained from Tsai et al. (2013) and reanalysed as described in the "Materials and methods" section. PC1, primary cells

2 days old; PC2, primary cells 11 days old; MC-noBC, metacystodes without brood capsules; MC-lateBC, late metacystode vesicles with brood capsules; PS-nonact, non-activated protoscoleces; PS-act, activated protoscoleces; Ad-pregrav, pregravid adult worms; Ad-grav, gravid adult worms

E. multilocularis reference genome from WormBase ParaSite (WBPS7) using the Hisat2 v2.0.5. Reads for each transcript (annotations from WormBase ParaSite and a manual annotation for *emfabp5*) were counted using the HTSeqCount v0.7.1 with a minimal quality score of 30 to filter out multiple-mapped reads, allowing qualitative distinction between *emfabp1.1* and *emfabp1.2*. As this method is not suitable to calculate realistic quantitative expression levels for genes with identical sequences, we used a second approach to estimate those. RNAseq data from (Tsai et al. 2013) and predicted *E. multilocularis* transcripts from WormBase ParaSite (WBPS7) together with the sequenced transcript for *emfabp5* were used to estimate expression levels with the Kallisto v0.43.1.

Parasite material

Parasite material was maintained in Mongolian jirds as described in Spiliotis et al. (2008) and Spiliotis and Brehm (2009) with approval from the ethics committee of the

Government of Lower Franconia (permit no. 55.2-2532-2-354) according to German and European regulation on animal protection (*Tierschutzgesetz*). *E. multilocularis* primary cells were obtained from in vitro-cultured metacystodes, as previously described (Kozioł et al. 2014).

RT-PCR and cloning

RNA from primary cell cultures (2 days cultures) was extracted employing Isol-RNA Lysis Reagent (5Prime) according to the manufacturer's instructions, and treated with DNase (RQ-1 RNase-free DNase, Promega). cDNA was synthesised employing poly-dT oligonucleotides and PrimeScript Reverse Transcriptase (TaKaRa) as recommended by the manufacturer. The obtained cDNA was employed as a template for PCR amplification of the coding sequences of the predicted FABP genes. PCRs were performed employing Platinum Pfx DNA polymerase (Invitrogen) and the corresponding primers for each sequence: EmFABP1.1-CDS-Fw, atggagggcgttcctcggta; EmFABP1.1-CDS-Rv,

ttacgacaccttgagtaggttc; EmFABP2-CDS-Fw, atggagccattcatcggtta; EmFABP2-CDS-Rv, ttacatccctcttgagtaggttcg; EmFABP3-CDS-Fw, atggatgactttctgggcacct; EmFABP3-CDS-Rv, tcagtcctttactcgacgataca; EmFABP4-CDS-Fw, atggatgaatttctgggacatctg; EmFABP4-CDS-Rv, ttattttgtcgaattagtattatccaa; EmFABP5-CDS-Fw, atggaggcattcctaggcac; EmFABP5-CDS-Rv, ctactccactcgtttgaagtt. It is important to note that CDS sequence of EmFABP1.2 is exactly the same as EmFABP1.1. After purification, the amplified DNA fragments were A-tailed and ligated to pGEM-T Easy vector (Promega), according to the protocol supplied by the manufacturer. The inserts of plasmids were sequenced at Macrogen, Inc. (Korea), employing SP6 promoter and T7 promoter universal primers. The obtained sequences were aligned to the predicted sequences employing the tool CLUSTALW and visual inspection.

Sequence alignment and phylogenetic analysis

In order to define the relatedness of the proteins across the species, a phylogenetic analysis was performed using cestode and model species FABPs. FABPs sequences were aligned using CLUSTALW; the sequences were adjusted with manual edition when needed. The phylogenetic tree was inferred using maximum likelihood method based on the JTTmatrix-based model. The bootstrap consensus tree was inferred from 500 replicates. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The analysis involved 72 amino acid sequences. There were a total of 117 of the final dataset. Evolutionary analysis was conducted in the Mega 7 (Kumar et al. 2016).

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Compliance with ethical standards

Conflict of interests The authors declare that they have no conflict of interest.

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