ISSN 1672-9145 **Acta Biochimica et Biophysica Sinica** 2005, **37**(6): 386–390 **CN** 31-1940/Q

Dose-dependent Inhibition of Gynecophoral Canal Protein Gene Expression *in Vitro* in the Schistosome (*Schistosoma japonicum*) by RNA Interference

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Abstract The gynecophoral canal protein gene *SjGCP* of *Schistosoma japonicum* that is necessary for the pairing between the male and female worms is specifically expressed in the adult male worm. This protein is widely distributed in the adult female worm after pairing. Reverse transcription-polymerase chain reaction (RT-PCR) and immunofluorescence were employed to analyze the relationship between the RNAi effect and dsRNA dosage in the parasites. The results revealed that the inhibition of *SjGCP* expression by siRNA is dose-dependent. RT-PCR analysis showed that the *SjGCP* transcript level was reduced by 75% when 100 nM dsRNA was applied.

Key words RNA interference; gynecophoral canal protein gene; *Schistosoma japonicum*; dosage

Schistosomiasis caused by schistosomes is a major public health problem in China and Southeast Asia mainly because a high percentage of schistosome eggs are retained in the liver of the final host where they elicit inflammatory immune responses [1]. The consequent formation of granuloma and fibrosis are the major pathological effects of schistosomiasis. A unique trait of schistosomes is their sexually dimorphic character. It has been proven that female schistosomes from single-sex infection are stunted in size and sexually immature [2]. Successful schistosome development depends on the correct signaling between male and female parasites during pairing [3–8], which suggests that schistosomes have a developmental system that requires signaling from the male schistosome to either directly or indirectly activate a number of female-specific gene expressions [9–14].

The gynecophoral canal protein SjGCP of *Schistosoma japonicum* is a cell-surface glycoprotein and is gender-specific in the male worm. It contains multiple short, conserved and repeated regions with sequence similarity to the developmentally regulated neural cell adhesion molecule fasciclin I. SjGCP is widely distributed on the cell surfaces of the adult female worm after pairing with the male worm [15]. We have previously shown that SjGCP is necessary for the pairing between the male and female worms *in vitro* (data not shown).

To further investigate the role of SjGCP during the pairing process, we conducted an investigation into RNA interference targeting of *SjGCP*.

Materials and Methods

Major materials and reagents

RPMI 1640 medium, Lipofectamine™ reagent and rabbit serum were supplied by Invitrogen Life Technologies (Invitrogen, Carlsbad, CA, USA). Lactalbumin hydro-

Received: January 18, 2005 Accepted: March 17, 2005

This work was supported by the grants from the Shanghai Important Science and Technology Key Program of China (No. 03DZ19231), the Shanghai Program for Phosphor Plan of China (No. 04QMX1462) and the National Natural Science Foundation of China (No. 30270311)

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DOI: 10.1111/j.1745-7270.2005.00058.x

lysate was obtained from Becton & Dickinson Company (San Jose, CA, USA). Bovine insulin, hydrocortisone, 5-hydroxytryptamine, hypoxanthine, penicillin and streptomycin were purchased from Sigma-Aldrich China Inc. (St. Louis, MO, USA). The Access reverse transcriptase-polymerase chain reaction (RT-PCR) system was obtained from Promega Corporation (Madison, WI, USA). Trizol was purchased from Shanghai Sangon Biological Engineering Technology & Service Company (Shanghai, China). Other chemicals and materials were of analytical grade and obtained from commercial sources. Fluorescence-conjugated goat anti-rabbit IgG was obtained from Sino-American Biotechnology Company (Shanghai, China). The electrophoresis image analysis system and Smartview analysis software were purchased from FuRi Science and Technology Company (Shanghai, China). The primers for the RT-PCR analysis were synthesized by Shanghai Saibaisheng Company (Shanghai, China). The siRNA was synthesized by the Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences, Shanghai, China.

Preparation of parasites

The life cycle of the schistosome (Anhui isolate) was maintained in the laboratory by New Zealand rabbits and Oncomelania snails. Schistosomulumes aseptically obtained from New Zealand rabbits were infected with 6000 cercariaes after 12 days. Then, approximately 100 schistosomulumes were cultured in 4 ml of RPMI 1640 medium containing 10% (*V*/*V*) rabbit serum, 0.1% (*W*/*V*) lactalbumin hydrolysate, 0.2 U/ml bovine insulin, 1 μM hydrocortisone, 1 μM 5-hydroxytryptamine, 10^{-6} M hypoxanthine, 100 U/ml penicillin, 100 μg/ml streptomycin and 80 μl of rabbit red blood cells. All parasites were cultured at 37 °C in an atmosphere of 5% CO_2 and 95% air for 7 days. The maintenance medium was changed every 3 days.

