**Stage-specific Expression of a *Schistosoma mansoni* Polypeptide Similar to the Vertebrate Regulatory Protein Statmin**

(Received for publication, November 5, 1998, and in revised form, September 6, 1999)

Cristiana Valle, Alfredo Festucci, Anna Calogero‡, Paola Macrı`, Barbara Mecozzi, Piero Liberti, and Donato Cioli§

*From the Institute of Cell Biology, National Research Council, 43 Viale Marx, 00137 Rome, Italy*

The ubiquitous vertebrate statmin is expressed and phosphorylated in response to a variety of external and internal signals. Statmin, in turn, controls cell growth and differentiation through its capacity to regulate microtubule assembly dynamics. This is the first report on the molecular cloning and characterization of a stathmin-like protein (SmSLP) in an invertebrate, the human blood fluke *Schistosoma mansoni*. SmSLP is first synthesized at high levels in the intermediate molluscan host and completely disappears 48 h after penetration into the mammalian host. The protein is preferentially iodinated in intact immature parasites using the Bolton-Hunter reagent, can be quantitatively extracted in high salt buffers, and remains soluble after boiling. Native SmSLP was partially sequenced, and its complete structure was derived from the cloning and sequencing of its cDNA. The sequence is up to 26% identical to vertebrate statmin sequences and contains two potential phosphorylation sites. Native SmSLP is indeed phosphorylated because phosphatase digestion shifts its mobility in electrofocusing gels. SmSLP associates with tubulin, as suggested by immune co-precipitation results. *In vitro* experiments demonstrated that SmSLP inhibits tubulin assembly and causes the depolymerization of preassembled microtubules, thus probably fulfilling regulatory roles in critical steps of schistosome development.

Statmin is a ubiquitous and highly conserved phosphoprotein, the synthesis and phosphorylation of which vary in close association with growth and differentiation of vertebrate cells (1, 2). Although statmin-like proteins have been recently identified in plants (3), no similar proteins have been reported for invertebrates.

We describe here a protein of the blood fluke *Schistosoma mansoni* that bears structural and functional similarity with statmin. Parasitic trematodes of the genus *Schistosoma* are the causative agents of schistosomiasis, a disease currently estimated to affect 200 million people in tropical and subtropical countries. Schistosomes have a complex life cycle involving a mammalian host in which adults of the two sexes mate and deposit eggs, a free aquatic stage (miracidium) derived from eggs excreted into the environment, a molluscan stage with active asexual multiplication, and another free aquatic stage (cercaria) that is capable of infecting the mammalian host by rapidly penetrating through intact skin. The newly penetrated larva (schistosomulum) migrates to its final location in the venous system of the mammalian host and differentiates into adult male and female worms. The life cycle is characterized by dramatic morphological transformations and by rapid physiological adaptations, such as those required by the drastic temperature and osmolarity variations that schistosomes encounter in their changes of host and environment. Practically nothing is known of the molecular mechanisms that govern these processes, e.g. of the signals exchanged between hosts and parasites, between the environment and the parasite, and between the two sexes of the parasite.

The *S. mansoni* statmin-like protein (SmSLP)1 is abundantly expressed during a very short and critical period of the parasite life cycle, whereas it is undetectable at other stages. We propose that SmSLP plays important regulatory roles during the life cycle of schistosomes.

**EXPERIMENTAL PROCEDURES**

**Parasites—**A Puerto Rican strain of *S. mansoni* was maintained by serial passages in outbred Swiss mice and the aquatic mollusc *Biomphalaria glabrata* (4). Adult schistosomes were obtained by portal perfusion of 7-week-infected mice. Three-hour schistosomula were produced by mechanical transformation of cercariae (5), whereas later schistosomulum stages were obtained by *in vitro* maintenance of 3-h schistosomula.

**Labeling Procedures—**Labeling with the Bolton-Hunter reagent and lactoperoxidase-catalyzed iodination were performed as described previously (6). For metabolic labeling at the sporocyst stage, infected snails were exposed for 4 h to [35S]methionine (25 μCi/ml in water), and aliquots of the snail hepatopancreas were boiled in sample buffer and used for polyacrylamide gel electrophoresis (PAGE) analysis.

**PAGE, Electrophoresing, Autoradiography, and Western Blotting—**PAGE was performed according to Laemmli (7). Isoelectric focusing gels contained a total amount of 2% ampholines, of the pH ranges 7–9, 9–11, and 3–10 in the proportion 2:2:1. The gel was run overnight at 1 mA and separated proteins were blotted to nitrocellulose after an incubation of 10 min in transfer buffer containing 0.2% SDS. Autoradiographic procedures have been previously described (6). Western blots were performed using a 1:1,000 dilution of the anti-SmSLP serum, followed by goat anti-rabbit serum (1: 5,000) conjugated to alkaline phosphatase.

