Identification of Ras, MAP kinases, and a GAP protein in *Schistosoma mansoni* by immunoblotting and their putative involvement in male–female interaction

P. SCHÜßLER, C. G. GREVELDING *and* W. KUNZ*

Institut für Genetik, Genetische Parasitologie und Biologisch–Medizinisches Forschungszentrum, Heinrich-Heine-Universität, Universitätsstraße 1, D-40225 Düsseldorf, Germany

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SUMMARY

The maturation of female *Schistosoma mansoni* depends on pairing with a male which induces mitotic activities in the reproductive organs of the female worm. Since in other organisms cell proliferation is regulated by well-conserved signal transducing molecules, we looked for such molecules on immunoblots of schistosomes, using antibodies against conserved epitopes of Ras, GAP and MAP kinases. We identified all 3 molecules in schistosomes and found that they are developmentally regulated. Furthermore, there is evidence for their involvement in the male-directed maturation of the female.

Key words: *Schistosoma mansoni*, signal transduction, Ras, GAP, MAP kinase, male–female interaction, pairing-dependent gene expression.

INTRODUCTION

Signal transduction pathways are well conserved in the whole animal kingdom and the molecules involved are equally conserved. Signalling molecules fulfil essential functions in the control of cell proliferation, and they provide the molecular basis for development by governing differentiation processes (for review see Pawson, 1991; Blenis, 1993). Among this class of molecules, the small G protein Ras plays a major role (McCormick, 1994). From cell-surface receptors stimulated by growth or differentiation factors, Ras is activated by tyrosine kinases, and it mediates the signal to downstream targets like mitogen-activated protein (MAP) kinases (McCormick, 1994). In order to transmit signals to the MAP kinases, Ras has to interact with its specific GTPase activator protein, the Ras–GAP, which converts Ras back to its inactive state, ready to receive the next signal (Parsons, 1990). GAP possesses SH2 and SH3 domains which are responsible for the interaction with tyrosine kinases (Schlessinger, 1994). The MAP kinases act consecutively in a cascade and transport the signal by serine/threonine phosphorylation into the nucleus, which finally leads to changes in gene expression (Marshall, 1994). Components of this pathway are highly conserved among yeast, flies, nematodes and mammals.

Adult schistosomes are closely paired with each other for their entire life. Female worms that are not in contact with a male, like those obtained by a unisexual infection, are inhibited in their development and remain stunted (Erasmus, 1973). Furthermore, in well-developed females that have been separated from their male partners, the vitelline cells degenerate (Popiel, 1986*b*). These observations show that pairing is essential for the final differentiation and maturation of the reproductive organs in female worms (Popiel, 1986*a*). The question arises of how the male stimulates such developmental processes in the female? Classical experiments suggest that the male transfers an inductive signal of chemical nature to the female during pairing (Popiel, 1986*b*). Histological studies have shown that DNA synthesis increases when unisexual females are paired with males, indicating mitotic activity (Den Hollander & Erasmus, 1985). Cell proliferation, induced by the male, has been observed during the development of the vitelline gland (Erasmus, 1986). All these observations led to our hypothesis that the male signal acts as a growth factor or stimulates such a factor that finally leads to mitotic proliferation of progenitor cells in the reproductive organs (Kunz *et al*. 1995). As a consequence of mitoses, a terminal differentiation programme of vitelline cells is started involving the expression of pairing-dependent genes (see the accompanying paper by Grevelding *et al*. 1997).

The male stimulus that induces vitelline cell proliferation in female schistosomes has to be propagated via signal transduction. Since this signalling results in a mitogenic programme, molecules must be involved that are known in other organisms to regulate cell proliferation events. In this paper, we

* Corresponding author. Tel. and Fax: +49 211 81 12333. E-mail: Werner.Kunz@uni-duesseldorf.de

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report the identification of the signal transduction molecules Ras, GAP and MAP kinases in *S. mansoni*. Furthermore, it is demonstrated that these molecules exhibit a developmentally regulated expression pattern. Beyond that, the expression of one of these molecules is influenced by pairing.

