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The unusual lipid binding proteins of parasitic helminths and their potential roles in parasitism and as therapeutic targets ☆

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A B S T R A C T

In this review paper we aim at presenting the current knowledge on structural aspects of soluble lipid binding proteins (LBPs) found in parasitic helminths and to discuss their potential role as novel drug targets. Helminth parasites produce and secrete a great variety of LBPs that may participate in the acquisition of nutrients from their host, such as fatty acids and cholesterol. It is also postulated that LBPs might interfere in the regulation of the host's immune response by sequestering lipidic intermediates or delivering bioactive lipids. A detailed comprehension of the structure of these proteins, as well as their interactions with ligands and membranes, is important to understand host–parasite relationships that they may mediate. This information could also contribute to determining the role that these proteins may play in the biology of parasitic helminths and how they modulate the immune systems of their hosts, and also towards the development of new therapeutics and prevention of the diseases caused by these highly pathogenic parasites.

1. Introduction

Parasitic helminths cause serious and difficult to treat diseases in humans, animals and plants. The majority of these worms are members of round worms (Phylum Nematoda) and the so called flukes (Trematoda) and tapeworms (Cestoda), both of which are classes within the Phylum Platyhelminthes. A high percentage of the Earth's human population, mainly in developing countries, suffers from helminth infections, and in Latin America almost 40% of the population is infected [1]. These diseases are usually related to poverty and social classes with limited economic resources and poor sanitary conditions. Unfortunately, only limited improvements in prevention strategies and treatments have been made against diseases caused by helminths in the last 50 years, thereby defining them as neglected diseases worldwide. According to the World Health Organization (WHO), eight out of 17 tropical neglected diseases are caused by parasitic helminths. Also of considerable interest to WHO is a subset of the neglected diseases called zoonotic neglected diseases, among which taeniasis/cysticercosis, echinococcosis and foodborne trematodiases are considered priority. Moreover, infections of domestic animals are of major economic interest and are important for animal health companies undertaking drug discovery programs [2].

Given their hydrophobic nature, lipids must be transported by other molecules in aqueous environments. In nature this problem has been solved by the existence of lipid binding proteins (LBPs) that act as transporters. In general terms there are two main types of LBPs found in nature: those that carry lipids through aqueous compartments such as blood, pseudocoelomic fluid and hemolymph of invertebrates, or in the cytosol; and those that transport lipids across cell membranes. The soluble lipid binding proteins are highly diverse in amino acid sequence, structure, nature of lipid ligands and binding stoichiometry. It is reasonable to think that diverse animal groups such as nematodes, trematodes and cestodes may express different types of lipid binding proteins from those of other animal phyla that may indicate helminths-specific adaptations.

In order to develop new therapeutic strategies and diagnostic tests we need further knowledge of these parasites' biology that means a better understanding of parasite genomes, host–parasite relationships and the molecular biology of parasites themselves. Parasitic helminths undergo quite drastic metabolic changes when they enter their final host. The adult stages usually present an

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anaerobic metabolism; hence they downregulate the oxygen-dependent pathways required for the synthesis of fatty acids (FA) and sterols, and must acquire simple and complex lipids from their hosts for energy metabolism, membrane construction, and lipid-based signaling, the latter possibly also encompassing modifications of the host's immune and inflammatory defense systems [3]. These organisms produce and release an unexpectedly wide range of lipid binding proteins (LBPs) that are structurally distinct from those of their hosts. Some of these helminth LBPs may be involved in internal functions typical of all multicellular organisms, although specific to the particular cell and structural organization of helminth types. Some will be associated with specialized external functions, including acquisition and distribution of nutrients. Yet others will be involved in modulation of the host's local tissue environment, and its innate and acquired immune systems by secreted lipids and carrier proteins. Although poorly understood, helminth LBPs are often immunodominant in infection, some attract allergic-type antibody responses and are associated with protective immunity. They are major components of helminths' secretions, so they are presumably important for parasite success, and some are abundant in certain specialized tissues of parasites [4–7]. Understanding their roles in parasitism requires a range of biochemical, immunological, cell biological and parasitological approaches.

