Identification, isolation and characterization of a Fyn-like tyrosine kinase from *Schistosoma mansoni*

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SUMMARY

Growth and development of adult schistosomes requires permanent communication processes of the parasites with their specific host environment and, additionally, between the two genders. Accumulating evidence suggests that, at the molecular level, the mandatory interactions are mediated by signal transduction processes. During recent years, a considerable interest has emerged in the identification of signalling molecules from this parasite and to elucidate their roles during development. In this organism, a number of different molecules have been identified which belong to diverse classes of evolutionary conserved signal transduction cascades. However, up to now no representative of the conserved family of cellular tyrosine kinases has been identified. In this study we present a suitable approach to identify this class of molecules and demonstrate the successful cloning and molecular characterization of one of the isolated genes, the tyrosine kinase 5 (TK5). An unexpected finding was that in the Liberian strain of *Schistosoma mansoni* the TK5 gene exhibits an allelic polymorphism.

Key words: *Schistosoma mansoni*, cellular tyrosine kinase, Src, Fyn, signal transduction, polymorphism.

INTRODUCTION

Tyrosine kinases are key molecules in a great variety of signal transduction pathways of eukaryotes (Hanks, Quinn & Hunter, 1988). There are 2 main classes, receptor tyrosine kinases (RTKs) and non-receptor tyrosine kinases, also called cytoplasmic or cellular tyrosine kinases (TKs). Both are involved in different signalling cascades which control diverse biological processes such as adhesion, cytoskeletal alteration, migration, proliferation, differentiation and survival (Erpel & Courtneidge, 1995). At the cellular level, TKs function as transmitter molecules which forward incoming signals to a variety of downstream targets within the cell. In this function, they fulfil essential tasks during internal cell communication processes mediating the response of the cell to external stimuli (Brown & Cooper, 1996).

The identification of signalling molecules and the elucidation of signal transduction processes are one of the most challenging tasks in understanding the biology of the dioecious trematode *Schistosoma mansoni*. This blood fluke exhibits 2 outstanding features. First, it is able to survive for decades in the blood-stream of its host, causing bilharzia in humans. Secondly, the sexual maturation of the female essentially depends on a close contact with the male (Erasmus, 1973). Both phenomena require molecular communication processes for which the first evidence has been obtained during the last few years. McKerrow (1997) reviewed the data suggesting that schistosomes use host-derived molecules like TNFα as a signal for egg production and propagation. Furthermore, Wolowczuk *et al.* (1999) provided experimental evidence for reduced growth and fecundity of schistosomes in an IL-7-defective host-environment, indicating that 1L-7 might play an essential role during the growth of schistosomes. Finally, results from Schüßler, Grevelding & Kunz (1997) indicated that signal transduction processes occur during the male–female interaction and may be involved in the process leading to female maturation.

Recently, a variety of signalling molecules have been identified in schistosomes including transmembrane and cellular receptors (Shoemaker *et al.* 1992; Escriva *et al.* 1997; Davies, Shoemaker & Pearce, 1998; Freebern *et al.* 1999; Inal, 1999), small G-proteins (Loeffler & Bennett, 1996; Kampkötter *et al.* 1999; Osman, Niles & LoVerde, 1999) and the 14-3-3 protein (Schechtman *et al.* 1995).

Since TKs have not yet been described in schistosomes, the present study was aimed at the identification of this class of signalling molecules. We report on an approach to specifically isolate TK sequences and on the characterization of one of the identified molecules.†

MATERIALS AND METHODS

Parasite stock

A Liberian strain of *Schistosoma mansoni* was

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maintained in *Biomphalaria glabrata* as intermediate host and in Syrian golden hamsters (*Mesocricetus auratus*) or gerbils (*Meriones unguiculatus*) as final hosts (Grevelding, 1995). Adult worms were obtained by perfusion at day 42 post-infection. Females and males were separated with a fine brush and stored under liquid nitrogen until used.

Isolation of nucleic acids

DNA from female and male worms was obtained by a proteinase K-based extraction method described elsewhere (Grevelding, 1995).

Poly(A)$^+$ RNA was obtained by a magnetic-particle based procedure (Schüßler *et al*. 1995) using 100 males or 150 females. Total RNA was isolated using commercially available kits following the instructions of the manufacturer (RNeasy Kit, QIAGEN).

RT–PCR procedures

Degenerate primers (purchased from Life Technologies) were designed according to conserve sequences obtained from GenBank: P1 [VHRD]: 5′-GGAATTCCATMGWGATYTDGC-3′, P2 [SDV]: 5′-GGAATTCCAWAWSHCCAMACRTC-3′ and P3 [DFG]: 5′-GGAATTCRARATNNSNGATTTYGG-3′ [M = A + C, W = A + T, Y = C + T, D = G + A + T, S = G + C, H = A + T + C, R = A + G, N = A + C + G + T].

RT–PCR was performed as a one-tube reaction in a total volume of 50 μl using 10 × RT-*Taq*-buffer (500 mM KCl, 100 mM Tris–Cl, pH 8·0, 25 mM $MgCl_2$, 1 mM DTT, in diethyl-pyrocarbonate (DEPC)-treated dH_2O), 5 μl gelatine (0·2 %), 2·5 μl W1-detergent (Boehringer Mannheim), 5–20 ng poly(A)$^+$ RNA from female schistosomes, 1 mM of each deoxynucleotide (dATP, dTTP, dCTP, dGTP; Pharmacia), 5 μM of each P1 and P2 primer, 20 units Reverse Transcriptase (M-MuLV, Boehringer Mannheim) and 5 units *Taq* polymerase (Appligene). After an initial denaturation of the mRNA for 5 min at 65 °C, cDNA first-strand synthesis was done for 25 min at 37 °C. Subsequent temperature cycling was performed as follows: 2 min at 95 °C, followed by 30 sec at 93 °C, 90 sec at 54 °C and 90 sec at 72 °C for 30 cycles and a final step for 5 min at 72 °C (PTC-100™, MJ Research).

