RESEARCH BRIEF

Schistosoma mansoni: Cloning and Characterization of the Ras Homologue

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In an attempt to identify and characterize molecules thought likely to play important roles during the development of schistosomes, we started to search for signal transduction molecules in Schistosoma mansoni. Ras, a small GTP-binding protein (G protein) and a key component of diverse signal transduction pathways, was chosen since it plays an essential role in growth and development (McCormick 1993; Maruta and Burgess 1994; Johnson and Vaillancourt 1994). Homologues of Ras have been characterized in a variety of eukaryotes, where they have been shown to function in signaling pathways which control mitosis, meiosis, tissue development, and differentiation (Barbacid 1987; Lowy and Willumsen 1993; Pronk and Bos 1994; Wassarman et al. 1995; Kayne and Sternberg 1995; Lee et al. 1996). These pathways are initiated through the binding of ligands such as growth factors and protein hormones to cell surface receptors of the tyrosine kinase-associated and serpentine classes and trigger cytoplasmic signal transduction cascades that converge at Ras. Subsequent activation of the multifunctional enzyme complex of MAP kinases results in the 91, 280±283. q 1999 Academic Press

Index Descriptors and Abbreviations: Schistosoma mansoni; ras; regulation of gene expression and other effects (Maruta and Burgess 1994; Johnson and Vaillancourt 1994; McCormick 1994). In schistosomes, signaling pathways have yet to be characterized, but single components have been identified, including other G proteins and an epidermal growth factor receptor (Shoemaker et al. 1992; Iltzsch et al. 1992; Loeffler and Bennett 1996).

Since the Ras homologues that have been characterized to date demonstrate significant sequence conservation, a nested RT±PCR approach, utilizing degenerate primers, was performed in order to isolate the Ras gene from S. mansoni. Primers were designed for highly conserved regions that were identified by alignment of Ras protein sequences from GenBank, taking the preferred codon usage of S. mansoni (http://www.dna.affrc.go.jp/ nakamura/codon.html) into consideration for the 5' region of each primer. In total, four primers were synthesized, the flanking primers 5.1 and 3.1 and the nested primers 5.2 and 3.2 (5.1: 5'-TGTTGATGARTAYGAYCC-3'; 3.1: 5'-TAATTCCACAYTTRTNCC-3'; 5.2: 5'-TCATAYCGNAARCRT-3'; 3.2: 5'-CGCATATATTGNTCNCCG-3'; where N = A/C/G/T; Y = C/T; R = G/A). For these experiments, a Liberian strain of S. mansoni (maintained in Biomphalaria glabrata and Syrian hamsters) was used (Grevelding 1995). Initial RT±PCR were performed on total RNA (50 ng) from either male or female adult worms. First strand cDNA was synthesized using 1 μM primer “3.1” and 200 units of reverse transcriptase (SuperScript, Gibco BRL) following the manufacturer’s protocol. PCR cycling was carried out in a total volume of 20 μl, containing 10% of the synthesized cDNA, 200 μM each deoxynucleotide (dATP, dTTP, dCTP, dGTP; Pharmacia), 1 μM each oligonucleotide primer, and 2.5 units of AmpliTaq polymerase (Perkin-Elmer, Norwalk, CT). PCR products were separated by gel electrophoresis, and the 280±283 sizes of the products were determined by comparison to standards run simultaneously. The sequence data reported herein have been submitted to GenBank and assigned the Accession Nos. U53177 for smras1 and Y11479 for MA018036.NA.

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of *Taq* polymerase (Appligene). After an initial denaturation step at 95°C for 5 min, temperature cycling was performed at 93°C, 45 s, 50°C, 60 s, 72°C, 30 s for 30 cycles, followed by a final elongation step at 72°C for 5 min (MJ Research MiniCycler).

