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Histamine signalling in *Schistosoma mansoni*: Immunolocalisation and characterisation of a new histamine-responsive receptor (SmGPR-2) ☆

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ABSTRACT

In parasitic platyhelminthes, including *Schistosoma mansoni*, biogenic amines play several important roles in the control of motility, metabolism and reproduction. A bioinformatics analysis of the *S. mansoni* genome identified approximately 16 full-length G protein-coupled receptors (GPCRs) that share significant homology with aminergic receptors from other species. Six of these sequences are structurally related to SmGPR-1 (formerly SmGPCR), a previously described histamine receptor of *S. mansoni*, and constitute a new clade of amine-like GPCRs. Here we report the cloning of a second member of this clade, named SmGPR-2. The full-length receptor cDNA was expressed in *Saccharomyces cerevisiae* and shown to be activated by histamine and 1-methylhistamine, whereas other common biogenic amines had no significant effect. Antagonist assays showed that SmGPR-2 was inhibited by classical biogenic amine antagonists but the pharmacological profile was unlike those of known mammalian histamine receptors. Confocal immunolocalisation studies revealed that SmGPR-2 was expressed in the nervous system and was particularly enriched in the subtegumental neuronal plexus of adult *S. mansoni* and larvae. The ligand, histamine, was found to be widely distributed, mainly in the peripheral nervous system including the subtegumental plexus where the receptor is also expressed. Finally, SmGPR-2 was shown to be developmentally regulated at the RNA level. Quantitative PCR studies showed it was up-regulated in the parasitic stages compared with cercaria and expressed at the highest level in young schistosomula. The widespread distribution of histamine and the presence of at least two receptors in *S. mansoni* suggest that this transmitter is an important neuroactive substance in schistosomes.

1. Introduction

Schistosoma mansoni (Platyhelminthes, Trematoda) is a major cause of human schistosomiasis, a disease that afflicts over 200 million people worldwide. *S. mansoni* exists where its intermediate host, the freshwater snail *Biomphalaria glabrata*, is available, notably in Africa, the Middle East, South America and the Caribbean. Praziquantel is the drug of choice for treatment of schistosomiasis but drug-resistant strains have emerged and thus alternative chemotherapeutic agents should be designed and tested (Fallon and Doenhoff, 1994; Ismail et al., 1994; William et al., 2001). Many pharmaceutical drugs exert their effects by interacting with G protein-coupled receptors (GPCRs) (Wise et al., 2002; Eglen, 2005), in particular Family A (Rhodopsin-like) GPCRs, which include the vast majority of small transmitter and hormone receptors. While a few GPCRs have been cloned from schistosomes (Hoffmann et al., 2001; Hamdan et al., 2002; Pearson et al., 2007; Taman and Ribeiro, 2009), there are many more predicted sequences in the *S. mansoni* gene database that have yet to be characterised (Berriman et al., 2009). These GPCRs are potentially good targets for new anti-schistosomal drugs, especially if their pharmacological profiles prove to be parasite-specific.

Biogenic amines (BAs) are derivatives of amino acids (tryptophan, tyrosine or histidine) and act as neurotransmitters, hormones and modulators. They include such ubiquitous substances as serotonin (5-hydroxytryptamine, 5HT), catecholamines (dopamine and noradrenaline) and histamine (HA). In platyhelminthes, BAs play many vital roles in metabolism, the control of motility and therefore survival within the host (Ribeiro et al., 2005; Maule et al., 2006; Ribeiro and Geary, 2010). The most widespread and best studied BA is 5HT. Serotonergic neurons are distributed abundantly in the CNS and peripheral nervous system (PNS) of every flatworm tested to date, including *S. mansoni*. Moreover, 5HT is strongly myoexcitatory (Day et al., 1994; Pax et al., 1996; Walker et al., 1996; Ribeiro et al., 2005; Maule et al., 2006) and there is evidence both for endogenous biosynthesis (Hamdan and Ribeiro, 1999) and carrier-mediated transport (Boyle and Yoshino, 2005; Patocka and Ribeiro, 2007). By comparison, little is known about other BAs, particularly HA. HA is variably distributed among

☆ Nucleotide sequence data reported in this paper are available in the GenBank database under the Accession No. GQ397114.