siRNA sequences

The gynecophoral canal protein gene of the schistosome (GenBank accession No. AF519183) was selected for RNA interference assay. The dsRNA molecule had s1 sense (1309) 5′-GUGGUGGUCAACAUAUUCAdTdT-3′, s1 antisense 5′-UGAAUAUGUUGACCACCACdTdT-3′. The dsRNA from the severe acute respiratory syndrome (SARS) virus of sense 5′-UUGCGAAUGGCCGGACACUCCdTdT-3′, antisense 5′-GGAGUGUCCGGCCAUUCGCAAdTdT-3′, was set as an irrelevant control. dsRNA was prepared according to the method described by Elbashir *et al.* [16]. The solution of siRNA duplexes was stored in a refrigerator at –80 °C.

RNAi treatment

The dsRNA of *SjGCP* was added to mediums with final concentrations of 12.5 nM, 25 nM, 37.5 nM, 50 nM, 62.5 nM, 75 nM, 87.5 nM, 100 nM, 125 nM and 200 nM respectively, and the dsRNA of the SARS virus was added to a medium with a final concentration of 200 nM in which the parasites had been cultured for 7 days.

RT-PCR analysis of *SjGCP* and image analysis

Cultured parasite RNA was isolated using Trizol reagent, following the manufacturer's instructions. For RT-PCR, a total reaction volume of 50 μl containing total RNA (3 μg), 10 μl of 5×reaction buffer, 1 μl of dNTP (10 mM), 2 μl of $MgSO_4$ (25 mM), and primers of *SjGCP* (15 μM) (sense: 5′-GGATCCAAGAGCTACACAGACAACAATT-3′; antisense: 5′-GACTCAATAAGTGTAACCGTTGTTTCAC-3′) was processed at 37 °C for 5 min and 48 °C for 45 min, following 32 cycles at 94 °C for 1 min, at 58 °C for 1 min and at 68 °C for 1.5 min. The oligos (sense: 5′-AGGCGGGACAGTGTGGTAAT-3′; antisense: 5′-TTGGAGAAGGAACTACTGAA-3′) for β-tubulin of the schistosome (GenBank accession No. AF220475.2) were amplified in each reaction as a form of constitutively expressed gene control. A relatively low number (32) of amplification cycles was used to keep the PCR reaction in the semi-quantitative range. The PCR products were separated on 1.5% agarose gel, scanned by the electrophoresis image analysis system and quantified by the Smartview analysis software.

Immunofluorescence

The parasites were first fixed in ice-cold acetone for 5 min, dried and washed with PBS 3 times, then incubated in PBS containing 1% goat serum for 30 min. The rabbit anti-*SjGCP* antibodies were directed against the recombinant gynecophoral canal protein expressed in *E. coli*. The parasites were incubated with anti-*SjGCP* antibodies at 1:10 dilution for 1 h at room temperature, and then washed 3 times in PBS for 10 min each time. A 1:300 dilution of fluorescence-conjugated goat anti-rabbit IgG was then added and incubated for 30 min. After multiple washings as described above, the parasites were mounted on slides that were covered with 90% glycerol, PBS, 2% 1,4-diazabicyclo (2,2,2) octane and examined by fluorescence microscopy.

Results

RT-PCR analysis of RNAi effect

The RNAi effects with different doses of dsRNA were analyzed by RT-PCR analysis and the results are shown in **Fig. 1**. The results show that the suppression of the target gene depends on the dosage of dsRNA. The transcript abundance level of *SjGCP* was reduced by 75% with the final concentration of 100 nM dsRNA. The higher concentrations of dsRNA did not further reduce the transcript level of the gynecophoral canal protein gene. It indicates that the inhibition of target gene expression by siRNA is dose-dependent.