Using the flanking primers (5.1, 3.1), three main amplification products were obtained irrespective of the gender of the adult worms providing the RNA. One of these products was of the expected size, predicted from the original sequence alignment, whereas the other two were larger and not considered for further analysis (result not shown). To verify the authenticity of this primary product, it was isolated and reamplified using the four possible combinations of flanking and nested primers. In all cases, the expected product appeared among the main amplification products (result not shown). The fragment corresponding to the predicted size of a genuine Ras product, generated by reamplification using the flanking primers, was recovered from an agarose gel and cloned into pUC18 plasmid by blunt-end ligation (SureClone Ligation Kit, Pharmacia, according to the manufacturer's protocol). Clones containing inserts of the expected size were individually sequenced by automated dye terminator, dideoxy sequencing (A.L.F., Pharmacia). One clone (ras 1.18) showed significant homology to Ras molecules of different organisms according to BLASTX and FASTA alignments (Menrath et al. 1995). A primary screen identified 16 positive clones, of which 6 were confirmed by rescreening and then sequenced (as above). Three of these (ras 1.18/2, 1.18/9, and 1.18/10) showed strong homology to Ras, as revealed by sequence analysis. These clones differ

![FIG. 1. Comparison of Ras protein sequences from eukaryotic species. The deduced amino acid sequence of SmRas1 (U53177) is shown aligned with Ras protein sequences from the SwissProt database: H. sapiens [HumKRas 4B (P01118), K-Ras 4A (P01116), H-Ras (P01112), and N-Ras (P01111)], D. melanogaster [DmelRas1 (P08646) and Ras2 (P04388)], C. elegans [CelLet (let 60; P22981)], and S. cerevisiae [ScerRas1 (P01119) and Ras2 (P01120); the C-terminal sequences of both yeast proteins have been omitted for easier comparison]. Conserved domains possessed by small eukaryotic GTPases are indicated above the alignment: G-1, binding domain for the α and β phosphates of GTP or GDP; G-2, effector domain that changes its conformation following GTP binding; G-3, binding domain for the γ-phosphate of GTP and for the catalytical Mg²⁺ ion; G-4, binding domain for the guanine ring; G-5, domain for the stabilization of GTP/GDP binding (Bourne et al. 1991). Identical amino acids are shown against a black background and homologous amino acids against gray.](image-url)
in length, but possess 100% identity in their overlapping regions. When assembled, they cover the entire translated and 3' nontranslated regions of the transcript. Clone ras 1.18/2 extends 14 bp 5' of the first ATG (methionine) codon, identified as the translation start point by a Ras protein sequence alignment (Fig. 1).

In a parallel approach, expressed sequence tag (EST) analysis of an *S. mansoni* cDNA library (Egyptian strain, mixed sex, adult worm cDNA library, prepared in Lambda ZapII and excised into Bluescript) revealed a clone (MA018036.NA) which was identified as a ras homologue by BLASTN analysis. This clone was also sequenced (dye terminator, dideoxy DNA sequencing, Applied Biosystems 373A).

A remarkable characteristic of both Liberian and Egyptian strain Ras sequences is the presence of a long, 3' nontranslated region (larger than the actual coding region), a feature that is uncommon in schistosome genes. The Liberian strain sequence (GenBank Accession No. U53177) differs from the Egyptian strain sequence (GenBank Accession No. Y11479) at three positions (434, 660, and 1120 bases from the start of the Liberian sequence). One of these differences lies within the coding region but leads to a synonymous substitution, while the other two occur in the 3' nontranslated region of the transcript (result not shown). Alignment of the deduced amino acid sequence of schistosome Ras (SmRas1) with other Ras proteins (from human, *Drosophila melanogaster*, *Caenorhabditis elegans*, and *Saccharomyces cerevisiae*) reveals the greatest similarity to the human K-Ras 4B (80% identity, 92% homology over 184 amino acids). The SmRas1 sequence exhibits all of the typical domains possessed by other eukaryotic Ras proteins (Bourne et al. 1991, see Fig. 1).

