

Conservation and developmental expression of ubiquitin isopeptidases in *Schistosoma mansoni*

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Several genes related to the ubiquitin (Ub)-proteasome pathway, including those coding for proteasome subunits and conjugation enzymes, are differentially expressed during the Schistosoma mansoni life cycle. Although deubiquitinating enzymes have been reported to be negative regulators of protein ubiquitination and shown to play an important role in Ub-dependent processes, little is known about their role in S. mansoni. In this study, we analysed the Ub carboxyl-terminal hydrolase (UCHs) proteins found in the database of the parasite's genome. An in silico analysis (GeneDB and MEROPS) identified three different UCH family members in the genome, SmUCH-L3, SmUCH-L5 and SmBAP-1 and a phylogenetic analysis confirmed the evolutionary conservation of the proteins. We performed quantitative reverse transcription-polymerase chain reaction and observed a differential expression profile for all of the investigated transcripts between the cercariae and adult worm stages. These results were corroborated by low rates of Z-Arg-Leu-Arg-Gly-Gly-AMC hydrolysis in a crude extract obtained from cercariae in parallel with high Ub conjugate levels in the same extracts. We suggest that the accumulation of ubiquitinated proteins in the cercaria and early schistosomulum stages is related to a decrease in 26S proteasome activity. Taken together, our data suggest that UCH family members contribute to regulating the activity of the Ub-proteasome system during the life cycle of this parasite.

Key words: deubiquitinating enzymes - ubiquitin C-terminal hydrolase - differential expression - deubiquitination activity

Ubiquitin (Ub) is a small (~8 kDa) protein that can be covalently attached to target protein substrates in a process called ubiquitination. This post-translational modification has emerged as a critical regulatory process in virtually all aspects of cell biology, including signal transduction and protein stability (Finley et al. 2004, Kerscher et al. 2006). The Ub molecule is first activated by the Ub-activating enzyme E1 and then transferred to the Ub-conjugating enzyme E2 and attached to a specific substrate with the assistance of Ub-ligase E3 (Hershko & Ciechanover 1998). The covalent attachment of polyubiquitin to a protein serves as a highly specific degradation tag that mediates trafficking to the proteasome. Ub has seven internal lysine residues, any of which can be polyubiquitinated to create Ub chains of distinct linkages. A Ub code exists whereby proteins tagged with K6, K11, K27, K29 and K48-linked polyubiquitin chains are targeted for proteasomal degradation, whereas K63-linked chains are preferentially involved in the lysosomal pathway (Ikeda & Dikic 2008, Dammer et al. 2011).

Protein ubiquitination is a reversible process involving deubiquitinating enzymes (DUBs), which are able to process linear polyubiquitin precursor proteins, such as ribosomal fusion proteins, into single Ub molecules (Wing 2003). Second, DUBs recycle Ub by processing polyubiquitin chains to generate free monomers that can enter the pool for subsequent conjugation events. This is a critical process because free polyubiquitin chains can inhibit the competitive binding of polyubiquitinated substrates to the 26S proteasome. Finally, DUBs remove Ub from ubiquitinated substrates, which antagonises Ub conjugation by E3 ligases (Komander et al. 2009). DUBs have been subdivided into six families based on sequence and structural similarity: Ub-specific proteases (USPs), Ub carboxyl-terminal hydrolases (UCHs), ovarian-tumour proteases (OTUs), Machado-Joseph disease protein domain proteases, JAMM/MPN domain-associated metalloproteases and monocyte chemotactic protein-induced protein (Fraile et al. 2012).

UCHs were the first DUB family to be structurally characterised. These enzymes only target small peptides from the C terminus of Ub because they have a confined loop that precludes the processing of polyubiquitin chains and folded target proteins (Komander et al. 2009). There are four UCHs in humans: UCH-L1, UCH-L3, UCH-L5 (UCH37) and BAP-1 (binding to the wild-type BRCA1 RING finger domain). The additional C-terminal extension present in UCH-L5 facilitates the trimming of polyubiquitin chains from conjugated proteins, whereas

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the C-terminal extension of BAP-1 interacts with the N-terminal ring finger of BRCA1 (Jensen et al. 1998, Koulich et al. 2008). Although USP-5 is a member of the USP family, this protease is an isopeptidase with four domains: a ZnF UBP domain, a USP/UBP domain and two UBA domains. USP-5 specifically disassembles unanchored polyubiquitin through its multiple Ub-binding domains (Reyes-Turcu & Wilkinson 2009).