**High Salt Extraction—**Cercariae or 3-h schistosomula were suspended in a Tris-buffered high salt solution (1.2 μ NaCl, 83 mm Tris-HCl, pH 7.4) for 1 h at 4 °C and then for another 1 h at 37 °C (6). The supernatant containing SmSLP was removed at the end of the incubation by letting the parasites settle at the bottom of the tube. When used for *in vitro* tubulin assembly experiments, SmSLP was further purified

---

1 The abbreviations used are: SmSLP, *S. mansoni* statmin-like protein; PAGE, polyacrylamide gel electrophoresis; MESS, 4-morpholineethanesulfonic acid.

The Journal of Biological Chemistry, Vol. 274, No. 48, Issue of November 26, pp. 33869–33874, 1999
Printed in U.S.A.
© 1999 by The American Society for Biochemistry and Molecular Biology, Inc.

This paper is available online at http://www.jbc.org
by molecular sieving on a Sephadex G-100 column in 0.15 M NaCl, 0.01 M Tris, pH 7.4.

**Antiserum**—Using the eluate of a pool of SmSLP bands cut from PAGE runs of schistosomulum high salt extracts, a rabbit was injected intradermally in 30 different sites with about 50 µg of protein in complete Freund's adjuvant, followed 1 month later by two additional injections of the same antigen in incomplete adjuvant. The serum obtained 1 week after the last immunization was capable of specifically precipitating the radioactive SmSLP band labeled in vivo and of recognizing the same band in Western blots of schistosomulum extracts.

**Amino Acid Sequencing**—A purified preparation of SmSLP was obtained by isolating the corresponding band from a PAGE run of a high salt extract of schistosomula, eluting a pool of such bands, and rerunning the eluate on a second gel. The protein was transferred to a Problot membrane (Applied Biosystems) and N-terminally sequenced using a Perkin-Elmer AB 476A apparatus (sequencing courtesy of Prof. D. Barra, University of Rome).

**Primers, Screening, Sequencing, and Expression**—Degenerate primers were designed on the N-terminal sequence obtained from the protein and were used in polymerase chain reaction to amplify the DNA obtained from a agt11 sporocyst library kindly supplied by Dr. J. Mc Kerrow (University of California, San Francisco). The same library was screened according to standard procedures (8), and the plaque-purified clones were sequenced after subcloning in pUC-18. The vector pQE9 (Qiagen) was used for expression, and the expressed proteins were analyzed by PAGE and Western blotting.

**Phosphatase Digestion**—A high salt extract from 104 schistosomula was incubated for 90 min at 37 °C with 10 units of alkaline phosphatase attached to oxirane-activated macroporous acrylic beads (Sigma), in a buffer containing 20 mM Tris-HCl, pH 8.0, 0.05% Triton X-100, and 0.1% SDS in a final volume of 80 µl. The reaction was terminated by freezing, and the sample was prepared for isoelectric focusing together with an identically treated aliquot incubated in the absence of phosphatase.

**In Vitro Microtubule Assembly**—The turbidimetric polymerization assay (9) was performed using bovine brain tubulin (Cytoskeleton, Denver, CO; catalog no. M113) and monitoring the A540 at 37 °C. The polymerization buffer contained 0.1 M MES, pH 6.6, 1 mM EGTA, 0.5 mM MgCl2, 3 M glycerol, 1 mM GTP.

**RESULTS**

**SmSLP Is Preferentially Labeled with the Bolton-Hunter Reagent**—In the course of a study aimed at the identification of surface larval proteins of *S. mansoni*, we noticed that, using the Bolton-Hunter reagent on 3-h schistosomula, a number of labeled bands could be detected by SDS-PAGE, but the most prominent concentration of radioactivity was in the 19-kDa region (Fig. 1A, lane 1). We have previously described an 18-kDa polypeptide that was preferentially iodinated in 3-h schistosomula using lactoperoxidase (6). We initially thought that the same protein also became labeled using the Bolton-Hunter reagent, but on closer examination, we realized that the relative mobility of the protein labeled with the latter reagent is slightly different (about 19 kDa, Fig. 1A, lane 2) and that the two molecules have different biochemical characteristics. In addition, although the radioactivity of SmSLP is exactly superimposed on a discrete protein band detected by Coomassie Blue staining, the radioactivity obtained by lactoperoxidase labeling does not coincide with a detectable band in the protein pattern of whole schistosomula (not shown).

