An unusual receptor tyrosine kinase of *Schistosoma mansoni* contains a Venus Flytrap module

Jérôme Vicogne a, Jean-Philippe Pin b, Vinca Lardans a, Monique Capron a, Christophe Noël a, Colette Dissous a, *

a Unité INSERM 547, Institut Pasteur Lille, 1 rue du Pr. Calmette, 59019 Lille Cedex, France  
b CNRS UPR 9023, 34094 Montpellier Cedex 5, France

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Abstract

Previous studies have suggested that successful development of the parasitic helminth *Schistosoma mansoni* must be dependent on an adaptative molecular dialogue with its hosts and on the existence of receptors for growth factors and hormones. Attempts to identify a homolog of the insulin receptor (IR) have led us to characterize a new receptor tyrosine kinase (RTK) molecule in *S. mansoni*. SmRTK-1 is an integral membrane protein with a single membrane-spanning sequence separating an extracellular ligand-binding domain and a cytoplasmic TK domain. Structural and phylogenetic analyses of the kinase domain of SmRTK-1 confirmed its similarity to IR catalytic domains. However, sequence analysis of the extracellular domain of SmRTK-1 revealed similarity with various proteins (such as drug receptors) that share a structure known as the Venus Flytrap (VFT) module. Alignment with other VFT modules for which the structure has been solved was used to generate a 3D model of the putative VFT module of SmRTK-1. Phylogenetic analysis indicated that the SmRTK-1 VFT module was closer to that of the GABAB receptor. Numerous RTK genes recently discovered in vertebrate and invertebrate species code for large families of modular proteins with diverse structures and ligand-binding specificities. SmRTK-1 probably represents a new class of RTK whose function remains to be determined. RTKs are present in all metazoans and associated with the control of metabolism, growth and development. The preferential localization of SmRTK-1 in sporocyst germinal cells and ovocytes could be in favor of its function in schistosome growth and differentiation.

Keywords: *Schistosoma mansoni*; Trematode; Receptor tyrosine kinase; Venus Flytrap module

1. Introduction

Development and differentiation of schistosomes have been shown to be dependent on host-derived signals. Several works have reported the importance of thyroid and steroid hormones in the survival, growth and differentiation of schistosomes in rodents [1,2] but no receptor for these hormones has been identified in the parasite. However, membrane-bound receptor proteins have been characterized in *Schistosoma mansoni* that are susceptible to transmit growth signals in the parasite. A protein with kinase activity specific for serine and threonine that belongs to the TGF-β receptor family, has been identified at the surface of adult worms [3]. In addition, a homologue to the epidermal growth receptor with a tyrosine kinase (TK) activity has been shown to be expressed in adult parasite muscles [4,5]. Signaling pathways mediated through receptors with tyrosine kinase (RTK) activity regulate metabolism, growth and development in metazoans. One of these pathways, activated by insulin and insulin-like molecules, plays a central role in invertebrates, affecting cell division and differentiation in arthropods, molluscs and in more ancient metazoan phyla such as cnidaria and sponges [6,7]. Conflicting results have been reported on the effect of insulin on helminth parasites. In schistosomes, however, this growth peptide has been shown to increase oxygen and glucose uptake [8] as well as in vitro viability of parasite larvae [9], suggesting that insulin receptors exist in these organisms. Attempts to characterize in the *S. mansoni* genome, sequences encoding homologs of members of the insulin receptor (IR) family, have led us to...
clone two RTKs containing the conserved catalytic domain of IR members. One of these RTK (SmRTK-2) presented all the features of IR with a conserved ligand-binding domain (Vicogne et al., GenBank AF 314754) whereas the other one (SmRTK-1) possessed an unexpected extracellular domain with a structure similar to the Venus Flytrap (VFT) module of bacterial periplasmic binding proteins (PBPs) formed by two lobes that close upon ligand-binding. PBPs have provided a model for the structures of binding modules in different receptor families [10]. VFT modules are thus present in the extracellular N-terminus of the family 3 G-protein coupled receptors that includes the γ-aminobutyric acid type B (GABA_B) receptor [11,12], the metabotrope glutamate receptors (mGluR) [13] and the Ca^{2+} sensing receptor [14] as well as in the extracellular domain of guanylate cyclase receptors such as the natriuretic peptide receptors [15,16].

