Toward Understanding the Functional Role of Ss-riok-1, a RIO Protein Kinase-Encoding Gene of Strongyloides stercoralis

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Abstract

Background: Some studies of Saccharomyces cerevisiae and mammals have shown that RIO protein kinases (RIOKs) are involved in ribosome biogenesis, cell cycle progression and development. However, there is a paucity of information on their functions in parasitic nematodes. We aimed to investigate the function of RIOK-1 encoding gene from Strongyloides stercoralis, a nematode parasitizing humans and dogs.

Methodology/Principal Findings: The RIOK-1 protein-encoding gene Ss-riok-1 was characterized from S. stercoralis. The full-length cDNA, gDNA and putative promoter region of Ss-riok-1 were isolated and sequenced. The cDNA comprises 1,828 bp, including a 377 bp 5’-UTR, a 17 bp 3’-UTR and a 1,434 bp ORF encoding a protein of 477 amino acids containing a RIOK-1 signature motif. The genomic sequence of the Ss-riok-1 coding region is 1,636 bp in length and has three exons and two introns. The putative promoter region comprises 4,280 bp and contains conserved promoter elements, including four CAAT boxes, 12 GATA boxes, eight E-boxes (CANNTG) and 38 TATA boxes. The Ss-riok-1 gene is transcribed throughout all developmental stages with the highest transcript abundance in the infective third-stage larva (iL3). Recombinant Ss-RIOK-1 is an active kinase, capable of both phosphorylation and auto-phosphorylation. Patterns of transcriptional reporter expression in transgenic S. stercoralis larvae indicated that Ss-RIOK-1 is expressed in neurons of the head, body and tail as well as in pharynx and hypodermis.

Conclusions/Significance: The characterization of the molecular and the temporal and spatial expression patterns of the encoding gene provide first clues as to functions of RIOKs in the biological processes of parasitic nematodes.

Introduction

Strongyloides stercoralis is a parasitic nematode infecting human beings and dogs, and causes a fatal, disseminated hyperinfection in immuno-compromised patients [1,2]. The life cycle of S. stercoralis, like other members of Strongyloides and related genera, is more complicated than that of most obligatory parasitic nematodes. S. stercoralis can execute both parasitic and free-living generations of development. Parasitic female adults (P Female) live in the host intestine and produce sexually differentiated eggs by mitotic parthenogenesis. Eggs of S. stercoralis hatch in the host intestine and in immune-competent hosts, newly hatched post parasitic first-stage larvae (PP L1) are passed in the feces. Once in the environment, female post-parasitic L1 can either develop directly (homogonically) to infective third stage larvae (iL3) and infect a host or develop heterogonically to free-living female adults (FL Female). Male PP L1s invariably develop via the heterogenic route to the free-living male adults (FL Male). Post-free-living L1 (PFL L1) produced by FL Female and FL Male are all female and develop to iL3. Female PP L1 of S.
Reproduction of functions of the essential genes that regulate the development and life cycle of this parasite, the understanding of molecular factors mised host, with geometric expansion of parasite numbers and may proceed for sequential generations in an immuno-compro-
somatic tissues and ultimately establish as a new generation of P
within the intestine, penetrate the intestinal wall, invade the
nematode diseases.

Interventions for Strongyloides and other related parasitic
diseases. We found that S. stercoralis which causes canine and human
diseases. We found that Ss-RIOK-1 has high sequence identities (50–65%) to its homologues from both verte-
brates and invertebrates. It also has abilities of phosphor-
ylation and auto-phosphorylation in vitro. Ss-riok-1 tran-
script is present in all stages of S. stercoralis with more abundance in the parasitic stages than in the free-living
stages, along with the gene expression in neuron system of post free-living L1 and body muscle of iL3, indicating that it plays important role in the development and infection of S. stercoralis. The findings have important implications for understanding the function of RIOK-1 in the development of parasitic nematodes.

Stercoralis may develop precociously to autoinfective L3 (ail3) within the intestine, penetrate the intestinal wall, invade the somatic tissues and ultimately establish as a new generation of P Female in the primary host intestine. This process of autoinfection may proceed for sequential generations in an immuno-compro-
mised host, with geometric expansion of parasite numbers and involvement of multiple body tissues, possibly leading to a fatal outcome for such immuno-compromised hosts [3].

In contrast to the relative wealth of information on the complex life cycle of this parasite, the understanding of molecular factors regulating its developmental biology is limited. Elucidating the functions of the essential genes that regulate the development and reproduction of S. stercoralis could facilitate the discovery of novel interventions for strongyloidiasis and other related parasitic nematode diseases.