Schistosomes are parasitic worms that are a prime example of a complex multicellular pathogen that affects humans. The development of *Schistosoma mansoni* in a vertebrate host requires several coordinated alterations of its body morphology and biochemical changes that guarantee adaptation (Stirewalt 1974, Fishelson et al. 1992, McKerrow & Salter 2002). Recently, our group showed that the Ub-proteasome proteolytic pathway in *S. mansoni* plays a crucial role in regulating the activity of the proteasome during parasite development (Guerra-Sá et al. 2005, Castro-Borges et al. 2007). However, despite their potential importance, little is known about DUBs in this parasite. In the present study, we identified UCH-L3, UCH-L5 (UCH37), BAP-1 and USP-5 (isopeptidase T) in *S. mansoni* and found that these DUBs exhibit differential gene expression profiles during development. In addition, we evaluated the activity of *Sm*UCH-L3 and *Sm*USP-5 using the fluorescent substrate Z-Arg-Leu-Arg-Gly-Gly-AMC and report high levels of ubiquitinated proteins in the cercaria, early schistosomulum and egg stages.

MATERIALS AND METHODS

Ethics statement - All experiments involving animals were authorised by the Ethical Committee for Animal Care of the Federal University of Ouro Preto (protocol 2011/55). These procedures were conducted in accordance with the accepted national and international regulations for laboratory animal use and care.

Parasites - The *S. mansoni* LE strain was maintained by routine passage through *Biomphalaria glabrata* snails and BALB/c mice. Infected snails were induced to shed cercariae under light exposure for 2 h, followed by recovery of the larvae by sedimentation on ice. Adult worm parasites were obtained by liver perfusion of mice after infection for 50 days. The mouse livers were triturated in phosphate buffer (pH 8.2); trypsin was added and the homogenate was incubated for 2.5 h at 37°C in a water bath. The eggs were recovered in saline solution after sequential sieving through 360- and 180-µm mesh. Mechanically transformed schistosomula (MTS) were prepared as described by Harrop and Wilson (1993). Briefly, cercariae were recovered and washed in RPMI-1640 medium (Invitrogen, São Paulo, Brazil) and then vortexing at maximum speed for 90 s; the cercariae were immediately cultured for 3.5 h at 37°C and 5% CO₂. The recovered schistosomula were washed with RPMI-1640 until no tails were detected. For the subsequent incubations, the parasites were maintained in M169 medium supplemented with 10% foetal bovine serum, penicillin and streptomycin (100 µg/mL) and 5% Schneider's medium (Basch & DiConza 1977) at 37°C with 5% CO₂ for 3.5 h, 24 h, 48 h and 72 h and 5, 8 and 10 days.

Analysis of UCH genes - UCH genes were identified by mining *S. mansoni* sequences in the GeneDB (genedb.org/genedb/smansoni/) and MEROPS (merops.sanger.ac.uk/) databases (Rawlings et al. 2008, 2010) using BLASTp and queries of known *Homo sapiens* proteins (BAP1, GeneDB ID: NP_004647.1 and MEROPS ID: MER003989; UCH-L3, GeneDB ID: NP_005993.1 and MEROPS ID: MER000836; UCH-L5, GeneDB ID: NP_057068.1 and MEROPS ID: MER005539). Reference proteins from other species were searched in the National Center for Biotechnology Information database to obtain a full set of putative homologue proteins to compare with the *S. mansoni* proteins. The BLASTp algorithm, underpinned by Pfam (v26.0), allowed for the detection of conserved protein domains or motifs from *S. mansoni* sequences. The entire protein sequences were used to perform multiple sequence alignments using the programme CLUSTALX 2.0 with the default settings (Larkin et al. 2007). Phylogenetic trees were inferred using the neighbour-joining method and the Jones-Taylor-Thornton model (Saitou & Nei 1987). A bootstrap consensus tree inferred from 1,000 replicates was used to represent the evolutionary history of the taxa analysed. The molecular phylogenetic analyses were conducted using MEGA 5 software (Tamura et al. 2011). The sequences used as queries obtained from the *H. sapiens* database, i.e., UCH-L5 (Li et al. 2001, Nishio et al. 2009, Maiti et al. 2011, Burgie et al. 2012), UCH-L3 (Johnston et al. 1997, Larsen et al. 1998) and BAP-1 (Sanchez-Pulido et al. 2012), were experimentally supported.

