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Molecular and functional characterisation of the heat shock protein 10 of *Strongyloides ratti*☆

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ABSTRACT

Strongyloides stercoralis and *S. ratti* are intestinal parasitic nematodes infecting rats and humans, respectively. Both present extraordinary life cycles comprising a free-living generation in addition to parasitic stages. In search of molecules possibly involved in parasite–host interaction, we performed mass spectrometry to identify excretory/secretory products of *S. ratti*. Amongst others we detected homologs of the heat shock proteins HSP10 and HSP60 (*Sr*-HSP10 and *Sr*-HSP60). HSPs are well known as chaperones involved in stress responses of cells, but recent studies suggest additional roles of small HSPs for parasite biology including immune modulation. To characterise *Sr*-HSP10, we cloned its full-length cDNA, analysed the genomic organisation, tested its presumptive role as an interaction partner of *Sr*-HSP60, studied its transcription in the parasite, and expressed the protein to test its immune responses. The cDNA contains an open reading frame of 330 bp encoding a polypeptide of 110 amino acids with an approximate molecular weight of 10 kDa. The *Sr*-HSP10 protein is highly homologous to that of the human pathogen *S. stercoralis* with only eight amino acid substitutions. Analysis of the genomic organisation of the *Sr*-HSP10 locus revealed that the gene is linked head-to-head to the gene encoding *Sr*-HSP60, and both share a bidirectional promoter. RT-PCR experiments indicated potential independent expression of the *Sr*-HSPs genes. *In situ* hybridisation results demonstrate *Sr*-HSP10 transcription in the gut area. Mammalian and yeast two-hybrid assays show dimerisation of *Sr*-HSP10, but no binding to recombinant *Sr*-HSP60. Immunisation experiments finally revealed a strong immunogenicity of *Sr*-HSP10 and provided evidence for a role in regulating the host–parasite interaction.

1. Introduction

Nematodes belong to the most prevalent organisms, and they are relevant pathogens of humans, animals and plants. Worldwide more than 2 billion humans are infected by gastrointestinal or tissue nematodes and 3.5 billion are exposed to them [1,2]. An estimated 100–200 million people are infected with *Strongyloides stercoralis* (threadworm) [3,4]. The study of host defence-modulating molecules in parasitic systems may help to better understand the host–parasite relationship and reveal new principles of their function.

Heat shock proteins are highly conserved molecules that play vital roles in all cells [5]. Based upon their function as chaperones, they were originally identified on the basis of their increased expression in heat-shocked and stressed cells. However, there is growing evidence that they play a key role also in many other cellular processes [6]. For instance, *Plasmodium falciparum* expresses chaperones and co-chaperones not only for housekeeping functions, but also for highly regulated and finely tuned events specific to its parasitic life cycle [7]. In *Brugia malayi*, the HSP Bm12.6 is able to bind to the IL-10 receptor, providing evidence of its immune modulatory role [8].

The human HSP10 is known as a co-chaperone for HSP60, and exerts immunosuppressive activity [9]. A circulating protein nearly

Abbreviations: gDNA, genomic DNA; HSP, heat shock protein; sHSP, small heat shock protein; iL3, infective larvae; E/S, excretory/secretory; EPF, early pregnancy factor; LC–MS/MS, liquid chromatography–tandem mass spectrometry; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis; EST, expressed sequence tag; SL, spliced leader; w/v, weight/volume; ORF, open reading frame; IPTG, isopropyl-thio-β-D-thiogalactoside; HBSS, Hanks balanced salt solution; ELISA, enzyme-linked immunosorbent assay.

☆ *Note*: Nucleotide sequence for *Strongyloides ratti* heat shock protein 10 (*Sr*-HSP10) plus *Sr*-HSP60 has been deposited in the GenBank Database under the accession number FJ694974.

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identical to HSP10 was originally identified as human extracellular early pregnancy factor (EPF), a principal immunosuppressive agent during pregnancy [10]. About 70% of the human platelet-derived EPF amino acid sequence has been identified [11]. Except for a single residue, the sequence is identical to that of rat and human HSP10. Furthermore, the human EPF and rat mitochondrial HSP10 were found to be functionally interchangeable *in vitro* [12].

In this study we identified and characterised *S. ratti* HSP10 (*Sr*-HSP10) as an excretory/secretory (E/S) product of several life stages of this parasite. Our results show that the cDNA of *Sr*-HSP10 and its genomic organisation are highly conserved including the regulatory region. Furthermore, evidence is provided for the dimerisation of *Sr*-HSP10, and for transcriptional activity in subcuticular tissue, mainly the gut area. The data presented in this study contribute to the elucidation of HSP10 activity in *Strongyloides* and to the growing awareness of parasite HSPs as candidate molecules involved in the parasite–host interaction.

2. Materials and methods

2.1. Parasites

For the isolation of infective larvae (iL3) of *S. ratti*, faecal pellets were collected on days 6–16 following subcutaneous infection of male Wistar rats (Charles River) with 2000 iL3. Charcoal coprocultures were incubated at 26 °C for 120 h. The standard Baermann method was used for recovery of the parasites [13]. Infective L3 were washed three times in sterile Hanks Balanced Salt Solution (HBSS) supplemented with 100 μg/mL penicillin, 100 units/mL streptomycin (Sigma–Aldrich Chemie, Steinheim, Germany). For the recovery of parasitic females, male Wistar rats were sacrificed on day 6 post infection with 2000 iL3. The small intestine was removed, cleaned and opened longitudinally. After collection using the Baermann method the parasitic females were extensively washed in HBSS.