For reamplification by nested PCR (using the primers P2 and P3), the standard reaction buffer and 2·5 units *Taq* polymerase (Appligene) were used in a final volume of 25 μl. After an initial denaturation step for 5 min at 95 °C, the following hot-start temperature cycling was applied: 60 sec at 93 °C, 60 sec at 60 °C and 60 sec at 72 °C for 30 cycles, followed by 5 min at 72 °C.

RT–PCRs with specific primers were done stepwise in separate reactions. About 200–300 ng total RNA were used, and the synthesis of cDNA first strands was initiated with 1 μM modified Oligo(dT) primer (5′-CCATCGATGGACTAGTCGGATCCTTTTTTTTTTTTTTTT-3′; BioTez, Germany). Using one tenth of the reverse transcription reaction, amplification reactions were performed. In a total volume of 25 μl, 1 μM of each primer (TK5-1: 5′-AGTACTGGTTATCGCATGCC-3′, position 1590–1609 and TK5-2: 5′-ACTGTCGACATTGATCGACG-3′, position 1894–1913; TK5-3: 5′-TTCCTTCTCCTCTTTTCTGG-3′, position 2738–2756 and TK5-4: 5′-GATTGAATCATTATTGGAGTC-3′, position 2967–2987; BioTez), 200 μM of each deoxynucleotide (dATP, dTTP, dCTP, dGTP; Pharmacia) and 2·5 units *Taq* polymerase (Appligene). After an initial denaturation step at 95 °C for 5 min, temperature cycling was performed at 93 °C, 60 sec; 54 °C, 60 sec; 72 °C, 60 sec for 30 cycles. The same time-profile but 60 °C annealing temperature was applied for PCR reactions with DNA as template using the primer combination TK5-1/TK5-2.

Southern and Northern blotting

Blotting procedures were performed according to standard protocols (Sambrook, Fritsch & Maniatis, 1989). For Southern blot analyses, DNA (3 μg) was digested with appropriate restriction enzymes and separated by 1 % agarose gel electrophoresis. For Northern blots, 20 μg total RNA was size-fractionated by 1·5 % denaturing agarose gel electrophoresis. Nucleic acids were transferred onto Hybond N™ membranes (Amersham) and fixed by UV cross-linking. Appropriate plasmid-derived DNA probes (see results), were gel-purified and labelled with [α-^{32}P]dCTP by random priming following the instructions of the manufacturer (NEBlot™ kit, Biolabs). Using homologous probes, Southern blots were hybridized at 65 °C and washed under stringent conditions. Hybridization with heterologous probes was performed at 32 °C with 50 % formamide. The filters were washed twice with 2 × SSC, 0·1 % SDS at hybridization temperature and finally in 1 × SSC, 0·1 % SDS for 30 min at 42 °C. Northern blots were hybridized at 42 °C in the presence of 50 % formamide and washed up to a stringency of 1 × SSC, 0·1 % SDS.

Cloning of PCR products

PCR amplification products were purified from agarose gels and cloned by blunt-end ligation into pUC18 plasmid (SureClone™ Ligation Kit, Pharmacia) following standard protocols. Preparation of plasmid DNA was done by chromatography using commercially available kits (QIAGEN).

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cDNA-library screening

For screening of a λ-ZAPII library (Menrath, Michel & Kunz, 1995), duplicate replica filters were hybridized at 65 °C in 6 × SSC for 20 h with the appropriate probe. The filters were washed at 65 °C under a stringency of 1 × SSC, 0·1 % SDS and subjected to autoradiography for 4 days. *In vivo* excision was done as described elsewhere (Menrath *et al*. 1995). The resulting phagemids were used to infect SOLR cells.

DNA sequencing

Cloned PCR products of the expected size were individually sequenced on both strands with an ABI 373 DNA-sequencer from Applied Biosystems, using the dye terminator cycle sequencing kit with AmpliTaq DNA Polymerase FS (Perkin–Elmer). Sequencing of TK5 cDNAs following subcloning of various restriction fragments into pBluescript SK^- (Stratagene) was performed by the method of Sanger using the ^{T7}Sequencing^{TM} Kit (Pharmacia) and standard primers or gene-specific primers.

Computer-based analyses of nucleic acids and proteins

For the computer-based analyses of nucleic acids and protein, the following tools have been used that are available via Internet: Swiss-Prot (http://www.expasy.ch/sprot/sprot-top.html), ClustalW (http://www.clustalw.genome.ad.jp/), Codon Usage Database (http://www.kazusa.or.jp/codon/cgibin/showcodon.cgi?species = Schistosoma+mansoni+[gbinv]), BLAST (http://www.ncbi.nlm.nih.gov/BLAST/), MOTIF (http://www.motif.genome.ad.jp/) and Compute pI/Mw (http://www.expasy.ch/tools/pi_tool.html).

RESULTS

Identification and cloning of partial tyrosine kinase sequences from S. mansoni

Alignments of tyrosine kinase (TK) protein sequences from distantly related organisms showed the highly conserved regions within the TK domain. Using a modification of the approach of Wilks (1991), degenerate primers were designed against 3 regions (P1, HRDL; P2, SDVW; P3, DFGL; Fig. 1 A), taking the preferred codon usage of *S. mansoni* into consideration.

Following reverse transcription of poly(A^+) RNA from adult female worms with primer P2, PCR amplification was performed using the primer combination P1 and P2. A 220 bp amplification product was obtained (Fig. 1 B) as predicted according to the conserved distance between HRDL and SDVW. To prove that TK sequences are among the amplified products, Southern blot and nested PCR experiments were carried out. For Southern blot analysis, the agarose gel shown in Fig. 1 B was blotted, and the filter was hybridized with a heterologous probe representing the highly conserved tyrosine kinase domain of the melanoma receptor tyrosine kinase from *Xiphophorus maculatus* (Wittbrodt *et al*. 1989). A specific hybridization signal was obtained (Fig. 1 C). Reamplification of the 220 bp amplicon with primer P2 and the nested primer P3 (Fig. 1 A) resulted in a PCR product of the expected size of 160 bp (not shown). Both, Southern blot and nested PCR confirmed the authenticity of the original amplification product.