Protein kinases are a large group of enzymes that are crucial in the regulation of a wide range of cellular processes, including cell-cycle progression, transcription, DNA replication and metabolic functions [4]. Based on their structures, protein kinases can be classified into eukaryotic protein kinases (ePKs) and atypical protein kinases (aPKs) [5]. The ePKs contain a conserved catalytic domain that phosphorylates enzymes of signal transduction pathways that regulating many biological processes. The aPKs are active kinases containing kinase domains with limited sequence similarity to the conserved catalytic domain of ePKs. According to their characteristics in their kinase domains and functions in different biological processes, the aPKs have been divided into 13 families, one of which contains the RIO kinases. There are currently four members of the RIOK family, RIOK-1, RIOK-2, RIOK-3 and RIOK-B [6,7]. RIOK-1 and RIOK-2 are strongly conserved from archaea to human, whereas RIOK-3 is only found in metazoans, and RIOK-B is restricted to cubacteria [6]. RIOK-1 controls cell cycle progression and chromosome maintenance in yeast [8–10] and participates in aspects of ribosomal biogenesis including 20S rRNA cleavage and maturation of ribosomal small subunits in both yeast and human cells [8,11]. The genome of the free-living nematode Caenorhabditis elegans also encodes RIOK-1 [12]. A large-scale double-stranded RNA interference (RNAi) study of C. elegans showed that the silencing of C-riok-1 leads to embryonic lethality and arrest of larval development [13–17]. This finding suggests that RIOK-1 is essential for development and growth of nematodes. In spite of the functional importance of this molecule in C. elegans, there is no published information on the functions of RIOK-1 in any related parasitic nematodes, other than DNA sequence characterization and bioinformatic analyses of RIOK encoding genes of the ovine parasitic nematodes Trichostrongylus vitrinus [18] and Haemonchus contortus [19]. These studies revealed that riek-1 of T. vitrinus is transcribed at the highest level in iL3 and proposed that riek-1 of H. contortus is a potential drug target. However, almost nothing is known about the function of this gene for any parasite.

Transgenesis, which is very useful for functional genomic studies in C. elegans [20], was successfully established in S. stercoralis [21,22], thus providing us with a technical platform to investigate the functions of genes in this parasite [21–23]. Because of the potential of this parasitic nematode for functional genomic studies, we aimed to isolate and characterize Ss-riok-1 and to explore the temporal and spatial expression patterns of this gene, with a view towards uncovering its function. Information on the function of Ss-
riok-1 will contribute to an evaluation of RIOKs as potential targets of drugs directed against S. stercoralis and related parasitic nematodes.

Materials and Methods

Ethics statement

The S. stercoralis (UPD strain) was maintained in prednisolone-treated Beagles in accordance with protocol (Permit Number: SYXK-0029) approved by the Committee on the Ethics of Animal Experiments of Hubei Province. The care and maintenance of animals were in strict accordance with the recommendations in the Guide for the Regulation for the Administration of Affairs Concerning Experimental Animals of P.R. China.

Parasite maintenance and culture

The UPD strain of S. stercoralis was maintained in prednisolo-
treated dogs and cultured as described [24,25]. RNA and genomic DNA were extracted from iL3s concentrated from charcoal coprocultures using the Baermann funnel technique [26] after 7–10 days of incubation at 22°C. The iL3s were washed several times with a sterile buffered saline called BU buffer [24,27] to reduce bacterial contamination. Free-living adult S. stercoralis for micro-injection were isolated from charcoal coprocultures using the Baermann funnel, incubated for two days at 22°C and then placed on Nematode Growth Medium (NGM) agar plates seeded with Escherichia coli OP50 [24].

DNA and cDNA preparation

Total genomic DNA was extracted from ~10,000 iL3 larvae using a small-scale sodium proteinase K extraction [28] followed by mini-column (Promega) purification. Total DNA of S. stercoralis was extracted from ~30,000 iL3 by Trizol reagent extraction (Life Technologies). RNA yields were estimated spectrophotometrically (NanoDrop Technologies, Thermo). Total 5’-ends cDNA and 3’-ends cDNA were synthesized by Smart RACE Kit (BD Bioscience) following the manufacturer’s protocol; cDNAs were stored at ~20°C.