2.2. RNA/DNA isolation and reverse transcription

For RNA isolation, freshly isolated or frozen worms were defrosted on ice in Trizol LS buffer (Invitrogen Corp., Carlsbad, CA, USA), homogenized and the total RNA extracted according to a standard protocol. For the generation of cDNA, 5 μg of total RNA was reverse-transcribed using Superscript III (Invitrogen) and GeneRacer (Invitrogen) oligo (dT) primer according to the manufacturer's instructions. For DNA isolation, a pellet of approximately 250,000 *S. ratti* iL3 were digested overnight at 56 °C with proteinase K (Qiagen, Hilden, Germany) under constant agitation. The sample was then precipitated and stored at 4 °C.

2.3. Preparation of excretory–secretory (E/S) proteins and soluble somatic extracts from infective larvae and parasitic females

After extensive washing, the iL3 and parasitic females were either (i) homogenized for preparation of soluble somatic extracts or (ii) incubated for generation of E/S products. Homogenisation (i) of the worms was performed by fast agitation of the worms in the presence of a single steel ball (2.8 mm diameter) using a Precellys Steel Kit (PeqLab Biotech., Erlangen, Germany); this technique is based on the one described by Laney et al. [14]. Subsequently, the proteins were extracted in the presence of proteinase inhibitors. For the generation of E/S products, (ii) iL3 (4×10^4/ml) and parasitic females (100/ml) were cultured at 37 °C using previously described protocols [15]. The incubation times were 24 h for the iL3 and 72 h for parasitic females with changes of media every 24 h. E/S products were supplemented with protease inhibitors (Roche Diagnostics, Mannheim, Germany) and concentrated 250× (Amicon Ultra 10.000 MWCO filters; Millipore GmbH, Schwalbach, Germany). In experiments involving inhibition of the protein synthesis, cycloheximide (Sigma–Aldrich) was added to the culture medium with a final concentration of 70 mM. As metalloproteinase activity was only transiently observed in the culture and no proteinase activity was observed in a substrate gel after exposure of the nematodes to cycloheximide, a general leakiness of the parasites is unlikely; instead, an active secretion of the released proteins is more likely (data not shown).

2.4. Liquid chromatography–tandem mass spectrometry (LC–MS/MS) analysis

The concentrated E/S proteins were subjected to SDS-PAGE followed by staining with colloidal Coomassie (Invitrogen). The entire lane was cut into 36 gel blocks of equal size. All gel blocks were then digested with trypsin using published procedures [16]. Peptides derived from in-gel digested proteins were analysed by online microscale capillary reversed-phase HPLC coupled to a linear ion trap mass spectrometer (LTQ, Thermo Scientific, San Jose, CA, USA). Samples were loaded onto an in-house packed 100 m i.d. × 15 cm C18 column (Magic C18, 5 m, 200 Å, Michrom Bioresource, Auburn, CA, USA) and separated at approximately 500 nl/min with 30 min linear gradients from 5% to 40% acetonitrile in 0.4% formic acid. After each survey spectrum, the six most intense ions per cycle were selected for fragmentation/sequencing.

All MS datasets were searched against combined protein sequence database containing EST sequences from *S. ratti* and *S. stercoralis* as well as the RefSeq protein sequences for *Caenorhabditis elegans* and *C. briggsae*. Searches were performed using the ProteinPilot search engine (v2.0). The following search parameters were selected: Sample Type: Identification; Cys. Alkylation: Iodoacetamide; Digestion: Trypsin; Instrument: LTQ; Special Factors: Gel-based ID; ID Focus: Amino acid substitutions; Search Effort: Thorough. Proteins were identified based on a minimum of 4.00 'unused score' equivalent to two or more unique peptides of confidence 99.

2.5. Completion of Sr-HSP10 cDNA, Southern blot and gene sequence

A nucleotide search for the designated *S. stercoralis* chaperonin-like EST (GenBank accession number BE580000) was performed using a NCBI nucleotide database. Degenerate *S. stercoralis* chaperonin-like EST primers and cDNA of *S. ratti* infectious larvae were used in RT-PCR. RT reactions without reverse transcriptase served as a negative control. The RT-PCR product was cloned into the TOPO TA vector and sequenced. To obtain the 5′ and 3′ regions and spliced leader sequences were obtained by [17] (SL)-RT-PCR and RACE, respectively. The full-length *Sr*-HSP10 cDNA was amplified using HSP10 forward primer 5′-CTT CTT TCC GCT GTT CG-3′ and HSP10 reverse primer 5′-TTA GTT AGT GAG TTT GGC-3′. DNA sequencing was performed using the Sanger method [18] by the service laboratory of the Center for Molecular Neurobiology Hamburg (Germany) using an ABI Prism 377 automated DNA sequencer (Applied Biosystems) and the prism dye terminator cycle sequencing ready reaction kit (PerkinElmer). Sequence alignment was performed by ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html). Nucleic acid sequences were translated to amino acid sequences using EMBOSS-Transeq (http://www.ebi.ac.uk/Tools/emboss/transeq/index.html). N-terminal mitochondrial targeting peptides were predicted by (iPSORT) (http://hc.ims.u-tokyo.ac.jp/iPSORT). The deduced amino acid sequences were submitted for similarity searches against non-redundant protein sequence databases at the National Center for Biotechnology Information using BlastP software.