The P1-P2 PCR product was gel-purified and cloned. Out of a large number of clones, a total of 384 were analysed by Southern blotting and subsequent hybridization with the heterologous probe from *X. maculatus*. A total of 17 clones reacted positively (not shown). Sequence analyses revealed the conserved DFGL region within 10 of these clones, and thus identified them as candidates for tyrosine kinases. These 10 clones represented 4 different tyrosine kinases. Computer-associated comparison with databases indicated that 2 of them may belong to the Src family, a class of cellular tyrosine kinases that has not yet been described in schistosomes. One of the two molecules (TK5) is described here, whereas the second one is the subject of another study.

Cloning of the TK5 cDNA

The cloned TK5-fragment was used as a probe for hybridization of duplicate replica filters representing 500000 phage plaques of an *S. mansoni* female λ-ZAPII cDNA-library. Two positive signals were obtained, and the recombinant phages were plaque-purified. After *in vivo* excision, the cDNA insert of one of these clones (TK5-32) was sequenced. TK5-32 is 2320 bp in length containing a 24 bp poly(A) tail. It contains an open reading frame of 927 bp which starts at the 5′ end of the cDNA. Since no N-terminal methionine residue was found in this sequence, the library was re-screened to obtain a full-length clone. The screening of 945000 phage plaques with a 252-bp fragment from the 5′ end of the TK5-32 sequence as a probe resulted in the identification of 9 further clones.

Sequence analysis revealed that 7 of the 9 are incomplete TK5 clones of variable lengths, whereas the remaining 2 clones (TK5-34 and TK5-35) contain identical cDNAs of 3381 bp. They cover an open reading frame of 1944 bp coding for a deduced protein of 647 amino acids (GenBank accession no. AF232691). The protein sequence is initiated by an ATG codon which occurs 48 nucleotides downstream of the 5′ end. An in-frame stop codon in the 5′ untranslated region (UTR) and the existence of a

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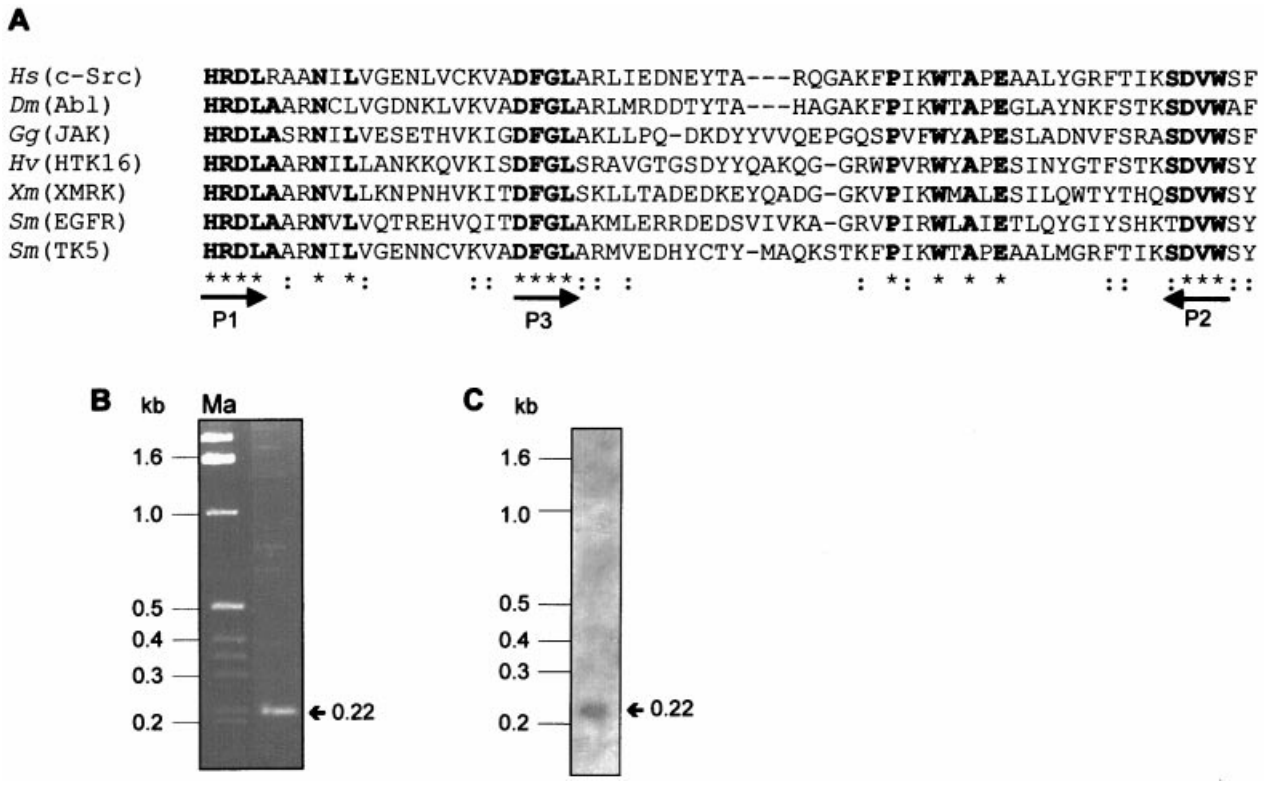

Fig. 1. (A) Alignment of part of the catalytic tyrosine kinase domains of cellular tyrosine kinases from 4 different organisms: *Homo sapiens* (*Hs*) c-Src, *Drosophila melanogaster* (*Dm*) Abl, *Gallus gallus* (*Gg*) JAK and *Hydra vulgaris* (*Hv*) HTK16. XMRK from *Xiphophorus maculatus* (*Xm*) and EGFR from *Schistosoma mansoni* (*Sm*) represent corresponding regions from receptor tyrosine kinases. The alignment also includes part of the newly identified tyrosine kinase 5 (TK5) of *S. mansoni*. Highly conserved amino acids are given in bold, identical amino acids are marked by asterisks and similar amino acids are marked by dots. Sequences used for primer design are indicated by arrows (HRDL = P1, DFGL = P3 and SDVW = P2). (B) RT–PCR amplification of poly(A^+)RNA from adult female schistosomes using the primer combination P1 and P2. Amplification products were separated on a 2% agarose gel and stained with ethidium bromide. Ma, size marker (1 kb ladder, Life Technologies). (C) The gel in B was blotted and the resulting filter hybridized with a radioactively labelled tyrosine kinase probe from *X. maculatus* (Wittbrodt *et al*. 1989).

