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Peroxiredoxin-1 from *Schistosoma japonicum* functions as a scavenger against hydrogen peroxide but not nitric oxide

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ABSTRACT

Three peroxiredoxins (Prxs) are expressed during most of the developmental stages in the schistosome. Prx-1 is localized on the surface of the schistosomula and adults of *Schistosoma japonicum*, while Prx-2 is localized in the sub-tegumental tissues, parenchyma, vitelline glands, and gut epithelium, but not on the surface of the worms. We applied RNA interference techniques to suppress the specific genes of *S. japonicum* Prxs. Schistosomula of *S. japonicum* were cultured together with long-dsRNA encoding Prx-1 and Prx-2 of *S. japonicum* (the soaking method). The transcription level of each Prx gene was reduced by an RNA interference (RNAi)-mediated effect specifically. Although neither Prx was the essential protein for survival of *S. japonicum* schistosomula, Prx-1 dsRNA-treated larvae were susceptible to hydrogen peroxide. Moreover, these larvae were also susceptible to *t*-butyl hydroperoxide and cumene-hydroperoxide. However, the knockdown of neither Prx-1 nor Prx-2 influenced the resistance against nitric oxide generated from DETA/NO. Prx-1 may work as a scavenger against reactive oxygen species (ROS) generated outside of the schistosomes to prevent the oxidation of the bodies and/or the attack by immune cells producing the ROS. These findings suggest that Prx-1 may become a novel target of drugs and vaccines for schistosomiasis.

1. Introduction

Schistosoma japonicum, which is a species of Asian schistosome, has a complex life cycle, surviving in the veins of the final host without destruction from the immune system [1]. The worms are exposed to oxidative stresses due to reactive oxygen species (ROS), which are released from host effector cells adhering to the antibody-coated worms. However, several studies have reported that the antioxidants from schistosomes might allow the worms to survive in the bloodstream by removing the ROS generated in the veins and those released from host immune cells [2–4].

Peroxiredoxin (Prx) is a family of novel antioxidant proteins containing two conserved Cys residues, which are essential for the enzymatic scavenging of hydrogen peroxide [5]. The molecular cloning and characterization of Prxs from *S. mansoni* have been reported [6–9]. Recently, Prx-1 and Prx-2 were also found in the sporocysts and in excretory-secretory proteins from sporocysts of *S. mansoni* [10]. We previously characterized three Prxs: Prx-1, Prx-2, and Prx-3, from *S. japonicum*, as well as the expression of each Prx throughout the life cycle [11]. Because Prx-3 alone contained the mitochondria targeting sequences, it was thought to work as a scavenger against ROS in mitochondria. We found that Prx-1 was not only expressed in the tegument, but was also present in the excretory/secretory products of the schistosomula and adult worms. Moreover, Prx-2 was mainly detected in the sub-tegumental tissues, parenchyma, vitelline glands, and gut epithelium of the adult worms, but was not detected in the tegument of adults or in the schistosomula. These data suggest that Prx-1 acts to protect the parasite against the ROS generated within the host vein.

RNA interference (RNAi) has been applied to the study of a wide range of organisms, including schistosomes. This method makes it possible for the targeted organisms to down-regulate their gene expressions specifically. Several studies have reported gene suppression in the schistosomula and the sporocyst of *S. mansoni* by RNAi [12–15]. One recent study reported that the down-regulation of Prx-1 by RNAi in *S. mansoni* was lethal, because it increased its sensitivity to hydrogen peroxide [16].

In the present study, we performed RNAi against the skin-penetrated schistosomula of *S. japonicum* to suppress the gene

Abbreviations: Cys, cysteine; DETA/NO, diethylenetramine with nitric oxide; dsRNA, double-stranded RNA; MBP, maltose-binding protein; NO, nitric oxide; Prx, peroxiredoxin; RNAi, RNA interference; RNS, reactive nitrogen species; ROS, reactive oxygen species; RT-PCR, reverse transcription polymerase chain reaction; TPI, triose-phosphate isomerase; Trx, thioredoxin.

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expression of Prx-1 and Prx-2. After the treatment was applied to the schistosomula with the double-stranded RNA (dsRNA) coding each Prx, larvae were subjected a killing assay with several ROS and reactive nitrogen species (RNS) to confirm the essential functions of Prxs in this worm.

2. Materials and methods

2.1. Preparation of the parasite at each stage

S. japonicum, isolated at Yamanashi, Japan, was maintained in the laboratory, using female ICR mice (SLC, Hamamatsu, Japan) and its snail host, *Oncomelania hupensis nosophora*. Cercariae were shed from the crushed snails, collected and used for infection and other experiments, as previously described [17]. Schistosomula were prepared by the previously described method [11]. Briefly, anesthetized ICR mice were percutaneously infected with more than 1000 cercariae through shaved abdominal skin. The mice were then killed, and the skin regions containing the schistosomula were peeled off and cultured in RPMI1640 medium supplemented with 5 mM L-glutamate, 200 U/ml penicillin, and 200 μg/ml streptomycin at 37°C in 5% CO_2 overnight. The schistosomula were released into the medium (skin-penetrated schistosomula), following which the skin was removed. The schistosomula were used in RNAi experiments. For the killing assay with NO, another batch of schistosomula was prepared by shearing off their tails mechanically with repeated passages through a 20-gauge needle. These transformed schistosomula were incubated in RPMI1640 medium at 37 °C in 5% CO_2 for 3 h (3 h m-schistosomula), and 24 h (24 h m-schistosomula), respectively. The animal experiments were performed under the control of the Ethics Committee of Animal Care and Experimentation in accordance with The Guiding of Principles for Animal Care Experimentation, University of Occupational and Environmental Health, Japan, and the Japanese Law for Animal Welfare and Care (No. 221).

