A SINGLE PARATHYROID HORMONE RECEPTOR-LIKE MEMBER OF FAMILY B1 G-PROTEIN COUPLED RECEPTORS IN *FASCIOLA GIGANTICA*

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Abstract. Intercellular signaling through cell membrane receptors has not only attracted basic research but also has been of high interest in the area of drug development. Family B1 G-protein coupled receptors (B1 GPCR) represent a small subfamily within the GPCR and they interact with peptide hormones. Their biological roles have mainly been studied in mammals with few functional analyses in invertebrates. Remarkably, based on current molecular sequence data trematode genomes also carry and express only a single B1 GPCR gene. In the present study we have characterized this trematode GPCR in the tropical liver fluke *Fasciola gigantica*. Sequence comparison, phylogenetic analysis and structure modeling indicate that it shares a common ancestor with chordate parathyroid receptor, known to be important for calcium homeostasis. RT-quantitative PCR of transcript from adult parasites showed sensitivity of this receptor-encoding gene to external calcium. It is speculated that the receptor has a role in the regulation of calcium ion levels in the parasite.

Keywords: *Fasciola gigantica*, calcium, G-protein coupled receptor, parathyroid hormone, Trematoda

INTRODUCTION

Liver flukes *Fasciola gigantica* and *F. hepatica* (Platyhelminthes, Trematoda, Digenea) are large tissue parasites in mammals. Their endemic areas overlap in Asia and Africa with *F. gigantica* generally associated with tropical regions, with *F. hepatica* also present in the Americas, Australia and Europe (Torgerson and Claxton, 1999). Importantly, they infect livestock, *eg*, cattle, water buffalo, goat and sheep grazing close to water sources harboring infective metacercariae but are also an accidental human parasite, with substantial numbers of reported and suspected human fascioliasis cases and epidemiological spread of the infection (Mas-Coma *et al*, 2009; Cabada and White, 2012).

Research on these parasites has focused mainly on the abundant released antigens or on surface antigens affecting host/parasite interaction and their practical application, *ie*, immunodiagnosis, drug and vaccine development (Robinson and Dalton, 2009; Cabada

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and White, 2012; Toet *et al*, 2014). Recent analysis of the parasite transcriptome (Young *et al*, 2010; *ibid*, 2011) has made it feasible to investigate metabolic pathways and signaling cascades following the lead of model organisms, such as the nematode *Caenorhabditis elegans* or the fruit fly *Drosophila melanogaster*, but progress is still limited by a lack of tools to access gene function.

A full set of G-protein coupled receptors (GPCRs) in *Schistosoma mansoni* (Platyhelminthes, Trematoda, Digenea) and *Schmidtea mediterranea* (Platyhelminthes, Turbellaria, Tricladida) were identified and classified based on their sequence conservation (Zamanian *et al*, 2011) and mining of the available genome/transcriptome data (Robb *et al*, 2008; Berriman *et al*, 2009; Bocchinfuso *et al*, 2012). In *S. mediterranea* and in *S. mansoni* 460 and 117 GPCRs, respectively were identified, with the majority (418 and 105 sequences, respectively) belonging to family A rhodopsin-like GPCRs (Zamanian *et al*, 2011). In a more recent in silico approach, Campos *et al* (2014) obtained comparable results. Other GPCRs include putative serotonin receptors, a FMRF (Phe-Met-Arg-Phe) amide-like peptide receptor and another serotonin receptor in *Turbellaria* spp (Saitoh *et al*, 1997; Omar *et al*, 2007; Nishimura *et al*, 2009); and a developmentally regulated rhodopsin receptor, possibly involved in photoreception (Hoffmann *et al*, 2001), a histamine receptor (Hamdan *et al*, 2002), two dopamine receptors (Taman and Ribeiro, 2009; El-Shehabi *et al*, 2012), and a serotonin receptor (Patocka and Ribeiro, 2013; Patocka *et al*, 2014) in *S. mansoni*. Receptors not associated with the nervous system have not been analyzed; Zamanian *et al* (2011) identified only three partial family B secretin-like receptor sequences, two in *S. mansoni* and one in *S. mediterranea*. The GPCR database (http://www.gpcr.org/7tm/) lists 34 members in family B comprising peptide hormone binding receptors (including secretin receptor), adhesion receptors and Methusalah-like receptors.

The small number of putative family B1 GPCR sequences identified in flatworm genomes/transcriptomes is likely due to limited tissue complexity and absence of a circulatory system in these parasites. The objectives of this study were to analyze the single family B1 GPCR in *F. gigantica* for its evolutionary relation to the vertebrate receptors, its expression pattern, and to suggest its function for the parasite.