Isolation of Ss-riok-1 cDNA and promoter region

Degenerate primers 1F and 2R (Table S1) were designed based on the alignment of riek-1 homologues of H. contortus (GenBank
were constructed employing Genome-Walker Kit (BD Bioscience), technology) and sequenced. To isolate the promoter sequence, four genomic DNA libraries were constructed employing Genome-Walker Kit (BD Bioscience), technology) and sequenced. To isolate the entire promoter region, four genomic DNA libraries were examined separately on agarose gels, and the assemblies according to the sequence obtained through 5'- and 3'-RACE PCR. Then a pair of primers with restriction sites (Ss-riok-1-BamHI and Ss-riok-1-XhoI, Table S1) were designed to amplify the coding region of Ss-riok-1 using the following cycling conditions: initial 94 °C, 5 min; then 94 °C, 30 s, 60 °C, 30 s, 72 °C, 2 min for 30 cycles; final extension at 72 °C for 10 min. The PCR product was then cloned into pMD19-T vector (Takara Biotechnology) and sequenced.

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Bioinformatic and phylogenetic analyses

The sequence of Ss-riok-1 was compared by BLASTx [30] with sequences in non-redundant databases from NCBI (http://www.ncbi.nlm.nih.gov/) to confirm the identity of genes isolated. The translation of cDNA of Ss-riok-1 into predicted amino acid sequences was performed by free software Bioedit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html#downloads). The protein motifs of Ss-riok-1 were identified by searching the databases PROSITE [31] (www.expasy.ch/tools/scanspit1.html) and Pfam [32] (www.sanger.ac.uk/Software/Pfam/). Ss-riok-1 was aligned with the homologues from selected species using the program MAFFT 7.0 [33] (http://mafft.cbrc.jp/alignment/software/), and the functional domains and subdomains were identified in the protein alignment. Promoter elements in the 5'-UTR were predicted using the transcription element search system Matrixcatch (http://www.gene-regulation.com/cgi-bin/mcatch/MatrixCatch.pl) [34].

For phylogenetic analysis, the amino acid sequences of 27 homologues were retrieved from GenBank databases and the alignment of protein sequences was carried out by Clustal X [35] and manually adjusted. The species selected were nine nematodes, including Ascaris suum (ERG07004.1), Brugia malayi (EPI30009.1), Caenorhabditis briggsae (CAP24959.2), Caenorhabditis elegans (CCD67367.1), Caenorhabditis remanei (XP_003090834.1), Haemonchus contortus (ADW23592.1), Loa loa (NP_005135673.1), Trichostygus vitrinus (CAR64255.1), Watcheria bancrofti (EJW82234.1), and 14 non-nematode species, including Aedes aegypti (XP_001661999.1), Arabidopsis thaliana (NP_0511000.1, AAPM5700.1, NP_1800711.1), Caenorhabditis elegans (NP_001109981.1, AAH79173.1), Saccharomyces cerevisiae (CAA99317.1), Xenopus laevis (NP_001116165.1), Xenopus tropicalis (XP_004915351.1). The phylogenetic analysis was conducted using the neighbor-joining (NJ), maximum parsimony (MP) and maximum likelihood (ML) methods based on Jones-Taylor-Thornton (JTT) model in the MEGA v.5.0 [36]. Confidence limits were assessed by bootstrapping using 1,000 pseudo-replicates for NJ, MP and ML, and other settings were obtained using the default values in MEGA v.5.0 [36]. A 50% cut-off value was implemented for the consensus tree.
overnight and then concentrated by centrifugation at 10000 rpm/min for 2 min. The bacteria were re-suspended in 50 mM Tris-Cl with 0.1 M NaCl, passed through a 0.45 μm filter and loaded onto a 1 mL GST rap 4B affinity columns (GE Healthcare). The bound Ss-RIOK-1 was eluted with 50 mM Tris-HCl, 40 mM reduced glutathione, pH 8.0. The elution was concentrated using an Ultra-15 50 KD centrifugal filter devices (Millipore). The final concentration was 1 mg/mL. As a control, E. coli Transetta cells were transformed with null pGEX-4T-1 vector, incorporating a GST tag. The GST protein was purified using the same method as described above.

Kinase assays

All assays were performed in 20 μL reaction volumes containing 25 mM Tris pH 7.5, 50 mM NaCl and 2 mM MgCl2 [46]. 10 μg purified GST-Ss-RIOK-1 were added into the autophosphorylation reaction; 2 μg GST-Ss-RIOK-1 and 9 μg myelin basic protein (MBP) were added to each phosphorylation reaction. In the control group, the GST-Ss-RIOK-1 was replaced with GST. All components were mixed prior to the addition of 1 μCi [γ-32P] ATP.