This paper describes the characterization and the structural analysis of the SmRTK-1 receptor which unusually associates an N-terminal VFT module with a TK domain and thus probably belongs to a new class of RTK. A potential role of this original molecule in parasite signaling and development is discussed.

2. Materials and methods

2.1. Parasites

A Puerto-Rican strain of S. mansoni was maintained by passage through albino B. glabrata snails and Mesocricetus auratus hamsters. Miracidia, cercariae and schistosomula were prepared as previously described [17]. Miracidia were transformed in vitro into sporocysts by incubation in Minimum Salt Medium at 28°C for 18 h [18]. Adult schistosomes were collected by portal perfusion from infected hamsters.

2.2. Cloning of SmRTK-1 and sequence analysis

Total RNA was isolated from S. mansoni adult worms by the method of Chirgwin et al. [19] and purified by centrifugation through a cesium chloride gradient. The poly(A)^+ RNA was purified by passage through an oligo(dT) -cellulose column (Pharmacia). The first-strand cDNA synthesis was performed using the ThermoScript™ RT-PCR System (Invitrogen) and the oligo(dT) primer. A pair of degenerate primers (INRB: 5'-GGN TCG TTY GGN A TG GTD TA Y and INRR: 5'-GGN TCN TTY GGN AFG GTD TAY VAR GG-3') and INRB: 5'-ATR TCH CKD GYC ATD CCR AAR TCD CC-3') complementary to conserved sequences from subdomains I and VII of the tyrosine kinase (TK) domains of various IR sequences was used to amplify schistosome TK sequences from parasite cDNA. PCR was carried out using the Taq Gold polymerase (Perkin-Elmer) under the following conditions: 10 min at 94°C, 40 cycles (1 min at 94°C, 1 min at 50°C, 1 min at 72°C). PCR products were cloned into the pCR™ II vector (Invitrogen) and sequenced. The full-length sequence was cloned from a sporocyst cDNA library constructed from 1 μg of poly(A)^+ RNA using the Marathon cDNA amplification kit (Clontech). Adaptor primers were used in combination with internal primers to amplify cDNA overlapping fragments. Sequencing reactions were performed using the dye terminator cycle sequencing kit and analyzed on an ABI Prism 377 DNA sequencer (Perkin-Elmer Biosystems). Sequence analysis was performed using the DNASTAR program.

2.3. Molecular modeling

A 3D model of part of the extracellular domain of SmRTK-1 (from residue 379 to 858) was generated in order to validate its structural similarity with the Venus Flytrap module of the bacterial periplasmic amino acid binding proteins (PBPs) of the class 3 [20]. First, a sequence alignment of VFT modules for which the structure has been solved was generated. These proteins include the leucine/isoleucine/valine binding protein (LIVBP, pdb code 2LIV [21]), the leucine binding protein (LBP, pdb code 2LBP [22]), the amide binding protein (AmIC (pdb code 1PEA [23])), the extracellular domain of the atrial natriuretic peptide receptor A (ANPA receptor, pdb code 1DP4 [15]) and the extracellular domain of the mGlu1 receptor (pdb code 1EWK [24]). Because the sequence identity between these proteins is less than 17%, no accurate alignment could be obtained using ClustaW [25]. The alignment was therefore deduced from the superposition of these structures using Swiss-PdbViewer. The sequences of the extracellular domain of the GABA_B receptors (GB1 and GB2) were aligned on top of these sequences based on our previous work [11,12]. The putative secondary structure elements of the VFT module of SmRTK-1 were identified using the IPRED2 server (http://pura.ebi.ac.uk:8888/submit.html [26]). The sequence of the VFT module of SmRTK-1 was aligned on these template sequences using the profile command of ClustaW (1.6) with the default parameters, and then manually modified using SeqView (The Garvan Institute of Medical Research, Australia) to superpose the secondary structure elements. Gaps were excluded from the putative secondary structure elements unless necessary. The 3D models were then generated using Modeler [27] in the Insight-II environment (Molecular Simulation Inc., San Diego, CA, USA) and the above mentioned structures as templates. The models were checked for validity using the Verify 3D algorithm [28] (Profile 3D in the Insight-II environment) and a slide window of 21 residues. The alignment corresponding to regions of the model with low 3D/1D scores according to Verify 3D was modified to improve the 3D/1D scores.