MATERIALS AND METHODS

Preparation of *F. gigantica* nucleic acids

Adult and juvenile (2-, 4- and 6-week old) *F. gigantica* were collected as previously described (Siricoon *et al*, 2012). In brief, adult parasites were collected from naturally infected cattle sacrificed at a local slaughterhouse and juvenile parasites were obtained from experimentally infected ICR mice. Total RNA was isolated from homogenized parasite tissue using TRIzol reagent (Invitrogen, Carlsbad, CA) and mRNA fraction from adult stage total RNA was subsequently isolated using an Ambion® Poly(A)Purist™ Kit (Invitrogen). All isolated RNA samples were dissolved in DEPC-treated RNase-free water and kept at -80°C until use. Genomic DNA was phenol-chloroform extracted from adult parasites powdered in liquid nitrogen as previously described (Adisakwattana *et al*, 2007), dissolved in sterile distilled water and kept at -20°C until use. Concentration of nucleic acids was measured using a Nanodrop analyzer (ND-1000, ThermoFisher, Wilmington,

DE) at 260 nm and integrity further verified by agarose gel-electrophoresis.

Molecular cloning and sequence analysis

A partial cDNA (Fhep09c11.q1k, 814 bp) encoding the C-terminal region of a putative 7-transmembrane receptor was detected using TBLASTN screening of a *F. hepatica* EST library available at the Sanger Institute (http://ftp.sanger.ac.uk/pub/databases/Trematode/Fhep). Primers were designed based on this cDNA sequence (forward primer: 5′-CGCGTGATATTTGTGAAAATGC-3′; reverse primer: 5′-TTGATCTCCGATGTGACCTC-3′) and used to amplify a homologous 275 bp cDNA fragment from total RNA of *F. gigantica* adult stage by RT-PCR (reagents: ThermoFisher Scientific,Vilnius, Lithuania). Reverse transcription was conducted by incubating a mixture containing 1 μl total RNA (1 μg/μl), 2 μl of reverse primer (10 μM), 10 μl of H_2O, 4 μl of 5X RevertAid™ reaction buffer, 0.5 μl of RevertAid™ M-MuLV Reverse Transcriptase (200 U/μl), 2 μl of dNTPs (2.5 mM), and 0.5 μl of RiboLock™ RNase inhibitor (40U/μl) at 42°C for 1 hour. PCR mixture consisted of 2 μl of the reverse transcription product, 5 μl of 10X $(NH_4)_2SO_4$ PCR buffer, 1 μl of each forward and reverse primers (10 μM), 5 μl of dNTPs (2.5 mM), 5 μl of $MgCl_2$ (25 mM), 0.5 μl of *Taq* DNA polymerase (5U/μl), and 31.5 μl of H_2O. Thermocycling was conducted in a Mastercycler Gradient 22331 (Eppendorf, Hamburg, Germany) as follows: 95°C for 5 minutes; 30 cycles of 95°C for 1 minute, 50°C for 1 minute and 72°C with 1 minute; then a final step at 72°C for 10 minutes. Amplicon was separated by 1% agarose gel-electrophoresis, stained with ethidium bromide and purified using a QIAquick Gel Extraction Kit (QIAGEN, Limburg, The Netherlands). The partial cDNA fragment was used as a digoxigenin (DIG)-labeled probe (PCR DIG labeling kit, Roche Diagnostics, Mannheim, Germany) for screening of a *F. gigantica* adult stage cDNA library (Meemon *et al*, 2004). The nucleotide sequence of the longest cDNA was determined (1st BASE, Singapore) and found to be incomplete at the 5′ end. A 5′-RACE system (Invitrogen, Carlsbad, CA) then was used to obtain the complete sequence. In brief, first strand cDNA was synthesized from adult stage *F. gigantica* mRNA by reverse transcription using the reverse primer 5′-AAACATAGCAGAAGCGAAAC-3′ and used as a template for 5′-tailing using terminal transferase. The 5′-tailed cDNA was amplified using a primer complementary to the introduced cDNA 5′-tail (Abridged Anchor Primer, Sigma-Aldrich, Singapore) and the cDNA specific primer 5′-GCAAAACAAGAAAACCCTGGA-3′. PCR was conducted as described for the partial *F. gigantica* cDNA above but with the annealing temperature set at 55°C. The RACE product was sequenced (1st BASE) to determine that the complete cDNA fragment was obtained. Existence of a continuous open reading frame was then confirmed by isolation of a full-length cDNA by RT-PCR as described for the partial *F. gigantica* cDNA above. TBLASTN screens (http://www.schistodb.net/schisto/) using the deduced FgPTHRL amino acid sequence revealed genomic DNA and mRNA (EST) sequences encoding orthologous receptors in *S. haematobium* (*Sha_100625*) and *S. mansoni* (*Smp_170560*). Primers 5′-AGTTTACACATAGTC TTAGG-3′ and 5′-TTCTTATTCTGTTGTGATTGC-3′ based on the partial ESTs, sm_m28-57b12.q1k and MF001B04.NK3 (GenBank: AI111094) were used to amplify a 1642 bp cDNA fragment containing the complete coding sequence (CDS) and parts of the untranslated sequences of *S. mansoni* PTHRL by

RT-PCR from 1 μg of adult stage total RNA as described for the partial *F. gigantica* cDNA above.