Knowing the structures and functions of the unusual proteins produced and released by helminths, as well as the host–parasite interactions that they may mediate, is clearly pertinent for preventing and treating helminth infections, and for the ultimate amelioration and prevention of the diseases they cause. In this review we present advances in the structural and functional analysis of a variety of LBPs and their interaction with ligands. Additionally, we will try to address the emerging characteristics of these proteins that make them interesting in both practical and theoretical terms.

2. Diversity of helminth LBPs

Proteins discussed in this review originate from different parasites of considerable medical and veterinary importance in South America and globally. Platyhelminthes present two types of LBPs described so far, these are members of the cytoplasmic fatty acid binding proteins (FABPs) family described for vertebrates and the hydrophobic lipid binding proteins (HLPBs). The latter are also of considerable and global importance and treated in another review of this special issue. On the other hand, nematodes produce two different types of small, helix rich, retinol and fatty acid-binding proteins (14–20 kDa), neither of which have recognizable counterparts in other animal groups. Additionally, nematodes also present the so called nemFABPs which present a structure similar to FABPs but with distinctive features (Fig. 1).

2.1. Fatty acid and retinol binding proteins (FARs) of nematodes

Genes encoding FAR proteins have been described in many nematode species, both free living and parasitic forms [8,9], each species produces several isoforms. FARs might be involved in host parasite interactions and pathogenesis since they are present in parasite excretory/secretory products. Ac-FAR-1 of the intestinal hookworm *Ancylostoma caninum* is a major ES product of the parasitic stages [10]. FARs have been reported to bind fatty acids and retinol [5,9–13] but our recent studies indicate that FARs bind a wider range of lipidic compounds such as diacylglycerol and phospholipids (unpublished data).

Recently the crystal structure of a FAR protein from the free-living nematode *Caenorhabditis elegans* has been reported [12]. This protein, however, has a low sequence identity to FARs of parasitic nematodes [8], for example Na-FAR-1, which was identified among the genes transcribed by the blood-feeding parasite of humans, *Necator americanus* [14]. Another example is some FAR genes that are transcribed at a notably high level in plant-parasitic nematodes [13,15]. The three dimensional structure of Na-FAR-1 has recently been solved by our group [16,17,18, unpublished data]. Na-FAR-1 presents 11 helices of diverse lengths arranged to enclose a hydrophobic internal cavity. The overall structure is a flattened ellipsoid which seems to adopt different degrees of expansion depending whether ligands are absent or bound to the central cavity.

2.2. Nematode polyprotein antigens/allergens (NPAs)

The NPAs are small, helix-rich lipid binding proteins confined to nematodes [6]. NPAs are synthesized as large precursors comprising tandemly repeated units that are cleaved posttranslationally into multiple ~15 kDa protein units of similar or divergent amino acid sequence, depending on the species [6,18,19]. These proteins bind small lipids such as fatty acids and retinol (vitamin A), and are synthesized in the intestinal cells and then exported to the pseudocoelomic fluid and secretions of the worms [20].

Several species of disease-causing nematodes, including *Ascaris lumbricodes* and *Brugia malayi* of humans, and *Dictyocaulus*

