Parasitol Res (2015) 114:1145–1152
DOI 10.1007/s00436-014-4292-z

ORIGINAL PAPER

Isoforms of Hsp70-binding human LDL in adult *Schistosoma mansoni* worms

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Received: 24 October 2014 / Accepted: 23 December 2014 / Published online: 10 January 2015

Abstract *Schistosoma mansoni* is one of the most common parasites infecting humans. They are well adapted to the host, and this parasite's longevity is a consequence of effective escape from the host immune system. In the blood circulation, lipoproteins not only help to conceal the worm from attack by host antibodies but also act as a source of lipids for *S. mansoni.* Previous SEM studies showed that the low-density lipoprotein (LDL) particles present on the surface of adult *S. mansoni* worms decreased in size when the incubation time increased. In this study, immunocytochemical and proteomic analyses were used to locate and identify *S. mansoni* binding proteins to human plasma LDL. Ultrathin sections of adult worms were cut transversely from the anterior, medial and posterior regions of the parasite. Immunocytochemical experiments revealed particles of gold in the tegument, muscle region and spine in male worms and around vitelline cells in females. Immunoblotting and 2D-electrophoresis using incubations with human serum, anti-LDL antibodies and anti-chicken IgG peroxidase conjugate were performed to identify LDL-binding proteins in *S. mansoni*. Analysis of the binding proteins using LC-MS identified two isoforms of the Hsp70 chaperone in *S. mansoni*. Hsp70 is involved in the interaction with apoB in the cytoplasm and its transport to the endoplasmic reticulum. However, further studies are needed to clarify the functional role of Hsp70 in *S. mansoni*, mainly related to the interaction with human LDL.

Keywords Schistosoma · *Schistosoma mansoni* · Lipoproteins · LDL · Hsp70

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Introduction

Schistosomiasis is an important parasitic disease in tropical and subtropical countries (deWalick et al. 1995; Van Hellemond et al. 2007; WHO 2013) caused by trematode worms of the genus *Schistosoma* (Gryseels et al. 2006; Castro-Borges et al. 2011). One of the three main species that infect humans is *Schistosoma mansoni*, which is found in sub-Saharan Africa, parts of the Middle East, Brazil, Venezuela and some Caribbean islands (Berriman et al. 2009). This parasite has a complex life cycle in invertebrate and vertebrate hosts, becoming sexually mature at approximately 4 weeks postinfection in humans and other susceptible animals (Cheng et al. 2005). The final habitat of the male and female adult worms is the mesenteric vasculature of the vertebrate host, where it remains in the bloodstream for decades, despite being permanently exposed to the host immune system. They have an effective method for evading the immune system that depends on the properties of the parasite tegument (Abath

and Werkhauser 1996; Mulvenna et al. 2010; Schramm and Haas 2010).

The incorporation of lipids by the worms is an important element in determining the properties of the parasite membranes. However, schistosomes are incapable of synthesising cholesterol and fatty acids (Meyer et al. 1970). Endogenous receptors may perform important functions, such as the absorption of lipids from host plasma lipoproteins for use in growth, development, maintenance and synthesis of parasite membranes and also host-immune evasion through the accumulation and display of host lipids on the surface tegument (Rumjanek et al. 1985; Calvo et al. 1997; Tempone et al. 1997).

Several studies demonstrated the expression of proteins in protozoa, helminths, schistosomula and adult worms of *Schistosoma* that can bind to low-density lipoprotein (LDL), very low-density lipoprotein (VLDL) and apoprotein B (apoB) (Rumjanek et al. 1983, 1985; Chiang and Caulfield 1989a, b; Rogers et al. 1989; Tempone et al. 1997). Via binding to apoB-100, the LDL receptor mediates the clearance of LDL in human plasma (Zhou et al. 1995). After binding of the extracellular components on the cell surface receptors, clathrin vesicles are formed and these vesicles play an important role in the internalisation of proteins and lipids (Young 2007).

