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In vivo and in vitro knockdown of *FREP2* gene expression in the snail *Biomphalaria glabrata* using RNA interference

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Abstract

RNA interference (RNAi) is reported here for the first time for *Biomphalaria glabrata*, the snail intermediate host for the human parasite *Schistosoma mansoni*. The fibrinogen-related protein 2 (FREP2) gene, normally expressed at increased levels following exposure to digenetic trematode parasites, such as *S. mansoni* or *Echinostoma paraensei*, was targeted for knockdown. Double-stranded RNA (dsRNA) corresponding to specific regions of the *FREP2* gene was introduced into snails by direct injection into the hemolymph, 2 days prior to exposure to trematodes, or added to co-cultures of *B. glabrata* embryonic (Bge) cells and *E. paraensei* sporocysts. After introduction of *FREP2* dsRNA, expression levels of *FREP2* were significantly reduced, to 20–30% of control values. In addition, we were able to disrupt expression of the house-keeping *myoglobin* gene, further confirming the feasibility of RNAi for *B. glabrata*. Cross-reactivity in RNAi has not been observed either among four *FREP* gene subfamilies or between *FREP2* and *myoglobin*. Establishment of RNAi techniques in *B. glabrata* provides an important tool for clarifying the function of genes believed to play a role in host-parasite interactions, specifically between *B. glabrata* and its trematode parasites, including *S. mansoni*.

Keywords: RNA interference (RNAi); Fibrinogen-related protein (FREP); *Biomphalaria glabrata*; *Echinostoma paraensei*; *Schistosoma mansoni*; Host–parasite interactions; Innate immunity; Comparative immunology

1. Introduction

RNA interference (RNAi) is a process by which the introduction of exogenous double-stranded RNA (dsRNA) corresponding to a specific messenger RNA (mRNA) sequence results in a significant reduction in level of the targeted mRNA [1–3]. This technique is a powerful post-transcriptional gene silencing technique that is providing insight into gene function in plants and animals [4–8]. RNAi has been used to assess gene function in invertebrates and vertebrates under in vivo and in vitro conditions. Most in vivo RNAi studies have been performed with invertebrates, mainly arthropods, with the aim of directly assessing phenotype changes. These invertebrates include *Caenorhabditis elegans* [9,10], digenetic trematodes [11,12], silkworms [13], moth *Spodoptera liturs* [14], *Drosophila* [15–17], mosquitoes [18,19], honeybee [20], and ticks [21]. In vitro RNAi assays have also been widely

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applied to cell lines from humans and model organisms such as *C. elegans* [10], *Drosophila* [22,23], mosquitoes [24], and mice [25,26].

Successful application of RNAi in a broad range of eukaryotic organisms suggests that it may be possible to employ this promising technique in mollusks. Indeed, RNAi has been reported to disrupt neuronal nitric oxide synthase (nNOS) gene function in the pond snail *Lymnaea stagnalis* [27], and to inhibit the CCAAT/enhancer-binding protein gene (ApC/EBP) in the marine mollusk *Aplysia* [28]. However, RNAi has not yet been reported in the snail *Biomphalaria glabrata*, a widely studied intermediate host of the trematode *Schistosoma mansoni*. This helminth infects 83 million people worldwide [29]. *B. glabrata* is used as a model to explore the nature of the defense responses of snails to digenetic trematodes like *S. mansoni* and *Echinostoma paraensei*. A large, diverse family of hemolymph proteins termed fibrinogen-related proteins, or FREPs is present in *B. glabrata* [30,31]. Previous work suggests that FREPs are involved in molluscan internal defense because they are capable of precipitating soluble trematode antigens and binding to trematode sporocysts. Although considerable data on the structure, diversity, and expression of *FREPs* have accumulated recently [32–36], the precise roles of *FREPs* in defense and perhaps other aspects of snail physiology require further clarification. Classical genetic knockout techniques are not presently available in mollusks, nor are they likely to soon be developed. RNAi could serve as a powerful alternative tool to assess snail gene function in both in vivo and in vitro studies. Towards this end, we here report our studies to develop RNAi to knockdown expression of *FREP* genes in *B. glabrata*, as a necessary prelude to exploring their role in anti-trematode defense.

FREP2, a member of *FREP* gene family, was chosen as a target gene because its transcripts appear in greater abundance in individuals of the M line strain of *B. glabrata* after exposure to either *E. paraensei* or *S. mansoni*, and in snails of the BS-90 (also called Salvador) strain after exposure to *S. mansoni* [36]. In addition, *FREP2* has a relatively simple gene structure with one immunoglobulin superfamily (IgSF) domain [32]. Moreover, like all *FREPs* currently known, *FREP2* is comprised of both a specific IgSF region and a more conserved fibrinogen (FBG) region. This provides a useful opportunity to assess the specificity of dsRNAs targeted to these regions in the RNAi experiments described below.

2. Materials and methods

2.1. Parasites, Bge cells and snails

The life cycles of *S. mansoni* and *E. paraensei* were maintained in the laboratory as described by Stibbs et al. [37] and by Loker and Hertel [38], respectively. The *B. glabrata* embryonic (Bge) cells originally established by Hansen [39] were obtained from the American Type Culture Collection (ATCC CRL 1494). Bge cells were maintained at 26 °C in complete Bge cell medium [39], supplemented by 50 μg/μl gentamicin sulfate (Sigma) and 5% fetal bovine serum (FCS) (Sigma) as described by Coustau et al. [40]. The BS-90 and M line strains of *B. glabrata* were maintained in the laboratory [39].

2.2. Co-culture of E. paraensei miracidia/sporocysts and Bge cells

Bge cells were seeded at 2×10^5 cells per well (500 μl culture medium) in a 24-well plate for 1 day prior to addition of dsRNA (or 5 μl nuclease-free water for the control). Miracidia were added 2 days after the addition of dsRNA or nuclease-free water. To maintain physical separation between the Bge cells and parasites, *E. paraensei* miracidia were added in sterile plastic inserts (0.4 μm membrane pore size; Corning Incorporated, NY, USA), and co-cultured with Bge cells in the same well. After co-culture for a specified number of days, the parasites were removed and Bge cells were collected for RNA extraction.

2.3. dsRNA synthesis

FREP2 dsRNA was synthesized following the method of Clemens et al. [22]. Sequence-specific primers were designed from *FREP2* cDNA sequences (GenBank accession No. AY012700). The primers used for generating *FREP2* dsRNA (537 bp) were: forward 5′-TAATACGACTCACTATAGGGTCGCTACCACTTCGACTTGTT-3′, reverse 5′-TAATACGACTCACTATAGGGCGTGGGACTGGCTCTTGATAT-3′.The sequence underlined is T7 promoter sequence (same for *myoglobin* primers below). The primers span the region that encodes the signal peptide (SP), IgSF, and interceding region (ICR) of *FREP2*, a region known to be specific to this particular *FREP* gene [35]. A full-length *FREP2* cDNA was cloned from M line snails and the sequence was confirmed by