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parasitic flatworms. Some species are capable of endogenous HA biosynthesis and have very high tissue levels of the amine (Mettrick and Telford, 1963; Eriksson et al., 1996), whereas in other parasites HA is present at low levels and may be entirely of host origin (Yonge and Webb, 1992). The biological role of HA in flatworms is unclear but it probably affects the musculature and the outcome is concentration-dependent. It was reported that HA significantly modulates movement in the posterior region of the strobila in *Hymenolepis diminuta* (Sukhdeo et al., 1984) and stimulates motility in *S. mansoni* (Ercoli et al., 1985). HA-containing neurons innervate the somatic musculature and the suckers in some species (Wikgren et al., 1990; Eriksson et al., 1996), which further supports a role in the control of muscle function and movement. The distribution of HA neurons in *S. mansoni* has not been investigated.

Previously, a GPCR from *S. mansoni*, named SmGPR-1 (formerly SmGPCR), was cloned in our laboratory and was shown to be selectively activated by HA (Hamdan et al., 2002). Further analysis of this receptor revealed that it was expressed in the tegument and musculature of larval and adult parasites (El-Shehabi et al., 2009). Following completion of the *S. mansoni* genome project, we detected several new sequences that are structurally related to SmGPR-1. Bioinformatics analyses suggest these sequences have evolved from a common ancestor and constitute a new structural type of BA receptor. Given their novelty, we have adopted the system of classification used for human orphan GPCRs and designated these sequences as *S. mansoni* GPR receptors (SmGPR). In the present study, we report the cloning, functional analysis and immunolocalisation of a new member of this clade, named SmGPR-2. The results indicate that SmGPR-2 is a second histaminergic receptor of *S. mansoni* and is expressed in close proximity to HA-containing neurons in the subtegumental neuronal plexus. We further demonstrate that histaminergic neurons are abundantly distributed in schistosomes, suggesting that HA is an important neuroactive system in this parasite.

2. Materials and methods

2.1. The parasite

B. glabrata snails infected with a Puerto Rican strain of *S. mansoni* were kindly provided by Dr. Fred Lewis, Biomedical Research Institute, Rockville, MD, USA. *S. mansoni* cercaria were collected 35–45 days p.i. (Lewis et al., 1986, 2001) and were mechanically transformed to produce schistosomula (Basch, 1981) as described by El-Shehabi et al. (2009). In vitro transformed schistosomula were cultured at 37 °C and 5% CO_2 in OPTI-MEM I medium (Invitrogen) supplemented with 10% FBS, streptomycin 100 μg/ml, penicillin 100 U/ml and fungizone 0.25 μg/ml (El-Shehabi et al., 2009). To obtain adult parasites, 28-day-old female CD-1 mice were infected with 150 cercaria/animal by skin penetration. Adult *S. mansoni* worms were recovered 6–7 weeks p.i. by perfusion of the liver (Basch and Humbert, 1981), washed extensively and either flash-frozen in liquid nitrogen for subsequent RNA extraction or fixed in 4% paraformaldehyde (PFA) for immunolocalisation experiments. Animal care was conducted according to the protocol approved by the Animal Care Committee of McGill University, Canada (Protocol No. 3346).

2.2. Cloning of S. mansoni SmGPR-2

The full-length SmGPR-2 cDNA was cloned from adult *S. mansoni* based on a predicted coding sequence (Smp_043340) obtained from the *S. mansoni* Genome database (*S. mansoni* GeneDB; http://www.genedb.org/genedb/smansoni/). Total RNA was purified from 25 to 30 adult *S. mansoni* worms (Qiagen RNeasy kit) and was oligo-dT reverse-transcribed with M-MLV reverse transcriptase (Invitrogen), according to standard procedures. To clone SmGPR-2, we designed primers that targeted the beginning and end of the predicted coding sequence. The primer sequences were as follows: 5′-ATGAAACAAGTGTTTTTAAATGACAACAG-3′ (sense) and 5′-TTATATATTCCTTCCAATATGTAATAAACG-3′ (antisense). A proof-reading Platinum *Pfx* DNA polymerase (Invitrogen) was used to amplify the cDNA in a standard PCR reaction (35 cycles of 94 °C/15 s, 55.6 °C/30 s and 68 °C/90 s). The resulting amplicon (1656 bp) was gel excised, purified (QIAquick spin kit, Qiagen), ligated to a pGEM-T Easy vector (Promega) and verified by DNA sequencing.