Immunofluorescence

To confirm the RT-PCR analysis, immunofluorescence was employed to examine the expression of *SjGCP* at the gynecophoral canal in the treated and control parasites after 7 days. As shown in **Fig. 2**, in the control parasites or those parasites with irrelevant dsRNA, very strong fluorescence signals were observed in the segment of the gynecophoral canal compared to other tegumental surfaces [**Fig. 2(A,B)**]. In contrast, the fluorescence signal was varied in the same location of schistosomulumes that were treated with different concentrations of dsRNA [**Fig. 2 (C–L)**]. When the parasites were treated with a low dose of dsRNA (12.5 nM), the fluorescence signal from their gynecophoral canal was comparable to that of the control parasites [**Fig. 2(C)**]. As the concentration of dsRNA increased from 25 nM to 50 nM, the fluorescence signal from the parasites was significantly suppressed [**Fig. 2 (D–F)**]. Parasites treated with final dsRNA concentrations ranging from 62.5 nM to 87.5 nM showed very little fluorescence [**Fig. 2(G–I)**]. Moreover, when the final concentration of dsRNA continued to increase, the fluorescence signal from the gynecophoral canal almost disappeared [**Fig. 2(J–L)**]. The immunofluorescence results show that dsRNA indeed inhibits the target gene expression in a dose-dependent manner, which is consistent with the *SjGCP* transcript levels obtained by RT-PCR analysis.

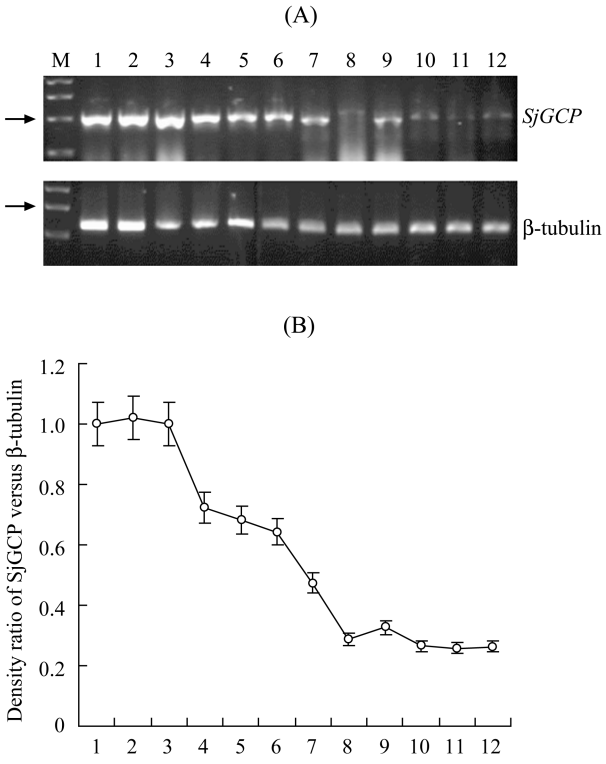

Fig. 1 RT-PCR analysis of treated and control parasites

PCR products (obtained using cDNA derived from parasites that were treated with different doses of dsRNA), irrelevant dsRNAs and the control are shown. β-Tubulin was amplified as the constitutively expressed gene control. (A) The image was analyzed by Smartview software. Lane M contains molecular size markers (arrow indicates 500 bp). (B) The values indicate the ratio of the optical density of *SjGCP* and β-tubulin.

Discussion

A unique trait of schistosomes is their sexually dimorphic character. Pairing between male and female schistosomes is the key process for successful development because there may be some signal molecules that are transducted between the male and female worms. *SjGCP* is specifically expressed in the adult male worm and its protein is widely distributed in the adult female worm after pairing. From previous studies, it has been found that *SjGCP* is necessary for the pairing between the male and female worms (data not shown) and RNA interference targeting the gynecophoral canal protein gene in the schistosome shows that *SjGCP* plays a critical role in the development of the male worm.

The relationship between dsRNA dosage and RNAi effect was examined by RT-PCR and immunofluorescence analysis. Our results indicate that the inhibition of target gene expression by dsRNA is dose-dependent and that the transcript level of *SjGCP* is reduced by 75% when the final concentration of dsRNA is 100 nM.

RNAi leads to a significant reduction of the level of transcription by introducing double-stranded RNA corresponding to a specific region of the targeted gene [17]. A limited amount of dsRNA per affected cell can efficiently