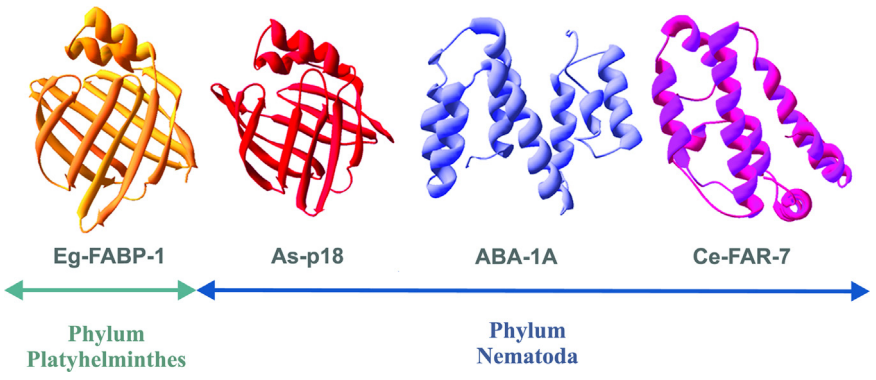

Fig. 1. Soluble lipid binding proteins from parasitic helminths. EgFABP1 (PDB: 108V) is from the highly pathogenic tapeworm that causes hydatid disease in humans and other mammals and is closely similar in overall structure to FABPs of humans. *As-p18* is found in the extracellular fluid surrounding the developing embryos of the nematode *Ascaris suum* (PDB: 1AS1 theoretical model based on A-FABP). We have now obtained an empirical structure for this protein that shows two α-helixes, 10 β-strand FABPs but with unusually extended loops (unpublished data). *ABA-1A* (PDB: 2XV9) is a single unit of the ABA-1 polyprotein of *Ascaris suum* of pigs and the *Ascaris lumbricoides* roundworm of humans. *Ce-FAR-7*(PDB: 2W9Y) is a FAR protein of the free-living nematode *Caenorhabditis elegans*. We now have an empirical structure for a FAR protein from the blood-feeding nematode of humans, *Necator americanus*, and this protein has some overall similarities to Ce-FAR-7 but also illustrates the structural diversity of this group of proteins (unpublished).

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viviparus, *Ostertagia ostertagi*, *Haemonchus contortus* and *Dirofilaria immitis* of domestic animals, express NPAs that are the targets of strong immune responses, often of a type associated with hypersensitivities [21]. Additionally, there is some evidence that NPAs merit attention for inclusion in vaccines [22].

Using high-resolution protein nuclear magnetic resonance (NMR), the structure of a single unit of this family of parasite allergens in solution was solved, the ABA-1A allergen of *Ascaris suum* [23]. It was demonstrated that the protein adopts a novel seven-helical fold comprising a long central helix that participates in two four-helical bundles on either side. There are two discrete hydrophobic ligand-binding pockets, one in each of the N-terminal and C-terminal bundles. One site is specific for fatty acids while the other might be for hydrophobic compounds of yet unknown nature. It is important to note that this is the first structure of a unit of any tandemly repetitive polyprotein yet reported. Moreover, work is being performed in our group in order to solve the ligand-free form of ABA-1A (unpublished data). Knowing the structure of the apo-protein will give detailed information about the structure of the unusual binding cavities of these proteins and test our current hypotheses about ligand binding mechanism by comparison to the holo-protein whose structure we have already determined.

Given the abundance of NPAs in some parasites, it would seem important to examine the hydrophobic ligands that bind to them in a biological context. A lipidomic analysis of a sample of ABA-1A, which had been affinity purified from *Escherichia coli*, but without performing prior lipid extraction, revealed that this protein is able to bind fatty acids as well as other molecules that might be important for lipid metabolism.

Another fascinating and difficult to address aspect of NPAs is the reason for their production as polyproteins. The individual units of ABA-1 (the parent polyprotein of ABA-1A) are flattened in tertiary structure with very short linkers (typically about four amino acids) [23,24]. This might suggest that the units, when still in the polyprotein form, stack or associate face-to-face. To date, the structure and ligand-binding characteristics of these proteins have been demonstrated only for single units of the polyprotein, but not for two or more units in tandem from the same polyprotein array. We have designed and expressed a tandem repeat of the unit ABA-1A with the intention of understanding whether and how NPA units interact before separation (unpublished data).

2.3. Fatty acid binding proteins (FABP) from platyhelminthes

Unlike the FARs and NPAs, FABPs of the FABP/CRBP/CRABP family [25,26] are widely distributed in nature. These proteins constitute a family of intracellular lipid binding proteins of low molecular mass (14–15 kDa) with the putative general function of lipid trafficking [27]. Although many of these proteins (mainly from vertebrates) have been studied extensively, their precise physiological functions are still unclear [27], though it is widely accepted that they are important in intracellular transport and targeting of fatty acids to specific membranous organelles and metabolic pathways. In spite of their differences in amino acid sequence, every known FABP structure (from mammals to flat worms) shares a similar β-barrel fold that resembles a clamshell. This β-barrel, which encloses the ligand binding cavity, consists of two five-stranded β-sheets arranged in a nearly orthogonal orientation [28].