Clathrin-mediated endocytosis in humans is a complex process involving accessory and regulatory proteins, including heat shock protein 70 (Hsp70), a chaperone that has been the subject of extensive research because it is involved in a number of cellular processes (Brodsky et al. 2001; Lemmon 2001). It is responsible for disrupting clathrin–clathrin interactions, leading to the uncoating of molecules (Brodin et al. 2000). Hsp70 is a cytoplasmic protein that targets the hydrophobic domains of substrate polypeptides, guiding proteins to cell organelles (Walter et al. 1984; Walter and Lingappa 1986; Zhou et al. 1995; Frydman et al. 1994; Mayer and Bukau 2005). Hsp70 is expressed during some of the stages of the *S. mansoni* life cycle, the miracidium, sporocyst, schistosomulum and adult worm (Levy-Holtzman and Schechter 1996). The present study identified two proteins belonging to the Hsp70 family that bind to human LDL. Further studies are needed to shed light on the host–parasite relationship and to identify the functional role of Hsp70 in the lipid metabolism of *S. mansoni.*

Materials and methods

Reagents

Chicken anti-LDL affinity-purified polyclonal antibody was purchased from Chemicon International (New Jersey, USA). Agarose, Coomassie blue, polyvinyldifluoride (PVDF) membranes, sodium dodecyl sulphate (SDS), urea, thiourea, 3-[(3-cholamidopropyl)-dimethylammonium]-propane-sulphonate (CHAPS), protease inhibitor mix, IPG buffer, 13-cm linear IPG strips pH 3–10 and Tris were obtained from GE Healthcare (New Jersey, USA). Trypsin was purchased from Promega (Southampton, Hampshire, UK). Acetonitrile (ACN), trifluoroacetic acid (TFA), formic acid, ethanol, hydrochloric acid (HCl) and glycerol were obtained from Merck (Darmstadt, Germany). The other reagents used in the experiments were from Sigma–Aldrich (St. Louis, USA).

Maintenance of the parasite

The SLM strain of *S. mansoni* (São Lourenço da Mata, Brazil) was maintained in *Biomphalaria glabrata* snails and male Swiss mice. Animals aged 7–9 weeks, weighing 35–45 g, were housed in cages (30×20×13 cm) containing a sterile bed of wood shavings. A standard diet (Labina®, Ralston Purina Ltda, São Paulo, Brazil) and water was available ad libitum. The room temperature was maintained at 22±2 °C with a 12:12 h light–dark cycle. The mice were infected by exposure to a *S. mansoni* cercariae suspension containing approximately 100 cercariae using the tail immersion technique (Olivier and Stirewalt 1952). After 8 weeks of infection, the *S. mansoni* adult worms were recovered by perfusion of the hepatic portal system (Duvall and Dewitt 1967). The worms were stored at −20 °C for crude extract preparations or were fixed for electron microscopy experiments. The experimental protocols were in accordance with the requirements of the Animal Experiments Ethics Committee of the Federal University of Pernambuco, Brazil.

Immunocytochemical localisation by electron microscopy

Freshly perfused adult parasites were fixed with 0.1 % (*v/v*) glutaraldehyde and 4 % (*v/v*) PFA in 0.1 M sodium cacodylate buffer pH 7.2 overnight at 4 °C. The parasites were washed with the same cacodylate buffer, dehydrated in a graded series of ethanol [30, 50, 70, 90 and 100 % (*v/v*), 20 min each] and then infiltrated and embedded in L.R. White resin. The material was placed in gelatine capsules, and the resin was allowed to polymerise at 50 °C. Ultrathin sections were cut transversely from the anterior, medial and posterior regions of the parasite. The sections were subsequently incubated with blocking solution (1.5 % (*w/v*) BSA, 0.01 % (*v/v*) Triton 20 in PBS) for 40 min at room temperature and washed in the same solution.

The grids were exposed to human serum diluted 1:10 in blocking solution for 90 min at room temperature, followed by washing in the same solution and incubation with the chicken anti-LDL antibody diluted 1:1000 in blocking solution for 5 h. Finally, the grids were incubated with protein A conjugated to 20-nm gold particles diluted 1:50 in PBS for 30 min. As a

negative control, the sections were incubated with protein A conjugated to colloidal gold particles only.