Expression analysis of DUB enzymes - Total RNA from cercariae, schistosomula, adult worms and eggs was obtained using a combination of the Trizol reagent (Sigma, Belo Horizonte, MG, Brazil) and chloroform for extraction and the RNA was column-purified using the SV total RNA Isolation system (Promega, Belo Horizonte, MG, Brazil). The preparation was treated with RNase-free DNase I in three different rounds with decreasing enzyme concentrations (RQ1 DNase; Promega). The RNA was quantified using a spectrophotometer and an aliquot containing 1 µg of total RNA was reverse transcribed using an oligo dT primer from the ThermoScript reverse transcription-polymerase chain reaction (RT-PCR) System (Invitrogen), as described by the manufacturer. The efficiency of DNase I treatment was evaluated by PCR amplification of the cDNA reaction mix without the addition of the ThermoScript enzyme. *S. mansoni*-specific primers were designed using the programme GeneRunner®. The sequence accessions and primer pairs are shown in Supplementary data. RT-cDNA samples were used as the templates for PCR amplification with the SYBR Green Master Mix UDG-RÖX® (Invitrogen) and 7300 Real-time PCR System (Applied Biosystems, Rio de Janeiro, Brazil). Specific primers for *S. mansoni* EIF4E were used as an endogenous control (GeneDB ID: Smp_001500) (forward, 5'TGTTCCAACACGGTCTCG3'; reverse, 5'TCGCCTTCCAATGCTTAGG3') (Liu et al. 2012). The efficiency of each pair of primers was evaluated according to the protocol developed by Applied Biosystems

RESULTS

Conservation of UCH in *S. mansoni* - An *in silico* analysis revealed six sequences representing three putative UCH sequences in the *S. mansoni* genome: Smp_168800.1/2/4 (UCH-L3), Smp_083200.1/2 (UCH-L5) and (BAP-1). Three similar sequences for UCH-L3 were found, which are most likely the result of alternative splicing and are annotated as Smp_168800.1, Smp_168800.2 and Smp_168800.4. Among them, the Smp_168800.2 sequence showed an insertion of 43 amino acids in the N-terminal region of the protein sequence. We also observed a putative alternative splicing event for UCH-L5, with two similar sequences that were annotated as Smp_083200.1 and Smp_083200.2.

Analyses using the Pfam protein domain database (v26.0) were performed to confirm whether these predicted proteases can be considered to be cysteine-protease family members. For all UCH entries, a conserved domain (Pfam: PF01088, Peptidase_C12) was identified in the three *S. mansoni* UCH homologues (Fig. 1). The SmUCH-L3, SmUCH-L5 and SmBAP-1 sequences contain two catalytic residues represented by a cysteine at

positions 115, 85 and 153, respectively, and a histidine at positions 189, 160 and 231, respectively. We observed that UCH-L5 showed high similarity (78%) and UCH-L3 and BAP-1 showed low similarities (55% and 26%, respectively) to their human orthologues.

We also evaluated the conservation of these proteins during evolution using a phylogenetic analysis. Our results showed that each UCH was grouped into a distinct clade close to *Schistosoma japonicum*, which reinforces the structural conservation among the orthologues (Fig. 2). Given the high sequence similarity, the three *S. mansoni* proteases were grouped in the same branch of putative UCH proteins from *S. japonicum*. Furthermore, it was noted that UCH-L3 occupies a distinct clade relative to the other two UCHs, BAP-1 and UCH-L5. Indeed, the UCH-L3 sequences are more closely related, whereas SmUCH-L5 and SmBAP-1 show divergence from their respective orthologues.

