

Molecular Cloning and Characterization of Growth Factor Receptor Bound-Protein in *Clonorchis sinensis*

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Abstract

Background: *Clonorchis sinensis* causes clonorchiasis, a potentially serious disease. Growth factor receptor-bound protein 2 (Grb2) is a cytosolic protein conserved among animals and plays roles in cellular functions such as meiosis, organogenesis and energy metabolism. In the present study, we report first molecular characters of growth factor receptor bound-protein (CsGrb2) from *C. sinensis* as counter part of Grb2 from animals and its possible functions in development and organogenesis of *C. sinensis*.

Methodology/Principal Findings: A CsGrb2 cDNA clone retrieved from the *C. sinensis* transcriptome encoded a polypeptide with a SH3-SH2-SH3 structure. Recombinant CsGrb2 was bacterially produced and purified to homogeneity. Native CsGrb2 with estimated molecular weight was identified from *C. sinensis* adult extract by western blotting using a mouse immune serum to recombinant CsGrb2. CsGrb2 transcripts was more abundant in the metacercariae than in the adults. Immunohistochemical staining showed that CsGrb2 was localized to the suckers, mesenchymal tissues, sperms in seminal receptacle and ovary in the adults, and abundantly expressed in most organs of the metacercariae. Recombinant CsGrb2 was evaluated to be little useful as a serodiagnostic reagent for *C. sinensis* human infections.

Conclusion: Grb2 protein found in *C. sinensis* was conserved among animals and suggested to play a role in the organogenesis, energy metabolism and mitotic spermatogenesis of *C. sinensis*. These findings from *C. sinensis* provide wider understanding on diverse function of Grb2 in lower animals such as platyhelminths.

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Introduction

Clonorchiasis sinensis (*C. sinensis*) presents a serious health problem that is epidemic in East Asian countries, such as, China, Korea, Vietnam and Thailand. It has been reported that about 35 million people were infected worldwide [1]. Mammals are the final hosts of *C. sinensis* and are infected by consuming raw or uncooked freshwater fish containing metacercariae. After being swallowed, metacercariae pass through stomach to the duodenum, and then the newly excysted juveniles migrate to the intrahepatic bile duct where they develop to adult worms [2]. *C. sinensis* causes pathological changes in the biliary tree, such as hyperplasia and inflammation of biliary mucosa, periductal fibrosis and ductal wall thickening. Patients with clonorchiasis experience epigastric pain, indigestion, abdominal distension, diarrhea, edema, weight loss, and hepatomegaly. Furthermore, *C. sinensis* has been classified as a biological carcinogen by the International Agency for Research on Cancer due to its association with cholangiocarcinoma [3].

During its growth from the metacercarial to the adult form, many genes might be expressed and contribute to organogenesis. Growth factor receptor-bound protein 2 (Grb2) is a protein that is present in cytoplasm, and is composed of one SH2 domain flanked by two SH3 domains. As an adaptor protein in signal transduction

pathways, Grb2 is involved in many cellular functions and pathological processes. Phosphotyrosine residues in activated receptor tyrosine kinase and adaptors such as Shc and FRS-2 are recognized by SH2 domain in Grb2. The SH2 domain of Grb2 also recognizes and binds to proline-rich motifs in target proteins such as Sos [4,5]. For example, EGFR in the cell membrane dimerizes upon ligand (EGF) binding, and this event leads to phosphorylations of receptor tyrosines and the binding of the SH2 domain of Grb2 to EGFR. The SH3 domains then recognize and bind to their target proteins [6]. As a result, Ras is activated by Grb2/Sos complex in response to growth factors in many types of cells, such as fibroblasts, neural cells and lymphocytes. Furthermore, Grb2 has been identified in diverse organisms. In *C. elegans*, Sem-5 homologous with Grb2 was found to be involved in the signal transduction pathway that controls vulva development and sex myoblast migration. In mice, Grb2 is present in many organs and participates in organogenesis and development. Grb2 also promotes the re-initiation of meiosis in *Xenopus* oocytes. In addition, Grb2 has even been reported to be associated with carcinogenesis [7,8,9].

However, no report has been issued about the molecular character and biological function of Grb2 in trematodes. By

bioinformatic searching of the *C. sinensis* transcriptome database, a cDNA clone (CsGrb2) encoding a putative polypeptide homologous with Grb2 was identified and retrieved. In the present study, we describe the molecular character of CsGrb2 and its possible function in *C. sinensis* organogenesis and development.