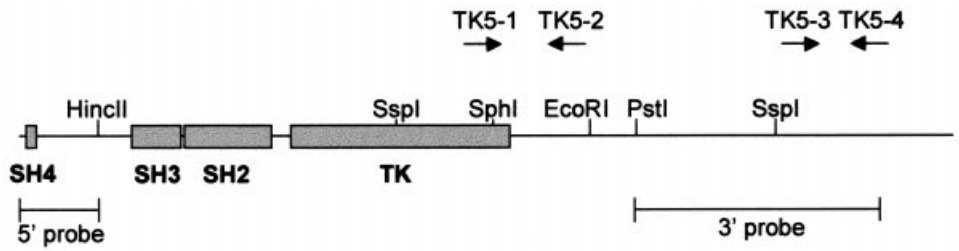

Fig. 2. Diagram of the structure of the TK5 cDNA. The hallmark domains SH4, SH3, SH2 as well as the catalytic tyrosine kinase (TK) domain are boxed, and important restriction sites are indicated. DNA probes used for Southern- and Northern-blot analyses are marked below. Locations of the primers for PCR and RT–PCR are shown by arrows above the diagram.

Kozak consensus sequence (Kozak, 1989) support the assumption that this ATG represents the start codon. The 3′ UTR has a length of 1390 bp terminating with a poly(A) tail of 19 bp, which is preceded by the cannonical polyadenylation signal (AATAAA) 16 bp upstream of the poly(A) tail. The diagram of the structure of the TK5 cDNA is shown in Fig. 2.

TK5 amino acid sequence analysis

Database comparisons revealed striking homology of the TK5 protein-sequence to Src-like tyrosine kinases. This family of cellular proteins consists of several subfamilies (Brown & Cooper, 1996; Thomas & Brugge, 1997). At the protein level, TK5 exhibits highest homologies to members of the Fyn subfamily

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leading to a multi-alignment which is presented in Fig. 3. According to the BLAST program the consensus sequence totals 453 amino acids, the identity among all 6 sequences is about 55%, and the similarity is about 74%.

As expected for members of the Src family, TK5 contains all the hallmark domains (Brown & Cooper, 1996), the SH4 domain (containing the conserved glycine residue), the SH3 domain, the SH2 domain and the catalytic tyrosine kinase domain (Figs 2 and 3). Within the tyrosine kinase domain, further characteristic motifs can be found. There is a putative ATP-binding region and a conserved tyrosine residue. Another conserved tyrosine occurs in the so-called tail (Fig. 3).

Summarizing these features, we conclude that *S. mansoni* TK5 is an Src-like tyrosine kinase. The predicted relative molecular mass of this molecule is 73 kDa, and the theoretical isoelectric point is 8·5.

TK5 is a single-copy gene in S. mansoni

To further characterize TK5, a genomic Southern blot analysis was performed. DNA of adult worms was digested with different restriction enzymes, size-separated by gel electrophoresis and blotted. The resulting filter was probed with a 1 kb fragment from the 3′ UTR (Fig. 2, 3′ probe). Fig. 4 shows that a single signal was obtained with DNA digested by *Eco*RI or *Hin*dIII, whereas 2 signals were obtained with *Ssp*I-digested DNA. This result is consistent with the absence of an *Eco*RI or *Hin*dIII-site in the area of hybridization and the occurrence of an *Ssp*I-site located within this region (see Fig. 2). These data suggest that TK5 represents a single-copy gene in *S. mansoni*.

Transcription analyses of TK5

To analyse the transcription profile of TK5, a Northern blot was performed with total RNA from adult male and female worms. For hybridization, a 270 bp fragment from the 5′ end (Fig. 2, 5′ probe) of TK5 was used as the probe. A distinct signal of approximately 3·4 kb was observed in both sexes occurring with similar intensity (Fig. 5A). The size of the signal coincides with the sizes of the cDNA clones found in the library.

To characterize the developmental occurrence of TK5 transcripts, RT–PCR experiments were performed starting with total RNA from adult worms and the larval stages (miracidia and cercariae). Reverse transcription was done with a modified oligo(dT) primer. To amplify the synthesized first-strand cDNAs the gene-specific primers TK5-3 and TK5-4 (see Fig. 2) were used. A specific amplicon of the expected size (250 bp) was obtained from all templates (Fig. 5B).

Thus, Northern blot and RT–PCR results indicate that TK5 is gender-independently expressed in the adults and, furthermore, occurs in the free-living larval stages.

Allele-specific polymorphism of TK5

Further PCR and RT–PCR experiments with 2 additional gene-specific primers (TK5-1, TK5-2; see Fig. 2) enclosing a region of 324 bp, unexpectedly showed 2 closely migrating amplicons of similar quantity (Fig. 6A). The larger amplicon, about 320 bp, was of the expected size and confirmed the occurrence of TK5 transcripts in adult worms. The smaller product was about 300 bp in size. Both amplicons reproducibly occurred under stringent PCR conditions, also in the larval stages (not shown). To clarify the origin of these products, they were recovered from the gel, cloned and sequenced. As expected, the sequence representing the larger product confirmed its origin as part of the TK5 full-length cDNA. The smaller product also originated from TK5, however, it exhibits an 18 bp difference leading to an amplicon of 306 bp. This difference occurs within an array of 13 asparagine residues (position 584–596 of the amino acid sequence) and the smaller product differs in exactly 6 asparagines without causing a frameshift of the TK5 sequence (Fig. 6B). Since TK5 is suggested to be a single-copy gene in *S. mansoni*, this finding indicates an allele-specific polymorphism.