2.4. Phylogenetic analyses

For phylogenetic analysis of the VFT domain of SmRTK-1, a set of sequences of various VFT modules were aligned together with that of SmRTK-1 on top of the struc-
tural alignment described above, using the profile command of the ClustalW (1.6) program with the default parameters. The alignment was manually modified in order to introduce the gaps in the loops rather than in the predicted secondary structure elements. The phylogenetic analysis and the bootstrapping were performed using ClustalW (keeping the gaps, and with the default parameters), and the tree was drawn using TreeView (version 1.6.2).

For the construction of the TK phylogenetic tree, sequences were aligned with DNASTAR MegAlign 4.0 by the ClustalW method and 163 residues were retained for the phylogenetic analyses. Unrooted tree was calculated by the PHYLIP (Phylogeny Inference Package) version 3.57c developed by Felsenstein, using the Dayhoff PAM matrix. The bootstrap resampling method was applied with 1000 replications using SEQBOOT included in the PHYLIP package. The output was further subjected to the PROTDIST and NEIGHBOR procedures and the calculated tree was analyzed by the Consense method.

2.5. Production of antibodies

Anti-SmRTK-1 antibodies were produced in rabbits against three synthetic peptides (P1: MSQVSALITEQSDC, P2: CYNGHFTNNYDDSPS, P3: CESVRFAKRYQYPYF) derived from the N-terminal sequence of the protein and conjugated to ovalbumin (OVA) (Syn:tem, France). Rabbits were immunized with a pool of 250 μg of each OVA-peptide in complete Freund’s adjuvant, and boosted 3 weeks later with the same quantity of peptides in incomplete Freund’s adjuvant. Serum specificity and antibody titers were determined using ELISA tests developed, respectively, against the P1, P2 and P3 peptides.

2.6. Immunolocalization

Miracidia and adult worms were fixed in Immunohistofix (ULB, Belgium) for 4 days at 4°C, then washed twice in phosphate buffered saline (PBS) and dehydrated by incubation in successive 30, 50, 70, 90 and 100% ethanol baths, 15 min each. Specimens were embedded in Immunohistowax (ULB, Belgium) at 37°C overnight and stored at 4°C until use. Sections cut at a thickness of 3 mm were deposed on Superfrost Plus slides (O. Kindler GmbH & Co., Germany), deparaffinized by two 5 min washes in acetone and rehydrated in PBS. Slides were saturated for 30 min in 4% bovine serum albumin (BSA) in PBS containing 1% decomplemented goat serum, then washed in PBS. This step was followed by a 2 h incubation at room temperature with anti-SmRTK-1 rabbit antibodies diluted (1:400) in PBS—1% BSA. After three washes in PBS, slides were incubated for 1 h at room temperature with Alexa Fluor 488-conjugated goat anti-rabbit IgG (Molecular probes) at 1:1000 dilution, then placed in a counterstaining solution of 0.0001% Blue Evans in PBS. After washing in PBS, slides were mounted using the Fluoromount G reagent (Southern Biotechnology Associated). Green fluorescence was detected using a 488 nm filter UV light microscope (Leitz DM RB, Leica).

2.7. Western blot analysis

Crude membrane fractions were prepared from sporocysts and adult worms. Briefly, parasites were resuspended in PBS, sonicated and centrifuged at 100,000 × g for 1 h. Membrane pellets were resuspended in PBS and centrifuged once more in the same conditions and analyzed by SDS–PAGE and blotting. Following a blocking step with 5% powdered non-fat milk in PBS, nitrocellulose sheets were incubated with anti-SmRTK-1 rabbit serum (dilution 1/200) for 2 h, then with Horse-Radish Peroxylase (HRP)-conjugated anti-rabbit mouse antibodies (Sigma) for 1 h. The enzyme was detected using the SuperSignal West Pico Chemiluminescent Substrate (Percin Bio Science).