MATERIALS AND METHODS

Parasite stocks

An *S. mansoni* stock from Liberia was maintained in *Biomphalaria glabrata* and in Syrian hamsters or gerbils. Recovery of worms was by perfusion (Köster *et al*. 1988). Male and female worms were separated with a fine brush and stored in liquid nitrogen. To obtain unisexual females, the intermediate snail hosts were infected with a single miracidium to produce cercariae of one sex for final host infection.

In vitro *culture of schistosomes*

Schistosomes were collected freshly after perfusion and maintained in an *in vitro* culture system for up to 3 weeks following the protocol described in the accompanying paper by Grevelding *et al*. (1997).

Protein extraction

The preparation of protein extracts from mature male, female and unisexual female schistosomes was based on a method described earlier (Schüßler *et al*. 1995*a*). For the preparation of protein extracts from *in vitro*-cultured female schistosomes, the combined method was used which also permits the isolation of nucleic acids (Schüßler *et al*. 1995*b*). To minimize putative host–protein contamination, all batches of schistosomes were intensively washed in perfusion buffer before they were used for protein extraction. Protein extracts from mouse tissue and proteins from yeast were prepared as described elsewhere (Linder, Bammer & Heinlein, 1995).

Immunoblotting

Isolated proteins from *S. mansoni*, yeast or mouse were separated on SDS–PAGE. After electrophoresis, proteins were blotted onto Immobilon PVDF membranes (Millipore) or 'nitrocellulose extra' membranes (Sartorius, Göttingen). One part of the membranes was stained with amido black as described previously (Köster *et al*. 1988), the other part was blocked for 30 min to 1 h at room temperature in PBS containing either 2% polyvinyl pyrolidone 360000 (for nitrocellulose) or 1% bovine serum albumin (BSA) and 0·05% Tween 20 (for PVDF). Antibody incubation was performed overnight at room temperature in PBS containing 0·3% BSA and 0·05% Tween 20. Detection of bound antibodies was achieved with alkaline phosphatase-conjugated goat anti-mouse antibodies (Dianova), and colour was developed with naphthol-AS-MX-phosphate and Fast Brown or Fast Red (Sigma) (Köster *et al*. 1988). Quantification of immunoblots was carried out with a ScanJet 4c (Hewlett Packard) and the BioScan© software (Viper, Germany) by integration.

To demonstrate that equal amounts of proteins were used for Western blot analysis, a re-incubation was done using a monospecific antibody directed against *S. mansoni* paramyosin (Schmidt *et al*. 1996). Detection and cross-staining were achieved as described above.

Antibodies

In a computer-assisted alignment, peptide sequences were determined that were located within particularly conserved regions of the signal transduction molecules Ras, GAP and MAP kinases. Commercial antibodies were used that were directed against such domains. Different batches of the respective antibodies were used. To ensure that their immunodetection would only be based on the primary amino acid sequence of the epitope, antibodies were chosen that have been produced against synthetic peptides.

The monoclonal anti-H-Ras antibody (DAKO) is a mouse IgG, directed against a synthetic peptide (Glu-Ser-Arg-Gln-Ala-Gln-Asp-Leu-Ala-Arg-Ser-Tyr-Gly-Ile-Pro-Tyr) corresponding to the human H-Ras sequence. This peptide is 100% identical within the H-Ras proteins of different vertebrates. The identity to Ras molecules of invertebrates is 50% at the amino acid level, and the similarity is 80% (e.g. slime mould or *Drosophila*).

The monoclonal anti-GAP antibody (Gibco BRL) which is a mouse IgG, is directed against a synthetic peptide (Thr-Pro-Gly-Asp-Tyr-Ser-Leu-Tyr-Phe-Arg) corresponding to the bovine and human GAP sequence. Again, the identity of the peptide in different vertebrate sequences is 100%. In invertebrates, the identity is 40%, and the similarity 90% (e.g. *Caenorhabditis elegans*).