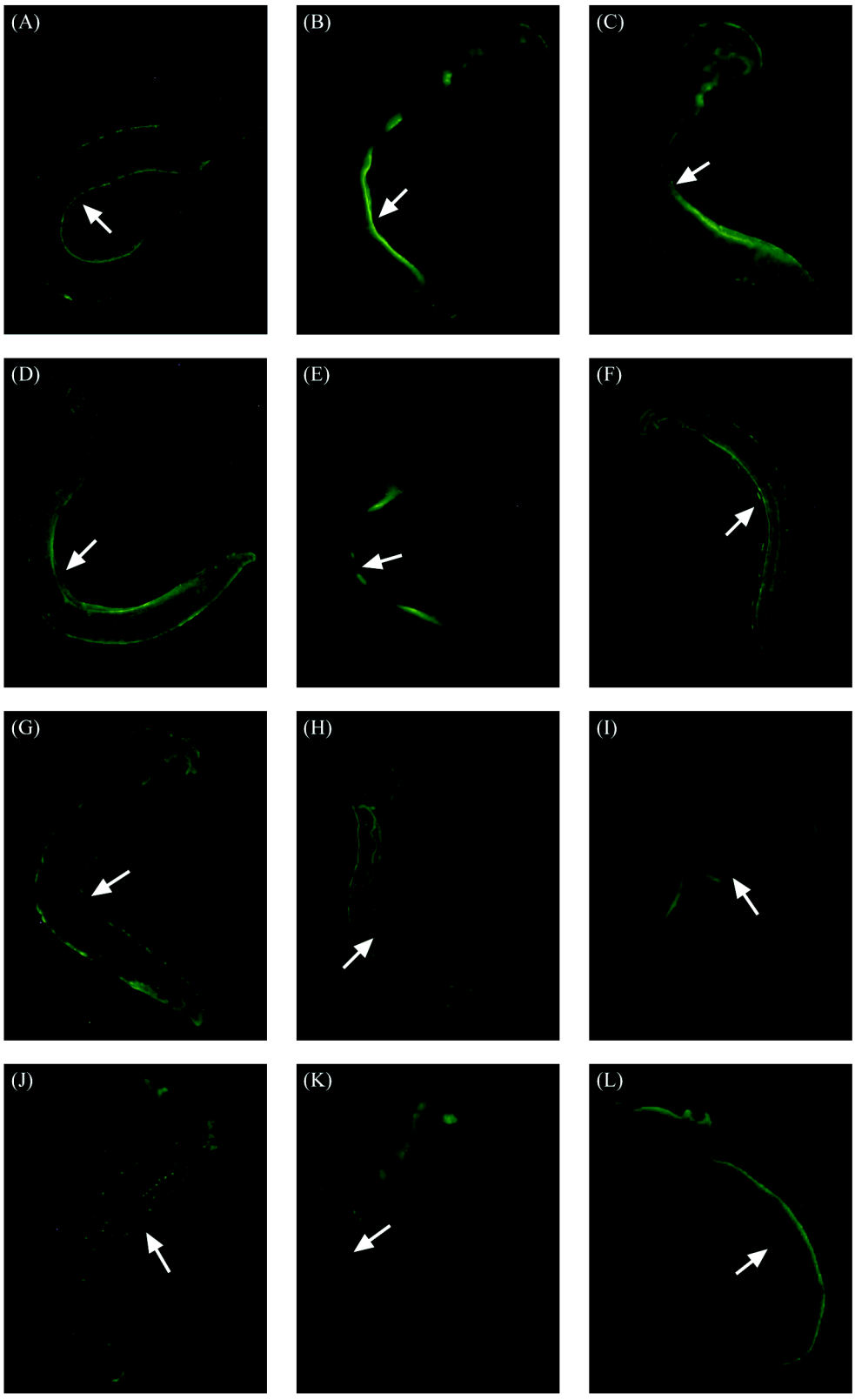

Fig. 2 Fluorescence patterns in treated and control schistosomes

(A) Control. (B) Irrelevant control. (C) 12.5 nM dsRNA. (D) 25 nM dsRNA. (E) 37.5 nM dsRNA. (F) 50 nM dsRNA. (G) 62.5 nM dsRNA. (H) 75 nM dsRNA. (I) 87.5 nM dsRNA. (J) 100 nM dsRNA. (K) 125 nM dsRNA. (L) 200 nM dsRNA. The arrow shows the gynecophoral canal of the schistosomes.

interfere with the expression of the targeted gene in *Caenorhabditis elegans* [17,18]. In this study, a simple and convenient soaking method was employed to transfect dsRNAs into parasites. However, as shown above, the transcript level of *SjGCP* was not significantly suppressed with a low dose of dsRNA (12.5 nM) compared to a high dose of dsRNA (100 nM). It is possible that the soaking method affected the validity of dsRNA entry in the schistosome. We also attempted to transfect dsRNAs into the parasites by using Lipofectamine™ reagent. However, upon transfection, RT-PCR analysis showed that the *SjGCP* transcript levels did not decrease further (data not shown).

In conclusion, the inhibition of *SjGCP* expression by dsRNA is dose-dependent. The most effective inhibitory concentration of dsRNA was found to be 100 nM, which causes a 75% reduction in the transcript level.

Acknowledgement

We thank Dr. Shi-Shan YUAN for revising the manuscript and for the valuable suggestions.

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