DUB enzymes are differentially expressed in *S. mansoni* - The gene expression profiles of *Smuch-l3*, *Smuch-l5*, *Smbap-1* and *Smusp5* were determined using quantitative RT-PCR during the cercaria-schistosomulum transition and also in adult worms and eggs (Fig. 3). We observed that *Smuch-l3*, *Smuch-l5*, *Smbap-1* and *Smusp5* transcripts were expressed at basal levels in cercariae. Conversely, the level of *Smuch-l3* transcript was significantly higher in adult worms and was approximately five and three-fold higher in MTS-3.5 h compared to the levels in cercariae and during schistosomulum development (MTS-24 h, MTS-48 h and MTS-72 h), respectively. With regard to *Smuch-l5*, we observed lower expression in cercariae (2-fold) compared to schistosomula, adult worms and eggs. Furthermore, a two-fold difference was observed in adult worms when compared to the other stages and *Smbap-1* transcripts were also less abundant during this stage of the parasite's life cycle. The levels of *Smusp-5* transcripts were significantly higher (3-fold) in the early schistosomulum compared to the other stages.

SmUCH-L3 and SmUSP-5 enzyme activities - In vitro endopeptidase assays were performed using crude extracts from cercariae, schistosomula, adult worms and eggs (Table). The UCH-L3 and USP-5 enzyme activities were slightly higher in the MTS-72 h and adult worms compared to cercariae and eggs. The enzyme activities were also measured in the presence of a commercially available UCH inhibitor, NEM and significant differences were observed in all the analysed stages, which were at least two-fold lower relative to the extracts without the inhibitor.

Ub conjugates in *S. mansoni* - To confirm the presence of Ub conjugates in the parasite, WB was used to examine crude extracts from cercariae, MTS-3.5 h, MTS-5 d, MTS-8 d, MTS-10 d, adult worms and eggs. The molecular mass distribution of Ub conjugates is presented in Fig. 4, with the majority of the conjugates appearing within the mass range of 20-100 kDa. It was observed that Ub conjugates accumulate in the cercariae and early schistosomula though appear at low levels in late schistosomula

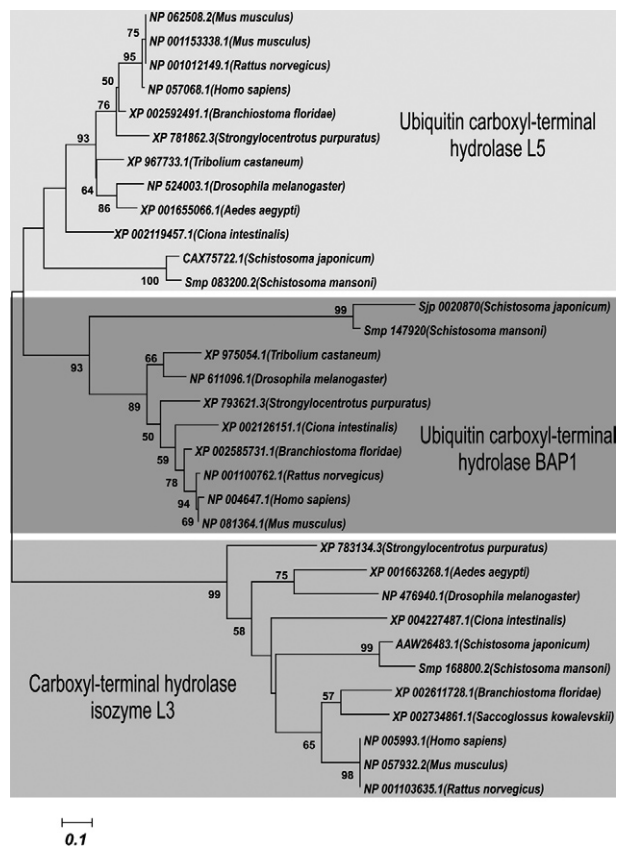


Fig. 2: consensus phylogenetic tree based on amino acid sequences of SmUCH-L3, SmUCH-L5 and SmBAP-1. Tree construction and bootstrap analysis were performed using CLUSTALX 2.0 and MEGA 5.0. For the consensus tree and reliability of the branches formed, a phylogenetic bootstrap analysis using 1,000 replicates for each sequence was used with a 50% the minimum for considering branch reliably.

and adult worms. In contrast, the egg stage exhibited the most pronounced increase in the levels of Ub conjugates relative to all the other stages examined.