Finally, the monoclonal anti-MAP2 kinase antibody MK12 (Gibco BRL) is a mouse IgG_1, directed against a synthetic peptide (Thr-Asp-Glu-Pro-Val-Ala-Glu-Glu-Pro-Phe-Thr-Phe-Asp-Met-Glu-Leu-Asp-Asp-Leu-Pro-Lys) corresponding to the rat and hamster MAP2 kinase sequence. The homology of the peptide in different vertebrate and invertebrate sequences is extremely high, e.g. 95% identity to human MAP2 kinase and 90% identity and 95% similarity to *Aplysia californica*, a mollusc. In mammals, there exists the isoform MAP1 kinase which is 90% identical with the MAP2 kinase (Blenis, 1993). A cross-reaction with this second isoform is expected (product information of Gibco BRL).

The production and purification of the monospecific anti-*S. mansoni* paramyosin antibody was described by Schmidt *et al*. (1996).

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RESULTS

In females, Ras is developmentally regulated

Total protein of mature and unisexual female schistosome extracts was separated on a 15% SDS–PAGE. After blotting, one part of the membrane was stained with amido black to document equal loading (Fig. 1, left). The other part was incubated with the anti-Ras antibody in a final concentration of 1:100 that resulted in the detection of a single protein band with a molecular mass of 23 kDa (Fig. 1). Ras was also found in protein extracts of males (data not shown). As a positive control, proteins extracted from 2 distantly related organisms, mouse and yeast, have been treated with this anti-Ras antibody. In both cases, a single protein band with the expected molecular mass of about 22 kDa was observed, demonstrating the specificity of the antibody (McCormick, 1994) (Fig. 1, right).

Furthermore, it was examined whether Ras exhibits a developmentally regulated expression. Protein extracts from unisexual and mature female schistosomes were prepared in 2 parallel experiments and probed with anti-Ras antibody. The signal intensity was at least 5 times stronger in mature than in unisexual females as evaluated by densitometric quantification and integration (Fig. 1, centre). In male worms, Ras was found to be equally expressed in unisexual and mature parasites (data not shown). No differences were found between 2 different batches of the antibody.

A re-incubation and cross-staining of the same Western blot with a monospecific anti-*S. mansoni* paramyosin antibody confirmed that equal amounts of protein were loaded (Fig. 1, centre).

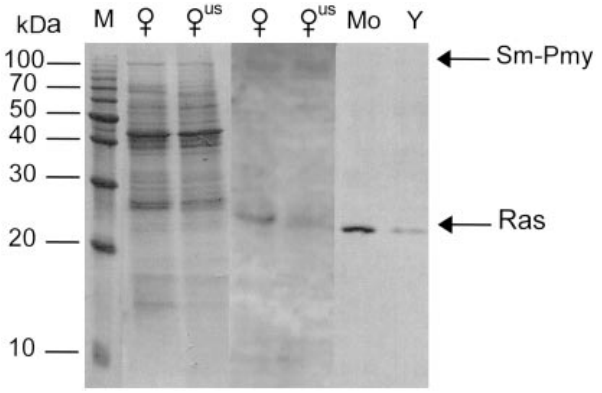

Fig. 1. Immunoblot analysis with an anti-Ras antibody. The samples containing 20 μg of protein extracts from mature and unisexual (us) females, and 40 μg of protein from mouse liver (Mo) and yeast (Y) were separated by 15% SDS–PAGE. After blotting, the left part of the membrane was stained with amido black and the right part was incubated with the anti-Ras antibody at a final concentration of 1:100 for *Schistosoma mansoni* protein and of 1:500 for mouse and yeast protein. For re-incubation and cross-staining, an anti-*S. mansoni* paramyosin antibody was used (Sm-Pmy). The 10 kDa ladder (Gibco BRL) has been used as marker (M).

The expression of Ras–GAP in females depends on pairing with the male

In signal transduction pathways, Ras interacts with the GTPase activator protein (GAP), which converts Ras back to the inactive state. In order to find this molecule in schistosomes, total protein extracts from males and females were separated on a 7·5% SDS–PAGE. After Western blotting, the membrane was incubated with the anti-GAP antibody in a final concentration of 1:500. We detected a single band of about 90 kDa (Fig. 2). The same results were obtained using 3 different batches of this antibody. This molecular weight is in the same size range of a Ras–GAP isoform in rat and *C. elegans* and, therefore, we believe that we identified the GAP homologue in *S. mansoni*. As a control, the protein extract from a mouse kidney was probed with the antibody and, as expected, 2 bands at 100 and 120 kDa were detected (Fig. 2, right). They represent 2 isoforms of GAP (Parsons, 1990).