2.8. RT-PCR and in situ hybridization

SmRTK-1 transcripts were detected in different developmental stages of the parasite by RT-PCR. Total RNA was extracted using the Chirgwin’s technique [19] from miracidia, sporocysts, cercariae, schistosomula and adult male and female worms and reverse transcribed using the ThermoscriptTM RT-PCR System (Invitrogen). cDNAs were used as templates for PCR amplification using the Taq Gold Polymerase (Perkin-Elmer). Primers corresponding to bp 632–652 and the complementary sequence of bp 1625–1650 were used for amplification of a part of the SmRTK-1 sequence.

For in situ hybridization, parasites were fixed and embedded in the conditions used for immunocytochemistry. Deparaffinized sections were treated with proteinase K (1 μg ml⁻¹), dehydrated and processed for hybridization. A cDNA insert corresponding to the C121, V135 fragment of SmRTK-1 was cloned in the pBlueScript II SK + vector. Recombinant plasmid was used as a template for T3 and T7 RNA polymerases (Boehringer Mannheim) to produce sense and antisense RNA probes, respectively. Probes were labeled by digoxigenin (Dig)-11-dUTP in the presence of Dig RNA labeling Mix (Roche Laboratories) and used at a concentration of 25 μg ml⁻¹ for hybridization in 50% formamide, 5× SSC, 1× Denhardt’s solution, 0.5% Tween 20, 100 μg ml⁻¹ E. coli tRNA, 200 μg ml⁻¹ herring sperm DNA. Hybridization was carried out at 52°C overnight. Successive washings were carried out for 15 min with 2× SSC containing 0.1% Tween, 1× SSC, 0.5× SSC and 0.1× SSC. Slides were saturated in MBS (100 mM maleic acid pH 7.5, 150 mM NaCl) containing 4% blocking reagent (BR) (Boehringer Mannheim) and allowed to react for 2 h with alkaline phosphatase conjugated anti-Dig antibodies (Boehringer Mannheim) diluted 1:2000 in the MBS-BR solution. Slides were developed using the Fast Red reagent (Sigma) as substrate for alkaline phosphatase.
3. Results

3.1. Molecular cloning of SmRTK-1

Insulin has been shown to improve the viability and the resistance to antibody cytotoxicity of S. mansoni schistosomula in vitro [9]. In preliminary experiments, we also demonstrated that human or bovine insulin was able to stimulate in vitro both protein and DNA synthesis in sporocysts (unpublished data). These data suggested the existence of a receptor for insulin in S. mansoni. IRs have been conserved throughout evolution. Their tetrameric structure comprise two α-subunits with the extracellular domain for ligand-binding and two β-subunits with a tyrosine kinase catalytic domain which is highly conserved in vertebrates and invertebrates. On the basis of this identity, we designed degenerate oligonucleotides (see Experimental procedures) to conserved sequences delimiting the TK core domain of human [29], amphioxus [30], nematode [31], insect [32] and mollusc [33] IR sequences. Reverse and forward primers were used to amplify a 454 bp fragment from adult worm RNA by RT-PCR, that was homologous (about 40% identity) to the TK domains of other IR members. Further PCR experiments performed on a Marathon (Clontech) library of adult worm RNA using S. mansoni gene specific primers allowed us to obtain a complete cDNA sequence (SmRTK-1) of 6022 bp containing a C terminal poly A tail (GenBank accession number AF101194) and with a unique and continuous open reading frame of 1579 amino-acid residues starting at the ATG codon (position nt 120–122). Southern blot experiments performed with cercarial genomic DNA confirmed the S. mansoni origin of SmRTK-1. Northern blot experiments also indicated that a 6 kb mRNA was specifically recognized by an SmRTK-1 probe and confirmed that the SmRTK-1 sequence corresponded to the full length cDNA molecule (not shown).

Hydrophobic analysis of the predicted amino-acid sequence revealed the presence of two hydrophobic segments in the protein (Fig. 1). The first segment found in the N-terminal (first 24 residues) could represent the signal peptide of the protein while the second one between residues V942–V962 may represent a transmembrane domain. Research of homology indicated that SmRTK-1 was closely related to the members of IR family but this homology was restricted to the putative TK domain located C terminal to the central hydrophobic domain. The N-terminal part of the protein was completely divergent from that of IR but similar to VFT modules including that of the GABAB receptor. Also, the conserved tetrasubasic sequence generally used as a cleavage site in IR precursors was not found upstream from the putative transmembrane domain of SmRTK-1.