Materials and Methods

Ethics statement

BALB/c mice (female, 7-week-old) and rabbits (New Zealand White, male, 2.2–2.5 kg) were handled in an accredited Chung-Ang University animal facility (Accredited Unit, Korea FDA; Unit Number 36). Approval for animal experiments was obtained from Institutional Animal Care and Use Committee of Chung-Ang University (Permit Number: CAU-2011-0052 and CAU-2011-0053). This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the US National Institutes of Health.

Bioinformatic analysis of CsGrb2 cDNA sequence and phylogenetic tree

The *C. sinensis* transcriptome database was analyzed using bioinformatics tools and found an EST clone encoding a polypeptide homologous with of Grb2 of other animals. The EST clone (CSA03475) was retrieved from *C. sinensis* transcriptome glycerol stock [10] and sequenced to reconfirm to encode the putative polypeptide deduced previously. The putative amino acid sequence was predicted using ExPASy translate tool with six reading frames (<http://web.expasy.org/translate/>). Homologues of CsGrb2 were searched in NCBI using BLASTx. Grb2s of nematodes, insects and other vertebrate animals were retrieved from GenBank. Sequence alignment was done by comparing CsGrb2 with Grb2s of other animals and functional domains of the CsGrb2 were determined using Clustal X [11] and manually edited in GeneDoc. The tertiary structure of CsGrb2 was predicted using Phyre2 [12]. Phylogenetic tree was constructed as of Neighbor-Joining format with CsGrb2 and 18 selected Grb2s using Mega 5.2 software [13,14]. Bootstrap value was estimated as of 1000 replicates.

Expression and purification of recombinant full-length CsGrb2 protein

A cDNA region corresponding to the CsGrb2 open reading frame was amplified by polymerase chain reaction (PCR) using *C. sinensis* cDNA as a template. Specific forward (5'-CATATG-TAGTCGACATGGAAGCTGTGGCAGTTC-3') and reverse (5'-GTATATCAGCGGCCGCTCATGTAGCACGTGATGGA-TG-3') primers containing *SalI* or *NdeI* restriction enzyme site were designed. Amplification was conducted at 95°C for 5 min followed by 30 amplification cycles (95°C for 1 min, 65°C for 30 sec, 72°C for 2 min) and a final extension at 72°C for 10 min. PCR products were purified and digested with the restriction enzymes *SalI* and *NdeI*, and then subcloned into prokaryotic expression vector pGEX-4T-3. Sequencing of the recombinant plasmid was done and revealed the insert cDNA CsGrb2 was in frame to the tag protein. After transforming *E. coli* BL21 (DE3) pLysS (Invitrogen, Carlsbad, CA, USA), sj26GST-CsGrb2 fusion protein was induced by isopropyl β -D-thiogalactopyranoside (IPTG) at a final concentration of 1 mM at 37°C for 4 hours. *E. coli* cells were harvested and lysed by sonication. Lysates were treated with 2 M urea to solubilize insoluble recombinant protein and dialyzed against 1 × phosphate buffered saline. Recombinant fusion protein was absorbed to GSH Sepharose 4B (GE Healthcare, Uppsala, Sweden) column and CsGrb2 was cleaved from sj26GST tag

protein with thrombin overnight at 4°C. The cleaved CsGrb2 was eluted with PBS and subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The purified target protein concentration was measured using Bio-Rad protein Assay (Bio-Rad, Philadelphia, USA).

Production of polyclonal antibody against CsGrb2

BALB/c mice, female, 7-week-old, were purchased from Samtako BioKorea Inc. (Osan, Seoul, Korea). Recombinant CsGrb2 protein (50 μ g per mouse) was emulsified with Freud's complete adjuvant and injected into mouse intraperitoneally. Two weeks later, mice were injected intraperitoneally with a booster dose of 50 μ g recombinant protein emulsified with Freud's incomplete adjuvant. Two weeks after this second injection, the mice were boosted with 20 μ g of recombinant protein through tail vein. Anti-CsGrb2 mouse sera were obtained one week after this third immunization.

Animal infection and recovery of *C. sinensis* adult worms

C. sinensis metacercariae were collected from fresh water fish *Pseudorasbora parva* (Shen yang, China) by artificial digestion. Rabbits, New Zealand, were infected orally with the *C. sinensis* metacercariae and the adult flukes were recovered from the bile duct 6 weeks after the infection. The adult flukes were washed using 1 × PBS 3 times and kept in -80°C freezer until use.

Identification of native CsGrb2 from *C. sinensis* adult crude extract by western blotting

C. sinensis adult worms were washed using 50 mM PBS containing 1% Triton X-100 and 1 × CompleteTM mini 3 times and homogenized in 2 volumes of PBS/CompleteTM mini then kept at 4°C overnight. The suspension was centrifuged at 1500 rpm for 5 min at 4°C. The supernatant was saved and centrifuged at 13000 rpm for 60 min at 4°C. The second supernatant was used as the cytosolic crude extract of *C. sinensis* adults. Concentration of the extract was determined by Bio-Rad protein assay (Bio-Rad, Philadelphia, USA).

