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A PAL for *Schistosoma mansoni* PHM

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ABSTRACT

Parasitic helminth neuromuscular function is a proven target for chemotherapeutic control. Although neuropeptide signalling plays a key role in helminth motor function, it has not yet provided targets for known anthelmintics. The majority of biologically active neuropeptides display a C-terminal amide (NH_2) motif, generated exclusively by the sequential action of two enzymes, peptidylglycine α-hydroxylating monooxygenase (PHM) and peptidylglycine α-amidating lyase (PAL). Further to our previous description of a monofunctional PHM enzyme (SmPHM) from the human blood fluke *Schistosoma mansoni,* here we describe a cDNA encoding *S. mansoni* PAL (SmPAL). SmPAL is a monofunctional enzyme which, following heterologous expression, we find to have functionally similar catalytic activity and optimal pH values, but key catalytic core amino acid substitutions, when compared to other known PALs including those found in humans. We have used *in situ* hybridisation to demonstrate that in adult schistosomes, SmPAL mRNA (*Sm-pal-1*) is expressed in neuronal cell bodies of the central nervous system, consistent with a role for amidated neuropeptides in *S. mansoni* neuromuscular function. In order to validate SmPAL as a putative drug target we applied published RNA interference (RNAi) methods in efforts to trigger knockdown of *Sm-pal-1* transcript in larval schistosomula. Although transcript knockdown was recorded on several occasions, silencing was variable and inconsistent and did not associate with any observable aberrant phenotype. The inconsistent outcomes of RNAi suggest that there may be tissue-specific differences in the applicability of RNAi methods for *S. mansoni*, with neuronal targets proving more difficult or refractory to knockdown. The key role played by schistosome amidating enzymes in neuropeptide maturation make them appealing as drug targets; their validation as such will depend on the development of more robust reverse genetic tools to facilitate efficient neuronal gene function studies.

1. Introduction

Diseases caused by helminth parasites remain one of the most significant public health problems facing society. The most prevalent and chronic flatworm infection of humans, resulting in a major, poverty-related problem in the developing world is schistosomiasis. *Schistosoma mansoni*, designated a Neglected Tropical Disease, is a major cause of human schistosomiasis, having a global prevalence of over 200 million in an estimated 74 developing countries [1]. The difficult situation facing health organisations involved in the reduction of morbidity due to schistosomiasis is aggravated by over-reliance on a single drug, praziquantel (PZQ). Although resistance does not appear to be widespread, it has been generated in the laboratory [2], and has occurred transiently in field situations [3,4]. These observations, coupled with increasing distribution and usage of PZQ, provide circumstances known to foster the development of drug resistance. As a result of this dependence and the consequent risk of inadvertently selecting for drug-resistant parasites, there is a pressing need to exploit and develop new drug targets and to develop novel chemotherapeutic agents for the treatment and control of schistosomiasis [5].

The neuropeptidergic system is central to flatworm motor control—neuropeptide signalling molecules appear to be involved in the modulation of a range of biological processes throughout the phylum Platyhelminthes [6–11]. So far, we have identified 18 distinct amidated peptides in *S. mansoni* [12,13], several of which have been localised to the CNS and PNS of larval and adult schistosomes [13–19]. Some of these peptides have also been shown to exert potent myoexcitatory effects on isolated muscle fibres from adult schistosomes, and as such are thought to be directly involved in muscle function [20]. Consequently, the neuropeptidergic component of the *S. mansoni* nervous system represents an attractive repository of novel drug targets.

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The majority of neuropeptides require a carboxy-terminal amide motif to confer biological activity, as demonstrated by the reduced activity of most non-amidated glycine-extended precursors (<10%) in comparison to their α-amidated counterparts [21–24]. Two key enzymes act sequentially in the only known mechanism for secretory peptide amidation: peptidylglycine α-hydroxylating monooxygenase (PHM), a copper, oxygen and ascorbate dependant enzyme which catalyses hydroxylation of the C-terminal glycine present on a peptide precursor; and peptidylglycine α-amidating lyase (PAL), a zinc dependant enzyme which catalyses dealkylation of the hydroxylated intermediate to form the α-amidated neuropeptide and glyoxylate [21,23]. This process occurs throughout the Metazoa, the importance of which is demonstrated in larval *Drosophila melanogaster* mutants, where the deletion of the PHM gene results in embryonic death [25].

In higher organisms PHM and PAL are commonly encoded by the same transcript, generating a bifunctional protein designated peptidylglycine α-amidating monooxygenase (PAM) [24]. In stark contrast, the genomes of a number of invertebrates including the flatworm, *Dugesia japonica*, encode single copies of monofunctional PHM and PAL [26], while others possess multiple monofunctional enzymes which can be membrane associated, soluble or even inactive [24,27]. Crucially, adult *S. mansoni* express monofunctional PHM (SmPHM), which is functionally divergent from the mammalian homologue [28]. These organisational and functional differences between the schistosome and mammalian amidating enzymes encourage the characterisation of schistosome amidating enzymes and their exploration as potential drug targets.

