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BINDING PROPERTIES AND IMMUNOLOCALIZATION OF A FATTY ACID–BINDING PROTEIN IN *GIARDIA LAMBLIA*

S. M. T. Hassan, M. Maache, R. Díaz de la Guardia, O. M. Córdova, J. R. Gil García, M. Galiana, D. Acuña Castroviejo, M. Martins*, and Antonio Osuna†
Instituto de Biotecnología, Universidad de Granada, Campus Universitario de Fuentenueva, E-18071 Granada, Spain. *e-mail: aosuna@ugr.es*

ABSTRACT: We describe here a fatty acid–binding protein (FABP) isolated and purified from the parasitic protozoon *Giardia lamblia*. The protein has a molecular mass of 8 kDa and an isoelectric point of 4.96. A Scatchard analysis of the data at equilibrium revealed a dissociation constant of 3.12×10^{-8} M when the labeled oleic acid was displaced by a 10-fold greater concentration of unlabeled oleic acid. Testosterone, sodium desoxycholate, taurocholate, metronidazol, and α-tocopherol, together with butyric, arachidonic, palmitic, retinoic, and glycocholic acids, were also bound to the protein. Assays with polyclonal antibodies revealed that the protein is located in the ventral disk and also appears in the dorsal membrane, the cytoplasm, and in the vicinity of the lipid vacuoles.

The flagellate protozoan, *Giardia lamblia*, infects the upper intestinal tract of many mammal species and is the most frequent cause of protozoan gastrointestinal infection in humans (Heyworth, 1996). *Giardia lamblia* can cause diarrhea, steatorrhea, malnutrition, and stunted growth. The course of the infection varies from asymptomatic infections to severe diarrhea (Hopkins and Juranek, 1991).

Like other intestinal protozoa, *G. lamblia* is incapable of de novo fatty acid synthesis. All known eukaryotic cells contain a number of proteins that bind to, and transport, fatty acids. These are the so-called fatty acid–binding proteins (FABPs); the sequence and structure of many are already known. They are generally of low molecular mass (around 14 kDa) and have been found in many vertebrate and helminth cells, such as *Fasciola hepatica* (Moser et al., 1991), *Schistosoma* sp. (Gobert, 1998), *Echinococcus* sp. (Alvite et al., 2001), *Moniezia* sp. (Jansen and Barret, 1995), and *Ascaris lumbricoides* (Kennedy et al., 1995), in which this protein family constitutes 2–3% of the total cytosolic protein involved in fatty acid metabolism (Frolov and Schroeder, 1997). FABPs are related to microsomal and mitochondrial membranes in the cytosol, where they intervene in the membrane synthesis of phospholipids (Murphy et al., 1998). FABPs bind to free fatty acids as well as to several hydrophobic molecules, including the hemo group, retinoic acid, and peroxisome proliferator (Cannon and Eacho, 1991). Their antigenic values constitute one of the most important characteristics currently being investigated among infections against the parasitic helminths, *E. granulosus* (Chabalgoity, 1997), *S. mansoni* (Brito et al., 2000), *Fasciola gigantica* (Sirisrio et al., 2002), and *F. hepatica* (Abane et al., 2000; Espino et al., 2001; Carballeira et al., 2003; Espino and Hillyer, 2003). We have recently described a positive saliva reaction to these proteins by immunoblotting in active giardiasis (Hassan et al., 2002).

Here, we report the purification and immunolocation of a FABP from *G. lamblia*. This polypeptide, with a relative molecular mass of ~8 kDa, was purified by affinity chromatography and characterized by SDS-PAGE electrophoresis, isoelectrofocusing, and western blotting. The protein showed highest affinity to hydrophobic molecules, including metronidazol and bile salts. The FABP in *G. lamblia* was located by electron microscopy in the suction disk, as well as in the cytosol and in the vicinity of the lipid vacuoles.

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* Fundação de Medicina Tropical-FMT/IMT-AM, Gerência de Parasitologia, Manaus-AM CEP: 69040 000, Brazil.
† To whom correspondence should be addressed.

MATERIALS AND METHODS

Trophozoite culture of *G. lamblia* and protein extract

Trophozoites of *G. lamblia* strains ATCC 30888 and 30957 were cultured as described elsewhere (Rosales et al., 1998) in TYI-S 33 medium (Keister, 1983) enriched with 10% heat-inactivated bovine serum. The parasites were cultured in glass flasks at 37 C for at least 72 hr. On reaching the logarithmic growth phase, they were centrifuged at 250 *g* for 10 min at 4 C. The pellet containing the protozoans was washed in phosphate-buffered saline (PBS; 0.15 M, pH 7.4) to eliminate the remains of the culture medium and serum. The flagellates were then suspended in PBS (pH 7.4) containing 1 mM EDTA, 1 mM DTT, and 1% (v/v) Triton X-100 (Campbell et al., 1994), with 0.003% (w/v) antipain-HCl protease inhibitors, 0.005% (w/v) bestatin, 0.001% (w/v) cimostatin, 0.003% (w/v) E-64, 0.005% (w/v) leupeptin, 0.005% (w/v) pepstatin, 0.003% (w/v) phosphoramidon, 0.2% (w/v) pefabloc SC, 0.005% (w/v) aprotinin, and 0.1% (w/v) EDTA (Roche Diagnostics GmbH, Manheim, Germany). The pellet containing the protozoans was frozen and thawed 3 times, followed by sonication (5 min at 60 cycles at 0 C) in a lysis buffer (20 mM PBS, pH 7.4, with 0.25 mM sucrose, 1 mM EDTA, 0.145 mM KCl, 1 mM DTT, 0.1% [v/v] Triton X-100) to break up the cells. The sonicated fraction was centrifuged at 22,000 *g* for 30 min, and the supernatant was collected. The supernatant was chromatographed through a Bio-Beads SM-2 column (Bio-Rad, Hercules, California) to eliminate the detergent used in the lysis buffer. The detergent-free extract was then passed through a Lipidex-1000 column (hydroxyalkoxypropyl Sepharose, Sigma, St. Louis, Missouri) as described elsewhere (Glatz et al., 1984; Lee et al., 1998) to delipidize the sample.