Purified recombinant CsGrb2 protein and *C. sinensis* adult cytosolic extract were electrophoresed in 10% SDS-PAGE and then transferred onto a nitrocellulose filter (NC filter). The membrane was blocked within 5% skim milk at room temperature for 1 hour and then incubated with anti-CsGrb2 mouse serum or negative control mouse serum at a dilution of 1:250 at 4°C overnight. After washing 3 times with PBS-T, the membrane was incubated with goat anti-mouse IgG alkaline phosphatase (AP)-conjugated antibody (Sigma, St. Louis, USA) at a dilution of 1:5000 at room temperature for 2 hours. After washing, target protein bands were visualized using a substrate BCIP/NBT (Sigma, St. Louis, USA).

Immunolocalization of CsGrb2 in *C. sinensis*

The *C. sinensis* in experimental rabbit liver and the metacercariae were fixed in 10% paraformaldehyde, embedded in paraffin and sectioned into ribbons. The ribbons were deparaffinized, rehydrated, and then incubated with anti-CsGrb2 mouse sera or naive mouse sera at a dilution of 1:300 for the adult flukes or 1:1000 for the metacercariae for 2 hours at room temperature. Subsequently, the ribbons were incubated with polymer-horse radish peroxidase (HRP) labeled anti-mouse IgG antibody (Dako Cytomation, Glostrup, Denmark). Color was developed using 3-amino-9-ethylcarbazole (AEC) as substrate and the ribbons were counterstained with hematoxylin.

Quantitative analysis of developmental expression of CsGrb2 mRNA

The frozen *C. sinensis* adult and metacercariae were ground into powder in liquid nitrogen. Total RNA was extracted using TRIzol reagent (Ambion, California, USA) according to the manufacturer's instruction. To remove trace of genomic DNA, total RNA preparation was treated with DNA-free kit (Ambion, California, USA). Quality of RNA sample was analyzed by measuring OD_{260/280} using a spectrophotometer (Ultrospec 3000, Pharmacia Biotech, Amersham, UK). Its OD_{260/280} ratio was above 1.9. First-strand cDNA was synthesized on the total RNA with oligo-d(T) primer using Power cDNA Synthesis kit (iNtRON Biotechnology, Gyeonggi-do, Korea) according to manufacturer's instruction.

A developmental transcription level of CsGrb2 between *C. sinensis* adults and metacercariae was measured by real time-PCR (qRT-PCR). Primers were designed using the Oligo 6 programme. For more stable comparison, three genes of β -actin, calyphosine and phosphoglycerate kinase were employed as reference genes [15]. An equal amount of total cDNA, 70 ng, of the adults and metacercariae and primers were mixed with 1 μ l of 10 \times the master mix (FasStart SYBR Green I Kit, Roche, Mannheim, Germany), making a final volume 10 μ l. Thermal cycle was performed using LightCycler 1.5 (Roche, Mannheim, Germany) as follow; at first step the reaction mix was heated to 95°C for 15 min, then followed by 45 cycles of 95°C for 10 sec, 60°C for 10 sec and 72°C for 30 sec. To plot a melting curve, the PCR products were heated to 95°C for 10 sec then cooled down to 65°C over 60 sec with increment 0.1°C/sec to 95°C. Data were analyzed using LightCycler program and transcription level was calculated using $2^{-\Delta\Delta C_t}$ method [16].

Enzyme-linked immunosorbent assay on recombinant CsGrb2

C. sinensis crude antigen and recombinant CsGrb2 were diluted in carbonate buffer to 5 μ g/ml and 2 μ g/ml, respectively. The antigen was aliquoted into a 96 well-plate at 200 μ l per well and incubated at 4°C overnight. After washing 5 times with PBS containing 0.1% Tween 20, the plate was incubated with platyhelminth-infected patients' sera or normal human sera (for crude antigen, 24 *C. sinensis*-infected and 24 normal human sera; for recombinant CsGrb2, 44 *C. sinensis*-infected, 10 *Paragonimus westermani*-infected, 10 *Schistosoma japonicum*-infected and 10 cysticercus cellulose-infected human sera, and 40 normal human sera) for 1 hour at 37°C. Secondary antibody (horseradish peroxidase-conjugated Goat anti-human IgG antibody (Sigma, St. Louis, USA) was added to the plate and incubated at 37°C for 1 hour. Substrate o-phenylenediamine was added to the plate and color development was achieved adding 8N H₂SO₄ solution. ODs were measured at 490 nm using microplate reader (Molecular Devices, California, USA). The experiment was repeated 3 times.