Two independent Southern blot experiments were performed on DNA restricted by different enzymes. One blot contained NlaIV-restricted DNA and the other blot DNA restricted by EcoRI or EcoRV (Fig. 2A). As hybridization probes, two different Ras cDNA fragments were used, one for each blot. One probe covered nearly the complete cDNA (positions 42–1575) and detected two bands with NlaIV-digested DNA. This was expected since one NlaIV-site occurs at position 591 of the cDNA. The other probe covered 850 bp (positions 97–964) and detected two bands with EcoRI-digested DNA and three bands with EcoRV-digested DNA. Within the cDNA sequence of Ras, there are three restriction sites for EcoRI (positions 174, 891, 1374); however, there was no site for EcoRV. This indicates the presence of at least one intron within the gene that contains these sites. The results suggest that ras is a single copy gene in *S. mansoni* as it is in *C. elegans* (Han and Sternberg 1990).

Northern blot analysis of poly(A)+ RNA from male worms revealed two distinct transcripts, one of about 1.8 kb, comparable to the full-length transcript, and the other of about 1.4 kb (Fig. 2B). Both bands were also detected with poly(A)+ RNA form females (result not shown). The smaller band probably represents a second transcript from the same gene which could have originated from alternative polyadenylation. There are three lines of evidence to support this proposition. First, among the positive clones identified by screening of the adult male cDNA library, one clone (1.18/9) of the appropriate size was found which possessed a poly(A) tail at position 1222 (from the start of the Liberian sequence). Second, analysis of the SmRas1 sequence revealed another presumptive polyadenylation site at position 1200, 23 bp upstream of the poly(A) tail of clone 1.18/9 (result not shown). Third, RT–PCR experiments, using an oligo(dT)17 primer and a 5' sense primer, confirmed the existence of the two transcripts that coincide with the transcripts detected by Northern blot analysis (results not shown). Unexpectedly, the RT–PCR experiment revealed a third transcript which was verified by Southern blot hybridization under stringent conditions. This transcript is about 60 bp smaller than the largest RT–PCR product, diagnostic for the 1.8-kb transcript, and could originate from a third presumptive polyadenylation site which occurs at position 1509. The size difference of about 60 bp cannot be resolved by Northern blot analysis, explaining why this additional transcript has not been detected before. Such findings correlate with data from the RHOA proto-oncogene, a Ras-related GTP-binding protein, which has three putative polyadenylation signals in the 3' UTR and transcripts of 1.8 and 1.5 kb (Moscov et al. 1994).

In the animal kingdom, the signal transducing molecule Ras plays a key role in protein hormone- and growth factor-mediated control of developmental processes such as mitogenesis, oogenesis, and differentiation (Wassarman et al. 1995; Kayne and Sternberg, 1995; Lee et al. 1996; Xu et al. 1996). Thus, it was tempting to speculate that in schistosomes Ras may be involved in similar processes. In this organism, numerous developmental changes occur, including the metamorphosis of the larval stages, the morphogenesis to the adults, and the male-dependent maturation of the female (Basch 1991). Therefore, the developmental expression of Ras was investigated by RT–PCR with RNA from adult and larval stages. Unexpectedly, all three transcripts were identified in paired and unpaired (single sex) females and males, as well as in cercariae and miracidia (result not shown). These results suggest that none of the transcripts plays an exclusive role in a specific developmental stage and indicate that all expression products may function during the whole development of schistosomes. However, it may be possible that quantitative differences in the level of these transcripts exist in the distinct life stages which cannot be detected by this RT–PCR approach. The assumption is confirmed by recently obtained immunoblotting data (Schüler et al. 1997). With a commercial antibody, which was directed against a highly conserved domain of Ras, quantitative differences were detected between paired and unpaired females.
Further studies will aim to analyze the function of Ras in schistosome development, focusing on its role during the male–female interaction.

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