EMBOSS 6.3 (Rice *et al*, 2000) was used for sequence editing, and Phobius (Käll *et al*, 2007), SignalP (Petersen *et al*, 2011), TMHMM (Krogh *et al*, 2001), and NetPhos (Blom *et al*, 1999) servers (http://www.cbs.dtu.dk/services/) were used for prediction of signal peptide, membrane topology, and phosphorylation sites. NCBI DELTA BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) was employed to screen for homologous proteins, Clustal Omega (Sievers *et al*, 2011) to calculate multiple alignments of GPCRs of the secretin family, TEXtopo (Beitz, 2000) to create a structural model of sequence conservation based on multiple alignment and resolved human glucagon receptor structure (Siu *et al*, 2013), Phyre2 for molecular modeling (Kelley *et al*, 2015), and PyMOL (The PyMOL Molecular Graphics System, Version 1.8 Schrödinger, LLC.) together with APBS (Baker *et al*, 2001) to create graphical representations of the computed models.

Phylogenetic analysis of parathyroid hormone (PTH) receptor sequences

Two sequence sets were used for analysis. The first set was described by Mirabeau and Joly (2013) (http://neuroevo.org/alignments/receptors/secretinR_final.aln) and contains sequences (116 amino acids) of protostome and deuterostome family B GPCRs including 15 human family B1 GPCRs (CALCR, CALRL, CRFR1, CRFR2, GHRHR, GIPR, GLP1R, GLP2R, GLR, PACR, PTH1R, PTH2R, SCTR, VIPR1, VIPR2). The sequences were edited to contain the conserved regions of the ligand-binding extracellular domain (starting at the N-terminal second conserved C) and helices (Mirabeau and Joly, 2013). In this analysis, the homologous regions of the *F. gigantica*, *Schistosoma mansoni*, and *Clonorchis sinensis* parathyroid hormone receptor-like (PTHRL) were added to the alignment and analyzed as previously described (Mirabeau and Joly, 2013) using PhyML 4.6.1 (Guindon *et al*, 2010) and a maximum likelihood approach. The second sequence set consisted of a larger number of predicted protostome and deuterostome PTH/PTH-like receptors (GenBank accession numbers shown in Fig 2B) and was analyzed using MrBayes 3.2.5 (Ronquist and Huelsenbeck, 2003) and a Bayesian approach with the following parameters: outgroup *H. sapiens* calcitonin receptor isoform 1 (NP_001158209), prset aamodelpr = mixed, and mcmc nchains = 4 ngen = 1000000.

Nucleic acid analysis

Southern hybridization. *F. gigantica* genomic DNA (50 μg) was digested *Bam*HI, *Pst*I, or *Bam*HI/*Pst*I (ThermoFisher Scientific, Lithuania), size-separated by 0.7% agarose gel-electrophoresis, denatured and transferred onto a nylon membrane (Amersham Hybond™ N+, GE Healthcare, Little Chalfont, UK) by capillary transfer and heated at 80°C for 1 hour. Lambda DNA/*Eco*RI+*Hin*dIII Marker (Fermentas Life Sciences, Vilnius, Lithaunia) was used as size standard. A 1418-bp DIG-labeled hybridization probe was generated by PCR (PCR DIG labeling kit, Roche Diagnostics, Mannheim, Germany), using FgPTHRL cDNA (nucleotides 220-1638) as template and primers 5′-GCGCAGTTTGATGGCGATATC-3′ and 5′-CTACACATTCTGCTGACAA-3′. Hybridization at 50°C and colorimetric detection was conducted as previously described (Adisakwattana *et al*, 2007).

Northern hybridization. The same hybridization probe was used to detect adult

stage *F. gigantica* total RNA (50 μg) and mRNA (10 μg). Heat-denatured RNA samples were resolved on a 1.2% agarose gel containing 2.2 M formaldehyde in MOPS buffer followed by capillary transfer onto a nylon membrane (Amersham Hybond™ N+, GE Healthcare) using 10X SSC as transfer buffer. RNA was fixed to the membrane as described above. Hybridization at 42°C and colorimetric detection were performed as described (Adisakwattana *et al*, 2007). RiboRuler™ High Range RNA Ladder (Fermentas Life Sciences) was used as standard size marker.

RT-PCR. Total RNA from 2-, 4-, 6-week-old juveniles and adult *F. gigantica* was used for RT-PCR as described for the partial *F. gigantica* cDNA above. The extracted RNA (1 μg each) was treated with DNase (Promega, Madison, WI) at 37°C for 30 minutes and DNase then inactivated by heating at 65°C for 10 minutes in the presence of 20 mM EGTA. DNase-treated RNA was reverse transcribed using oligo(dT)18 primer at 42°C for 1 hour, then subjected to PCR amplification of a 251-bp FgPTHRL cDNA fragment (nucleotides 1387-1638) using primers 5′-CGCACTCACCCACGGTCCATG-3′ and 5′-GCGGCTGCAGCTACACATTCTGCTGACAA-3′. PCR amplification was conducted as described above. Amplicons were size-separated by 1% agarose gel-electrophoresis and visualized by ethidium bromide staining.