2.2. Preparation of dsRNA

Double-stranded RNA molecules were prepared using the Hiscribe™ RNAi transcription kit (NEB, Ipswich, USA). Each complete coding region from the cloned pCR2.1-Prx-1 vector and pCR2.1-Prx-2 vector was amplified by PCR using primer sets with the addition of BamHI and HindIII restriction sites at the 5′ and 3′-ends, respectively. After checking the sequences, each fragment coding Prx-1 or Prx-2 was sub-cloned into the pLITMUS™ vector, containing the T7 RNA polymerase dual promoter sequence at each end. The PCR reaction with T7 primer was performed using the purified sub-cloned constructs as a template. To synthesize dsRNA, *in vitro* transcription by T7 RNA polymerase (Invitrogen, San Diego, USA) was performed using the amplified products as a template at 42°C for 6 h. The dsRNAs were treated with DNase and purified by ethanol precipitation. After checking the bands corresponding to each dsRNA by gel electrophoresis, their concentrations were determined by measurement of OD at 260 nm using a spectrometer. Using the same procedure, dsRNA was synthesized from the construct of the maltose-binding protein (MBP) from *E. coli* as a negative control.

2.3. RNA interference (RNAi)

The skin-penetrated schistosomula were washed with medium and approximately thirty larvae in 100 μl of medium were put into each well of a 96-well plate. Each dsRNA solution adjusted to the 200 nM concentration was added to each well at a final volume of 200 μl (final concentration was 100 nM). The treated schistosomula were cultured at 37°C in 5% CO_2 for 6 days without a medium change. At the same time, to examine the influence of RNAi on worm survival, approximately 50 larvae treated or non-treated by RNAi were incubated for 14 days, with 100 μl of fresh medium being added every 6 days. The number of surviving larvae was checked under microscopy daily and each experiment was performed in triplicate twice.

2.4. Preparation of total RNA and RT-PCR

Three days after RNAi treatment, total RNA was isolated from each treated group of the schistosomula using TRIzol reagent (Invitrogen). Complementary DNAs were synthesized with reverse transcriptase (Invitrogen) in a 20-μl reaction mixture using random hexamer oligonucleotides as primers. The resulted solutions were adjusted to 11 larvae per 20 μl with TE buffer. As a template, 1 μl of cDNA solution was applied to PCR in a 20-μl reaction mixture. The specific primer sets for each gene were as follows: Prx-1 (5′-TGGATTGGGTGACATGAGAA-3′, 5′-CGAATTGTACACTGCCATTCA-3′), Prx-2 (5′-TTCCTCGATTTCAAGTCAGTCA-3′, 5′-CCTAATCCACCAGCTTTTCG-3′), and triose-phosphate isomerase (TPI) (5′-ATGGCAGTAGAGCCGACAAC-3′, 5′-AACGCTTAGACCTCCTGCAA-3′). The last one was used as an internal control [18]. The PCR consisted of 30 cycles each at 95 °C for 20 s, 60 °C for 30 s, and 72 °C for 30 s. The PCR products were resolved by agarose gel electrophoresis, fand stained in ethidium bromide.

2.5. Schistosomula killing assay

Following schistosomula treatment with dsRNA, cell-free killing was performed by incubating the dsRNA-treated schistosomula or 3 h schistosomula with hydrogen peroxide (10 μM), *t*-butyl hydroperoxide (10 μM), cumene-hydroperoxide (1 μM), and DETA/NO (the adduct of diethylenetramine with nitric oxide; 0.5 mM), which is the chemical donor for generating NO in aqueous solution [19]. Trypan blue was added to the killing assay culture at a final concentration of 0.01%. After 48 h, the schistosomula mortality was assessed by counting the non-stained worms. All experiments were performed in triplicate three times.

2.6. Statistical analysis

The significance of the decrease in worm survival rate was determined by two-tailed Student's *t*-test.

3. Results

3.1. Specific suppression of expression from Prx-1 and Prx-2 by dsRNA treatment

To determine the function of each Prx from *S. japonicum*, we performed RNAi using the entire coding region of Prx-1 and Prx-2. Each dsRNA was prepared from the Prx-coding vector by *in vitro* transcription, and the purified dsRNA was checked by gel-electrophoresis. After performing the RNAi (soaking method) against the skin-penetrated schistosomula using each dsRNA, total RNA was recovered from each treated group. By RT-PCR, we confirmed that the gene expression was specifically suppressed depending on the dsRNA employed (Fig. 1). However, the dsRNA coding maltose-binding protein from *E. coli* as a negative control did not affect the Prx expressions. Furthermore, the treatment of the dsRNA rarely influenced the expression of TPI, which is expressed constitutively throughout developmental stages. Taken together, these results suggest that the specific RNAi was successful against the schistosomula using Prxs–dsRNA.