Even though neuropeptide signalling is a core component of schistosome neural function and neuromuscular modulation, it has not been a major focus for novel drug target discovery and validation in schistosomes, largely hindered by the absence of known neuropeptides and their cognate receptors. Recent proliferations in EST datasets and publication of the *S. mansoni* genome sequence [29] have fuelled the recent discovery of SmPHM and 18 schistosome neuropeptides, making neuropeptide signalling ripe for more detailed interrogation and validation as a source of suitable targets for schistosome control [13,28,29]. Further, genome level bioinformatics has uncovered a set of at least 24 putative schistosome neuropeptide receptors [29], which are appealing because of the proven druggability of many known G-protein coupled receptors (GPCRs) and their suitability for high throughput screens. We hypothesise that neuropeptide activation/processing provides an alternative and potentially powerful set of drug targets. Since the combined activities of PHM and PAL are the only known conduit to neuropeptide amidation/activation and we already know schistosomes possess multiple amidated peptide messengers, these enzymes have much appeal as drug target candidates. Previously we demonstrated that one half of the neuropeptide amidating pathway in *S. mansoni* is functionally distinct from that seen in vertebrates and, indeed, other invertebrates, such that we now switch focus to the second half of the amidation pathway, PAL. Here we report the identification, localisation, cloning and functional characterisation of a *S. mansoni* cDNA encoding a novel, monofunctional PAL enzyme. Further, we attempt to validate its candidature as a drug target through the application of RNA interference (RNAi).

2. Materials and methods

2.1. Schistosoma mansoni culture

Schistosome infected mice supplied by Dr. Fred Lewis, Biomedical Research Institute, Rockville MD, USA, were maintained and processed at Iowa State University (ISU). Adult schistosomes recovered from infected mice were either processed for whole-mount *in situ* hybridisation (WISH) experiments as previously described [30] or stored in RNAlater to allow subsequent RNA extraction, before being shipped to Queen's University Belfast (QUB) on dry ice. Schistosomes used in the RNA interference (RNAi) experiments were maintained at QUB in *Biomphalaria glabrata* snails; cercariae were shed from infected snails by photostimulus, and mechanically transformed to schistosomula by vortexing, schistosome bodies were isolated from tails by centrifugation over a solution of 30% Percoll in water (500 × *g* for 15 min at 4 °C), protocol adapted from [53]. Schistosomula were maintained *in vitro* in complete RPMI media [31] containing 20% serum, at 37 °C in a 5% CO_2 atmosphere.

2.2. Bioinformatics

BLAST methodology [32] was employed to uncover novel putative PAL encoding transcripts from *S. mansoni* EST and genomic datasets. BLASTn and tBLASTn tools were used at the National Centre for Biotechnology Information (NCBI) BLAST server (http://blast.ncbi.nlm.nih.gov/Blast) as well as the *S. mansoni* genome database, SmGeneDB (http://www.genedb.org/genedb/smansoni). Searches were performed on the 'est_others' database, had an expect value of >1000, and were limited by the query '*S. mansoni*'. Known PAL genes [25] were used as query sequences. Returns were translated in all six reading frames (http://www.expasy.org/tools/dna.html), and examined for both the presence of PAL specific motifs and similarity to other PAL genes/proteins (http://www.ebi.ac.uk/Tools/InterProScan) [33]. Sequences were also analysed for the presence of an N-terminal secretory signal peptide, and putative N-glycosylation sites, using the online SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP/) [34], and NetNGlyc servers (http://www.cbs.dtu.dk/services/NetNGlyc/) respectively.

2.3. RNA extraction and PCR analyses

Messenger RNA was extracted from between 10 and 50 mg of *S. mansoni* tissue using Dynabeads mRNA Direct™ kit (Invitrogen) according to the manufacturer's instructions. mRNA eluted in Tris–HCl was quantified using a NanoDrop™ 1000 spectrophotometer. Separate populations of 5′ and 3′ RACE-ready cDNAs were generated using the SMART™ RACE cDNA Amplification kit (Clontech/Takara), using ≤1 μg mRNA per synthesis according to the manufacturer's instructions. RACE cDNA was stored at −20 °C until use.

Gene specific primers (GSP) designed against the putative *S. mansoni* PAL (SmPAL) ESTs (GenBank accession numbers AM047261 and AM043268) were used in 50 μl PCR reactions (94 °C for 2 min; 40 cycles of: 94 °C 1 min, 55 °C 1 min, and 72 °C 1 min; 72 °C for 7 min) to confirm the presence of SmPAL in the following reaction mixture: 5 μl 10× PCR buffer (Invitrogen), 3 μl $MgCl_2$ (50 mM, Invitrogen), 1 μl dNTP mix (10 mM, Promega), 1 μl of primer SmPAL-F1 and SmPAL-R1 (20 μM; Table 1), 1 μl cDNA template, 0.3 μl Platinum® *Taq* DNA Polymerase (5 U/μl, Invitrogen), and ddH_2O to 50 μl. Reaction products were TOPO-TA cloned (Invitrogen) and at least 3 plasmids were sequence verified.

2.4. Functional SmPAL expression

The SmPAL open reading frame (1236 bp) was PCR-amplified using sense primer SmPAL-Xba1-F and antisense primer SmPAL-Bgl2-R (see Table 1) incorporating 5′ Xba1 and Bgl2 restriction sites respectively. Products were TOPO-TA cloned (Invitrogen) and sequence verified. Using Xba1, Bgl2, and pBS.rhodopsin, the rhodopsin tag (11 amino acids) was appended to the C-terminus of SmPAL. pCIS.SmPAL-rhod was then created using Xba1 and Hpa1. pBS.SmPAL-rhod was used as a template to PCR-amplify SmPAL-rhod without the native *S. mansoni* signal peptide (aa 1–18)