Transformation constructs and transformation of S. stercoralis

To make the plasmid for transgenesis, the promoter region of Ss-riok-1 was digested with restriction enzymes PsI and AgeI (Thermo) and gel-purified by TIANgen Gel purification kit (TIANgen Biotech). The purified product was then subcloned into the promoter-less vector pAA01 [29] to create a plasmid pRP1 (Fig. S1). The constructs was extracted by TIANpure Midi Plasmid Kit (TIANgen Biotech) and then was diluted to 30 ng/μL and stored at −20°C.

Adult FL Female S. stercoralis were transformed by gonadal micro-injection using an established approach [21]. Briefly, 30 ng/μL of plasmid Ss-riok-1p::gfp::Ss-era-1t [pRP1] were injected into the distal gonads of individual worms. Single females transformed with pRP1 were then paired with one or two FL adult males on an NGM+OP50 plate and incubated at 22°C for egg laying. F1 progeny were screened for fluorescence at 24, 48 and 72 h, respectively, after microinjection. S. stercoralis larvae were screened for expression of GFP fluorescent reporter transgenes using an Olympus SZX12 stereomicroscope with epifluorescence. Worms with GFP expression were examined in detail using an Olympus BX60 compound microscope equipped with Nomarski contrast (Olympus America Inc.). Specimens were immobilized on a 2% agarose pad (Lonza), anesthetized using 20–50 mM levamisole (Olympus America Inc.). Specimens were immobilized on a 2% agarose pad (Lonza), anesthetized using 20–50 mM levamisole (Olympus America Inc.). Specimens were immobilized on a 2% agarose pad (Lonza), anesthetized using 20–50 mM levamisole (Olympus America Inc.). Specimens were immobilized on a 2% agarose pad (Lonza), anesthetized using 20–50 mM levamisole (Olympus America Inc.). Specimens were immobilized on a 2% agarose pad (Lonza), anesthetized using 20–50 mM levamisole (Olympus America Inc.). Specimens were immobilized on a 2% agarose pad (Lonza), anesthetized using 20–50 mM levamisole (Olympus America Inc.). Specimens were immobilized on a 2% agarose pad (Lonza), anesthetized using 20–50 mM levamisole (Olympus America Inc.). Specimens were immobilized on a 2% agarose pad (Lonza), anesthetized using 20–50 mM levamisole (Olympus America Inc.). Specimens were immobilized on a 2% agarose pad (Lonza), anesthetized using 20–50 mM levamisole (Olympus America Inc.). Specimens were immobilized on a 2% agarose pad (Lonza), anesthetized using 20–50 mM levamisole (Olympus America Inc.). Specimens were immobilized on a 2% agarose pad (Lonza), anesthetized using 20–50 mM levamisole (Olympus America Inc.). Specimens were immobilized on a 2% agarose pad (Lonza), anesthetized using 20–50 mM levamisole (Olympus America Inc.). Specimens were immobilized on a 2% agarose pad (Lonza), anesthetized using 20–50 mM levamisole (Olympus America Inc.). Specimens were immobilized on a 2% agarose pad (Lonza), anesthetized using 20–50 mM levamisole (Olympus America Inc.). Specimens were immobilized on a 2% agarose pad (Lonza), anesthetized using 20–50 mM levamisole (Olympus America Inc.). Specimens were immobilized on a 2% agarose pad (Lonza), anesthetized using 20–50 mM levamisole (Olympus America Inc.). Specimens were immobilized on a 2% agarose pad (Lonza), anesthetized using 20–50 mM levamisole (Olympus America Inc.). Specimens were immobilized on a 2% agarose pad (Lonza), anesthetized using 20–50 mM levamisole (Olympus America Inc.). Specimens were immobilized on a 2% agarose pad (Lonza), anesthetized using 20–50 mM levamisole (Olympus America Inc.). Specimens were immobilized on a 2% agarose pad (Lonza), anesthetized using 20–50 mM levamisole (Olympus America Inc.). Specimens were immobilized on a 2% agarose pad (Lonza), anesthetized using 20–50 mM levamisole (Olympus America Inc.). Specimens were immobilized on a 2% agarose pad (Lonza), anesthetized using 20–50 mM levamisole (Olympus America Inc.).