To confirm that equal amounts of protein extracts from male and female schistosomes had been loaded

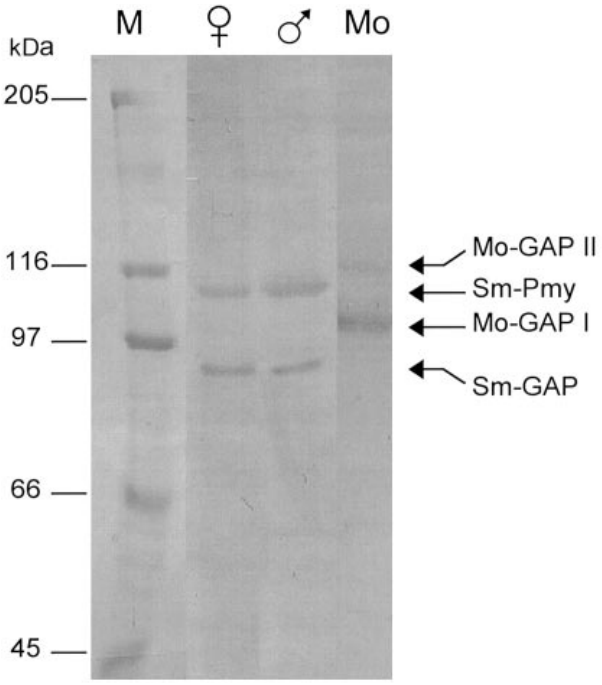

Fig. 2. Immunoblot analysis with an anti-GAP antibody. The samples containing 40 μg of female and male *Schistosoma mansoni* (Sm), and mouse kidney (Mo) protein extracts, were separated by 7·5% SDS–PAGE. After blotting, the lane with the high molecular weight markers (M) was stained with amido black and the protein extracts were incubated with an anti-GAP antibody at a final concentration of 1:500 for *S. mansoni* protein and of 1:2000 for mouse protein. The position of the GAP protein and its isoforms in mouse (I and II) are indicated by arrows. For re-incubation and cross-staining, an anti-*S. mansoni* paramyosin antibody was used (Sm-Pmy).

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on the gel, this Western blot was re-incubated and cross-stained with the monospecific anti-*S. mansoni* paramyosin antibody (Fig. 2, centre). The densitometric quantification of the immunoblot indicates that the signal intensity of the GAP in schistosomes is similar between females and males.

Since we have shown that Ras expression is increased in mature versus unisexual females, we were interested to investigate whether the expression of the Ras–GAP protein also may depend on contact with the male. We performed pairing experiments in an *in vitro* culture system (Fig. 3). Paired schistosomes were freshly collected after perfusion, and the following cultures were performed. One group of worms was cultured as pairs for a 6-day period (♀p). This was compared with another group of females that were separated from males and cultured *in vitro* for up to 6 days (d_0–d_6). A third group were females separated from the males for 6 days and then remated with males for another 7 days (♀r). This was compared with females that were kept separated from males for 13 days in culture (d_{13}).

After the appropriate periods, proteins were isolated from females (Schüßler *et al*. 1995*b*) and separated on a 7·5 % SDS–PAGE. Each lane of the gel contained approximately 30 μg protein extracts that were blotted and probed with the anti-GAP antibody in a final concentration of 1:500. Fig. 3 shows that GAP decreased in females during the first 3 days of separation and had totally disappeared after 6 days. It remained, however, distinctly visible in paired females. Remarkably, GAP was re-expressed in those females that had been separated from males and then remated.