Affinity chromatography by stearic Sepharose

Once delipidized, the sample was passed through an EAH-Sepharose 4B column (Amersham Pharmacia Biotech, Uppsala, Sweden) prepared according to the manufacturer's instructions. Briefly, epoxy-activated Sepharose 4B was swelled and washed extensively with deionized water. EAH-Sepharose 4B (45 ml) was incubated with 67 ml of 0.1 M stearic acid (previously solubilized in dioxane) as a ligand. The final concentration of stearic acid was 200 μmol/ml of the gel. The coupling buffer composition was 0.1 M $NaHCO_3$ and 0.5 M NaCl (pH 10.0) plus 2 g of 1-ethyl-3-(dimethylaminopropyle) carbodiimide. The mixture was stirred for 72 hr at 37 C. Surplus ligand was eliminated by successive washings with the 3 following solutions: a 50% (v/v) ethanol–coupling buffer, a 1:1 solution of $NaHPO_4$ in 0.075 M ethanol (pH 2.4), and a 1:1 solution of OHNa in 0.05 M ethanol. The resin was incubated overnight with 1 M ethanolamine to block the unreacted epoxide groups. A column (1.5 × 22 cm) containing 0.05% NaN_3 was prepared with the stearic agarose resin in 0.15 M PBS as preservative until used.

The delipidized extract sample was chromatographed at a linear flow rate of 0.1 ml/min, and the column was washed with 0.15 M PBS (pH

7.4). After eluting the FABP with 0.1 M Tris HCl buffer containing 7 M urea, the eluted fraction was dialyzed through distilled water. The protein concentration was determined by Bradford's (1976) method with bovine serum albumin (BSA) as the protein standard. The sample was distributed in aliquots for lyophilization.

Determination of the purification factor by fluorometry

After purification, the fatty acid–binding capacity in the *G. lamblia* extract was determined with 11-(dansylamino) undecanoic acid (DAUDA). This fluorescent fatty acid analog shows a wavelength maximum from 530 to 480 nm when it binds to FABP. The yield, or purification, was calculated by determining the fluorescence in the fraction and comparing it with the same protein concentration of the original trophozoite extract before purification. Measurements were made at 500 nm in polymetacrylate cuvets (Sigma) in a Hitachi F 2000 spectrofluorimeter, with its excitation source at an angle of 45° and excitation wavelength at 350 nm. Briefly, 4 μg of either the extract or the purified protein was added to 1 μM DAUDA (Molecular Probes, Eugene, Oregon) in 50 mM PBS (pH 7.2) to a final volume of 1 ml. The equilibration time was 10 min at 25 C. The relative values of DAUDA linked to FABP were calculated via the equation

$$DP = (F_m - F_f)/(F_p - F_f),$$

where DP is the relative value of DAUDA linked to FABP protein, F_m is the maximum fluorescence obtained by DAUDA (1 μM) linked to the protein (emission at 500 nm, excitation at 330 nm), F_p is the fluorescence obtained by DAUDA (1 μM; emission at 500 nm, excitation at 330 nm) in the presence of 4 μg of protein from the different samples obtained in the various purification steps, and F_f is the fluorescence obtained by DAUDA (1 μM; emission at 500 nm, excitation at 330 nm) in the absence of protein.

Sample preparation for MALDI-TOF-MS

Ethanol extract (20 μl) containing 4 μl of 50 mM octyl-β-glucopyranoside detergent was mixed with a matrix solution made up of 100 μl of saturated sinapinic acid in 30% aqueous acetonitrile and 0.1% trifluoroacetic acid; 0.5 μl of this mixture was then dripped onto a Bruker Multiprobe 2001.1 lathe-tooled stainless steel probe tip (Bremen, Germany) and allowed to dry at room temperature for 5 min (Camafeita et al., 1998). Samples were measured in a Bruker reflex II MALDI-TOF-MS system equipped with an ion source with visualization optics and a N_2 laser (337 nm). Mass spectra were recorded in linear positive mode at 30 kV acceleration voltage and 2 kV in the linear detector by accumulating 70 spectra of single laser shots under threshold irradiance. Only highly intense, well-resolved mass signals arising from 2–3 selected target spots were considered. The equipment was externally calibrated, employing single-, double-, and triple-charged signals from a mixture of BSA (66,430 Da) and cytochrome (12,360 Da).

SDS-PAGE and blotting

Samples taken from the affinity column were electrophoresed on 12.5 or 20% SDS-PAGE (Laemmli, 1970) with the PhastSystem (Amersham Pharmacia Biotech). The protein samples were prepared in a sample buffer (10 mM Tris/HCl; 1 mM EDTA, pH 8.0; 2.5% SDS and 17% glycerine and bromophenol blue at 0.01%) and heated to 98 C for 5 min. The gels for protein profiles were stained with silver (Heukeshoven and Dernick, 1985).

The isoelectric point of the purified proteins was determined by isoelectric focusing (Andrews, 1986). Separation and staining was done in the same PhastSystem (Amersham Pharmacia Biotech). Polyacrylamide gels were used within the pH range of 3.0–9.0 (PhastGel IEF 3–9, Amersham Pharmacia Biotech). The samples were dissolved in 10 mM Tris buffer (pH 8.0). Separation involved prefocusing at 2.5 mA and 75 volt/hour (Vh) followed by separation proper at 2.5 mA and 410 Vh. At the end of the process, the gel was stained with silver nitrate.

The gels were digitized and processed by QuantiScan software (Biosoft, Cambridge, U.K.) to calculate the relative molecular masses and the isoelectric point.