DISCUSSION

DUBs directly regulate the protein ubiquitination process. Most commonly, ubiquitination leads to protein degradation, whereas deubiquitination has a stabilising effect and actively increases protein levels in cells. In addition, these enzymes are involved in numerous biological processes, such as growth, differentiation and transcriptional regulation (Sridhar et al. 2007, Singhal et al. 2008, Fraile et al. 2012). Given that protein homeostasis and cell signalling often require tight temporal and spatial regulation, the DUBs affecting these pathways are also regulated in many different ways. Previous results from our group have demonstrated that the accumulation of ubiquitinated conjugates in cercariae correlated with decreased 26S proteasome activity at this stage (Guerra-Sá et al. 2005, Pereira-Júnior et al. 2012).

UCHs catalyse the hydrolysis of Ub in the C-terminal region and these enzymes play a key role in the maturation of Ub by processing its precursor in addition to recycling the free monomer by disassembling polyubiquitin chains (Fang et al. 2010). In the present study, the genes coding for at least three UCHs (UCH-L3, UCH-L5 and BAP-1) were identified in the *S. mansoni* genome. In contrast, our *in silico* analyses did not reveal

the presence of UCH-L1, a hydrolase that has been implicated in Parkinson's disease and neuronal function in humans (Reyes-Turcu & Wilkinson 2009). Although the specificity and function of UCHs in parasite biology remain elusive, each of these enzymes contains conserved catalytic domains (UCH-domains) that are critical for enzymatic activity. In addition, the *SmUCHs* domain contains two conserved cysteine and histidine residues, which constitute a signature for all UCH family members. Subsequent reports concerning the crystal structures of UCH-L3 and UCH-L5 have demonstrated three conserved residues (cysteine, histidine and aspartate) in UCH-domains (Johnston et al. 1997, Nishio et al. 2009). UCH-L5 and BAP-1, which are more similar to each other and have diverged from UCH-L3, occupy a distinct subclade and share a common ancestor (Sanchez-Pulido et al. 2012). This observation is corroborated by the fact that UCH-L5 and BAP-1 share 60 amino acids at their C-terminus (approximately 38%), which is designated the UCH37 like-domain (Eletr & Wilkinson 2011).

UCH-L5 can suppress proteasome-mediated degradation via the disassembly of distal polyubiquitin moieties (Lam et al. 1997, Koulich et al. 2008, Schreiner et al. 2008). However, a recent study supports the role of UCH-L5 in catalysing the selected degradation of specific proteasome substrates, such as nitric oxide synthase and I κ B (Mazumdar et al. 2010). These findings raise the possibility that UCH-L5 has a double regulatory role