DISCUSSION

Cellular tyrosine kinase (TKs) are widespread and well-characterized signal transduction molecules involved in a great variety of pathways (Brown & Cooper, 1996). One family of TKs which communicates with a variety of different receptors is the Src family of protein kinases (Thomas & Brugge, 1997). Since the detection of the first member of this family, the transforming protein v-Src of the oncogenic Rous sarcoma virus (Brugge & Erikson 1977), a large number of similar proteins were identified. According to their similarities, they have been classified into the following subfamilies: Src, Fyn, Yrk, Yes, Fgr, Hck, Lck, Lyn and Blk (Brown & Cooper, 1996; Thomas & Brugge, 1997). Members of these subfamilies share common features such as an SH4 domain (a short N-terminal membrane-anchor sequence) which is followed by a poorly conserved unique region, a conserved SH3 domain (a region that binds to proline-rich sequences), a conserved SH2 domain (a region that specifically binds phosphorylated tyrosines), a conserved TK domain (the catalytic domain with tyrosine kinase activity) containing a conserved tyrosine residue and, finally, a C-terminal tail-region containing

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HsFyn   1 MG-CVQ-------CKDKEATKLT-EERDGSLNQSSGYRYGTDPTP-------QHYPSFGV
XlFyn   1 MG-CVQ-------CKDKEATKLT-DERDNSLTQSLGYRYGTDPTP-------QHYPSFTV
XhFyn   1 MG-CVQ-------CKDKEATKLT-DDRDASISQGAGYRYGADPTP-------QHYPSFGV
TcFyn   1 MG-CVQ-------CKDKETTKTTVEERAGSIPQNPGYRYGPDPSP-------QHYPNFSV
GgYrk   1 MG-CVH-------CKEKISGKGQ-GGSGTGTPAHPPSQYDPDP----------TQLSGAF
SmTK5   1 MGNCFQGIVISMFVRDRAFTFGHGEISNSVGVKASRPMDDVSPFLPSRSLSSKARVSETF
          ** *.:        :::                          *

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HsFyn  45 TSIPNYNNFHAAGGQ-----GLTVFGGVNSSSHTGTLRTRGG---------TGVTLFVAL
XlFyn  45 TTIPNYNNFHATAGQ-----GLTVFGGVNSSSHTGTLRTRGG---------TGVTLFVAL
XhFyn  46 TAIPNYNNFHAPVGQ-----GVTVFGGVNTSSHTGTLRTRGG---------TGVTLFVAL
TcFyn  38 TGIPNYNSFHGTGGQ-----TLTVFGGVHTSSHTGTLRTRGGGSLT-----VGVTLFVAL
GgYrk  42 THIPDFNNFHAAAVS-----PPVPFSGPG-FYPCNTLQAHSSITG------GGVTLFIAL
SmTK5  61 QNSPPLVRCVKSKPQSCQPTNPTSNLAISPSHPSETQTVTTSKISGRSVKQLKPKHYVAL
            *                                 *                  ::**

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HsFyn  91 YDYEARTEDDLSFHKGEKFQILNSSEGDWWEARSLTTGETGYIPSNYVAPVDSIQAEEWY
XlFyn  91 YDYEARTEDDLSFQKGEKFQILNSSEGDWWEARSLTTGGTGYIPSNYVAPVDSIQAEEWY
XhFyn  91 YDYEARTEDDLSFRKGERFQILNSTEGDWWDARSLTTGGSGYIPSNYVAPVDSIQAEDWY
TcFyn  97 YDYDARTDDDLSFHKGEKFQILNNTEGDWWEARSLQTGGTGYIPSNYVAPVDSIQAEEWY
GgYrk  90 YDYEARTEDDLSFQKGEKFHIINNTEGDWWEARSLSSGATGYIPSNYVAPVDSIQAEEWY
SmTK5121 FDYNARTEEETFLSRRDEVLLLADTDSEWWLVQNLSSGRKGYVPSSFVAKKGSVEAEEWF
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HsFyn151 FGKLGRKDAERQLLSFGNPRGTFLIRESETTKGAYSLSIRDWD---DMKGDHVKHYKIRK
XlFyn151 FGKLGRKDAERQLLSFGNPRGTYLIRESETTKGAYSLSIRDWD---DMKGDHVKHYKIRK
XhFyn151 FGKLGRKDAERQLLSTGNPRGTYLIRESETTKGAFSLSIRDWD---DEKGDHVKHYKIRK
TcFyn157 FGKLGRKEAERQLLSVCNPRGTFLIRESETTKGAYSLSIRDWD---DTKGDHVKHYKIRK
GgYrk150 FGKIGRKDAERQLLCHGNCRGTFLIRESETTKGAYSLSIRDWD---EAKGDHVKHYKIRK
SmTK5181 MPMLSRKDSERLLLLEGNAQGVFLVRESETSQGSLTLSVRDEERGLSGIMNTVKHYRIKH
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HsFyn208 LDNGGYYITTRAQFETLQQLVQHYSERAAGLCCRLVVPCHKGMPRLTDLSVKTKDVWEIP
XlFyn208 LDNGGYYITTRAQFETLQQLVQHYSERAAGLCCRLVVPCHKGMPRLTDLSVKTKDVWEIP
XhFyn208 LDSGGYYITTRAQFDTLQQLVQHYSDRAAGLCCRLVVPCHKGMPRLADLSVKTKDVWEIP
TcFyn214 LDSGGYYITTRAQFETLQQLVQHYAERAAGLCCRLVVPCHKGMPRLADLSVKTKDVWEIS
GgYrk207 LDSGGYYITTRAQFDTIQQLVQHYIERAAGLCCRLAVPCPKGTPKLADLSVKTKDVWEIP
SmTK5241 PDYRYYYITTKCSFSSLQELIQFYSIDSHGLCCKLTRACLCPPPITSDLSVKTKDHWEIS
           *   *****:  * ::*:*:* *   : ****:*   *    *  :******** ***