Electroelution

Electroelution was carried out in a Gel Eluter (Amersham Biosciences Inc., San Francisco, California). After electrophoresis, slices from each side of the gel were stained with Coomassie blue, and the band with low molecular mass was cut horizontally. Gel slices were put into an elution tube assembled according to the manufacturer's instructions and filled with TAE (0.04 M Tris, 0.02 M acetic acid, 0.002 M EDTA, pH 8). The proteins were electroeluted for 1 hr at 100 V. After elution, all the samples were dialyzed against distilled water for 48 hr at 4 C and lyophilized. To check the molecular mass of the eluted protein, the samples were electrophoresed in SDS-PAGE, as already described, and the gel was stained with silver nitrate.

Study of binding properties

We carried out a radiolabeled fatty acid–binding assay with Lipidex 1000 (Nemecz and Schroedert, 1991). The assay mixture contained 0.1 μCi oleic acid [^{14}C(U)] (NEN Dupont, Boston, Massachusetts) in 25% ethanol in 50 μM PBS (pH 7.2) with or without 1 μg of the purified *G. lamblia* protein. Binding was studied at intervals of 0, 5, 10, 20, 30, 60, and 120 min at 20 C. After incubation, the tubes were chilled on ice for 10 min, and 150 μl of 50% (v/v) Lipidex was added to each, whereupon they were centrifuged at 10,000 *g* in 0.1 M PBS (pH 7.2). After vigorous stirring, the tubes were incubated at 4 C for 10 min and then centrifuged at 10,000 *g* for 4 min. Aliquots of the supernatant were removed, and their radioactivity was determined by liquid scintillation in a β-scintillation system (Beckman, Palo Alto, California) to calculate the number of DPMs. The scintillation liquid (EcoLite, ICB, Costa Mesa, California) comprised alkylbenzene, phenylxylethane, nonionic surfactants, 2.5-diphenyloxazole, and *p*-bis(*o*-methylstyril) benzene. The DPMs were used to calculate the association (K + 1) and dissociation constants (K − 1), the quotient of which gave the affinity constant (K_d). These calculations were made with the Ligand-PC program (Biosoft).

To study the time-dependent association and dissociation of binding between the protein and oleic acid, the protein was incubated with labeled ^{14}C oleic acid (ARC, American Radiolabeled Chemicals) with a specific activity of 55 mCi/mmol, at 37 C, and binding was studied at 0, 5, 10, 20, 30, 60, and 120 min after incubation began. At 60 min, a 100-fold excess of nonradioactive oleic acid was added, and its displacement versus time was studied at intervals of 0, 15, 30, 60, and 120 min. The mixture was then treated with Lipidex-1000 and measured in the scintillation spectrometer as described previously. Similarly, labeled oleic displacement was performed by incubating the protein-labeled oleic acid complex with a 100-fold excess of other substances (i.e., α-tocopherol, testosterone, butyric acid, arachidonic acid, palmitic acid, retinoic acid, glycocholate, taurocholate, and metronidazol). The proteins were then treated as described above, and the radioactivity was measured.

To demonstrate the capacity of the purified protein to bind to the fatty acids, the protein was separated under nonreducing conditions in polyacrylamide gels in SDS-PAGE and transferred to nitrocellulose (Towbin et al., 1979) before being incubated with oleic acid (ARC, American Radiolabeled Chemicals) at a concentration of 0.1 μCi/μg protein (Campbell et al., 1994). The area of the autoradiogram was calculated with the Quantiscan program by performing autoradiography on Kodak X-OMAT film and incubation on a Cronex Dupont intensifier screen for 4–5 days.

Production of anti-FABP–specific antibodies

To produce antibodies specifically against the fatty acid–binding protein, the 8-kDa bands of a 12.5% SDS-PAGE electrophoresis were cut out, ground and homogenized, and electroeluted in a Gel Eluter (Amersham Pharmacia Biotech). The protein was injected intraperitoneally into mice in 0.125 M PBS mixed with Freund's complete adjuvant, followed once weekly by 4 injections of incomplete Freund's adjuvant. Seven days after the last injection, blood was extracted, and the serum was collected and stored in aliquots at −20 C until use.

Immunoprecipitation

To determine the association between the antibodies and the protein purified from *G. lamblia* trophozoites, extracts were prepared in lysis buffer and subject to chromatography through Lipidex. They were then incubated with the antibody and 20 μl of protein A/G Sepharose beads for 16 hr at 4 C. The protein–bead complexes were washed 3 times

TABLE I. Purification of FABP from *Giardia.*

Sample	Total volume (ml)	Protein (mg/ml)*	Total protein (mg)*	Protein (%)	R Dp†	Yield (%)
Giardia lysate	8.5	2.45	20.825	100	0.1042	100
Supernatant	7.8	2.18	17.004	81.65	0.1095	96
Lipidex	6.6	0.71	4.686	22.50	0.091	83.1
Affinity chromatography	5.98	0.065	0.388	1.863	0.05817	53.13

Determined by Bradford's method.

† Relative fluorescence values obtained by the formula, DP = $F_m - F_f/F_p - F_f$. R DP = relative values of DAUDA linked to FABP protein. F_m = fluorescence obtained by DAUDA (1 μm), emission at 500 nm and excitation at 330 nm in the presence of 400 μg of FABP protein. F_p = * fluorescence obtained by DAUDA (1 μM), emission at 500 nm and excitation at 330 nm in the presence of 4 μg of protein from the different purification steps. F_f = fluorescence obtained by DAUDA (1 μM), emission at 500 nm and excitation at 330 nm in the absence of protein.

with glycine-HCl buffer (pH 4.5) before adding SDS loading buffer. The samples were resolved on SDS-PAGE.