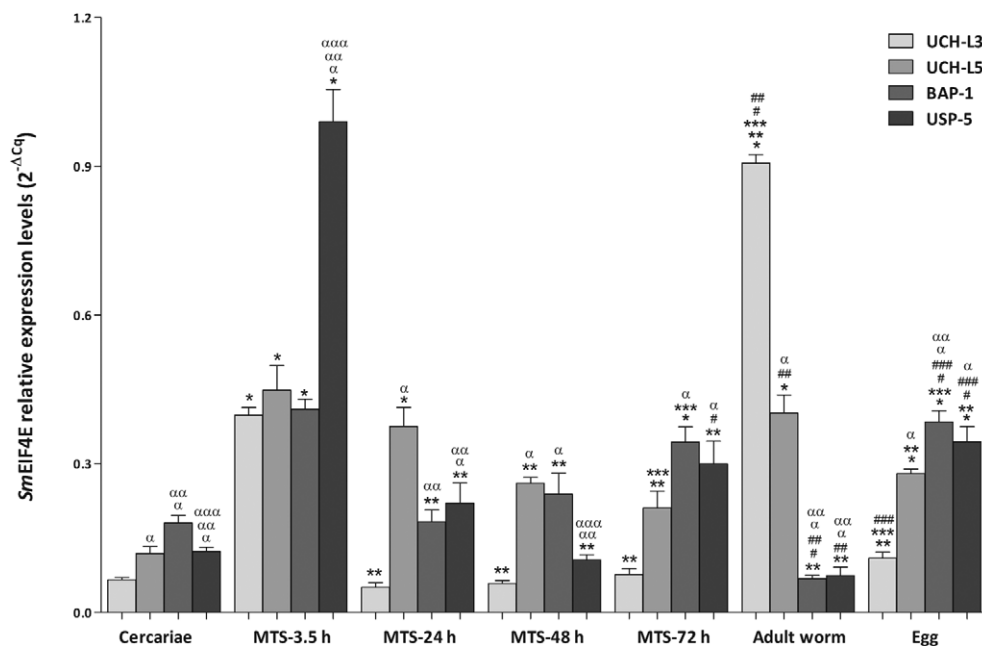


Fig. 3: *SmUCH-L3*, *SmUCH-L5*, *SmBAP-1* and *SmUSP-5* are differentially expressed throughout the *Schistosoma mansoni* life cycle. *SmUCH-L3*, *SmUCH-L5*, *SmBAP-1* and *SmUSP-5* mRNA expression levels were measured based on three replicates for each of the following stages: adult worms, cercariae and eggs as well as mechanically transformed schistosomula (MTS)-3.5 h, 24 h, 48 h and 72 h using quantitative reverse transcription-polymerase chain reaction. Expression levels were calibrated according to the comparative $2^{-\Delta C_t}$ method using the constitutively expressed *SmEIF4E* as an endogenous control (ANOVA followed by Tukey's pairwise comparison $p < 0.05$). *: different from cercariae; **: different from MTS-3.5 h; ***: different from MTS-24 h; #: different from MTS-48 h; ##: different from MTS-72 h; ###: different from adult worm; °: different from ubiquitin (Ub) carboxy-terminal hydrolases (UCH)-L3; °°: different from UCH-L5; °°°: different from BAP-1; USP: Ub-specific proteases.

TABLE
SmUCH-L3 and *SmUSP-5* enzymatic activities in *Schistosoma mansoni*

Deubiquitinating activity	Cercariae	MTS-3.5 h	MTS-24 h	MTS-48 h	MTS-72 h	Adult worms	Eggs
NEM (-)	110 ± 0.46	300 ± 0.87	315 ± 0.87	150 ± 0.58	835 ± 1.04	833 ± 32.59	270 ± 0.58
NEM (+)	8 ± 0.01	90 ± 0.29	70 ± 0.29	45 ± 0.17	169 ± 0.87	150 ± 0.46	85 ± 0.52

the fluorescence levels were measured based on three replicates. Statistical analysis was performed using t tests followed by the unpaired test $p < 0.001$. The fluorescence was expressed as indicated (see Materials and Methods). The results represent the average of three independent experiments $\pm$ standard error of the means. MTS: mechanically transformed schistosomula; UCH: ubiquitin (Ub) carboxy-terminal hydrolases; USP: Ub-specific proteases.

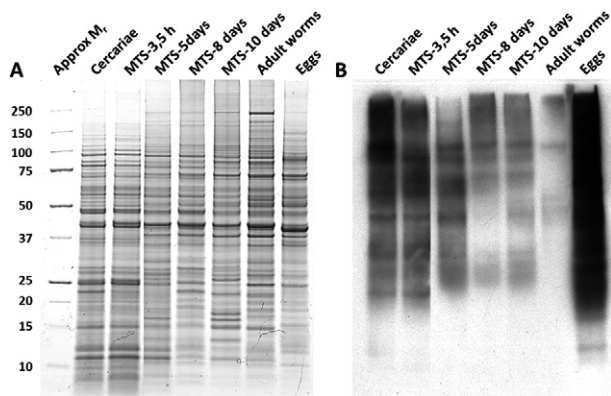


Fig. 4: the ubiquitination profile throughout the *Schistosoma mansoni* life cycle. A: gel stained in Sypro Ruby®; B: 10 µg of crude extracts were analysed by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis followed by immunoblotting using anti-ubiquitin antibody produced in rabbit.