Results

Characterization of the Ss-riok-1 cDNA

The full-length cDNA of Ss-riok-1 (GeneBank Accession No. KJ701282) is 5889 bp in length. The 377 bp 5'-UTR of cDNA is interrupted by two large introns of 711 bp and 3148 bp in size, respectively. The coding sequence of Ss-riok-1 encompasses 1,091 bp with the flavin domain-containing protein-encoding gene. The isolated 5'-upstream region of the start codon of Ss-riok-1 identified (Fig. 1). The Ss-RIOK-1 shares high sequence identity (50-65%) to RIOK-1s from a diverse range of organisms, including vertebrates, amphibians, fish, plants and nematodes, with the highest identity (63%) to Ar-RIOK-1 from A. suum.

Alignment of the amino acid sequences of Ss-RIOK-1 with the homologues from selected species (Fig. 1) shows that the conserved regions include the ATP binding motif (sub-domains I and II), the flexible loop, the hinge region (subdomain V), the active site (sub-domain VIb), the metal binding loop (DFG loop, subdomains VII and VIII) and other features of RIOK-1s, such as the C terminus of ATP-binding motif G-x-[ILV]-S-T-G-K-E and the altered I-D-V-[SAQ] in the metal-binding motif of Ss-RIOK-1. The key residues "Asp" and "Asn" essential for protein kinase activity in the active sites of RIOK-1s, which are involved in catalytic function and are conserved in all ePKs [4,9]. The amino acid sequences in regions external to these functional subdomains were more divergent than the sequences within them (Fig. 1).

Relationship of Ss-RIOK-1 with orthologues from other species

Results of phylogenetic analyses (Fig. 2) showed that there is concordance in topology among the MP, ML and NJ trees. Ss-RIOK-1 groups with orthologues from clade V nematodes [47] with strong (99%) nodal support. The RIOK-1s from parasitic nematodes representing clade III [47] grouped together with strong (93%) support; all nematode RIOK-1s formed a cluster with absolute support to the exclusion of 17 RIOK-1s from 13 non-nematode species. Among the 17 RIOK-1s representing 13 non-nematode species, RIOK-1s from plants, mammals or insects each grouped together, respectively with high bootstrap support (99–100%). The RIOK-1s from other vertebrates, including fish and amphibians, grouped with the RIOK-1s from mammals with strong bootstrap support respectively (100%).

Genetic structure of Ss-riok-1 and comparison with orthologues from C. elegans and H. contortus

The genomic DNA representing Ss-riok-1 (GeneBank Accession No. KJ701282) is 5889 bp in length. The 377 bp 5'-UTR from cDNA is interrupted by two large introns of 711 bp and 3148 bp in length, respectively. The coding sequence of Ss-riok-1 encompasses 1,636 bp, containing three exons of 284–387 bp in size and two introns of 64 bp and 138 bp in size, respectively. The 17 bp 5'-UTR of Ss-riok-1 follows the third exon of the coding sequence (Fig. 3). Comparison with Ce-riok-1 (MO1B12.2a, sequences were retrieved from WormBase) from C. elegans and Hc-riok-1 from H. contortus [19] showed that these two homologues contain more introns than Ss-riok-1. The coding sequence of Ce-riok-1 contains eight exons of 72–532 bp in size and seven introns of 58–857 bp in size, whereas Hc-riok-1 has 16 exons of 61–200 bp in size and 15 introns of 30–520 bp in size [19].

Analysis of the predicted Ss-riok-1 promoter

The isolated 5'-upstream region of the start codon of Ss-riok-1 is 4,280 bp in size (GeneBank Accession No. KJ701282). Bioinformatic analysis of transcriptomic and genomic data from S. stercoralis revealed that the region between Ss-riok-1 and the upstream gene was 6,854 bp. The gene upstream of Ss-riok-1 encoded a putative falvin domain-containing protein and is transcribed in the opposite orientation of Ss-riok-1. The putative promoter region of this gene was 3,665 bp; the 4,280 bp DNA region upstream of the start codon of Ss-riok-1 overlapped by 1,091 bp with the flavin domain-containing protein-encoding gene.
Comparison between the isolated 5'-upstream region of Ss-riok-1 and the homologous region in Ce-riok-1 (4,242 bp upstream of the start code of Ce-riok-1 retrieved from WormBase) showed a sequence identity of 42.7% (Fig. S2). The putative promoter regions of both genes are A+T rich, with the A+T content of 81.1% for Ss-riok-1 and 68.1% for Ce-riok-1, respectively. Further analysis failed to detect CpG islands in either promoter region, but found a GC box (GGCGG) in the promoter region of Ce-riok-1 that is absent from that of Ss-riok-1. This analysis highlighted several promoter elements, including 38 TATA boxes, four CAAT (CCAAT) or inverse CAAT (ATTGG), 12 GATA (WGATAR), 19 inverse GATA (TTATC) and eight E-boxes (CANNTG) in the promoter region of Ss-riok-1. With the exception of the GC-boxes, CAAT boxes and inverse CAAT boxes, there are generally fewer such elements in the promoter region of Ce-riok-1 than in that of Ss-riok-1. There are 10 CAAT (CCAAT) or inverse CAAT (ATTGG), one GC-box, two GATA (WGATAR), seven inverse GATA (TTATC), seven E-boxes (CANNTG) and six TATA boxes in the promoter region of Ce-riok-1. The four nucleotides preceding the start codon (ATG) are AAGG for Ss-riok-1 and AAAC for Ce-riok-1. The AAAC sequence observed in Ce-riok-1 differs from the adenine tract AAAA more frequently seen in C. elegans genes [48]. The predicted promoter elements are scattered across the promoter regions of the two genes, with no apparent pattern to their distribution.