Immunotransference with the serum obtained

To determine the specificity of the antibodies in the mouse immunoserum, we made immunoblots (Towbin et al., 1979). The protein fraction deriving from affinity chromatography, separated via SDS-PAGE in polyacrylamide gel (20% homogeneous), was transferred to PVDF membrane (Mini ProBlott Membranes, Applied Biosystems, Foster City, California). After blocking the nonspecific free sites of the membrane with a 0.4% gelatin solution and 0.2% Tween 20 in 0.125 M PBS (PBS-T), the membrane strips were incubated for 30 min at 37 C with the anti-FABP serum at dilutions of 1:50 and 1:100. A nonimmunized mouse serum was used as negative control. The membranes were incubated with the sera and then washed 5 times with PBS-T. The strips were incubated for 30 min at 37 C with anti-IgG mouse serum (Fc specific) marked with peroxidase (Sigma) diluted to 1:1,800 in PBS-T. After the final incubation, the strips were washed and incubated for 15–30 min at 37 C with the peroxidase substrate 0.5 mg/ml 3,3-diaminobenzidine diluted in 0.1 M Tris/HCl (pH 7.4) containing 1:5,000 H_2O_2. The membranes were finally washed with distilled water.

To study the amino acid composition, the PVDF membranes were stained with Coomassie Brilliant Blue R 250 (Sigma), and the transferred protein was cut from the membrane and analyzed (at the IBCP, Claude Bernard University, Lyon I, France). The total quantity of amino acids in the hydrolyzed samples was 0.20 μg.

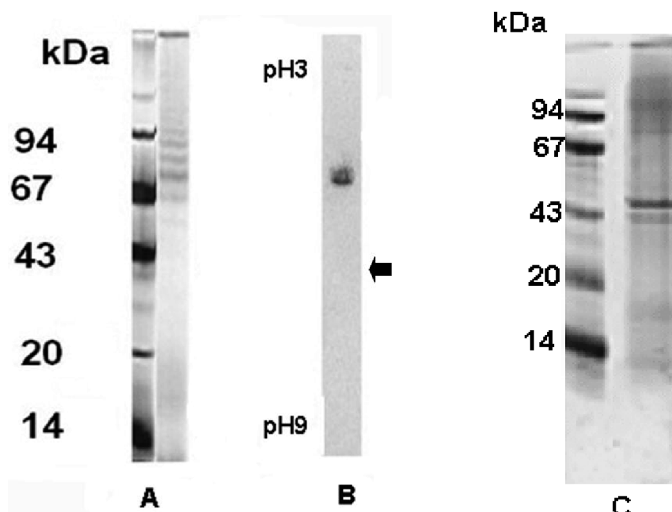

FIGURE 1. **a.** SDS-PAGE (gel 12.5% under nonreducing conditions) of the purified proteins of *Giardia lamblia*. On the left, the marker protein pattern and the molecular masses. **b.** Autoradiography of the isoelectric focusing (3–9) of the purified proteins of *G. lamblia* after incubation of the gel with ^{14}C oleic acid. The arrow shows the application point. **c.** 20% SDS-PAGE, under nonreducing conditions of the previously electroeluted ~8-kDa band. On the left, the marker protein pattern and the molecular masses.

Indirect immunofluorescence

For indirect immunofluorescence (IFI) analysis, a *G. lamblia* trophozoite suspension from the culture was washed by centrifugation with 0.125 M PBS, and a drop of the pellet was fixed to immunofluorescence slides with acetone at −20 C. The immunoserum previously obtained from mice was used at a dilution of 1:1,000 and allowed to react in the wet chamber for 30 min at room temperature. The slides were then washed 3 times with 1.25 M PBS and incubated for 30 min at room temperature with anti-IgG Fab mouse serum marked with fluorescein diluted to 1:100 in 0.125 M PBS with Evans blue (0.003%, fluorescein-isothiocyanate). The slide was mounted in buffered glycerine and studied under a fluorescence microscope.

Immunolocation of the fatty acid–binding protein

The *G. lamblia* trophozoites obtained from the cultures as described above were washed with 0.125 M PBS by centrifugation at 250 *g* for 10 min. The pellet containing the protozoans was washed and fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer with 0.1 M sucrose (pH 7.2) for 12 hr at 4 C. It was then embedded in LR white resin, sliced, and incubated with the anti-FABP immunoserum for 30 min at a dilution of 1:250 in 0.125 M PBS, washed with PBS, and then incubated with the protein A–colloidal–gold complex (Sigma) at a dilution of 1:125 in PBS buffer containing 0.5% albumin and 0.05%

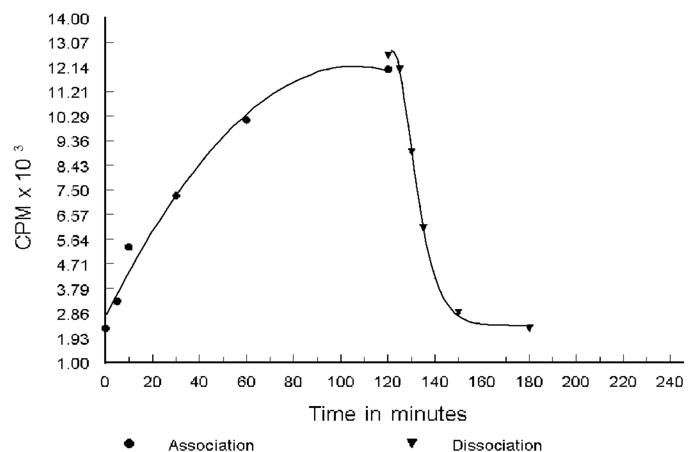

FIGURE 2. Association and dissociation of labeled oleic acid versus time. (●) The association kinetics measured by the area of the autoradiograph; (▼) the displacement (dissociation) of the labeled oleic acid by unlabeled oleic acid versus time, measured by the CPMs.