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HsFyn268 RESLQLIKRLGNGQFGEVWMGTWNGNTKVAIKTLKPGTMSPESFLEEAQIMKKLKHDKLV
XlFyn268 RESLQLIKRLGNGQFGEVWMGTWNGNTKVAIKTLKPGTMSPESFLEEAQIMKKLKHDKLV
XhFyn268 RESLQLIKRLGNGQFGEVWMGTWNGTTKVAVKTLKPGTMSPESFLEEAQIMKKLRHDKLV
TcFyn274 RESLQLIKRLGNGQFGEVWMGTWNGTTKVAVKTLKPGTMSPESFLEEAQIMKKLRHDKLV
GgYrk267 RESLQLLQKLGNGQFGEVWMGTWNGTTKVAVKTLKPGTMSPEAFLEEAQIMKRLRHDKLV
SmTK5301 KSSIVLIEKLGAGQFGEVWKGTWNGTTEVAVKTLKQGTMTKEDFLKEARIMRAAQHPKLV
          : *: *:::** ******* ***** *:**:**** ***: * **:**:**:  :* ***

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HsFyn328 QLYAVVSEEPIYIVTEYMNKGSLLDFLKDGEGRALKLPNLVDMAAQVAAGMAYIERMNYI
XlFyn328 QLYAVVSEEPIYIVTEYMSKGSLLDFLKDGEGRALKLPNLVDMAAQVARGMAYIERMNYI
XhFyn328 QLYAVVSEEPIYIVTEYMSKGSLLDFLKDGEGRALKLPNLVDMAAQVAAGMAYIERMNYI
TcFyn334 QLYAVVSEEPIYIVTEYMSKGSLLDFLKDGEGRVLKLPNLVDMAAQVAAGMAYIERMNYI
GgYrk327 QLYAVVSEEPIYIVTEFMSQGSLLDFLKDGDGRYLKLPQLVDMAAQIAAGMAYIERMNYI
SmTK5361 RLYAVCTEDPIYIVTELMCNGSLLQYLRDGPGKNLLINQLVDMMAQIANGMAYLEKEHYI
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Fig. 3. For legend see opposite.

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HsFyn 388 HRDLRSANILVGNGLICKIADFGLARLIEDN--EYTARQGAKFPIKWTAPEAALYGRFTI
XlFyn 388 HRDLRSANILVGNGLICKIADFGLARLIEDN--EYTARQGAKFPIKWTAPEAALYGRFTI
XhFyn 388 HRDLRSANILVGDNLVCKIADFGLARLIEDN--EYTARQGAKFPIKWTAPEAALYGRFTI
TcFyn 394 HRDLRSANILVGDNLVCKIADFGLARLIEDN--EYTARQGAKFPIKWTAPEAALYGRFTI
GgYrk 387 HRDLRAANILVGDNLVCKIADFGLARLIEDN--EYTARQGAKFPIKWTAPEAALFGKFTI
SmTK5 421 HRDLAARNILVGENNCVKVADFGLARMVEDHYCTYMAQKSTKFPIKWTAPEAALMGRFTI
          **** : *****:    *:*******  **:   * *:: :************ *:***

                ____________________________________________________________
HsFyn 446 KSDVWSFGILLTELVTKGRVPYPGMNNREVLEQVERGYRMPCPQDCPISLHELMIHCWKK
XlFyn 446 KSDVWSFGILLTELVTKGRVPYPGMNNREVLEQVERGYRMPCPQDCPISLHELMLNCWKK
XhFyn 446 KSDVWSFGILLTELVTKGRVPYPGMNNREVLEQVERGYRMPCPQDCPASLHELMLQCWKK
TcFyn 452 KSDVWSFGILLTELVTKGRVPYPGMNNREVLEQVERGYRMASPQDCPSSLHELMIQCWKK
GgYrk 445 KSDVWSFGILLTELVTKGRVPYPGMNNREVLEQVERGYRMQCPGGCPPSLHDVMVQCWKR
SmTK5 481 KSDVWSFGIVIYELITLGQVPYPSMNNTETLHQVSTGYRMPRPVNCPQPIYDMLLRIWDS
          *********:: **:* *:**** *** * * **  ****  *  **  ::::::  *

                ____________________
HsFyn 506 DPEERPTFEYLQSFLEDYFTATEPQYQPGENL----------------------------
XlFyn 506 DPEERPTFEYLQGFLEDYFTATEPQYQPGDNL----------------------------
XhFyn 506 DPEERPTFEYLQAFLEDYFTATEPQYQPGDNL----------------------------
TcFyn 512 DPEERPTFEYLQAFLEDYFTATEPQYQPGDNL----------------------------
GgYrk 505 EPEERPTFEYLQSFLEDYFTATEPQYQPGDNQ----------------------------
SmTK5 541 CPEKRPTFSSLFEFFEDYFVTSDANYKHANSALTGCSACITTVNNNNNNNNNNNNNSLHH
           **:****  *  *:**** ::: :*:  :