in suppressing or promoting the degradation of specific proteasome substrates. The DUB activity of UCH-L5 is tightly regulated and is dependent on its association with the Ub receptor Rpn13/ADRM1, which is located at the base of 19S RP (Hamazaki et al. 2006, Qiu et al. 2006, Yao et al. 2006, Lander et al. 2012). Although UCH-L5 can cleave polyubiquitin when bound to the proteasome, this reaction may require the participation of additional proteasome components to partially unfold the polyubiquitin chain (Eletr & Wilkinson 2011). We detected low levels of UCH-L5 expression in the cercaria stage, in agreement with the previous results from our group that demonstrated decreased 26S proteasome activity in extracts from cercariae relative to adult worms (Pereira-Júnior et al. 2012).

BAP-1, a tumour suppressor, is a nuclear DUB with an N-terminal UCH domain and two predicted nuclear localisation signals (NLSs), though only one is a classic NLS (Ventii et al. 2008). BAP-1 has been reported to influence cell proliferation at G1/S (Eletr & Wilkinson 2011). *Smbap-1* transcripts were up-regulated in MTS-3.5 h, MTS-72 h and eggs and are down-regulated in cercariae and adult worms, results that are consistent with a possible participation of *SmBAP-1* in parasite stages

during which cell proliferation is particularly required, as observed for the larval stage (Stirewalt 1974, Fishelson et al. 1992, McKerrow & Salter 2002).

We found that *Smuch-l3* transcripts were up-regulated in MTS-72 h, adult worms and eggs and down-regulated in cercariae. UCH-L3 is a Ub-protein hydrolase that is involved in the processing of Ub precursors and ubiquitinated substrates to generate free Ub. The enzyme recognises and hydrolyses isopeptide bonds at the C-terminal glycine of either Ub or Nedd8, a Ub-like protein (Hemelaar et al. 2004, Frickel et al. 2007). Although neither dimerisation nor ligase activity is observed for UCH-L3, this hydrolase can interact with Lys48-linked Ub dimers to protect it from degradation and inhibit its hydrolase activity (Fang et al. 2010). *SmUCH-L3* was highly expressed in adult worms, suggesting an important role at this stage.

A unique USP enzyme analysed in this study was USP-5, which is also an isopeptidase. The transcript levels of this protease showed higher expression in MTS-3.5 h and similar levels in cercariae and adult worms. USP-5 is responsible for the majority of unanchored polyubiquitin disassembly (Eletr & Wilkinson 2011) and another significant finding of this study was the correlation between the expression and activity of *SmUCH-L3* and *SmUSP-5*. Although the fluorogenic substrate utilised does not allow the discrimination between UCH-L3 and USP-5 activities, it is likely that the peptide hydrolysis observed in the cercariae was strongly related to UCH-L3, given its higher expression at this stage.

The WB analysis for the detection of ubiquitinated proteins in *S. mansoni* revealed the characteristic band smearing at all investigated stages, possibly reflecting proteins tagged with a varying number of Ub molecules (Carlson et al. 1987, Pickart et al. 1991). As each Ub moiety adds approximately 8 kDa to the target protein conjugate, the length of the Ub chain influences the apparent molecular mass of Ub conjugates. The accumulation of ubiquitinated conjugates in cercariae compared to adult worms is consistent with the results obtained by Guerra-Sá et al. (2005), showing the lowest activity of the 26S proteasome. Moreover, the most intense accumulation of ubiquitinated conjugates was observed in the egg stage. In this regard, Mathieson et al. (2011) showed development and morphology-specific characteristics of the proteasome-Ub pathway in *S. mansoni* eggs.

In summary, our study addresses the biological role played by this particular UCH sub-family during the *S. mansoni* life cycle and our results raise a number of questions concerning the regulation of proteasome activity and its role in schistosome biology. The recombinant versions of these proteins might allow for the assessment their substrate specificity and specific anti-*Sm*UCHs antibodies could provide a deeper understanding of the role of these enzymes in this parasite.

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