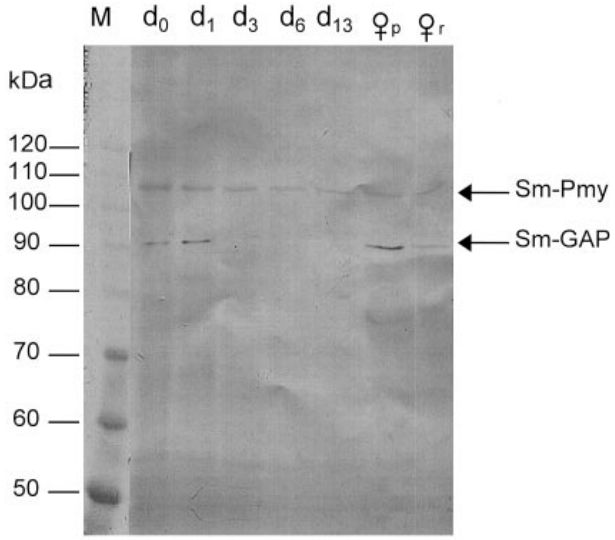

Fig. 3. Immunoblot analysis of the GAP-like protein from female *Schistosoma mansoni* (Sm) cultured *in vitro*. The samples containing 30 μg of protein extracts each of separated, paired (p), and remated (r) females were loaded onto on a 7·5 % SDS polyacrylamide gel and subjected to electrophoresis. The proteins of the separated females were prepared after zero (d_0), 1 (d_1), 3 (d_3), 6 (d_6), and 13 (d_{13}) days of *in vitro* culturing. After blotting, the 10 kDa-ladder marker lane (M) was stained with amido black and the protein extracts were incubated with an anti-GAP antibody at a final concentration of 1:500. The position of the GAP proteins is indicated by an arrow. As a control for the nearly equal amount of protein extracts loaded onto the gel, we used again the anti-*S. mansoni* paramyosin (Sm-Pmy) antibody.

The MAP1 and 2 kinases are preferentially expressed in females

In many signal transduction pathways, the Ras protein functions as a mediator point between a receptor and a serine/threonine kinase cascade. Members of this cascade are the MAP kinases. To prove the existence of such kinases in schistosomes, we separated equal amounts of protein from male and female worms on 15 % SDS–PAGE (Fig. 4, left). The immunoblot was treated with an anti-MAP2 kinase antibody at a final concentration of 1:75. This antibody reacted with 2 protein bands in the size range of 42 and 45 kDa (Fig. 4), suggesting the existence of MAP kinases in schistosomes. In a control experiment with protein extracted from mouse liver, we detected 2 bands corresponding in size with the MAP1 and 2 kinase isoforms (Fig. 4, right) (Boulton *et al*. 1991). The identity in size of the 2 bands in mouse and schistosomes shows that schistosomes also possess these 2 isoforms. The signal of the MAP1 kinase isoform (42 kDa) of *S. mansoni*, however, was much more intense than the signal of the other isoform (45 kDa). Interestingly,

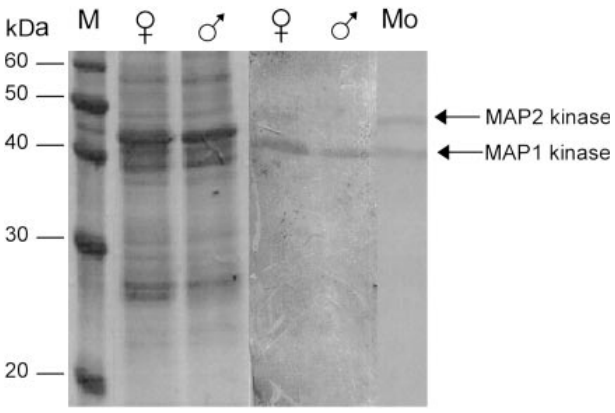

Fig. 4. Immunoblot analysis with an anti-MAP2 kinase antibody. The samples containing 40 μg of female and male *Schistosoma mansoni*, and mouse liver (Mo) protein extracts, were separated by 15 % SDS–PAGE. After blotting, the left part of the membrane was stained with amido black and the right part was incubated with an anti-MAP2 kinase antibody at a final concentration of 1:75 for *S. mansoni* protein and of 1:500 for mouse protein. The positions of the MAP1 and 2 kinase isoforms are indicated by arrows. The 10 kDa-ladder has been used as marker (M).