Results

Molecular characters of CsGrb2

A cDNA clone (CSA03475) retrieved from the *C. sinensis* transcriptome encoded a polypeptide of 311 amino acids, showing high sequence identity with Grb2 of invertebrates and vertebrate animals (Figure 1). By comparing with the secondary structure of Grb2 in other organisms, CsGrb2 was found to have one SH2 domain (with 5 conserved Grb2 motifs) flanked by two SH3 domains [17]. The residues in SH2 and SH3 domains for binding target molecules were highly conserved. A conceptual tertiary structure of this polypeptide showed that N-terminal SH3 domain

had 4 β -sheets, and C-terminal SH3 domain had 5 β -sheets, and SH2 domain consisted of 5- β -sheets and 2 α -helices. CsGrb2 showed a very long loop between the SH2 and N-terminal SH3 domains, which also existed in *Schistosoma mansoni* and *Schistosoma japonicum* Grb2s, but not in the other Grb2s (Figures 1 & 2). In the SH2 domain, residues binding to the phosphorylated tyrosine were highly conserved. In the N- and C-terminal SH3 domains, residues binding to the proline-rich proteins were highly conserved at the identical position to other Grb2s. The first and second loops on the C-terminal SH3 domain had several conserved residues of negative charge. Given this bioinformatics results, this cDNA clone was considered to encode a growth factor receptor bound protein of *C. sinensis* (CsGrb2; Fig. 1). A phylogenetic analysis showed that CsGrb2 was grouped with homologs of trematodes such as *Schistosoma mansoni* and *Schistosoma japonicum* (Figure 3). The mammalian Grb2s formed a major clade and the vertebrates the second. The insect Grb2s grouped a clade from others.

Recombinant and native CsGrb2 proteins

Recombinant fusion protein Sj26GST-CsGrb2 (60 kDa) was induced to be expressed in *E. coli* BL21 (DE3) pLysS (Figure S1). The *E. coli* were pelleted by spinning and lysed in 2 M urea to solubilize the fusion protein. After dialysis against PBS for 2 hours, the fusion protein bound efficiently to GSH-Sepharose beads. Recombinant CsGrb2 protein (34 kDa) was eluted from its tag-protein Sj26GST by thrombin treatment, the eluted at high purity with PBS (Figure S1). This purified recombinant CsGrb2 protein was used for immune sera production from mice. The CsGrb2 immune mouse sera recognized native CsGrb2 from *C. sinensis* soluble extract (Figure 4) showing an estimated molecular mass of its recombinant form, 34 kDa.

Developmental distribution of CsGrb2 in the *C. sinensis* adult and metacercaria

The CsGrb2 immune sera were used for immunohistochemical localization of CsGrb2 in *C. sinensis* adult worms and metacercariae. In the adult worms, strong color staining was found in the oral sucker and followed by the sperms in the seminal receptacle, the mesenchymal tissues, the intra-uterine eggs and the ovary (Figure 5). Weak staining was observed in the testes and the vitelline follicles (data not shown). In the metacercariae, color staining much stronger than in the adults, was observed in the tegument, subtegument, mesenchymal tissues, and the musculature of the oral and ventral suckers (Figure 6).

Expression of CsGrb2 in the developmental stages

Developmental expression of the CsGrb2 in the *C. sinensis* adults and metacercariae was analyzed by quantitative real time PCR using total RNA as template. A relative transcription level of the target gene CsGrb2 was from C_T-values of target and reference genes using $2^{-\Delta\Delta C_t}$ method [16]. The CsGrb2 level was 2.8 times higher in the metacercariae than in the adults (Figure 7A). To check PCR-amplification and relative quantification, PCR product were electrophoresed in an agarose gel. The amplified DNA bands of three reference genes [10] showed equal thickness in the two stages. Referred to the reference gene products, the amplified DNA band of the CsGrb2 revealed thicker in the metacercariae than in the adults (Figure 7B), supporting the relative quantification by qRT-PCR and $2^{-\Delta\Delta C_t}$ method [16].