HsFyn     ------------------------------------------------
XlFyn     ------------------------------------------------
XhFyn     ------------------------------------------------
TcFyn     ------------------------------------------------
GgYrk     ------------------------------------------------
SmTK5 601 LSNLTPLDTTSSSSTTSINVDSTHSAVSKCCSGRLLLRDKLLRQSTQ
```

Fig. 3. Multi-alignment of the *Schistosoma mansoni* tyrosine kinase 5 (SmTK5) with Fyn-like tyrosine kinases from distantly related organisms: *Homo sapiens* (*Hs*Fyn), *Xenopus laevis* (*Xl*Fyn), the teleost fish *Xiphophorus helleri* (*Xh*Fyn), the pacific electric ray *Torpedo californica* (*Tc*Fyn) and the Yrk protein from *Gallus gallus* (*Gg*Yrk). The 4 hallmark domains of these proteins are marked as lines above the alignment. SH4 (1–6, roof-like line), SH3 (112–173 in TK5, dotted line), SH2 (179–279 in TK5, interrupted line) and the catalytic tyrosine kinase domain (306–559 in TK5, continuous line). Furthermore, the putative tyrosine phosphorylation sites at positions 455 and 566 in TK5 are printed in bold. Asterisk, identical amino acids; dots, similar amino acids.

another conserved tyrosine residue. Beside their structural similarity, members of the Src subfamilies of tyrosine kinases are conserved in size, too.

Using the PCR approach in this study, we were able to isolate 4 different classes of TK-molecules. For one of them, TK5, computer-based analyses reproducibly showed striking homology to the Fyn subfamily of Src-like TKs, although not every feature of TK5 corresponds to this subfamily. Among the 5 molecules exhibiting highest homology to TK5 is Yrk, a member of a Src subfamily occurring in chicken. According to Pileup program data, Yrk is the closest relative of the Fyn-subfamily (Brown & Cooper, 1996).

TK5 shows all the characteristic hallmark domains mentioned above. However, there are some differences to other Src-like proteins which affect the length of TK5 and the structure of its domains. Compared to the conserved lengths of Src-like tyrosine kinases, the N-terminal region and especially the C-terminal region of TK5 are elongated. Within the N-terminal region, a structural difference affects the SH4 domain which contains the sequence motif for myristylation. This co-translational activity covalently links the 14 carbon saturated fatty acid myristate to the N-terminal glycine at position 2 which normally occurs in the consensus context Met-Gly-X-X-X-Ser/Thr (Resh, 1994). This modification is a prerequisite for membrane association and subsequent signalling activity. In the majority of Src-family members, membrane binding can be alternatively promoted by palmitylation of cysteine residues occurring at positions 3 or 6 and/or by basic amino acids contributing to membrane association. For TK5, a glycine occurs at position 2, but there is neither a Ser/Thr at position 6 nor a cysteine residue at positions 3 or 6. Instead, a cysteine occurs at position 4 as well as several basic amino acids in the

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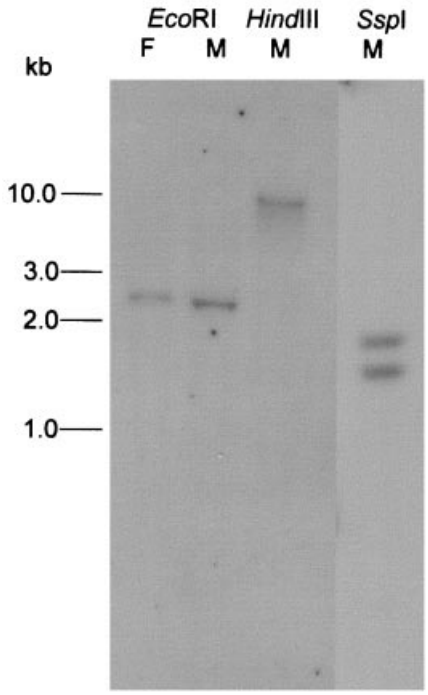

Fig. 4. Southern blot hybridization with similar amounts of female (F) or male (M) DNA digested by *Eco*RI, *Hin*dIII or *Ssp*I as indicated. A radioactively labelled 1 kb-fragment of the 3′-end of TK5 was used as the probe (see Fig. 2).

N-terminal region, which could promote membrane association. Therefore, we conclude that TK5 may be membrane anchored.

In contrast to all other subfamilies, members of the Fyn subfamily exhibit a high degree of homology concerning amino acid composition and length of their unique domains. Although computer-based analyses affiliated TK5 to the Fyn subtype, there are some uncommon features within its unique domain. First, this domain is longer. Second, there are 2 non-conserved cysteines which are not present in the unique domains of other Fyn proteins. In contrast, it is the Lck-subfamily of tyrosine kinases where it has been shown that 2 cysteine residues exist within the unique domain. They form disulfide bonds with transmembrane receptors (Shaw *et al*. 1989).

In contrast to the unique domain, the length of the SH3 domain meets the expectation. Within the Src family, the 5 N-terminal amino acids of SH3 domains are usually highly conserved, but TK5 differs in this region. This divergence probably does not influence the globular SH3 domain structure responsible for binding proline-rich ligands, because the key conserved residues forming the hydrophobic ligand-binding surface are not affected (Brown & Cooper, 1996). Within the SH3 domain of other Src-like tyrosine kinases, a tyrosine phosphorylation site occurs at position 136 (PSNYVASP). This site was shown to be the target of PDGF receptor activity (Broome & Hunter, 1997). An equivalent tyrosine phosphorylation site does not exist in TK5.

In the SH2 domain, a conserved tyrosine residue occurs at position 213 (position 247 for TK5). In other Src proteins this site has been shown to be phosphorylated in PDGF-treated cells (Stover, Becker & Lydon, 1996). Therefore, an interaction of TK5 with a putative PDGF-homologue in schistosomes may be possible. The length of the SH2 domain of TK5 slightly differs from that of other Src-type kinases. There are 3 additional amino acids

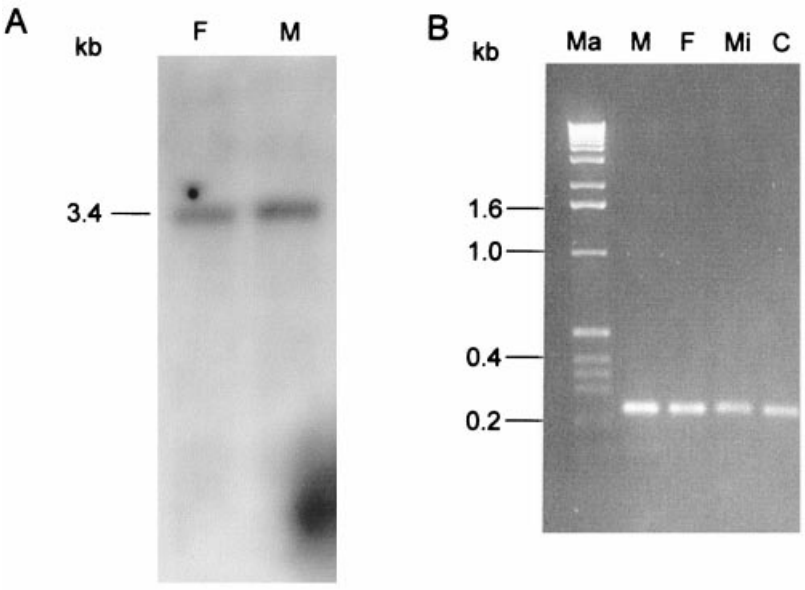