Two-dimensional electrophoresis

Adult worms (100–200 mg wet weight) were homogenised in 500 μL PBS containing protease inhibitor and centrifuged at 3000×*g* for 30 min at 4 °C. The supernatant was discarded, and the pellet was resuspended in 500 μL PBS plus protease inhibitor. After protein quantification (Lowry et al. 1951), 400 μg of protein was lyophilised and resuspended in rehydration solution (7 M urea, 2 M thiourea, 0.5 % (*w/v*) CHAPS, 0.2 % (*w/v*) DTT, 0.5 % (*v/v*) IPG buffer (pH 3–10) and 0.002 % (*w/v*) bromophenol blue).

For the first dimension, the proteins were subjected to isoelectric focusing on 13 cm linear IPG pH 3–10 strips using a Multiphor II system (GE Healthcare, New Jersey, USA) under the following conditions: 300 V (1 min), 300–3500 V (1 h 30 min) and 3500 V (4 h). The focused strips were incubated with 65 mM DTT in 10 mL equilibration buffer (50 mM (*w/v*) Tris/HCl, 6 M (*w/v*) urea, 30 % (*v/v*) glycerol and 2 % (*w/v*) SDS), followed by a further incubation with 135 mM iodoacetamide in the same buffer. Both incubations were performed for 15 min. For the second dimension, the strips were directly applied to 10 % homogeneous SDS-PAGE and overlaid with 1 % (*w/v*) agarose solution in electrophoresis buffer. SDS-PAGE was performed with a constant current (20 mA per gel) using a vertical system for 4 h. Analytical gels were stained with Coomassie blue. The pattern quality and reproducibility of each set of two-dimensional electrophoresis 2DE gels were evaluated with three independent experiments.

Immunoblotting

Proteins resolved using SDS-PAGE were transferred to PVDF membranes at a constant current of 100 mA for 1 h followed by incubation with PBS containing 3 % (*w/v*) casein for 3 h at 4 °C. The membranes were incubated for 2 h at 25 °C with human serum diluted 1:10 overnight at 4 °C, with anti-LDL antibody diluted 1:1000 and for 3 h at 4 °C and with anti-chicken IgY (IgG) peroxidase conjugate diluted 1:1000. All dilutions were made with PBS. The membranes were also washed with PBS between the incubations. Protein visualisation was performed with the TMB substrate. The reaction was stopped by the addition of distilled water. Analyses of the 2DE electrophoresis gel and immunoblotting were performed by digitisation of the images obtained with the Transilluminator L-Pix HE Scanner (Loccus Biotecnologia, São Paulo, Brazil) and analysed using Image Master 2D Platinum (GE Healthcare, New Jersey, USA).

Protein identification

The selected protein spots from the 2DE analysis were excised from the gels and destained with 200 μL 25 mM NH_4HCO_3 in 50 % (*v/v*) ACN pH 8.0 and then vortexed three times for 5–10 min each. The gels were washed with Milli-Q water, the supernatants discarded and the samples dried in a Speed-Vac (Concentrator Plus, Eppendorf, Brazil). The dried gels were rehydrated overnight with 50 mM NH_4HCO_3 in the presence of trypsin solution (20 ng/μL) at 37 °C. The peptide fragments digested by trypsin were extracted with 50 % (*v/v*) ACN/5 % (*v/v*) TFA, desalted using C18 reverse phase ZipTips and dried in a Speed-Vac. After this, 10 μL of each sample was injected into a nanoEase C18 (100 mm×100 μm) column (Waters, Milford, MA, USA) and eluted (0.4 μL/min) with a linear gradient [10–50 % (*v/v*)] of ACN containing 0.1 % (*v/v*) formic acid. Electrospray tandem mass spectra (ESI) were recorded using a Q-Tof quadrupole/orthogonal acceleration time-of-flight spectrometer (Waters, Milford, MA, USA) interfaced with the nanoACQUITY system capillary chromatograph. The ESI voltage was set at 3500 V using a metal needle, the source temperature was 100 °C and the cone voltage was 40 V. Instrument control and data acquisition were performed with the MassLynx data system (Version 4.1, Waters). The experiments were performed by scanning from a mass-to-charge ratio (*m*/*z*) of 200–2000 using a scan time of 1 s for the entire chromatographic process.