Recombinant CsGrb2 protein as serodiagnostic antigen

Antigenicity of the recombinant CsGrb2 protein was evaluated by ELISA using human sera infected with platyhelminths. The

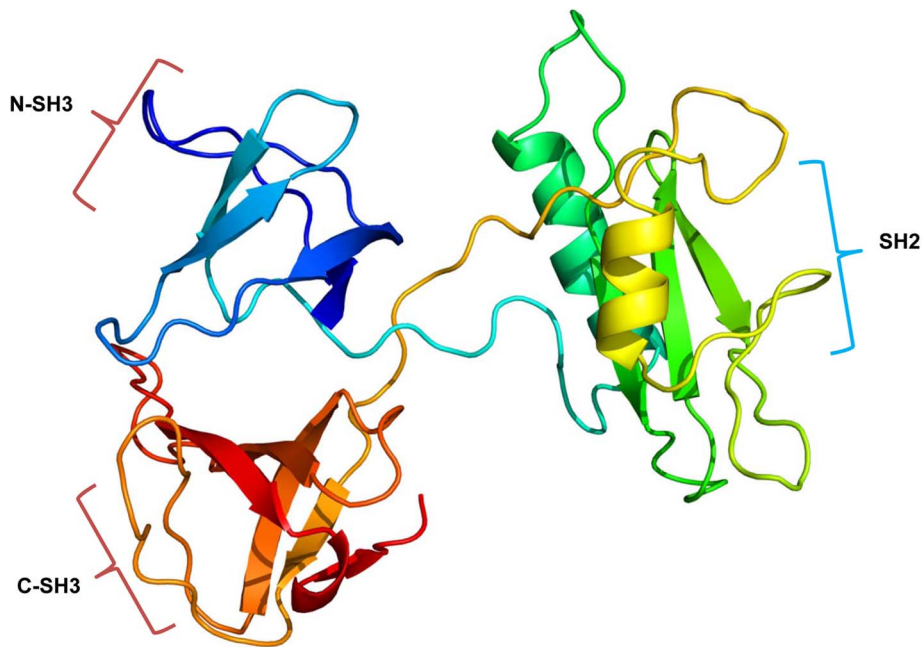


Figure 2. Putative three dimensional structure of *C. sinensis* growth factor receptor-bound protein (CsGrb2). The tertiary structure of CsGrb2 was predicted by online program of Phyre2. N- and C-SH3s are N- and C-terminal SH3 domains, respectively. The secondary structure of CsGrb2 was predicted to have α -helices and β -sheets.
doi:10.1371/journal.pone.0085577.g002