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both isoforms were clearly enriched in females. The MAP1 kinase isoform has been quantified to be 2·5 times more intense in females than in males. The MAP2 kinase isoform was weakly visible in female worms, but was not detectable in males. These results have been reproduced by using another batch of the antibody.

DISCUSSION

In this paper, we report on the identification of 3 signal tranducing molecules in *S. mansoni* which are known in other organisms to be involved in mitogenic processes: Ras, GAP and MAP kinases. These molecules have been detected with monoclonal antibodies on immunoblots. The antibodies were directed against highly conserved epitopes of these signal transduction molecules. They specifically detected only the respective molecules on Western blots of protein homogenates from mouse or yeast. Since only bands in the same size range were recognized on immunoblots of schistosomes, it is almost certain that Ras, GAP and MAP kinases do exist in *S. mansoni*.

To exclude contamination of the schistosome protein extracts with rodent host tissue, the worms were intensively washed. In addition, mouse proteins were used in our Western blot analyses as an indirect control for comparison. These controls show a different molecular size for Ras and GAP as well as differences in isoform distribution of MAP kinase in mouse, compared with schistosomes. Furthermore, differences in signal intensities between the sexes or developmental stages in *S. mansoni* argue against host origin of the detected molecules.

Interestingly, Ras, GAP and the MAP kinases are developmentally regulated. Ras occurs in higher quantities in mature females compared with immature ones. GAP is much more abundant in paired females than in unpaired females, and the MAP kinases are highly enriched in females compared to males. The function of Ras depends on the interaction with GAP. Activated Ras transmits signals to the MAP kinases (McCormick, 1994; Marshall, 1994). Since the presence of GAP depends on pairing and since Ras, GAP and the MAP kinases interact in the same signal transduction pathway, we expect that all 3 molecules in schistosomes are involved in the female maturation process.

The developmental control of the 3 molecules is inferred from their expression profile. It is the protein quantity that has been found to vary among the different stages. This shows that the molecules of the signal transducing pathway are synthesized *de novo* as a consequence of the male signal. Although it is known for several signalling pathways that the involved molecules are permanently expressed and are only temporarily activated by phosphorylation, there are numerous examples for regulation by *de novo* synthesis of the molecules of signal transducing pathways. In *Hydra*, Ras2 synthesis responds to head-specific signals, providing an example for Ras expression during a specific differentiation state (Bosch *et al*. 1995). In the mouse brain, 3 members of the Ras family are regulated in a developmental and regional pattern (Ayala *et al*. 1989).

In the literature, there are already a few examples of signal transduction molecules in schistosomes. The serotonin receptor and its signalling pathway have been characterized in *S. mansoni* (e.g. Iltzsch *et al*. 1992; Day, Bennett & Pax, 1994). This receptor is linked via G protein activation to an adenylate cyclase that catalyses the synthesis of the second messenger, cAMP. Serotonin acts in carbohydrate metabolism and causes stimulation of motility. Another example is represented by the small G protein Rab, known to be involved in secretory processes, that has recently been cloned in *S. mansoni* (Loeffler & Bennett, 1996). Furthermore, a number of calcium-binding proteins have been identified that regulate the level of calcium, an important second messenger which controls different physiological functions like muscle contraction, and which is involved in cell proliferation (e.g. Ram *et al*. 1989; Siddiqui, Podesta & Clarke, 1991). Wiest *et al*. reported on protein kinase C as well as phospholipase C activities in schistosomes (Wiest *et al*. 1992; Wiest, Kunz & Miller, 1994) and Shoemaker *et al*. (1992) identified the epidermal growth factor receptor. These molecules are known to participate in cell proliferation processes (Pawson, 1991). Due to the function, the sites and stages of expression of all these described molecules, however, it is unlikely that they are involved in the male–female interaction.

Further experiments are in progress to clone the genes for GAP and the MAP kinases in schistosomes. The gene encoding Ras has recently been identified (Kampkötter *et al*. manuscript in preparation). Localization of the expression products of these genes will provide more information on their role in the male–female interaction.

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