The mass spectra corresponding to each signal from the total ion current (TIC) chromatogram were averaged, allowing for accurate measurement of the molecular mass. The exact mass was determined automatically using the Q-Tof LockSpray™ (Waters, Milford, MA, USA). Data-dependent MS/MS acquisitions were performed on precursors with charge states of 2 and 3 over a range of 50–2000 *m*/*z* and below a 2 *m*/*z* window. A maximum of three ions were selected for MS/MS from a single MS survey. The adduct masses of Na^+ and K^+ were automatically excluded. Collision-induced dissociation (CID) MS/MS spectra were obtained using argon as the collision gas at a pressure of 13 psi, and the collision voltage varied between 18 and 45 V depending on the mass of the precursor.

The scan rate was 1 scan/s. All data were processed using the ProteinLynx Global server (version 2.0, Waters). The processing locks mass corrected the *m*/*z* scale of both the MS and MS/MS data using the lock spray reference ion. The MS/MS data were also charge state deconvoluted and deisotoped using a maximum entropy algorithm (MaxEnt 3, Waters).

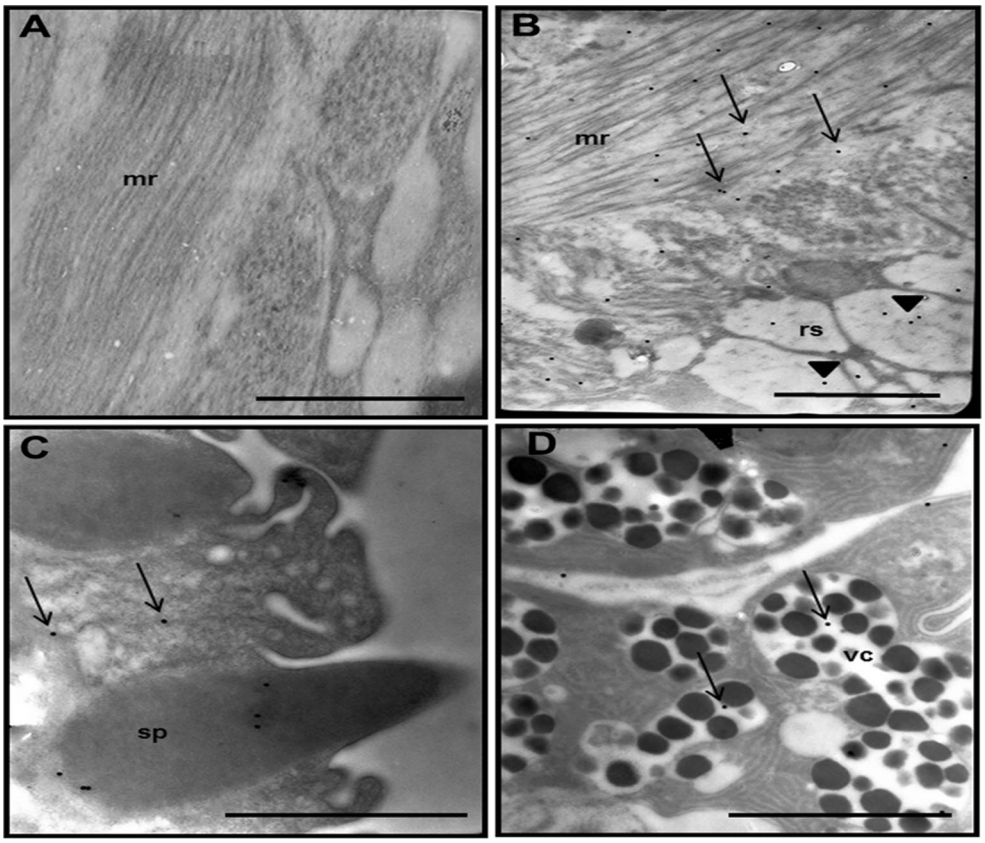

Fig. 1 Transmission electron micrographs of *Schistosoma mansoni* adult worms incubated with human serum (HS), anti-LDL antibody and protein-A-gold-complex. Ultrathin sections were exposed to HS diluted 1:10 for 90 min followed by incubations with chicken anti-LDL antibody (1:1000) for 5 h and protein A conjugated to 20-nm gold particles diluted 1:50 for 30 min. **a** Tegument without LDL incubation (control). **b** Label on muscular and syncytial regions. **c** Gold particles in and around spine. **d** Binding site in vitelline cells of females. *Bars*=1 μm; *mr* muscular region (mr), *rs* syncytial region, *sp* spine, *vc* vitelline cells