3.2. Structural and phylogenetic analyses of the kinase domain of SmRTK-1

Alignment with different IR β-chains provided evidence for the presence in SmRTK-1 of a conserved TK domain (from residues N90 to I1220) containing most of the residues expected to play a role in protein kinase activity (Fig. 2). The consensus sequence G905XGXXG910 essential for ATP binding is present as well as the conserved sequence V1024AVK-(16X)-E1045 implicated in ATP binding and necessary for its stabilization in the catalytic pocket [34]. The sequence H1129RDACRNC1137 fits with the consensus HRDAXXRNC found in the catalytic subdomain VI of many tyrosine kinases and in which aspartic acid is supposed to catalyze the phosphotransfer reaction. We could also note the presence of the triplet D1150FG required for magnesium binding. It represents in subdomain VII the most highly conserved short stretch in catalytic domains and is flanked on either side by hydrophobic G1149 and L1153 residues. The consensus P1174IRWMSPE sequence is also considered as a strong indicator of tyrosine substrate specificity [34]. Finally, Y1164 and Y1165 constitute the two putative autophosphorylation sites specific for IR.

![Fig. 1. Structural domains of the SmRTK-1 sequence. (A) Hydrophobicity plot according to Kyte and Doolittle with a window of 11 residues showing a prototypical signal peptide and one transmembrane domain (rounded position 950). (B) Regions of the sequence that share similarity with the Venus Flytrap (VFT) module and the tyrosine kinase (TK) domains are indicated. The N-terminal part of the VFT module highlighted with thick lines corresponds to the sequence that was identified as being similar to other VFT modules including that of the GABAB receptor using Blast. SP (signal peptide), TM (transmembrane domain).](image-url)
Fig. 2. Alignment of the catalytic domain of SmRTK-1 (AF101194) with the TK domains of Caenorhabditis elegans (AF012437), Drosophila melanogaster (U28136) and Mus musculus (P15208) insulin receptor sequences. Identical residues are indicated in red, conserved residues in bold letters and consensus sequences in italic letters. Arrows indicate the positions of INRF and INRR primers used for the initial amplification of parasite cDNA.

Fig. 3. Rooted tree showing the phylogenetic relationship between the kinase domain of SmRTK-1 (AF101194) and insulin receptor sequences from vertebrates (Rattus norvegicus A33837, Mus musculus P15208, Homo sapiens PO6213), cephalochordate (Branchiostoma lanceolata O02466) and invertebrates (Lymnaea stagnalis Q25410, B. glabrata AF101195, Aedes aegypti Q93105, Drosophila melanogaster U28136, Caenorhabditis elegans AF012437, fron raphanus type 1 Y17877, type 2 Y17878, S. mansoni AP144751, RFF receptor sequences from R. norvegicus (A33837) and H. sapiens (XO4434) were added). The catalytic domain of INR S. raphanus 1 was used as the outgroup sequence. Numbers indicate the levels of confidence as determined by bootstrap analysis.
Phylogenetic analysis of the SmRTK-1 kinase domain further demonstrated its similarity to IR catalytic domains from both invertebrates and vertebrates. The phylogenetic tree was constructed and rooted with the sequence of the TK domain of sponge IR (Fig. 3). Selected sequences of IR from vertebrates were grouped and clustered with their insulin growth factor (IGF) receptor homologs. The SmRTK-1 sequence was positioned on one branch of the tree together with the TK domain sequence of the other *S. mansoni* RTK (SmRTK-2) corresponding to the putative IR of this trematode (Vicogne et al., GenBank AF314754). This relationship is statistically solid as shown by the boot-strap values. Both schistosome proteins appear to be close to the *daf-2* sequence from the nematode *Caenorhabditis elegans*.