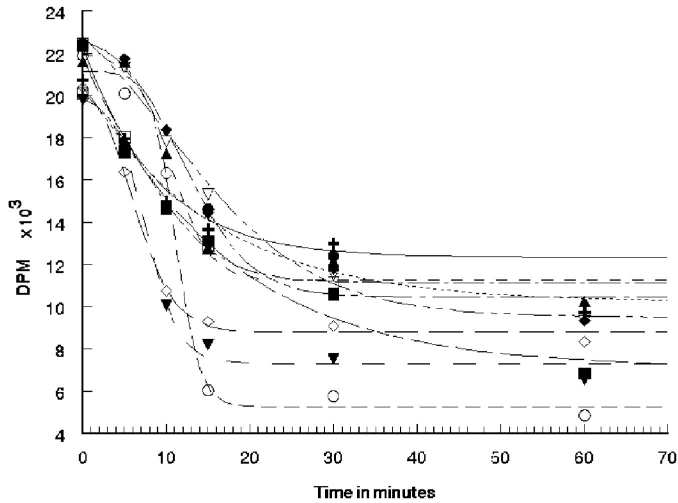

FIGURE 3. Displacement of the labeled oleic acid versus time by: (∇) arachidonic acid, (◆) palmitic acid, (▲) butyric acid, (+) retinoic acid, (●) α-tocopherol, (□) testosterone, (▼) taurocholate, (◇) glycocholate, (■) desoxycholate, (○) metronidazol.

Tween 20. A negative control was made by direct incubation with the protein A–gold without antibodies. Finally, staining was performed and ultrathin sections were examined under a Zeiss (6EM902) electron microscope (Osuna et al., 1993).

RESULTS

The results of purification and protein yield measured in terms of its capacity to bind to the DAUDA fluorescent probe are set out in Table I. After purification by affinity chromatography, the final yield was 53.13%. Electrophoresis of the fraction obtained in SDS-PAGE under nonreducing conditions provided at least 13 protein bands (Fig. 1a) with molecular masses ranging from 8 to 120 kDa (8, 25, 29, 41, 49, 55, 60, 65, 69, 78, 90, 120 kDa).

Nevertheless, autoradiography of the transferred membranes after incubating the proteins with labeled oleic acid or electroelution and subsequent binding with DAUDA showed that only one of the bands, that with a relative molecular mass of around 8 kDa, was able to bind to the labeled fatty acid. Figure 1b shows the isoelectrofocusing of the purified protein with a isoelectric point of 4.96 and the autoradiography after incubating the protein with labeled oleic acid. Electroelution of the ~8-kDa band followed by electrophoresis in 20% SDS-PAGE gel provided a band pattern similar to those obtained by affinity chromatography (Fig. 1c).

Figure 2 shows the capacity and specificity of the *Giardia* FABP protein to bind to oleic acid. The binding is saturable after 90 min of incubation. The analysis of the data at equilibrium revealed a K_d of 3.12×10^{-8} M. Figure 3 shows the displacement of the labeled oleic bound to the protein when they were incubated with arachidonic acid, palmitic acid, butyric acid, glycocholate acid, taurocholate, α-tocopherol, retinoic acid, testosterone, and metronidazol.

Molecular mass was determined by Malditoff spectrometry, a procedure that gives a mass accuracy of about 0.01% (Burlingame, 1993). The spectra show the presence of a single component with a molecular mass of 8,215.96 Da (Fig. 4).

Table II shows that the amino acid composition of the protein is rich in glycine (17.35%), glutamate (14.29%), valine (11.22%), aspartate (10.2%), and leucine (10.2%).

The immunoblot made with mouse immunoserum showed

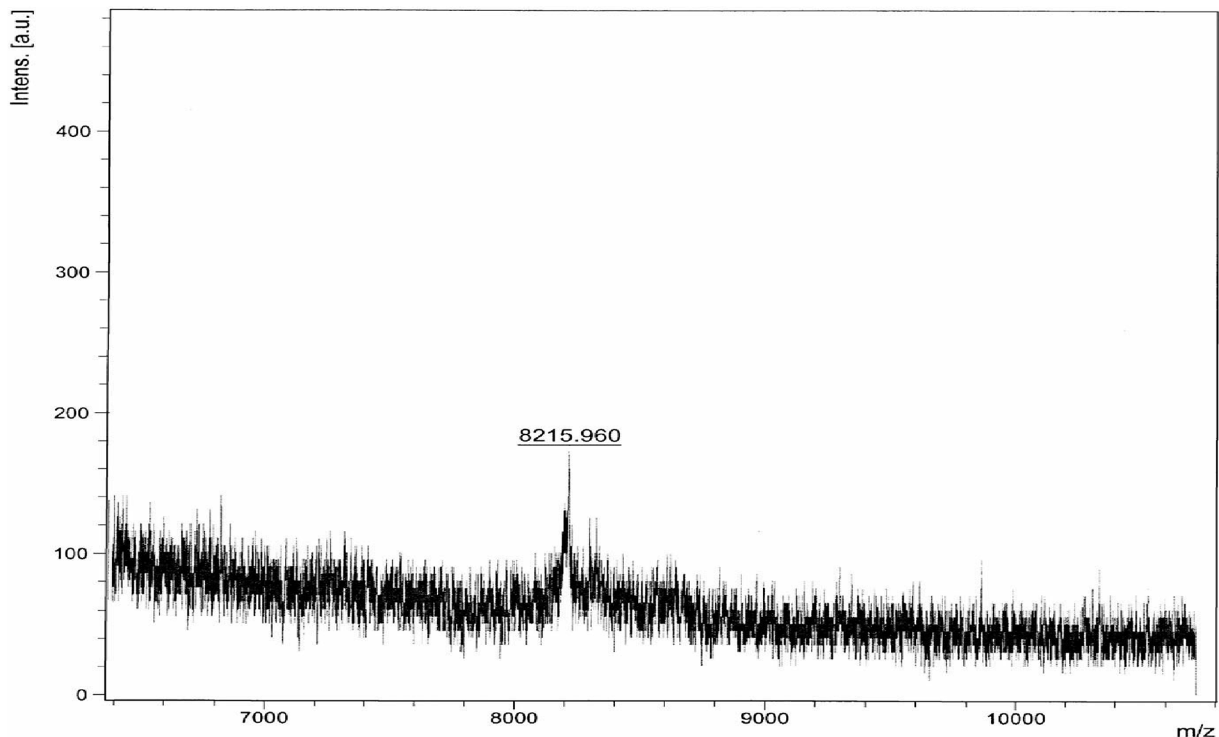

FIGURE 4. MALDI-TOF mass spectra of the affinity chromatography–purified protein (130 μg/ml).