Proteins were identified by correlation of tandem mass spectra and the NCBInr database of proteins (Version 050623, 2,564,994 sequences) using the MASCOT software (Matrix Science, version 2.1). They were assumed to be carbamidomethylated, and a variable modification of methionine (oxidation) was allowed. Identification was considered positive if at least two peptides matched with a mass accuracy of less than 0.2 Da.

Results

Immunolabeling of *S. mansoni* tegument incubated with human serum

When ultrathin sections from the anterior, medial and posterior regions of *S. mansoni* were incubated with human serum followed by incubations with anti-LDL antibody and protein-

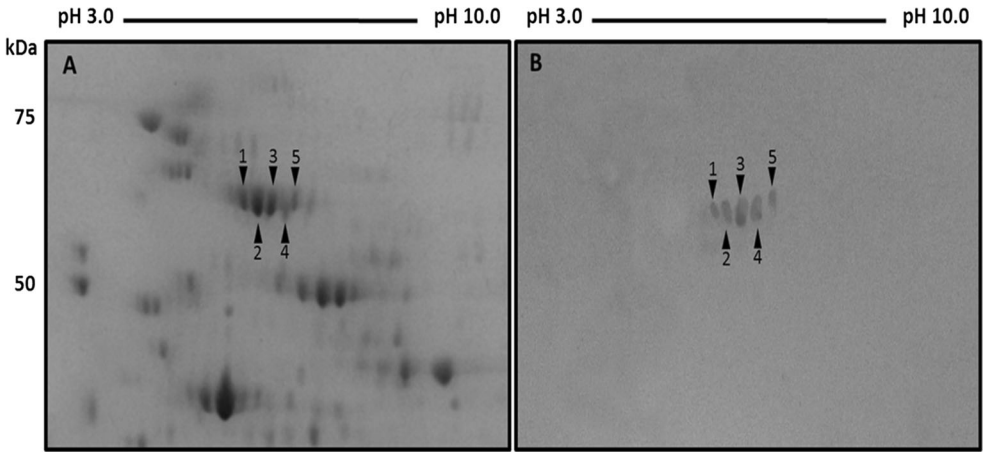

Fig. 2 Two-dimensional electrophoresis gel of *Schistosoma mansoni* adult worm extract and corresponding Western blotting. **a** Representative 2D electrophoresis using 400 μg of proteins, 13-cm pH 3–10 NL strip, 10 % SDS-PAGE and Coomassie staining. **b** Western blotting on PVDF membrane incubated with anti-LDL and anti-chicken IgY (IgG) peroxidase conjugate detected using TMB substrate. The numbers correspond to the Supporting Information, Table 1. The area of the gel and blotting shown in Fig. 2a, b corresponds approximately to pH 5.5 to 8.0

A-gold-complex, immunolabeling was detected in various parts of the male and female worms (Fig. 1a–d). In adult male parasites, the gold particles were observed in the tegument, muscular region and spine and were more abundantly distributed in the muscle fibre (Fig. 1b, c). Immunolabeling was detected in and around the vitelline cells in the females (Fig. 1d). No reactivity was observed in the male (Fig. 1a) and female controls in which incubations were performed with the protein-A-gold-complex alone without the anti-LDL antibody step.