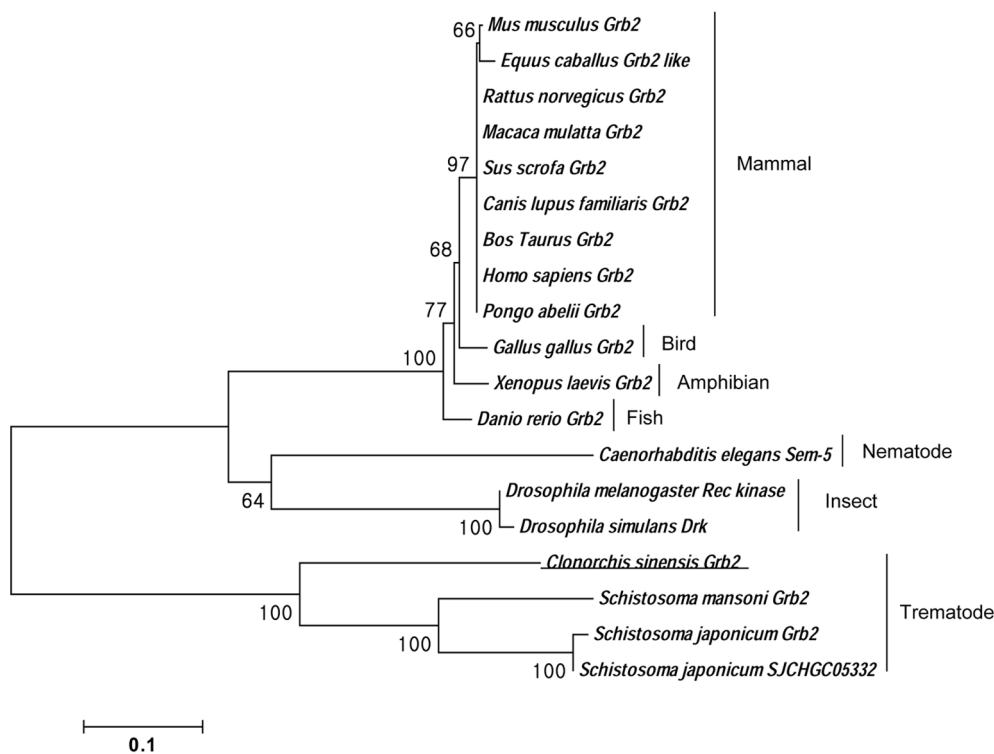


Figure 3. A phylogenetic tree of *C. sinensis* growth factor receptor-bound protein with those of other animals. Neighbor-Joining tree was constructed based on amino sequences of CsGrb2 and 18 Grb2 of vertebrates and invertebrates which were retrieved from GenBank. Number at each node shows bootstrap value (1000 replicates). (CAM21966.1, AEB61246, EDM06605, AFI36901, NP_001131100, XP_540431.2, DAA18091.1, CAG46740.1, NP_001126954, AAA16318, CAB59279.1, NP_998200, CCD63850.1, AAN17585, EDX06936.1, XP_002575772, CAX74268, and AAW26772) is GenBank accession number.
doi:10.1371/journal.pone.0085577.g003

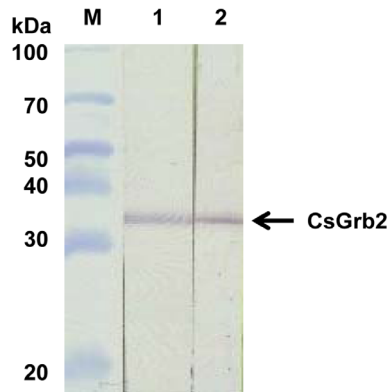


Figure 4. Immunoblotting of recombinant and native *C. sinensis* growth factor receptor-bound proteins (CsGrb2). Blots were probed with CsGrb2-immune mouse serum. Lane 1, recombinant CsGrb2; Lane 2, crude extract of adult *C. sinensis*. M, protein molecular weight marker.

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metacercariae. The metacercariae has exclusively somatic organs such as oral and ventral suckers, pharynx, esophagus and excretory bladder. A genital anlage is present as an indistinctive rudiment posterior to the ventral sucker. During development to adult *C. sinensis*, the anlage differentiates to reproductive organs such as ovary, vitelline glands, uterus, seminal receptacle, Mehlis' gland, testes and seminal vesicle. The high expression and wide distribution of CsGrb2 in the metacercariae may imply that it is required for development of *C. sinensis* from the metacercariae to the adults. As an adaptor, Grb2/Sem-5 is closely related to formation of vulva in *C. elegans* [22] and to organogenesis in mouse [30]. In *Drosophila*, Drk, a homologue of Grb2, is necessary for Sos-mediated Ras signal pathway leading a development of precursor cells to the eyes [23].

In adult *C. sinensis*, CsGrb2 was abundantly distributed in the somatic and reproductive organs, suggesting that CsGrb2 may have functions in these tissues. Adult *C. sinensis* produce a large

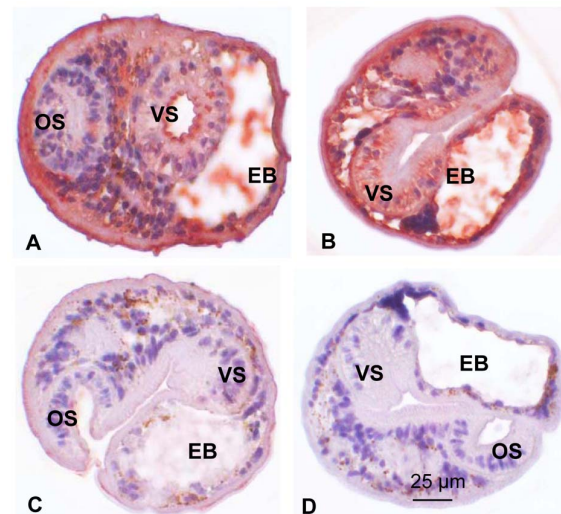


Figure 6. Localization of growth factor receptor-bound protein (CsGrb2) in *C. sinensis* metacercariae. Metacercariae were treated with anti-CsGrb2 mouse serum (A and B) or with normal mouse serum (C) at a dilution of 1:1000 or no serum (D). OS, oral sucker; VS, ventral sucker; EB, excretory bladder.

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mount of eggs every day through mitogenesis and meiosis. Grb2 is highly expressed as an adaptor in signal transduction pathway leading to mitogenesis and found to reinitiate meiosis in *Xenopus* oocytes [31]. In rat, Ash/Grb2 also links receptor PTKs to Ras, Rac and Rho which belong to the same family and lead to mitogenesis, membrane ruffle formation [32]. Besides the Ras signaling pathway, Grb2 binds to dynamin, a protein essential for endocytosis, synaptic transmission and neurogenesis, through its SH3 domain [33,34]. It is, therefore, suggested that CsGrb2 may be involved in meiosis during spermatogenesis and oogenesis of the adult *C. sinensis*.