TABLE II. Amino acid composition of the purified FABP from *Giardia lamblia.* The total quantity of amino acids in the hydrolyzed samples was 0.20 μg.

Amino acid	Residue
	(0.00)
Hyp	0.00
Aspartate	102.04
Threonine	30.61
Serine	91.84
Sar	0.00
Glutamate	142.86
Proline	0.00
Glycine	173.47
Alanine	40.82
Cysteine	ND
Valine	112.24
Methionine	40.82
Lle	71.43
Leucine	102.04
Tyrosine	0.00
Phenylalanine	30.61
Histidine	v0.00
H. Lys	0.00
Lysine	61.22
Tryptophan	ND
Arginine	0.00
β-Alanine	ND
Des + Isd	0.00
	1000

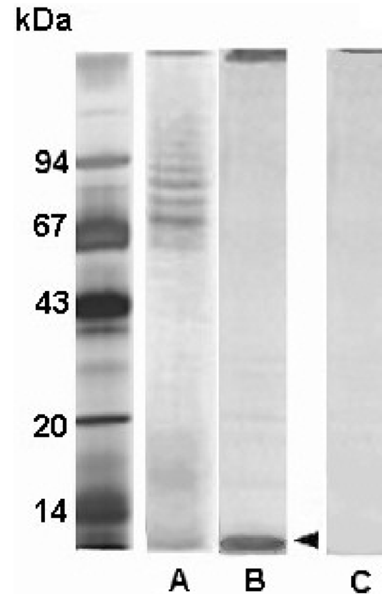

FIGURE 5. **a.** Pattern in SDS-PAGE of the purified protein by immunoprecipitation with the immunoserum obtained from the electroeluted ~8-kDa protein band. **b.** The arrow shows the positive recognition by immunoblotting of the purified 8-kDa protein by the immunoserum obtained from the electroeluted protein band. On the left, the marker protein pattern and the molecular masses. **c.** Immunoblot of the purified 8-kDa protein by serum from nonimmunized mice.

reactivity to the protein of 8.2 kDa, whereas the negative control serum showed no reactivity (Fig. 5).

Electrophoresis of the immunoprecipitate of the immunoserum obtained showed a band pattern similar to that given by affinity chromatography (Fig. 5a).

Immunolocalization studies of the FABP by IFI (Fig. 6) indicated fluorescence on the surface of the parasite, as well as in the ventral disk. This observation was corroborated by immunocytochemistry observed under an electron microscope (Fig. 7), in which the protein was seen to be located mainly in the suction disk, the wings of the disk, the dorsal membrane, and the vicinity of the lipid vacuoles.

DISCUSSION

The intestinal protozoan, *G. lamblia*, afflicts humans worldwide, particularly children, causing a wide range of intestinal maladies, including diarrhea followed by constipation, plus a number of syndromes that incapacitate digestion and interfere with the uptake of proteins, sugars, and especially fats, giving rise to the most characteristic disorder, steatorrhea (Korman et al., 1990). All of these processes result in malabsorption of nutrients and induce a general weight loss and stunted development in children (Farthing et al., 1986).

Its lack of mitochondria and other eukaryotic organelles, together with evidence provided by rRNA analysis, place *G. lamblia* at a crucial point in the evolution of eukaryotic organisms (Sogin et al., 1989). In the complete absence of oxygen *G. lamblia* trophozoites metabolize glucose into ethanol or alanine, whereas at subtoxic concentrations of oxygen, the final products are carbon dioxide and acetate (Paget et al., 1990, 1993). Energy metabolism in this flagellate is related to a number of amino acids, such as arginine, that constitute a potentially significant energy source, and alanine, which is the main end product of *G. lamblia* metabolism in its natural environment (Edwards et al., 1989). Although trophozoites possess a transport system for the entry of alanine, they can also produce alanine from pyruvate with the use of alanine amino-transferase. Under certain conditions, alanine can be a major energy source for this protozoan. *Giardia lamblia* trophozoites cannot incorporate lipids or fatty acids, glucose, threonine, or acetate; even the acetyl-CoA generated during glucose metabolism is not used in the lipid biosynthetic pathway (Jarroll et al., 1981). *Giardia lamblia* trophozoites appear in fact to rely on preformed lipids rather than synthesizing them de novo (Jarroll et al., 1989), capturing them by various kinetic means from lipoproteins and bile salt mycelles (Lujan et al., 1996). These latter authors show that lipids play an important part in regulating the life cycle of this protozoon. It is certainly the case that if lipoproteins are not available, *G. lamblia* is quick to express the proteins that induce its cyst form, but the addition of cholesterol, low-density, or very low density lipoproteins to the lipid-deficient medium inhibits the expression of these cyst wall proteins (Lujan et al., 1996). In this context, *G. lamblia* trophozoites appear to depend on preformed lipids rather than on de novo synthesis and can incorporate fatty acids such as arachidonic and palmitic acids into phospholipids and neutral lipids (Blair and Weller, 1987; Hiltpold et al., 2000). The main fatty acids found in *G. lamblia* are palmitic and stearic acids, as saturated fatty acids, and oleic and linoleic acids, as saturated acids (Das et al., 2002). The presence of the enzyme fatty acid desaturase allows the introduction of double bonds to the saturated fatty acids in