RT-quantitative (q)PCR assay

Parasite culture and total RNA isolation. Adult worms, freshly collected at a local slaughterhouse, were washed twice in phosphate-buffered saline pH 7.4 (PBS) and allowed to recover in pre-warmed RPMI 1640 culture medium (Life Technologies, Carlsbad, CA) without serum and antibiotic supplements at 37°C for 30 minutes. Five intact worms of comparable size were immediately collected after recovery, washed twice with pre-warmed PBS and total RNA was extracted from each worm using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Another set of five worms were cultured in PBS or PBS containing 2 nM $CaCl_2$ at 37°C under an atmosphere of 5% CO_2 for 10 hours. Culture media were replaced every hour and worm motility was observed every 30 minutes. Finally, worms were washed twice in pre-warmed PBS and total RNA was extracted from each worm as described above.

RT-qPCR. One μg from each of the 15 RNA samples was treated with RNase-free DNaseI (Promega) at 37°C for 30 minutes and then converted to cDNA using RevertAID™ reverse transcriptase (ThermoFisher Scientific) and oligo(dT)20 primer (Sigma-Aldrich, St Louis, MO) at 42°C for 1 hour. The cDNA synthesis was terminated by heating at 70°C for 10 minutes and concentration measured using a NanoDrop2000c spectrophotometer (ThermoFisher Scientific). A sample of cDNA (500 ng) was used as template for qPCR using iTaq™ Universal SYBR® Green Supermix (BioRad, Hercules, CA) in a CFX96 Touch™ Real-Time PCR machine (BIORAD). RNA of the *F. gigantica* genes encoding tubulin (TUB), PTHRL, calmodulin (CALM, EST FgA01171; R. Grams unpublished data), calreticulin (CALR, EST FgA01028; R. Grams unpublished data), heat shock protein 70 (HSP70, GenBank: EF506931) and glutathione S-transferase 1 (GST-1, GenBank: AF112567) were used as targets of the primers listed in Table 1. All qPCR samples were denatured at 95°C for 1 minute and then amplified for 40 cycles at the following conditions: 95°C for 30 seconds, 55°C for 30 seconds and

Table 1
Primer pairs used for reverse transcription quantitative real time PCR of the listed *F. gigantica* transcripts.

Gene	Forward primer	Reverse primer
FgTUB	TGAAGCCTGGGCTCGTTTGGACCACAA	TTAGTATTCTTCACCCTCGCCTTCACC
FgPTHRL	CGCACTCACCCACGGTCCATG	CTACACATTCTGCTGACAA
FgCALM	AAACTCGCGTAACGTTGTCC	GAATTCCACGGGTGTGTACC
FgCALR	AGACTGGGATGACGAAATGG	AAGTCAAACCCGACGTATGC
FgHSP70	GGATGTGGCACCTCTTTCAT	AGCTCAAACTTTCCGAGCAA
FgGST-1	TCTTCGGATGGGTTTTGTTC	CACTGTGGTGCCAAATAACG

72°C for 2 minutes; followed by a final heating at 72°C for 3 minutes. Amplifications were performed in triplicate. TUB mRNA was used as internal control for calculating fold changes in expression and was amplified in parallel to each set of experiments. Quantification of the gene expression levels was performed using the $2^{-\Delta\Delta CT}$ method (Schmittgen and Livak, 2008). Quality of the amplicons was determined by agarose gel-electrophoresis and direct sequencing using the forward and reverse primers (Table 1). QPCR was repeated with RNA as template (omitting the reverse transcription step) as negative control.

RESULTS

Detection and characterization of *F. gigantica* gene encoding parathyroid hormone receptor-like (FgPTHRL)

In a screen for cDNAs encoding a G-protein coupled receptor of *F. gigantica* we have isolated a 2,282 bp cDNA encoding a 1,527 bp open reading frame, 111 bp 5′ UTR, and 616 bp 3′ UTR (GenBank: KF498716). DELTA-BLAST search of the NCBI NR protein database with the deduced 508 amino acid sequence revealed a significant similarity with a 7-transmembrane receptor (secretin family, 7tm_2) (E-value of 9.58e-40) and with a hormone receptor domain (HRM) (pfam02793) (E-value of 8.66e-13). High ranking hits starting with an E-value of 1e-110 were found with members of the vertebrate family B secretin-like GPCRs; subsequent analysis suggested a common ancestry with vertebrate parathyroid hormone receptor (PTHR), a family B1 GPCR (detailed below) and accordingly, the parasite protein was named *F. gigantica* parathyroid hormone receptor-like (FgPTHRL).

SignalP predicted the presence of an N-terminal signal sequence ending at residue A37 and TMHMM the presence of seven transmembrane helices, with identical predictions obtained by Phobius. A multiple amino acid sequence alignment of FgPTHRL with twelve human B1 GPCRs was calculated and used together with the resolved structure of the human glucagon receptor (Siu *et al*, 2013) to obtain a model demonstrating sequence conservation among these proteins (Fig 1), in particular the conserved external C residues in the ligand-binding domain, TM3 and the second external loop, which are thought to be structurally important for these receptors. Conservation is highest with residues and short motifs in the seven transmembrane helices, internal loops and adjacent internal helix-8, important for structure, activation and G-protein

contact (Siu *et al*, 2013; Vohra *et al*, 2013). This contrasts with low conservation in the external loops and at both the terminal sequences. It is worth noting the high number of positively charged residues (mainly R) in the cytoplasmic tail (Fig 1).