3.3. Structural and phylogenetic analyses of the N-terminal part of SmRTK-1

Further sequence alignments did not reveal any homology between the N-terminal portion of SmRTK-1 and the extracellular domains of various IR. Secondary structure predictions also confirmed that SmRTK-1 did not exhibit the classical arrangement of the ligand-binding domain of IR members usually formed by two homologous domains separated by a cysteine-rich region. This indicates that SmRTK-1 belongs to an RTK class distinct from the class II. Surprisingly, searches in protein databases indicated a significant sequence similarity between the SmRTK-1 sequence (from residues N379 to V858) and part of the ligand recognition domain of the GABA₉ receptor (Blast Sum probability $5.5 \times 10^{-12}$). Other sequences with significant sequence similarity identified using PyrBlast were bacterial periplasmic amino acid binding proteins, operon regulator proteins, the extracellular portion of the metabotropic glutamate receptors, the extracellular domain of the natriuretic peptide receptors, a portion of the extracellular domain of the ionotropic glutamate receptor subunits either from mammals or plants (*Arabidopsis thaliana*).

The resolution of the structure of some of these proteins [15,16,21–24] as well as the generation of 3D models for others [11,12,35] is consistent with all these proteins having a similar 3D fold although their overall sequence identity is often less than 20%. These proteins are constituted of two lobes interconnected by three short linkers. Because they are in an open conformation that often closes upon binding of the ligand in the cleft, such protein domains are called Venus Flytrap (VFT) modules. In order to ascertain the possible VFT-like structure of one part of the extracellular domain of SmRTK-1, a large proportion of the N-terminal sequence of SmRTK-1 was aligned with that of VFT modules for
Fig. 5. Ribbon view of the 3D model of the VFT module of SmRTK-1. (A) The model was constructed using Modeller. The structures of the extracellular domain of the ANPA receptor (1DP4), LIVBP (2LIV), AmiC (1PEA) and the extracellular domain of mGlu1 receptor (1EWK) were used as templates with the alignment depicted in Fig. 1. The alpha helices are in red, the beta strands in yellow. The cysteine residues possibly involved in intramolecular disulfide bonds like in the GABA\textsubscript{B} extracellular domain are shown. (B) Plot obtained using the Verify 3D program and the coordinates of the 3D model of the VFT module of SmRTK-1. The $x$-axis numbering corresponds to the amino acid numbering of SmRTK-1. The $y$-axis gives the average 3D/1D scores for residues in a 21-residue sliding window.

which the structure is known (Fig. 4). For the alignment, both the sequence and the predicted secondary structure elements were taken into consideration. As shown in Fig. 4, residues 379-858 can be aligned with the five entire VFT modules used as templates, although the overall sequence identity is less than 17%.

This alignment was then used to generate a 3D model of the SmRTK-1 putative VFT module (Fig. 5A). According to this model, the two disulfide bonds predicted in the GABA\textsubscript{B} VFT modules [11], are conserved in SmRTK-1. This model was then assessed using the Verify 3D algorithm [28]. As shown in Fig. 5B, the 3D/1D scores of our model are always positive and are similar to those obtained with the template structures. The scores obtained are in the range of scores for highly refined correct X-ray structure determinations, indicating that this domain of SmRTK-1 can fold like a VFT module. A phylogenetic analysis was also performed with a wide range of VFT modules. The alignment generated above was extended to other similar proteins, and then used to build a phylogenetic tree. As shown in Fig. 6, the SmRTK-1 VFT module was found to be closest (Bootstrap value 793) to the GABA\textsubscript{B} VFT modules.