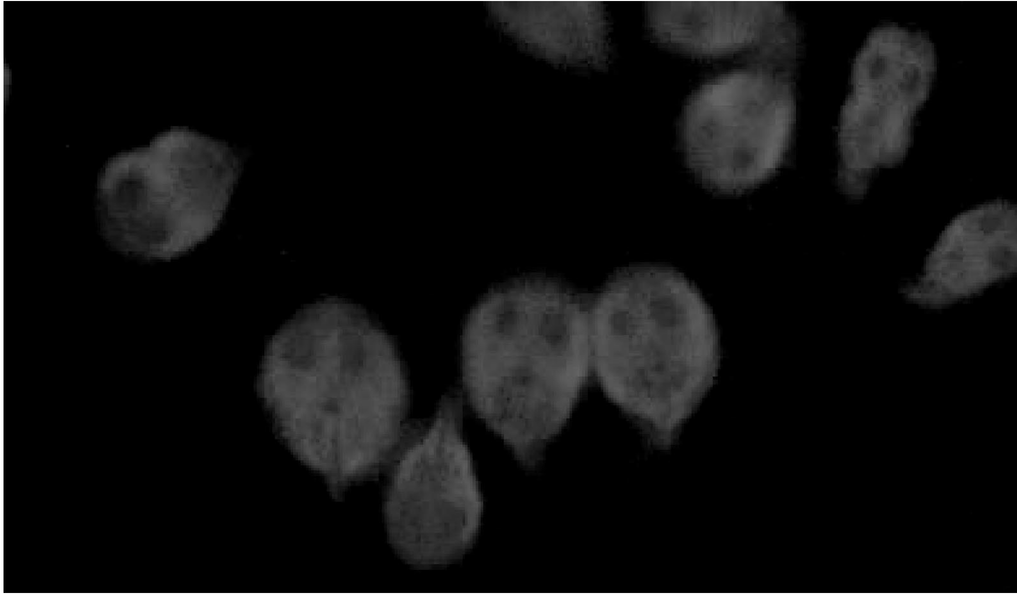

FIGURE 6. Positive indirect immunofluorescence (IFI) in the *Giardia lamblia* trophozoites incubated with anti-FABP–specific and anti-mouse antibodies labeled with fluorescein isothiocyanate (FITC).

the membrane phospholipids and to the cytosolic fatty acids, which allows *G. lamblia* to remodel, to some extent, the fatty acids it incorporates (Ellis et al., 1996).

Giardia lamblia incorporates up to 90% palmitic acid, whereas it can only incorporate 10% arachidonic acid (Gibson et al., 1999). It incorporates fatty acids in the phospholipids via a deacylation, reacylation, and head group exchange reaction (Stevens et al., 1997; Gibson et al., 1999; Das et al., 2001, 2002).

Apart from fatty acids, FABPs also transport other hydrophobic substances (i.e., hormones, vitamins, retinol, and bile salts; Veerkamp et al., 1995; Bass, 1993; Sha et al., 1993; Baier et al., 1995). FABPs participate in nuclear functions as well as in lipid metabolism (Bass, 1993).

Because of their location in membranes and their sequence variation in different species, FABPs play an important role in the biology of some parasitic helminths, as we have described elsewhere (Moser et al., 1991; Jansen and Barret, 1995; Gobert, 1998; Alvite et al., 2001; Espino et al., 2001; Espino and Hillyer, 2003). An analysis of the proteins purified by electrophoresis in SDS-PAGE enabled the identification of a total of 13 bands, corresponding to proteins with relative molecular masses of between 8 and 120 kDa (Fig. 1a).

The proteins that are purified by binding to the affinity column, but do not bind to this fatty acid, might be FABP aggregates that appear during the purification process, as described by Fournier and Richard (1990) for FABPs in mammals. It is possible that in aqueous solution, they are associated by their hydrophobic groups (giving rise to erroneous molecular masses) and are incapable of binding to fatty acids on finding the hydrophobic groups already occupied. Nevertheless, the band capable of binding to oleic acid always had a molecular mass of ~8 kDa and an isoelectric point of 4.96. It is curious to note that immunoblotting only revealed reactions for the band cor-

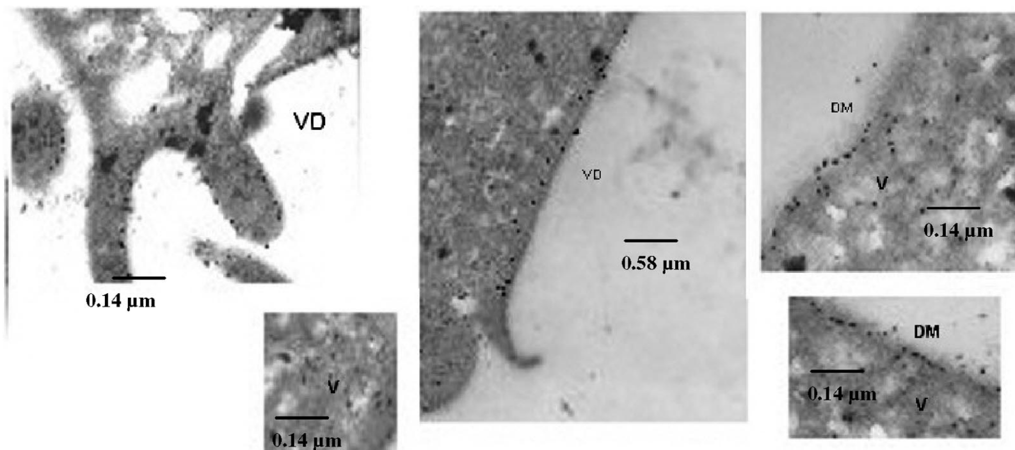

FIGURE 7. Immunochemical techniques applied to locating fatty acid–binding protein in *Giardia*. Transmission electron microscopy: the gold deposits appear in the ventral disk (VD), dorsal membrane (DM), and lipid vacuoles (VL).