The amino acid sequences of the uncharacterized orthologous receptors from *F. hepatica*, *S. haematobium* and *S. mansoni* were used to evaluate conservation among the trematodes. The sequence of *F. hepatica* PTHRL was kindly provided by Dr Neil Young (Faculty of Veterinary Science, The University of Melbourne, Victoria, Australia) and that of *Sha_100625* encoding *S. haematobium* PTHRL retrieved from a BLAST screen of the parasite's genome database (http://www.schistodb.net/schisto/). The sequence of *S. mansoni Smp_17056* (incomplete but supported at 5′ and 3′ ends by partial ESTs) finally was obtained from a SmPTHRL cDNA containing the complete ORF generated by RT-PCR from parasite adult stage total RNA (GenBank: KM459026) to correct the intron/exon predicted sequence. BLAST analysis of genomes of human liver flukes *Opisthorchis viverrini* and *Clonorchis sinensis* (DBJ: BADR02003637 and DBJ: BADR02003324, respectively) indicated that both carry an orthologous gene, and as in the case of *Fasciola* spp and *Schistosoma* spp, the query results did not support the presence of any additional B1 GPCR in these trematodes (data not shown). Intrageneric sequence conservation is very high at 97.2% identity for *Fasciola* spp and 93.0% identity for *Schistosoma* spp, while intergeneric conservation between *Fasciola* spp and *Schistosoma* spp is significantly lower (approximately 44% identity/64% similarity). As noted above, sequence conservation is highest in the seven transmembrane helices, internal loops and adjacent internal helix-8, while the C-terminal cytoplasmic region shows low conservation but is rich in hydrophilic residues, and contains several putative serine phosphorylation sites (NetPhos 2.0 Server) and a fully conserved terminal RRPSPLC motif containing one of these phosphorylation sites. All trematode sequences carry the full set of structural important cysteine residues and the highest conserved residue of each of the seven transmembrane helices as described for human family B secretin-like receptors (Wootten *et al*, 2013).

Phylogenetic analysis of protostome and deuterostome PTH/PTH-like receptors

In the absence of functional data for most of B1 GPCR family members in Protostomia and with such data limited to the higher evolved vertebrates in Deuterostomia, it is a challenging task to determine the exact evolutionary relationship between these family B1 GPCRs among different phyla. In an exhaustive study of family B1 receptors and their cognate peptide ligands Mirabeau and Joly (2013) noted the difficulty of detecting ligands outside the Chordata due to sequence divergence. Nevertheless, the authors presented a convincing phylogeny in which the parathyroid receptor is present in protostomes as previously suggested by Cardoso *et al* (2006). A repeat of the analysis of Mirabeau and Joly (2013) and amending their dataset of family B1 receptor sequences with the trematode receptor sequences showed the latter to be members of the protostome PTHR-like clade (Fig 2A). A second phylogenetic analysis based on Bayesian approach with sequences of additional PTHR/PTHRL and human calcitonin receptor as an outgroup confirmed this result (Fig 2B).