3.4. Expression of SmRTK-1 in developmental stages

Further experiments were concerned with the detection of SmRTK-1 transcripts in the different schistosome developmental stages. RT-PCR results first indicated the presence of SmRTK-1 mRNA in all larval and adult stages of the parasite (Fig. 7A). In situ hybridization experiments confirmed the presence of the transcripts in parasite tissues, revealing specific staining in male, female and larval stages. In male schistosomes, we observed a preferential localization of SmRTK-1 transcripts in parenchymal cells (Fig. 7B, b) whereas in female worms, an intense labelling was associated with oocytes contained in the ovary and in the ovary duct (Fig. 7B, c and e). In miracidia and newly-transformed sporocysts, a specific staining was detected in cells surround-
Fig. 6. Phylogenetic tree constructed using various VFT modules. These include the sequences of the GABA B receptor subunits (GABA B R1 from rat (Q9Z0U4) and D. melanogaster (GenBank AF318272), GABA B R2 from rat (O88871) and D. melanogaster (GenBank AF318273), and the GABA B R3 from D. melanogaster (GenBank AF318274)), the atrial natriuretic peptide receptors A (ANPA HUMAN, P16066 ) and C (ANPC HUMAN, P17342) from Human and the type C from Anguilla japonica (ANPC ANGJA, P55202), periplasmic amino acid proteins from E. coli (LIVJ ECOLI, P02917) and Pseudomonas aeruginosa (BRAC PSEAE, P21175) and from the cyanobacterium Synechocystis sp. (Q55387), the amid binding protein AmiC from Pseudomonas aeruginosa (P27017) and nitril hydratase regulator I from Boodleaeococcus thalassiae (Q36035), the bovine parathyroid Ca-sensing receptor (BoPCaR, P35384), the rat mGluR1 (P22385), the Drosophila mGluR1B (P91685), and two mGlu receptor sequences from C. elegans (identified in the cosmids F45H11 and ZC506), and the VFT module of ionotropic glutamate receptor subunits from C. elegans (YKQ4 CAEEL, P34299), Human (GLR1 (P42286) GLK1 (P39086) NMZ1 (Q06566), rat (MNE1, Q98959) and Arabidopsis thaliana (iGR-ARA, GenBank T51135). Unless otherwise indicated, the accession numbers are those of the Swissprot database.

Fig. 7B, g and h.

The presence of the SmRTK-1 protein in sporocysts and adult parasites was demonstrated by Western blot analysis (Fig. 8A) and immunocytochemistry (Fig. 8B) using a rabbit polyclonal antiserum generated against a pool of the three specific peptides from the N-terminal sequence of SmRTK-1. Blotting results indicated the presence in sporocyst and adult worm membrane extracts of the native molecule with an apparent molecular weight (250 kDa) larger than that of the translation product of SmRTK-1 sequence in reticulocyte lysate (170 kDa, results not shown).
Fig. 7. Detection of SmRTK-1 transcripts in various schistosome developmental stages. (A) Detection of SmRTK-1 RNA by RT-PCR in cercariae (1), miracidia (2), sporocysts (3), schistosomula (4), male (5) and female (6) worms obtained from bisexual infections. A band of 1 kb was amplified in all stages. (B) In situ hybridization in male (a and b), female worm (c, d, e), miracidium (f and g) and sporocyst (h and i) sections. Negative reactions (a, d, f and h) were performed using an SmRTK-1 sense probe. SmRTK-1 transcripts were detected in parenchymal cells (P) of male parasites (b) while in female sections (c and e) an intense labelling was associated with ovocytes in ovary (O) and ovary duct (D). In miracidia, the cells surrounding the neural mass (NM) were preferentially labelled (g). A similar observation was made in newly-transformed sporocyst sections (i).

and that predicted for the protein (calculated mass of 172 kDa). These data confirmed that SmRTK-1 was associated with parasite membranes and suggested that it was glycosylated as many other transmembrane receptors. The two minor lower bands detected in sporocyst extracts were probably due to degradation products inherent to the membrane preparation. Native SmRTK-1 was localized in sporocyst and male sections. In sporocysts (Fig. 8B, b) the protein was detected in the same cells that were shown to contain SmRTK-1 transcripts. In adult worm sections (Fig. 8B, d), an intense labelling of the parenchyma was observed, confirming the results of in situ hybridization.

Fig. 8. Detection of the SmRTK-1 protein in sporocysts and adult worms. (A) Identification of native SmRTK-1 in sporocyst and adult worm membrane preparations by Western blot analysis using the anti-SmRTK-1 rabbit immune serum (I). A non-immune serum was used as a control (NI). (B) Immunolocalization of SmRTK-1 in sporocyst (a and b) and adult male (c and d) sections. Positive fluorescent (green to yellow) cells were detected in the sporocyst body (b) as well as in the male parenchyma (d). Counterstaining by Blue Evans is responsible for red color in parasite sections.