Among the FABPs of flatworms the first one to be described was Sm14 from the blood fluke *Schistosoma mansoni* [29]. Since then, many other FABPs have been reported in the phylum Platyhelminthes, either trematodes or cestodes *e.g.* Sj-FABPc from *Schistosoma japonicum* [7,30], Fh15 from *Fasciola hepatica* [31], and EgFABP1 and EgFABP2 from the highly pathogenic and commonlly lethal tapeworm causing hydatid disease, *Echinococcus granulosus* [32,33]. Platyhelminthes' FABPs have been included in a group together with mammalian FABPs expressed in heart muscle (H-FABP), with which they share not only certain sequence similarity, but also structural features [34,35]. It is also remarkable that EgFABP1 and Sm14 (from *S. mansoni*), both bind fatty acids in a U-shaped conformation, as described for human H-FABP and other related FABPs [28]. Interestingly, residues involved in ligand binding in certain vertebrate FABPs are also conserved in the FABPs of platyhelminthes [36]. Moreover, Arg22, Arg30 and Lys31 from EgFABP1 could be considered equivalent to Lys21, Arg29 and Lys30 from the FABP expressed in adipocytes (A-FABP), which have been described as a nuclear localization signal (NLS) [37]. *In silico* ligand binding simulations performed for EgFABP1 also showed that Phe58 could adopt different conformations upon binding of certain ligands, which at the same time would trigger the closure of the portal region, and the exposure of NLS [38]. These changes are also observed for Phe57 in mammalian adipocyte fatty acid binding protein (A-FABP) [37].

In spite of the lack of detailed information at the atomic level, recent work proved that EgFABP1 undergoes certain conformational changes upon ligand binding. Moreover, it has been observed that oleic acid induces conformational changes distinct from those induced by palmitic acid, as assessed by circular dichroism and partial digestion with ArgC protease [39]. These results would be in accordance with that which has been proposed from *in silico* analysis [38]. Taken together, these observations would allow us to suggest that FABPs in *E. granulosus* (and also in other platyhelminthes) could play a more significant, signaling role, rather than merely transporting fatty acids within cells.

Another interesting aspect from EgFABP1 is that it has been reported as an excretory/secretory product [40,41] in spite of not having any known secretory N-terminal signal peptide. Similarly, it has been very recently demonstrated that A-FABP is secreted from adipocytes, as well as from macrophages, by a non-classical, but regulated, mechanism [42]. This latter FABP has already been proposed as an adipokine, *i.e.*, a bioactive peptide that influences body weight, lipid metabolism, insulin resistance, etc. [43]. Although it is not clear what would be the role of EgFABP1 as a secreted protein, these recent findings open a new set of questions that should be answered in order to achieve a better understanding of the host–parasite interaction in hydatid disease.

2.4. Nematode fatty acid binding proteins (NemFABPs)

The nemFABPs are also similar to vertebrate cytosolic FABPs, but have structural features unique to nematodes. NemFABPs are remarkable in the possession of secretory signal sequences and being released by the synthesizing cell, presumably through classical secretory pathways. Molecular modelling predicts additional inserts in the loop regions of nemFABPs that are not found in the FABPs of any other animal group [4]. NemFABPs have been found in the reproductive stages of nematodes, examples including As-p18 in the eggs of the large roundworm, *A. suum* [4,44], and Bm-FAB-1 of the developing microfilaria of the filariasis agent *B. malayi* [45], and a gene for a closely similar protein has been described from the infective stage of the riverblindness nematode *Onchocerca volvulus* (NCBI accession BF727540, see below). These nemFABPs are abundant in the fluid surrounding the embryos, bind lipids, and may therefore be essential to nutrient acquisition by, and/or maintenance of the eggshell, of the developing larvae. The NMR analysis of recombinantly produced As-p18 has also been completed in our laboratory, and is the first nemFABP structure to be elucidated and it has revealed novel modifications among the LBP superfamily [46]. In spite of presenting an overall structure in accordance with the canonical

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FABP fold – including a β-barrel and a helix-turn-helix domain – it exhibits two extended loops that could be implicated in protein–protein or protein–membrane interactions. There are also indications that the way the ligand is accommodated inside the binding pocket is somehow different from the mammalian counterparts (unpublished data). Structural peculiarities along with the extracellular localization constitute unique features that may reflect an adaptation to a specific function in nematode organisms. Studies of these exclusive features could contribute to the understanding of this protein's role in *A. suum* infective eggs, and in nematode reproduction [46], in addition to drug development.