Structure prediction

The sequence of FgPTHRL ectodo-

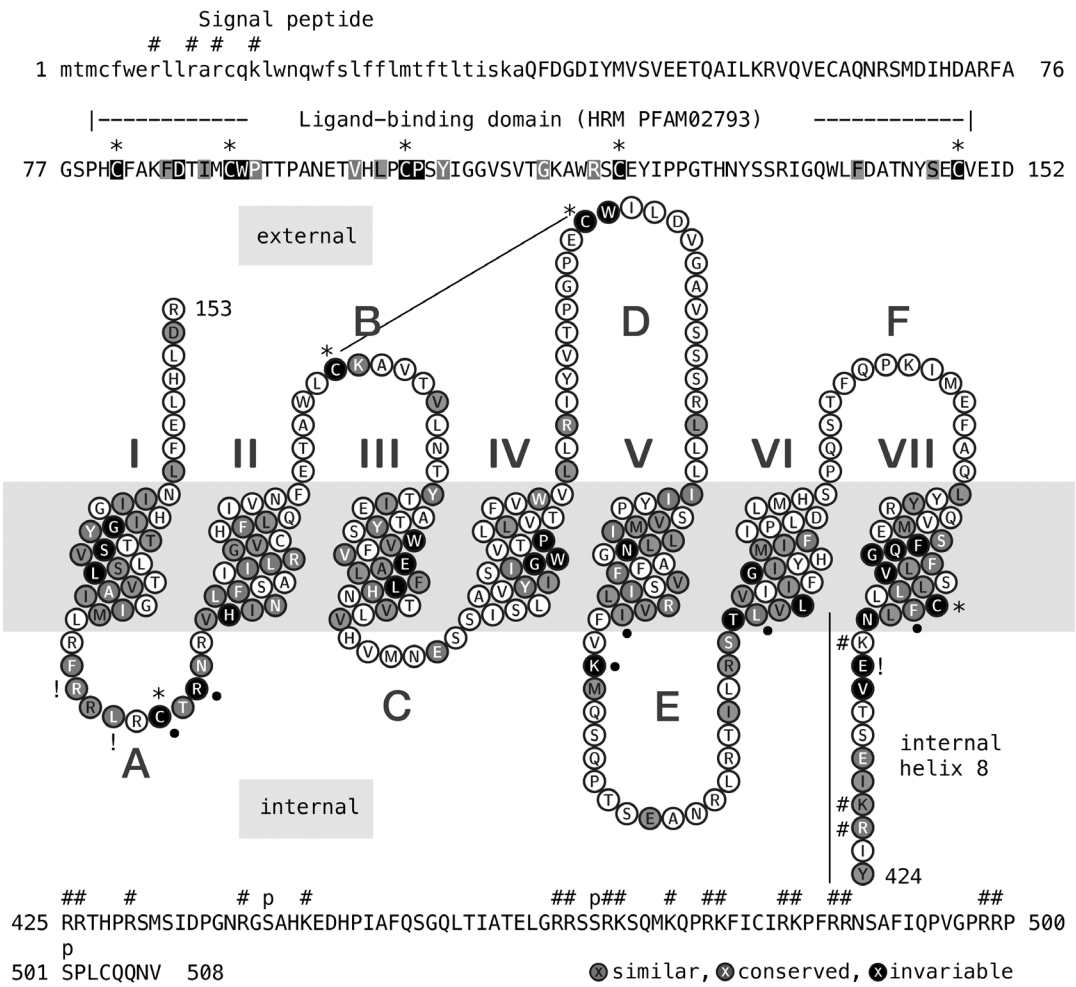

Fig 1–Membrane topology and sequence conservation of FgPTHRL. The 508 amino acid residues of FgPTHRL are shown with conservation to human family B1 GPCRs (SCTRL, PTHR2, CRHR2, GLP1R, VIPR1, VIPR2, GIPR, PACAP4, GCGR, GHRGR, CALCR, CALCRL) indicated by shading for invariable, conserved (residue present in >50% of the sequences), and similar positions (see shading codes at the figure bottom). The predicted N-terminal signal sequence is shown in lower case lettering. The external ligand-binding region, spanning residues P79 to I151, as defined by PFAM database entry pfam02793, hormone receptor domain (HRM, E-value: 8.66e-13) is indicated. Putative transmembrane helices (I-VII) and internal/external loops (A-F) are shown schematically folded with extension of helical structures based on the resolved structure of human glucagon receptor (Siu *et al*, 2013). Residues L160 to V405 encompass PFAM database entry pfam00002, 7 transmembrane receptor (secretin family, 7tm_2, E-value: 9.58e-40). Invariable C residues are indicated by asterisks (*). Conserved residues known to interact with G-proteins are indicated by a black circle (•), residues suspected in key interactions are indicated by an exclamation mark (!) (Vohra *et al*, 2013). Positively charged residues in the signal sequence and the internal C-terminal sequence are indicated by hash symbols (#). Three putative serine phosphorylation sites in the C-terminal region present in the orthologous schistosome proteins are indicated by lower case p characters.

Fig 2–Phylogenetic analysis of FgPTHRL. (A) Family B secretin-like GPCRs. Multiple alignment of secretin-like receptor sequences used by Mirabeau and Joly (2013) (http://neuroevo.org/alignments/receptors/_secretinR_final.aln) was amended with *F. gigantica*, *C. sinensis*, *S. mansoni* PTHRL sequences and used for phylogenetic analysis using PhyML. Invertebrate and Chordata PTHR/PTHRL are evolutionary related (shaded areas). (B) Phylogenetic tree constructed in MrBayes based on a multiple sequence alignment of putative orthologs of mammalian parathyroid hormone/parathyroid hormone-related peptide receptor. Species abbreviations are as follows. Annelida: *Capitella teleta* (Ct), Arthropoda: *Bombus impatiens* (Bi), *Cimex lectularius* (Cl), *Diachasma alloeum* (Da), *Halyomorpha halys* (Hh), *Microplitis demolitor* (Md), *Pediculus humanus corporis* (Pc), *Tribolium castaneum* (Tc); Platyhelminthes: *C. sinensis* (Cs), *F. gigantica* (Fg), *S. mansoni* (Sm); Mollusca: *Aplysia californica* (Ac), *Crassostrea gigas* (Cg), *Octopus bimaculoides* (Ob); Chordata: *Branchiostoma floridae* (Bf), *Ciona intestinalis* (Ci), *Gallus* (Gg), *Lepisosteus oculatus* (Lo), *Salmo salar* (Ss), *Thamnophis sirtalis* (Ts), *Xenopus tropicalis* (Xt). The human calcitonin receptor was used as an outgroup. Significant posterior probability values are indicated.