4. Discussion

In recent years, numerous efforts have been made to characterize molecular factors essential for the development and the differentiation of schistosomes. *S. mansoni* presents various larval and adult forms that are morphologically and biochemically distinct and perfectly adapted to the different environments encountered successively by the trematode during its life cycle. The success of such a complex life cycle must be dependent on an adaptive molecular dialogue with the intermediate and definitive hosts and on the receipt of appropriate host-derived signals. The permanent pairing of male and female schistosomes and the requirement for an intimate contact for the development of female genital organs, would also imply signal exchanges between the two partners [36]. However, at this time, ligands and/or receptors involved in host-parasite or male-female communications remain totally unknown.
A TGF-β serine/threonine kinase receptor I (SmTβR-1) was identified at the surface of *S. mansoni* [3] raising the question of a possible role of TGF-β-related ligands in host-parasite relationships. Further studies indicated the ability of human TGF-β to activate SmTβR-1 [37] as well as conservation of the downstream signaling pathway involving Smad2 homologue proteins as intracellular signal transducers [38,39].

Numerous growth factors stimulate cellular mitogenesis by interacting with cell-surface RTK. The RTK superfamily of signaling molecules is potentially unique to metazoa and regulates cellular and developmental processes by allowing cell–cell communication and tissue organization in animals [40]. RTKs are integral membrane proteins with a single membrane-spanning sequence separating an extracellular ligand-binding domain and a cytoplasmic ligand-sensitive TK catalytic domain which undergoes tyrosine autophosphorylation and triggers intracellular signalling. In *S. mansoni*, a homologue of the epidermal growth receptor has been characterized in adult parasite muscles [4,5] that shares the IR ortholog of schistosomes. The second sequence (SmRTK-2) encoded a protein with all the characteristic features of IR (typical cystein-rich ligand-binding domain and a cytoplasmic ligand-sensitive TK domains) that the nematode contains at least 40 different RTK, among which 11 remain unclassified with no identifiable mammalian counterpart [48]. Extracellular and cytoplasmic portions of RTK have independent functions and are supposed to have distinct evolutionary origins. The receptor TK domains are homologous to various cytoplasmic tyrosine kinases and one explanation for RTK evolution and diversity would be that these receptors have evolved by genetic combination of diverse archaic receptors lacking integral enzymatic function with exons encoding TK catalytic domains [41]. According to this hypothesis, SmRTK-1 would result from the combination of a pre-existing VFT module with the catalytic domain of a cytoplasmic TK.

Recently, the determination of the structure of the VFT of mGluR1 [24], and that of the natriuretic peptide receptors A and C [15,16] confirm they form dimers. Interestingly, binding of the ligand (glutamate in the case of mGluR1, and the natriuretic peptide in the case of the NPRC) revealed major conformational changes of the dimeric structure. This change in conformation is such that the C-terminal ends of the two VFT modules are closer in the presence of agonists than in the absence. In the case of the natriuretic receptors A and B, such a change in conformation is expected to allow the correct interaction of the intracellular guanylate cyclase domains of each subunit of the dimeric receptor, allowing the full activity of the enzyme. Such a model for receptor activation would very well fit with an RTK of class II, in which the change in conformation of the dimeric VFT domain would allow the correct interaction of the two intracellular TK domains leading to their activation.

As revealed by the phylogenetic analysis, the VFT module of SmRTK-1 is close to the GABA-A receptor. As shown in Fig. 4, all residues identified in the VFT of GABA-A1 as being important for GABA binding (S246, Y366, E465 and...
Homologues suggest a role for an insulin-like molecule in regulating growth and patterning in H. diminuta. The evolutionary conservation of SmRTK-1 and SmRTK-2 suggests a role for an insulin-like molecule in regulating growth and patterning in H. diminuta.

References

D7) [11,12] are conserved, or replaced by homologous residues in SmRTK-1 (S465, F585, D743 and E750). Accordingly, it is possible that SmRTK-1 constitutes an original GABA-activated RTK. However, more work is necessary to confirm this possibility. Another attractive possibility would be that SmRTK-1 is involved in the recognition of a male pheromone signal necessary for the development of the female ovary. Indeed, some mammalian pheromone receptors have a VFT module likely responsible for ligand-binding [45–47]. The presence of large amounts of smRTK-1 transcripts in ovocytes of female parasites and the necessity of their permanent pairing with male schistosomes for sexual differentiation will be two important steps towards elucidating the function of the signaling pathway potentially triggered by SmRTK-1.

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