Fig. 5. Transcription analyses of TK5. (A) Northern blot hybridization with similar amounts of total RNA from adult female (F) and male (M) worms. As probe, a radioactively labelled 270 bp-fragment of the 5′-end of TK5 was used (see Fig. 2). (B) RT–PCR with total RNA from adults or larval stages (miracidia = Mi or cercariae = C). Ma, size marker (1 kb ladder, Life Technologies).

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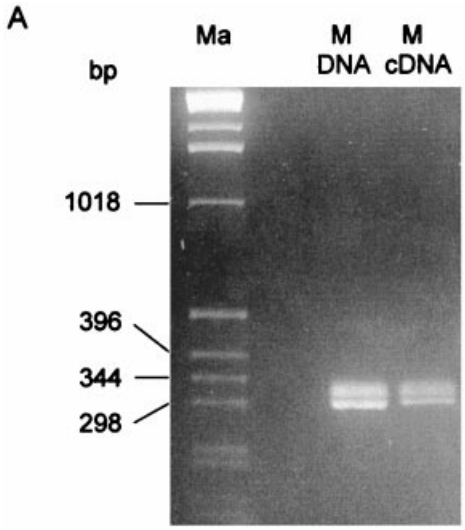

B

(1) CACA ACAGTTAATA ATAATAATAA TAATAACAAT AATAATAATA ATAATAGTCT ACACCATCTA
T T V N N N N N N N N N N N S L H H L

(2) CACA ACAGTTAATA ATAATAATAA TAATAATAGTCT ACACCATCTA
T T V N N N N N N N S L H H L

Fig. 6. (A) PCR and RT–PCR analyses using the primer combination TK5-1/TK5-2 (see Fig. 2). As templates, DNA and cDNA of males (M) were used as indicated. Amplicons were separated by 2·5 % agarose gel electrophoresis and stained with ethidium bromide. Ma, size marker (1 kb ladder, Life Technologies). (B) Sequence polymorphism of the TK5 gene occurring in the Liberian strain of *Schistosoma mansoni*; (1) part of the sequence including the complete asparagine stretch, (2) polymorphic variant of the same sequence area.

elongating this domain in a low conserved loop-area which probably does not influence its activity as a binding module for phosphorylated tyrosines.

Downstream of SH2, a linker region occurs connecting this domain with the catalytic domain. The different subfamilies of Src show only little sequence similarity in this region (Williams, Wierenga & Saraste, 1998). However, in the subfamily of Fyn-like tyrosine kinases, a high number of identical amino acids occurs within this region which is also found in TK5.

Among the members of the Src family, the catalytic domain is the most conserved domain and well conserved with other protein tyrosine kinases (Brown & Cooper, 1996). It determines substrate specificity affecting the phosphorylation pattern of proteins within a cell. The catalytic domain of TK5 is similar to other respective domains except for 2 additional amino acids, a tyrosine and a cysteine residue. They occur 2 amino acids upstream of the highly conserved tyrosine (position 455 in TK5) which is the major autophosphorylation site within the Src family (Smart *et al*. 1981). For other Src molecules, it was shown that this tyrosine residue is not phosphorylated when Src adopts an inactive conformation. Upon autophosphorylation of this site, a conformational change occurs leading to the active state of Src. Since this conserved tyrosine is located within the important activation loop area (Thomas & Brugge, 1997), it is surprising that schistosome TK5 has 2 additional amino acids within the activation loop which could influence its 3-dimensional conformation.

All vertebrate Src-family kinases have short C-terminal ends containing another conserved tyrosine residue which is a further major site of phosphorylation in the cell. In the inactive conformation of Src, this tyrosine residue is phosphorylated and intra-molecularly attached to the SH2 domain of the same molecule. Upon dephosphorylation, the inactive conformation turns into an active one allowing the phosphorylation of the conserved tyrosine residue in the catalytic domain and the access of other proteins to the SH2-and SH3 domains. In TK5, the conserved C-terminal tyrosine residue occurs at position 566. Because a conserved tyrosine residue with regulatory functions is found both in the catalytic domain and also in the C-terminus, we assume that the state of TK5 activity in schistosomes is probably controlled by the same conserved mechanisms as described for other Src/Fyn-like TKs.

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In contrast to the conserved length of the C-terminus of other Fyn proteins, TK5 contains additional 75 amino acids in this region including an unusual stretch of 13 asparagines. PCR analyses showed the existence of variable C-terminal sequences, which differ in length affecting 6 of the 13 asparagines without disturbing the reading frame. Since TK5 was shown to be a single-copy gene, this finding indicates that there are 2 different TK5 alleles in the Liberian strain of *S. mansoni*. The investigation of the occurrence of this polymorphism in other schistosome strains may provide a possibility for strain differentiation and may help to elucidate evolutionary aspects.

TK5 is a protein of 647 amino acids, which is about 100 amino acids longer than the Fyn tyrosine kinases described in vertebrates. In invertebrates, only a limited number of tyrosine kinases have yet been identified and characterized. None of them have been classified as a member of the Fyn subfamily. In this respect, TK5 probably represents the first invertebrate tyrosine kinase of the Fyn-type. According to database analyses, the closest invertebrate relative of TK5 is AcSrc1, a Src-like tyrosine kinase from sea urchin which seems to play a role during embryogenesis as well as gut formation and/or function (Onodera *et al*. 1999).

The receptor tyrosine kinase clone p17-2 from *Xiphophorus maculatus* was kindly provided by Professor Schartl (Würzburg). Furthermore, the authors thank Dr Karl Köhrer and Dr Sibylle Scheuring from the Center for Biological and Medical Research Düsseldorf for support with the automated sequencing, Stefanie Sudhop and Didina David for technical support, Dr Ralf Winnen for providing a Northern blot filter and the Deutsche Forschungsgemeinschaft (Grant Ku 282/13-3 and 15-1) for financial support.

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