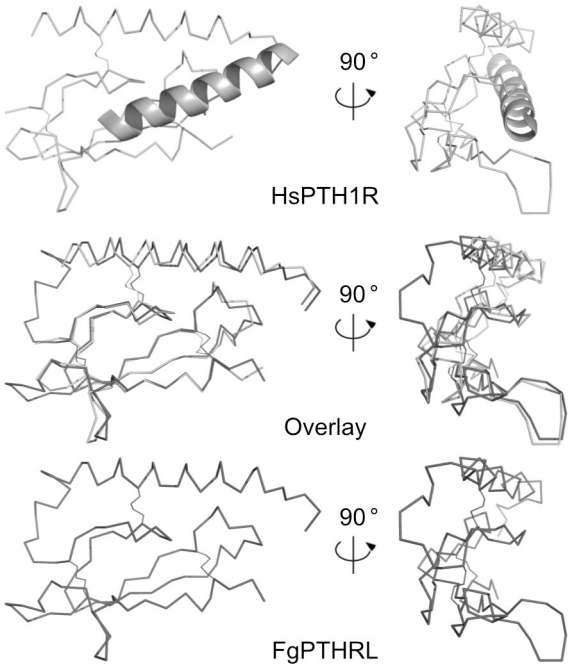

Fig 3–Structural model of extracellular ligand-binding domain of FgPTHRL generated using Phyre2 (Kelley *et al*, 2015). The model was based on the resolved structure of human PTH1R extracellular domain [PDB: 3C4M (Pioszak and Xu, 2008)]. Shown are the backbone traces of the extracellular domains of human PTH1R [residues 57-105 forming a disordered loop are not shown (Pioszak and Xu, 2008)] including bound PTH (residues 15-34), FgPTHRL, and an overlay of both extracellular domains.

main (amino acids 35-153) was analyzed for its tertiary structure by modeling in Phyre2 (Fig 3) and the first ranked match was to the ectodomain of human parathyroid hormone-related protein receptor (PTH1R), the structure of which had been resolved using a complex of PTH and parathyroid hormone-related protein (PTHrP) (Pioszak and Xu, 2008; Pioszak *et al*, 2009). The same result was obtained for SmPTHRL and is, in both cases, due to the higher sequence conservation with the human PTH1R ectodomain than to those of other B1 family GPCRs. However, it is also obvious that the putative ligand-binding site of the parasite receptor is different in surface structure and charge distribution from those of the human receptor.

Nucleic acid hybridization analysis

In order to confirm the origin of the identified sequence from *F. gigantica* genome, the full-length cDNA was subjected to Southern hybridization and the transcripts present in various developmental stages of the parasite were subjected to northern hybridization and to RT-PCR analysis. The results supported the existence of a gene encoding FgPTHRL and that it was transcriptionally active in juvenile and mature parasites (Fig 4).

Function of FgPTHRL

The human parathyroid hormone 1 receptor (PTH1R) regulates calcium homeostasis (Potts and Jüppner, 1997). Surmising that this is an evolutionary conserved function that is also present in FgPTHRL, then the transcriptional activity of its gene itself should be regulated by the external calcium concentration. Hence a comparative reverse transcriptase qPCR analysis was used to conclude gene activity in adult worms at low and high external calcium concentration and a 3.6 fold increase in FgPTHRL mRNA was observed in the presence of 2 nM external calcium (Fig 5). Calcium-dependent upregulation was observed for mRNA of the calcium sensor calmodulin (CALM) and calcium buffer calreticulin (CALR), but not with glutathione S-transferase 1 (GST-1) and heat

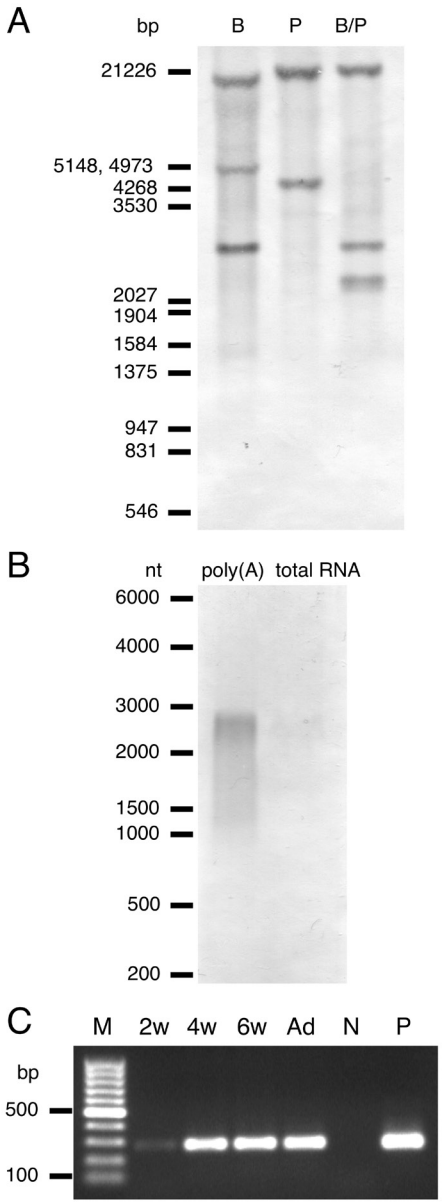

Fig 4–Nucleic acid analysis of FgPTHRL. (A) Southern hybridization. DNA (50 μg) per lane was digested with *Bam*HI (B), *Pst*I (P) and *Bam*HI/*Pst*I (B/P) and detected with a 1418-bp DIG-labelled FgPTHRL cDNA probe. (B) Northern hybridization. *F. gigantica* poly(A)+ RNA (10 μg) and total RNA (50 μg) were detected with a 1418-bp DIG-labelled FgPTHRL cDNA probe. (C) RT-PCR. Total RNA from 2, 4, and 6-week-old juveniles and adult *F. gigantica.* N, negative and P, positive control.

shock protein 70 (HSP70) (negative controls).