responding to ~8 kDa. The epitopes recognized by the antibodies (probably in the more hydrophobic parts of the protein) are inaccessible to the antibodies when the interaction occurs in a hydrophilic medium because the protein is aggregated via these hydrophobic parts (Fig. 1c). The electroelution of the ~8-kDa band and its subsequent electrophoresis gave rise to the same band pattern as that obtained by affinity chromatography (Fig. 5a) and similar to that resulting from immunoprecipitation with the IgGs obtained from immunization with the ~8-kDa band (Fig. 5a). We found in fact that after electroeluting all the bands, the only one to bind to the radiolabeled fatty acid was the ~8-kDa band (data not shown).

Differences in binding between the free and oligomeric species of FABP in vivo might be a way of regulating the sequestering of ligands, as suggested in H-FABP by Fournier and Richard (1988, 1990) and Janssen and Barrett (1995) in the *Moniezia* sp. LBP. This inability of protein aggregates to bind to fatty acids leads us to conclude that changes are induced in the aggregation process, in terms of the secondary structure of the protein, affecting accessibility to the binding center of the fatty acid and protein. It should be noted that treatment of the protein for SDS-PAGE electrophoresis did not affect its binding capacity to oleic acid, this protein–fatty acid bond being formed after electrofocusing.

The FABPs described in different animal species have a relative molecular mass of ~14 kDa. Although this protein appears to be related to a family of intracellular fatty acid–binding proteins, it might also be related to membrane proteins (Murphy et al., 1998). A FABP with a similar relative mass of ~7–9 kDa has been described in the sunflower, *Helianthus* sp. at 8 kDa (Arondel et al., 1990), *Avena* sp. at 8.7 kDa (Rikers et al., 1984), and *Triticum* sp. at 7 kDa (Castagnaro and Garcia-Olmedo, 1994), among others.

The binding was temperature dependent (data not shown), and an analysis of the data revealed an apparent binding site that is saturable at 90 min (Fig. 2). As with other FABPs (Campbell et al., 1994), this binding is specific to oleic acid and competitive in the presence of excess unlabeled oleic acid. Its displacement versus time is depicted in Figure 2. A Scatchard analysis of the equilibrium data revealed a K_d affinity constant of 3.12×10^{-8} M, and the labeled oleic acid was displaced with unlabeled oleic at a 100-fold higher concentration than that of the labelled form. This result indicates the strong affinity toward long-chain fatty acids, although it could also bind to hydrophobic substances, as it does with other proteins belonging to this family. The displacement kinetic of α-tocopherol was much slower than that observed with oleic acid. The displacement curves for the different hydrophobic substances assayed are set out in Figure 3. The high affinity shown for bile salts means that this FABP shares many characteristics with other FABPs. *Giardia lamblia* colonizes the upper intestine and, in some cases, the common bile duct and gall bladder. The importance of bile salts for the physiological well-being of *G. lamblia* was reported by Lujan et al. (1996), who demonstrated that culture media deprived of serum and supplemented with bile salts supported its growth as well as complete media, allowing *G. lamblia* trophozoites to capture compounds such as cholesterol. The capture of bile salts by *G. lamblia* can be prevented by analogs, and their uptake is temperature dependent (Halliday et al., 1995). The uptake of fatty acids by FABPs follows similar lines, being also subject to inhibition by analogs and temperature dependent (Abumrad et al., 1984).

These data, as in the case of the affinity for fatty acids, reflect the strong similarity with those found for FABPs described in other animal species (Maatman et al., 1994).

The location of the fatty acid–binding protein in *G. lamblia* trophozoites by means of immunochemical techniques, with the use of the specific polyclonal antibodies obtained, revealed its presence in the suction disk, the cytoplasm, the vicinity of the lipid vacuoles, and the dorsal area of the protozoan. The microtubular organization of the disk, together with the negative pressure exerted by the movement of the ventral flagella, causes a flow of intestinal fluid through the ventral channel, producing a suction cup effect that fixes the protozoan to the intestinal microvilli and enables nutrient uptake by pinocytosis. This appears to indicate that *G. lamblia* acquires fatty acids through the ventral disk and, to a lesser degree, through the dorsal zone. As mentioned above, *G. lamblia* cannot synthesize fatty acids de novo (Blair and Weller, 1987).

Several studies suggest that FABPs increase the solubility of fatty acids in cell cytoplasm, causing a net diffusion of fatty acids from the plasma membrane to the intracellular membrane compartments (Tipping and Ketterer, 1981). The presence of FABP in the lipid vacuoles of an organism that has no mitochondria might indicate the different roles that this protein can play in lipid breakdown. Undoubtedly, the detection of gold marks in the cell cytoplasm and the lipid vacuoles, which indicates the presence of antigens recognized by the antibodies, reflects the importance of this protein both for transporting fatty acids through the cytoplasm and for regulating lipid metabolism in the vacuoles. A similar location in the vacuoles of this type of protein has been observed previously in *Schistosoma japonicum* (Gobert, 1998).

The role of these FABPs should not, however, be considered solely in the context of the binding and incorporation of fatty acids, but also in that of binding to any hydrophobic substance, including some agents used in antiprotozoan chemotherapy, such as metronidazol. Metronidazol possesses specific activity for the treatment of some protozoan diseases, such as giardiasis, trichomoniasis, and amebiasis, as well as for anaerobic bacterial processes. The displacement of oleic acid labeled by metronidazol takes place quickly, which could perhaps serve as an important basis for future research into the role of this protein as a target or transporter for this drug or as a determinant in the study of parasite strains resistant to metronidazol.

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