Transcriptional analysis of Ss-riok-1

Ss-riok-1-specific transcripts were detected in all developmental stages of S. stercoralis examined (Fig. 4). Abundance of these transcripts increases significantly during the transition from PFL...
Figure 2. The Neighbor-joining tree of *Strongyloides stercoralis* Ss-RIOK-1 with 27 homologues from 23 selected species. These species contain nine nematode species, two plant species, two insects, three fish and amphibian species and six mammalian species. The RIOK-1 from *Saccharomyces cerevisiae* (CAA99317.1) is used as the outgroup. GenBank accession numbers of the homologous sequences are listed beside the species name. Bootstrap values are displayed above or below the branches. doi:10.1371/journal.pntd.0003062.g002

Figure 3. The gene structure of *Ss-riok-1* with comparison to its homologues from *Caenorhabditis elegans* and *Haemonchus contortus*. Black boxes indicate the exons, with the numbers above indicating the length of exon. Introns are indicated by slanted lines between the exons, with the numbers indicating the intron length. The 5′- and 3′- untranslated regions (UTR) of *Ss-riok-1* and *Ce-riok-1* are indicated with white boxes, with the numbers above the box indicating the length of the UTR. doi:10.1371/journal.pntd.0003062.g003
L1 to L3, and remains at a high level in the host-derived L3+. L3+ develop to the parthenogenetic P female during their migration in the host and reach the intestine; a significant decrease with the transcripts abundance of Ss-riok-1 \((p<0.05)\) was found during migration and development. The reduced abundance of Ss-riok-1 transcripts during development of PP L1s to FL females was also detected. The abundance of Ss-riok-1 transcripts in iL3 is significantly greater than in PP L3 \((p<0.001)\). By contrast, the abundance of Ss-riok-1 transcripts are significantly higher in P female and PP L1 than in FL female and PFL L1, respectively \((p<0.001)\).

**Protein kinase activity of recombinant Ss-RIOK-1**

The activities of many protein kinases include phosphorylation and auto-phosphorylation. It is reported that RIOK-1 could also phosphorylate the common protein kinase substrate MBP as well as RIOK-1 itself \[49\]. To assess the kinase activity of Ss-RIOK-1, recombinant GST-Ss-RIOK-1 with a GST tag (designated GST-Ss-RIOK-1) was expressed in E. coli (Fig. 5A). Purified recombinant GST-Ss-RIOK-1 incubated with \[^{32}\text{P}\] ATP only or in the presence of MBP showed radioactive signals associated with the GST-Ss-RIOK-1 and MBP, respectively, indicating that the GST-Ss-RIOK-1 is capable of both phosphorylation and auto-phosphorylation (Fig. 5B).

**Localisation of Ss-RIOK-1 expression**

To determine the anatomic expression pattern of Ss-riok-1, larval progeny of FL Female of *S. stercoralis* transformed with the construct pRP1 were screened for GFP expression. Some of the immature eggs had GFP expression, even when they were still in the vulva of the female adults (data not shown). After 24 h, newly hatched transgenic PFL L1s exhibited GFP expression throughout the body, with strongest expression at the boundary between pharynx and intestine (Fig. 6A and B). After 72 h, strong GFP
expression under the Ss-riok-1 promoter was seen in the nervous system, including some head neurons, body neurons and tail neurons as well as in pharynx and hypoderms of transgenic PFL L1s and PFL L2s (Fig. 7A and B). Processes of head neurons go through the body of these larvae, connecting to neurons in the body and tail. The nervous system of S. stercoralis has not been mapped in its entirety, so that a neural map of the free-living nematode C. elegans [50,51] was employed as a model to tentatively identify of neurons expressing GFP under the Ss-riok-1 promoter. Using this comparative approach, we concluded that body neurons expressing the Ss-riok-1-based reporter are likely sensory neurons and ventral nerve cord motor neurons (Fig. 7C, D, E and F). Furthermore, as PFL L1s developed towards L3s in the next 4–5 days in culture at 22°C, Ss-riok-1-specific reporter expression was localized to zones in the body wall muscle of the parasite (Fig. 6 C and D).