DISCUSSION

In the present work a *F. gigantica* FgPTHRL was identified as the single member of family B1 GPCRs in this trematode genus and potentially in other trematode genera, *eg*, *Schistosoma* and *Clonorchis*/*Opisthorchis*. Evolutionary conservation of structural and functional important motifs in B GPCRs (Siu *et al*, 2013; Vohra *et al*, 2013) indicates that not only the general structure but also regulation of activity and binding with a G-protein complex are conserved in FgPTHRL. The C-terminal region of the trematode receptor contains several conserved putative serine phosphorylation sites and the importance of C-terminal serine/threonine phosphorylation has been previously demonstrated for regulation of mammalian GPCR activity (Butcher *et al*, 2012). The mammalian members of family B1 GPCRs function as peptide hormone receptors, prominent ligands of which are glucagon and secretin (Couvineau and Laburthe, 2012).

FgPTHRL is related to PTHR of diverse species in other phyla. However, ligands resembling PTH or PTHrP have so far not been isolated and characterized in trematodes or any protostome and are thought to be too divergent to be identified by sequence comparison tools (Mirabeau and Joly, 2013). However, PTH was detected in snail ganglia in a study using an anti-bovine-PTH antibody but the

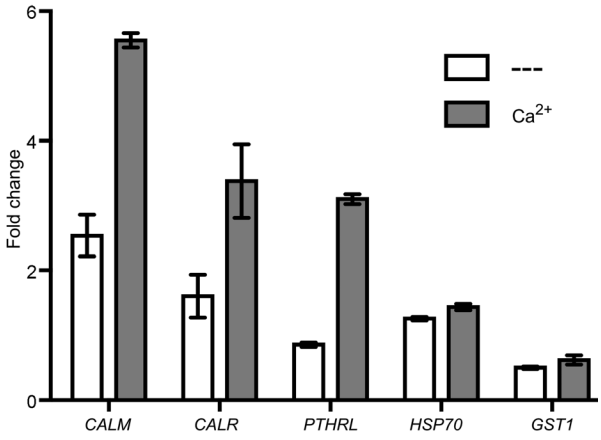

Fig 5–Transcript levels of *F. gigantica* calmodulin (CALM), calreticulin (CALR), PTHRL, heat shock protein (HSP70), glutathione S-transferase 1 (GST-1) genes in adult worms incubated in PBS without (---) and with 2 nM calcium (Ca^{2+}) for 10 hours. Quantification of RT-quantitative PCR was determined using tubulin mRNA as internal control. Each bar represents mean ± SEM of independent measurements in triplicate from five worms.

14 kDa antigen was not isolated to verify its putative property (Hull *et al*, 2006). Without a ligand it is difficult to assay a receptor function and to study its biological role(s) as has been achieved in trematodes for family A GPCRs with conserved ligands, *eg*, dopamine, serotonin **(**Taman and Ribeiro, 2009; El-Shehabi *et al*, 2012; Patocka and Ribeiro, 2013; Patocka *et al*, 2014**)**. Considering the absence of a circulatory system in trematodes, paracrine activation of the receptor is likely. Human PTH1R, for example, is stimulated in an endocrine fashion by PTH, but paracrine/autocrine stimulation by PTHrP, with PTH-activated pathways controlling serum calcium level, and PTHrP also being important during development (Gensure *et al*, 2005). It is of interest that transcriptional activity of the FgPTHRL gene was upregulated in the presence of external calcium indicating a feedback mechanism to regulate receptor abundance depending on calcium level. Phylogenetic analysis of PTH receptors demonstrated they belong to family B1 GPCRs that are conserved in many phyla (Mirabeau and Joly, 2013), which is understandable given the many cellular functions of calcium. Based on bioinformatics analysis, species in other protostome phyla have a richer set of B1 receptor family including PDF (involved in circadian rhythm) and DH31 and DH44 (involved in diuretic function) receptors (Mirabeau and Joly, 2013; Cardoso *et al*, 2014). Remarkably, in our screen for sequences related to FgPTHRL in protostomes we noticed an especially large but unexplored set of family B1 GPCRs present in *Octopus bimaculoides* (Mollusca, Cephalopoda) that at least matched the number of identified human family B1 GPCRs. Less complex body organization, physiology, and evolutionary adaptation to parasitic lifestyle might have led to the loss of all but most important family B1 GPCRs in trematodes.

In summary, we have characterized the single family B1 GPCR of *F. gigantica* as a distant homolog of the vertebrate parathyroid hormone receptor. Transcriptional activity of FgPTHRL gene was positively correlated with external calcium level and the gene was transcribed in the juvenile and mature parasite. Future research will focus on tissue-specificity and functional analysis of the receptor for its potential as a drug target.

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