3. Why are soluble lipid binding proteins from parasitic helminths interesting?

3.1. Anthelminthic resistance

There are several types of anthelmintic drugs: piperazine, thiabendazole, mebendazole, levamisole, and ivermectin among others. Although most of them act very effectively, resistance is becoming a serious problem. In veterinary practice, frequent treatment of closed populations has led to a serious problem of anthelminthic drug resistance which is now spreading and is largely irreversible [47,48]. Gastrointestinal nematodes are among the most important causes of production loss in farmed ruminants, and anthelmintic resistance is emerging globally. There are many factors in the establishment of resistant strains, high treatment frequency, single drug regimes, targeting and timing of mass treatments and underdosing [47]. Species of the genus *Haemonchus* are a case in point, representing a major cause of economic losses in livestock production in tropical and subtropical areas [49]. It is the most pathogenic gastrointestinal nematode parasite of small ruminants, feeds on blood, often resulting in severe anemia and death. Finding new drug targets to treat these infections is one strategy to try to solve anthelmintic resistance in gastrointestinal diseases caused by *H. contortus*. Recently, the existence of six different FAR proteins and an NPA in excretory/secretory products of adult stage *H. contortus* has been reported and their encoding genes were identified [18]. The Hc-NPA repeats are quite divergent in amino acid sequence. This level of divergence is also seen among the NPA repeats of several nematodes [6,50,51], but differs from *A. suum* NPA where six of the tandem repeats are almost identical in sequence [6].

3.2. LBPs as drug carriers

Given that drug resistance development is an already established problem for livestock helminths and is likely to become a problem for human helminths as well, finding new drug targets is of great importance [52,53]. Ideal drug targets are those proteins that participate in important metabolic pathways or critical biological processes such as cell division, development of the nervous system and reproduction.

Most anthelmintic drugs are hydrophobic molecules such as thiabendazole, levamisole and ivermectin, amongst others. It is reasonable to think that, given their capacities for binding lipid ligands, LBPs may interact with anthelmintic drugs. LBPs are thought to play an important role in lipid metabolism of helminth parasites by transporting and storing lipids inside the organism. In that case, the effect of anthelmintic drugs on LBPs would be the disruption of their normal physiological activity generating a clear damage to these organisms. Another hypothesis about LBPs functions is that they could protect cells and tissues from the toxic detergent effect of fatty acids since they are usually found to be expressed in large concentrations in tissues with very active lipid metabolism [28,54]. Hence, in the same way they do with fatty acids, LBPs might act as a reservoir for anthelmintic drugs which may affect the drug pharmacokinetics. Additionally, if parasite LBPs present high affinity for anthelmintics, they could be important in determining drug specificity and delivery to their targets [55].

FARs have been identified in the protein fraction that binds retinol and also ivermectin, a remarkably potent drug used in onchocerciasis control [9,56]. In the case of NPAs, competitive displacement assays showed that many anthelmintic drugs could not displace the fluorescent probe DAUDA from ABA-1A from *Ascaris lumbricoides* [57]. However, these data should be considered carefully since it is possible that ligands from *E. coli* bacteria in which NPAs were expressed could have remained bound to the proteins and interfered with the drug-binding assays. In order to solve this problem the complete removal of contaminating ligands from the bacterial expression system has been achieved by reverse-phase (RP) chromatography, followed by refolding in aqueous buffer [23].