Discussion

The crucial role that RIOK-1 plays in the development of organisms was initially deduced from investigations in yeast as well as in C. elegans [8,13–17]. In the present study, we laid the groundwork for functional studies of RIOK-1 in parasitic nematodes by isolating and characterizing the RIOK-1 encoding gene Ss-riok-1 from S. stercoralis, an important parasite causing disease in humans and dogs.

The present study revealed only one Ss-riok-1 transcript. By contrast, multiple riok-1 transcript variants, with shortened C-terminal and N-terminal ends, have been identified in C. elegans and humans, respectively. The presence of only one riok-1 transcript appears to be a common feature of parasitic nematodes as public database searches (results not shown) have failed to detect multiple riok-1 transcript variants in various species including A. suum, B. malayi, Dirofilaria immitis, H. contortus, L. loa, S. ratti, S. stercoralis, T. vitrinus and W. bancrofti. The functional significance of transcript variants encoding an incomplete RIOK-1 in C. elegans and human is yet unknown.

The main functional domains in RIOK-1 appear to be conserved among organisms studied to date, including Archaeoglobus fulgidus and humans. Previous studies in yeast and human cells revealed that RIOK-1s have several functional domains possessing different functions. RIOK-1s lack the substrate binding motif commonly found in ePKs, but have a flexible loop (between β3 and αC) which is absent from ePKs [7,52]. The conserved RIOK-1 signature sequence “STGKEA” in the ATP binding motif has higher similarity to the signature sequence “STGKES” in the ATP binding motif of RIOK-3 than to the analogous signature sequence “GxGKES” in RIOK-2. The Active site of RIOK-1 “LVHxDLSEYN” also has higher similarity to that of RIOK-3 “LVHxDLSExN” than to that of RIOK-2 “IHxDoNEFN”, and the two residues Asp (D) and Asn (N) in this motif are present in the active sites of all ePKs [7]. The active sites in ePKs are usually involved in the transfer of phosphate groups from adenosine triphosphate (ATP) to substrate proteins, and phosphorylation events are basic to signal transduction pathways regulating numerous cellular and metabolic processes [5,53]. Active site mutations that disrupt RIOK-1 kinase activity also interfere with recycling of two trans-activating factors (endonuclease hNobI and its binding partner hDim2) which are necessary for maturation of the human 40S ribosomal subunit [11]. Besides the active sites, the more divergent N-terminal and C-terminal regions of RIOK-1 also participate in some biological processes. The first 120 amino acids of the N-terminal region of human RIOK-1 interact with a complex consisting of protein arginine methyltransferase 5 (PRMT5) and methylosome protein 50 (MEP50), which are two components of the methylosome [54]. This RIOK-1-PRMT5 complex methylates the RNA binding protein nucleolin, which is involved in ribosomal maturation [55–58]. In addition to the active sites and the N-terminal region, the C-terminal region of yeast RIOK-1 is phosphorylated by the casein kinase 2 (CKII) to regulate the cell cycle in yeast [59]. The functions of the RIO domain and the N- and C-terminal regions of RIOK-1 in parasitic nematodes are unknown. In the present study, the predicted amino acid sequence alignment (Fig. 1) revealed that Ss-RIOK-1 shares common features with the RIOK-1 family. Ss-RIOK-1 has limited similarity in its N-terminal and C-terminal regions to yeast and human RIOK-1 homologues. In addition, Ss-RIOK-1 is capable of both
phosphorylation and autophosphorylation, which is a property of the RIOK-1s from *A. fulgidus*, *S. cerevisiae* and humans [9,11,46]. Taken together, these findings suggest that Ss-RIOK-1 is an active protein kinase but its biological functions may differ from those of its homologues in yeast and humans.