2.3. Yeast functional expression assays

The SmGPR-2 coding sequence was sub-cloned between the *Nco*I/*Xba*I restriction sites of the yeast expression vector Cp4258 (kindly provided by Dr. J. Broach, Princeton University, NJ, USA) and the resulting construct was confirmed by DNA sequencing. The functional expression assay was adapted from the protocol of Wang et al. (2006) as described by Kimber et al. (2009). The receptor was expressed in *Saccharomyces cerevisiae* strain YEX108 (*MATα* P_{FUS1}*-HIS3* P_{GPA1}*-Gαq(41)-GPA1-Gaq(5) can1 far1Δ 1442 his3 leu2 lys2 sst2Δ2 ste14::trp1::LYS2 ste18Δ6-3841 ste3Δ1156 tbt1-1 trp1 ura3*; kindly provided by J. Broach, Princeton University, NJ, USA). This strain expresses the *HIS3* reporter gene under the control of the *FUS1* promoter (Stevenson et al., 1992) and contains an integrated copy of a chimeric Gα gene in which the first 31 and last five codons of native yeast Gα (GPA1) were replaced with those of human Gαq (Wang et al., 2006). Strains carrying chimeras of GPA1 and human Gαi2, Gα12, Gαo or Gαs were also tested in preliminary experiments but were found to yield lower or no receptor activity compared with strain YEX108. *S. cerevisiae* were cultured in yeast YPD medium (1% yeast extract, 2% peptone and 2% dextrose), according to standard conditions and transformation was performed by the lithium acetate method (Gietz et al., 1995), using 200 μl mid-log phase cells, 200 μg carrier single stranded (ssDNA) (Invitrogen) and 1 μg Cp4258-SmGPR-2 or empty plasmid as a negative control. Positive transformants were selected on synthetic complete (SC) 2% glucose solid medium lacking leucine (SC/leu$^-$). For the agonist assay, single colonies of transformants carrying plasmid Cp4258-SmGPR-2 or vector alone (mock control) were cultured overnight in SC/leu$^-$ liquid medium at 250 rpm/30 °C. The next day, cells were washed three times in SC 2% glucose liquid medium that lacked both leucine and histidine (SC/leu$^-$/his$^-$). Cells were finally resuspended in SC/leu$^-$/his$^-$ medium supplemented with 50 mM 3-(N-morpholino)propanesulfonic acid (MOPS), pH 6.8 and 1.5 mM 3-Amino-1, 2, 4-Triazole (3-AT). 3-AT inhibits the gene product of *HIS3* and was used to reduce basal growth due to endogenous background signalling (Wang et al., 2006). Aliquots of cell culture containing approximately 3000 cells were added to each well of a 96-well plate containing test agonist or vehicle plus additional medium for a total reaction volume of 100 μl. The plates were incubated at 30 °C for 22–26 h, after which 10 μl of Alamar blue (Invitrogen) was added to each well. The plates were returned to the 30 °C incubator until the Alamar blue began to change to pink (approximately 1–4 h) and fluorescence (560 nm excitation/590 nm emission) was measured at 30 °C every 30 min for 3–4 h using a plate fluorometer (FlexStation II, Molecular Devices, USA) Antagonist assays were done in the same way, except that each well contained 10^{-4} M agonist (HA or 1-methylHA, as indicated) and the antagonist at the specified concentration. Data analyses and dose–response curve fits were performed using Prism v5.0 (GraphPad software Inc.).