Oral sucker is a very important locomotorium of *C. sinensis* with lots of myocytes. *C. sinensis* can move depending on strong hold of

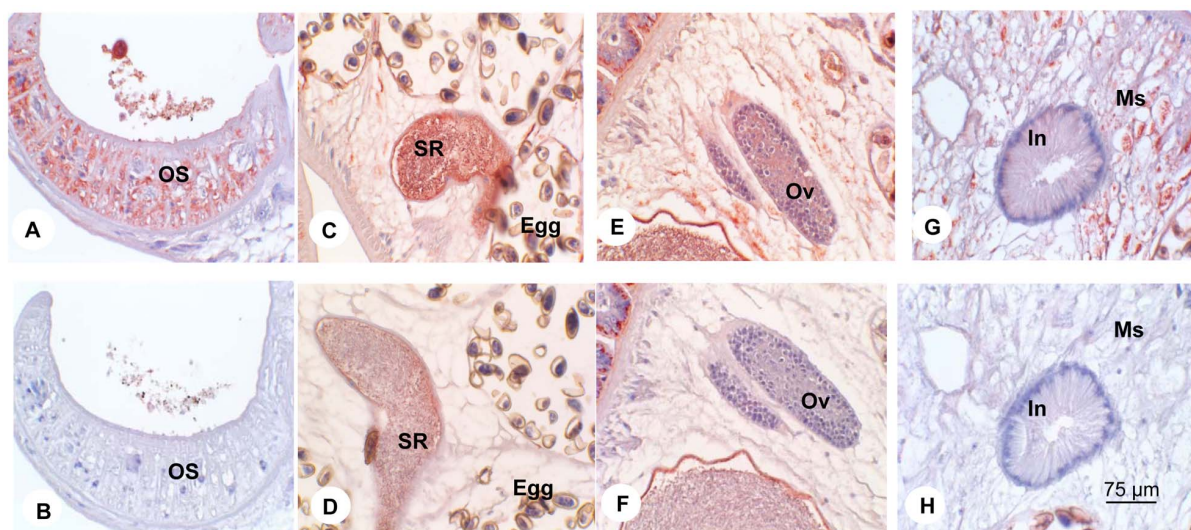


Figure 5. Localization of growth factor receptor-bound protein (CsGrb2) in *C. sinensis* adult. Panels A and B, oral sucker (OS). Panels C and D, seminal receptacle (SR); Panels E and F, ovary (Ov); Panels G and H, intestine (In) in mesenchymal tissue (Ms). Panels A, C, E and G were treated with anti-CsGrb2 mouse serum, and Panels B, D, F and H with normal mouse serum. Original magnification, $\times 400$.

doi:10.1371/journal.pone.0085577.g005

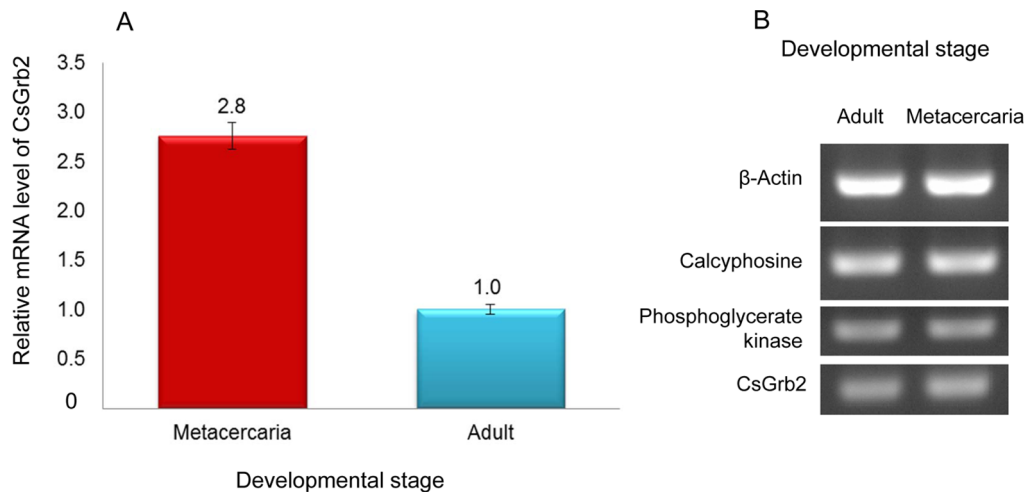


Figure 7. Relative transcriptional level of mRNA of *C. sinensis* growth factor receptor-bound protein in the developmental stages. Quantitative RT-PCR was performed on adult and metacercarial mRNA. A, relative mRNA level of *C. sinensis* adults and metacercariae. For quantification, refer to Materials and Methods; B, agarose gel electrophoresis of amplification products of quantitative RT-PCR. doi:10.1371/journal.pone.0085577.g007