Regarding FABPs from cestodes, there is almost no evidence of drug binding until now and reported cases showed very little affinity for bezafibrate or clofibrate drugs [58]. However, several A-FABP inhibitors have been synthesized, and they have been shown to bind with high affinity and selectively to this protein [28,59]. Particularly, BMS309403 interacts with residues Ser53, Arg106, Arg126 and Tyr128 of the fatty-acid binding pocket of A-FABP and it inhibits binding of endogenous fatty acids [60]. Moreover, it was demonstrated that chemical inhibition of A-FABP could be a potential therapeutic strategy against insulin resistance, diabetes, fatty liver disease as well as atherosclerosis in independent experimental models [28]. Additional work using another A-FABP inhibitor (HT501037) showed that it can reduce interaction of A-FABP with hormone sensitive lipase, decrease lipolysis and reduce the production of inflammatory cytokines, in agreement with the phenotype showed by A-FABP knockout mice [59]. Given the structural similarity of FABPs from cestodes with vertebrate FABPs, this drug might represent a starting point for drug binding assays and future drug design, and the same applies to nemFABPs.

Obtaining very accurate knowledge of these proteins' structures gives a strong base support to the refinement and improvement of already accepted anthelminthic drugs, and to the search for novel therapeutic techniques. X-ray crystallography and NMR are currently the most powerful methodologies with which to obtain structural information at the atomic level of proteins and can also be used to analyze protein–ligand interactions. Drug development can be achieved mainly by two strategies, high-throughput screening and structure-based methods. Rational drug design is perhaps the best option in terms of saving time and lowering monetary costs than conventional high-throughput screening protocols may generate [61]. Nowadays, *in silico* molecular docking, which is a powerful tool to predict the predominant binding mode of a ligand with a protein of known three-dimensional structure, is extensively used. But the most important attribute of molecular docking is that it can perform virtual screening on large libraries of compounds saving valuable time, although functional tests are ultimately always necessary [62].

3.3. Vaccines

The fact that many LBPs from parasitic helminths have no counterpart in other animal phyla makes them very good candidates for vaccine generation. One of the strategies recommended by the WHO to control soil-transmitted nematode infections is based on massive drug administration for children between the ages of 1 and 14 years who live in areas where the prevalence of

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these infections exceeds 20% [63]. However, due to high rates of post-treatment reinfection, lack of efficacy for single dose drug treatments, emerging drug resistance and poor sanitary conditions, these massive treatments do not seem to be sufficient [64]. Several antigens have been identified as good candidates for hookworm vaccines [65], and are in different stages of development [66]. It has recently been shown that immunization with a recombinant FAR from the blood feeding hookworm of humans *Ancylostoma ceylanicum* is associated with reduced worm burden in an animal model of hookworm infection [11]. Additionally, Ov-FAR-1 from the river blindness parasite *O. volvulus* was originally used to examine the immune response to onchocerciasis in humans [67,68]. It has shown strong direct immunomodulatory effects on immune cells [69] so could represent a valuable vaccine target.

4. Concluding remarks

Parasitic helminths are of global health and economic importance as agents of severe morbidity or mortality to humans, domestic animals and crop plants. During their life cycles they traverse a wide range of physical, chemical and immunological environments, which is especially true for those that have free-living stages, or have two or three different host species before infecting their final hosts. Nematodes are particularly remarkable because some parasitic species can survive even freezing or complete dessication in their dormant stages before encounter with a new host. Lipid-binding proteins of these parasites are of interest because they may be involved in interfering with the lipid-mediated defense and tissue differentiation processes in parasitized hosts. They have also been found to be immunodominant in human infections and as targets of allergic-type antibodies. Our main focus in this review has been that lipid-binding proteins of helminths represent structures that have only been found in these organisms, or that they have similarities with lipid-binding proteins found in all animals groups but with helminth-specific structural modifications. Understanding the structures of these unusual proteins could improve our knowledge of the biology and success of helminth parasites, especially nematodes, and thereby stimulate the development of therapeutic interventions now that inhibitors of lipid binding proteins are being developed in other contexts [59,70]. Inhibitory drugs combined with gene knock out systems for parasites would provide tools to test directly the above predictions regarding the function of lipid-binding proteins in the success of these parasites infections and their control over host tissues and immune systems to their advantage.

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