Ss-riok-1 contained fewer introns than its homologues from *C. elegans* and *H. contortus*. This reduction in intron number has been a consistent trend in comparisons of genes in *S. stercoralis* and their orthologs in *C. elegans* and its parasitic counterparts in clade V [21,37,60,61]. The comparison of 5’-UTRs in Ss-riok-1 and Ce-riok-1 revealed some shared promoter elements, though the sequence similarity was limited. The promoter elements included TATA box, CAAT, GATA box and E-boxes were all found in the regulatory region of Ss-riok-1 and Ce-riok-1. Along with the TATA box, the CAAT box is another common promoter element for protein-coding genes in eukaryotes [62]. The GATA box is recognized by GATA transcription factors and is necessary for regulation of eukaryotic development and reproduction [63–67]. E-boxes are recognized and bound by basic helix-loop-helix (bHLH) proteins which regulate a wide range of developmental process in eukaryotic organisms including neurogenesis and myogenesis [68–70]. 37 bHLH proteins have been identified in *C. elegans*, and some of them are associated with specification of neural lineages and differentiation of myogenic lineages [71,72]. E-boxes are also characterized as gene promoter elements in the parasitic nematode *H. contortus* [73] and, as demonstrated here, in *S. stercoralis*, suggesting that these elements are involved in regulating the development of parasitic nematodes.

Ss-riok-1 transcripts are present in all life stages of *S. stercoralis*, suggesting that this gene functions in the development of all stages of this parasite. The abundance of Ss-riok-1 transcripts varies during development, being higher in the iL3 and in parasitic and post-parasitic life stages, which are progressing towards the free-living adults (FL Female and FL Male) than in the FL female and
In conclusion, we have isolated and characterized the RIOK-1 encoding gene Ss-riok-1 from the zoonotic parasite S. stercoralis. Ss-riok-1 contains a RIO1 signature motif and has high similarity to a range of homologues from different species. Recombinant Ss-riok-1 has kinase activity. Ss-riok-1 transcripts are present throughout development in S. stercoralis with the highest abundance in iL3. The Ss-riok-1 promoter is active in head neurons, body neurons and tail neurons as well as in pharynx and hypodermis of S. stercoralis of PFL L1 and L2s and in body wall muscle of iL3. These findings suggest that Ss-riok-1 plays an important role in regulating development of S. stercoralis, particularly in the formation of the nervous system in PFL L1 and L2s and in morphogenesis of the iL3 which is crucial to the infective process. Future work should focus on ascertaining whether Ss-riok-1 function is essential for the development or survival of S. stercoralis and by what mechanisms it exerts its function.

Supporting Information
Figure S1 Diagram of Ss-riok-1 transcriptional reporter construct pRP1 used to transform S. stercoralis. The 4280 bp promoter of Ss-riok-1 was inserted into pAJ01 between the PviI and AgeI restriction sites. Length of gfp with artificial introns and Ss-era-1 3’ UTR are marked above them.

(DOC)
Figure S2 Alignment of promoter regions predicted from the 5’-UTRs of Ss-riok-1 and Ce-riok-1. Colour boxed represent the promoter elements: CAAT (CCAAAT) or inverse CAAT (ATTTG) motif (turquoise), inverse GATA (TTATGC) (green), inverse GATA (TTATGC) (green); GC box (yellow); E-box (CANNTG) (grey); TATA box (pink). The number represents the position of the nucleotide upstream of the start codon.

(DOC)
Table S1 The names and DNA sequences of primers used in the present study for isolating cDNA and promoter region of Ss-riok-1 and for constructing protein expression and transgenic plasmids.

(continued)

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Author Contributions
Conceived and designed the experiments: MH JBL. Performed the experiments: WY. Analyzed the data: WY JDS JBL MH. Contributed reagents/materials/analysis tools: WY JDS JBL MH RBG FF WQL RF YQZ JLL. Contributed to the writing of the manuscript: WY MH JBL RBG.

Acknowledgments
Sincere thanks to Hongguang Shao and Xinshe Li for assistance with the gonad injection of S. stercoralis. Special thanks to Qing Ye for the critical reading of the draft manuscript.
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Title:
Toward Understanding the Functional Role of Ss-riok-1, a RIO Protein Kinase-Encoding Gene of Strongyloides stercoralis

Date:
2014-08-01

Citation:
Yuan, W., Lok, J. B., Stoltzfus, J. D., Gasser, R. B., Fang, F., Lei, W. -Q., Fang, R., Zhou, Y. -Q., Zhao, J. -L.  &  Hu, M. (2014). Toward Understanding the Functional Role of Ss-riok-1, a RIO Protein Kinase-Encoding Gene of Strongyloides stercoralis. PLOS NEGLECTED TROPICAL DISEASES, 8 (8), https://doi.org/10.1371/journal.pntd.0003062.

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