Recognition of LDL-binding proteins in *S. mansoni* via 2D-electrophoresis

Two-dimensional electrophoresis of proteins from adult *S. mansoni* worms (Fig. 2a) and Western blotting analyses (Fig. 2b) were performed to identify LDL-binding proteins in the parasite. Coomassie-stained analytical gels revealed a total of 215 spots, of which five were identified using anti-LDL antibodies. The ImageMaster 2D Platinum software created a matching algorithm that compared images of the related spots. Parameters, such as MW and pI, were assigned to the images analysed. The results showed that the spots recognised by the anti-LDL antibodies ranged from pI 5.8 to 6.3 and MW 71 to 73 kDa (Fig. 2a, b). The LDL-binding spots identified were numbered and located in the corresponding 2D electrophoresis gel (Fig. 2a).

LC MS/MS analysis of the proteins recognised by anti-LDL antibody

Spots that consistently registered in the same position in the gel and blot were considered the same protein. Five spots were successfully identified using LC MS/MS. The tandem mass spectra generated by LC MS/MS were correlated with the NCBInr database using the MASCOT software. Two different forms of the chaperone heat shock protein 70 (Hsp70) of *S. mansoni* were identified (Table 1), showing high coverage and uniform distribution of amino acids. Partial sequences of the peptides were searched in the database using the BLAST bioinformatics program, resulting in a high degree of confidence in the proteins identified.

Discussion

Human schistosomiasis is often accompanied by perturbation of lipid homeostasis (Silva et al. 2002; Ramos et al. 2004). *S. mansoni* does not synthesise fatty acids and sterols but depends on such molecules for the formation of membranes (Meyer et al. 1970; Bennett and Caulfield 1991). To meet their metabolic needs and evade immune recognition, these

Table 1 Proteins involved in the interaction of the LDL with the *Schistosoma mansoni*

Sample	Accession number (NCBI BLAST)	Protein	Peptide matches	Number of unique peptides identified	Decoy (%)		Nominal mass (M_r)	Experimental mass	Calculated pI	Experimental pI	Score	Coverage (%)
					Identity	Homology						
1	gi|353229993	Heat shock protein 70 [*Schistosoma mansoni*] Smp_106930	42(7)	9	0	3	70215	73030	5.42	5.84	1098	46
2	gi|353229993	Heat shock protein 70 [*Schistosoma mansoni*] Smp_106930	47(14)	8	0	0	70215	72433	5.42	5.93	1269	44
3	gi|353229993	Heat shock protein 70 [*Schistosoma mansoni*] Smp_106930	49(8)	10	0	2	70215	71645	5.42	6.04	1113	46
4	gi|353229993	Heat shock protein 70 [*Schistosoma mansoni*] Smp_106930	22(4)	5	0	1	70215	72831	5.42	6.19	655	32
5	gi|256090832	Heat shock protein 70 [*Schistosoma mansoni*] Smp_106130.2	28(3)	14	0	0	71595	72038	6.09	6.30	831	35

parasites use host molecules such as serum lipoproteins, glycolipids from the blood groups, and histocompatibility antigens (Rogers 1991; Bryant 1993; Abath and Werkhauser 1996; Tempone et al. 1997; Loukas et al. 2001; Jones et al. 2004; Gan et al. 2006; Xiao et al. 2010).

Evidence suggests that mechanisms exist for transferring lipid or lipid precursors between the definitive host and the parasite (Bennett and Caulfield 1991; Tempone et al. 1997; Loukas et al. 2001; Fan et al. 2003). LDL is rich in sterols and phosphatidylcholine, which are required for the synthesis of membranes and also play major roles in cholesterol transport in human plasma (Chiang and Caulfield 1989a, 1989b). Previous studies showed that LDL is linked to a receptor on the surface of the *S. mansoni*, forming a protective cover on the parasite tegument (Chiang and Caulfield 1989a, 1989b; Caulfield et al. 1991; Xu and Caufield 1992; Tempone et al. 1997). The LDL receptor is a multi-domain protein and has been described in schistosomula and adult worms of *S. mansoni* and *S. japonicum* (Chiang and Caulfield 1989a, 1989b; Bennett and Caulfield 1991; Xu and Caufield 1992). There are reports of proteins that are synthesised constitutively and transported to the parasite surface at various stages in its life cycle (Rogers et al. 1989, 1990; Xu and Caufield 1992; Rumjanek et al. 1988).