the oral and ventral suckers and extension of the forebody. In present study, CsGrb2 was found abundant in the oral sucker, suggesting that CsGrb2 may play a role also in this tissue. In mice, Grb2 is necessary for migratory myoblasts and secondary fiber development by binding to Met receptor under the stimulation of hepatocyte growth factor [35,36]. To keep muscle cell viability, dystroglycan, a novel laminin receptor, interacts with Grb2 to link the extracellular matrix and sarcolemma in skeletal muscle [37]. In points of view, it is suggested that CsGrb2 may play a role for muscle development and action in the *C. sinensis*.

CsGrb2 was also found to be widely expressed in the mesenchymal tissues that composed of many adipocytes in *C. sinensis* adults. In rats and mice, Grb2 is widely distributed in many organs [29,38], suggesting that Grb2 was also important for basic

metabolism [39]. Grb2 has been found to bind to tyrosine-phosphorylated insulin receptor substrate-1, leading to the stimulation of DNA synthesis [40], increased expression and activity of the GLUT-1 glucose transporter [41]. GLUT-1 is present in many tissues and responsible for basal glucose uptake. In adipocytes, IRS-1/Grb2 also plays an important role in insulin-induced glucose transport and mitogenesis [42,43,44]. Glucose absorption and metabolism is critical for *C. sinensis* since it lives in an oxygen deficient environment [2]. Glucose transporters and sodium/glucose co-transporters were found to be expressed at high levels in the adult *C. sinensis* [10]. Glycolytic enzymes were also of the most abundantly expressed genes in the *C. sinensis* developmental transcriptome [10]. Based on these findings, we

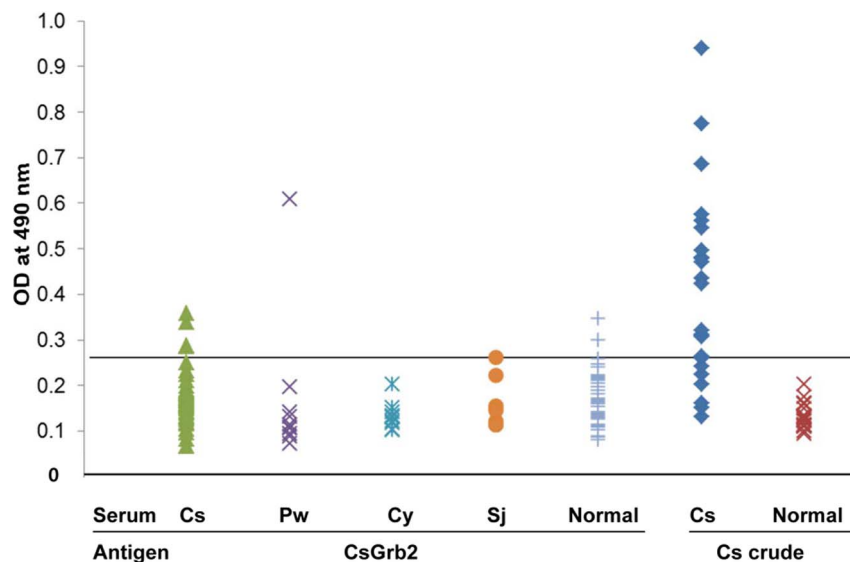


Figure 8. Antigenicity of recombinant *C. sinensis* growth factor receptor-bound protein towards IgG antibody in *C. sinensis* and helminth-infected human sera by ELISA. Cs, clonorchiasis sinensis; Pw, paragonimiasis westermani; Cy, cysticercosis cellulosae; Sj, schistosomiasis japonicum; Normal, uninfected human sera were used as a negative control. doi:10.1371/journal.pone.0085577.g008

suggest that CsGrb2 may play a regulatory function during energy metabolism of *C. sinensis*, which deserves further investigation.

For low antibody titers were elicited in *C. sinensis* infected people, antigenicity of recombinant CsGrb2 was not significant. As a serodiagnostic antigen, therefore the CsGrb2 recombinant protein seems less useful since this protein revealed a low sensitivity.

In conclusion, CsGrb2 was cloned, produced in recombinant protein and its biological characters were analyzed. The CsGrb2 may play roles in development, organogenesis, glucose transport and lipolytic metabolism of *C. sinensis*. The CsGrb2 deserves further studies to understand its molecular biological functions and to comprehensively widely parasitic and free-living platyhelminths.

Supporting Information

Figure S1 Purification and on-bead cleavage of recombinant *C. sinensis* growth factor receptor-bound

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