A previous study confirmed the interaction of LDL with the tegument of *S. mansoni* (SLM strain) using scanning electron microscopy. The size of the LDL aggregates decreased over the incubation period (0–120 min) (Pereira et al. 2011), suggesting that it occurred because of lipid transfer from the host lipoproteins to the worms.

The accumulation and release of lipids has different standards in male and female *S. mansoni* (Haseeb et al., 1984, 1985, 1986, 1989). In females, lipid accumulation was present only in vitelline cells (Haseeb et al. 1985). Similarly, we detected LDL-colloidal gold particles inside and around the vitelline cells, whereas in males, they were present in the muscle cells, syncytium and spines. Exposure of *S. mansoni* to human serum allows the LDL-linked protein to emerge on the surface (Rumjanek et al. 1983).

There is no evidence that *S. mansoni* worms present components forming part of the endocytosis machinery of the LDL receptor complex with clathrin-coated vesicles. In mammals, LDL binds to receptors on the cell surface, followed by internalisation of the complex with subsequent proteolytic degradation of LDL (Lehninger 2011). However, this process has not been demonstrated in *S. mansoni*.

Some authors suggest that human Hsp70 is involved in the interaction with apoB, present in the LDL, and its transport to the endoplasmic reticulum. Hsp70 may also disrupt clathrin–clathrin interactions, leading to the release of lipoprotein (Zhou et al. 1995; Gusarova et al. 2001). Kanehisa (2000) proposed a mechanism in which Hsp70 in *S. mansoni* is involved in disrupting the clathrin–clathrin interactions and transporting lipoprotein into the cell. Thus far, no protein from the Hsp family was identified as lipoprotein-binding. Hsp plays key roles in many organisms, including schistosomes (Maresca and Carratu 1992; Neumann et al. 1993; Smirlis et al. 2010; Yang et al. 2012). Of the Hsp family, Hsp70 is hypothesised to be the most conserved, acting as an intracellular chaperone and in the extracellular transport of immunoregulatory proteins and other molecules (Mayer and Bukau 2005; De Jong-Brink 1995; Martinez et al. 2004; Kimura et al. 2007). In 1996, the presence of Hsp70 in *S. mansoni* adult worms was reported (Levy-Holtzman and Schechter 1996). In this study, two isoforms of Hsp70 were identified as human plasma LDL-binding proteins. Hsp70 is a cytoplasmic protein capable of interacting with the hydrophobic domains of proteins, such as apoB (Zhou et al. 1995).

There is evidence of mechanisms for transferring lipids and/or proteins of LDL for the adult male worm of *S. mansoni* (Pereira et al., 2011). The exposure of the worm to human serum caused the appearance of LDL-binding proteins on the surface of the worm (Rumjanek et al. 1983; Chiang and Caulfield 1989a, b). However, previous studies do not favour the postulate that there is internalisation of the LDL in a manner similar to that of humans, although the transport of lipids into and out of the cells must occur via proteins because of the hydrophobic characteristics of these molecules. By contrast, apoB binds to cell surface receptors on *S. mansoni* (Chiang and Caulfield 1989a, b; Zhou et al. 1995). Therefore, it is possible that even without the full internalisation of the lipoprotein particle, there is a partial internalisation of its components. Further studies are required to shed light on the mechanism of action involved in the acquisition of lipids by *S. mansoni*.

Acknowledgments This study received support from the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES). The authors would like to thank Ana Lúcia Oliveira Carvalho from the Mass Spectrometry and Proteomics Unit (UEMP), Federal University of Rio de Janeiro, Brazil, for the technical assistance.

Conflict of interest The authors declare no conflicts of interest.

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