**SmSLP Can Be Extracted with a High Salt Buffer**—Because it was assumed that the preferential labeling of SmSLP could be due to its location at or near the surface of the schistosomulum, the production of surface membrane vesicles (10) seemed an appropriate method for the purification of the protein. When 3-h schistosomula were kept in high salt buffer for 1 h at 4 °C followed by 1 h at 37 °C, a band corresponding to the position of SmSLP was quantitatively extracted in the first supernatant (which does not contain vesicles), as shown by SDS-PAGE analysis (Fig. 1B). An identical experiment carried out using schistosomula labeled with the Bolton-Hunter reagent confirmed that the labeled band was also quantitatively extracted (not shown). Because this procedure yielded a limited number of released proteins, elution of SmSLP from acrylamide gels of extraction supernatants provided a fraction containing SmSLP in very high purity. The same extraction procedure used with schistosomula could be applied directly to cercariae.

**SmSLP Is Synthesized in the Snail Sporocyst**—In order to select the life cycle stage to be used as the source of SmSLP mRNA, we incubated 3-h schistosomula, adult worms, and infected snails with [35S]methionine and immunoprecipitated the labeled products with the specific anti-SmSLP serum. Whereas 3-h schistosomula and adult worms failed to show any incorporation of precursor in the SmSLP band (not shown), the immunoprecipitate of infected snails showed a labeled band of the 19-kDa mobility expected for SmSLP (Fig. 1C), demonstrating that the synthesis of this protein occurs in the sporocyst of infected snails.

**SmSLP Is Not Glycosylated**—Before undertaking immunoscreening of the cDNA library, we also made sure that our antiserum was not directed against carbohydrate determinants that could be possibly present in SmSLP. We could not detect any binding of labeled SmSLP to a number of lectins (concanavalin A, wheat germ, peanut, or lentil lectin). In addition, when labeled SmSLP was subjected to digestion with endoglycosidase F in a test tube containing unlabeled fetuin as a control, no modification in SDS-PAGE mobility could be detected for SmSLP, whereas fetuin was clearly shifted to lower molecular weights after digestion (results not shown). We could thus conclude that SmSLP is very unlikely to be glycosylated.

**Partial Amino Acid Sequence**—A purified fraction of SmSLP was prepared by extraction of schistosomula in high salt buffer, SDS-PAGE separation of the supernatant, and elution of the gel region corresponding to trace amounts of labeled SmSLP. The following N-terminal amino acid sequence was obtained by
Edman degradation of the purified fraction: TTLEAPHPSEKDEMELVYADYEKEEGGLK SIXNEIK.

cDNA Cloning—Based on the partial amino acid sequence, two degenerate primers were designed and synthesized (forward, 5'-GAA TTC GAR AAY AGC GCN CCN CAY CC-3'; reverse, 5'-GAA TTC CCY TTY TTY TCR TAY TC-3'). Polymerase chain reaction amplification using these primers on a template consisting of the DNA from a cDNA library of S. mansoni sporocysts yielded a 71-base pair product that had the sequence expected for the N-terminal region of SmSLP. The anti-SmSLP rabbit antiserum was used to screen an Agt11 cDNA expression library from S. mansoni sporocysts and led to the isolation of a clone corresponding to the 3'-region comprising the poly(A).

Sequential cloning of the DNA was obtained through polymerase chain reaction amplification and 5'-rapid amplification of cDNA ends using various combinations of the primers designed according to the sequences of the 5'- and 3'-regions of the gene and the Agt11 forward and reverse primers (Fig. 2).

Predicted SmSLP Amino Acid Sequence—The coding sequence contains at the N terminus a typical hydrophobic leader peptide of 22 amino acids, followed by the sequence originally determined by Edman degradation of the mature protein (Fig. 2, underlined). The open reading frame corresponds to a protein of 117 residues and is followed by three closely spaced termination codons. Downstream of 99 untranslated nucleotides, a polyadenylation signal is followed by 15 additional nucleotides and by the poly(A). The deduced protein is predominantly hydrophilic, is predicted to be mostly in 6-helical conformation, and has a theoretical PI of 8.0. No potential glycosylation sites are available, whereas two serine residues (positions 32 and 61) are present within protein kinase C phosphorylation consensus sequences. The mature protein (without leader peptide) has a calculated molecular weight of 11,345, which is considerably smaller than the size estimated from SDS-PAGE analysis (19 kDa). However, when the recombinant SmSLP without leader sequence was expressed in Escherichia coli, it showed a mobility of 19 kDa, which corresponded exactly to the Rf of high salt extracted and iodinated SmSLP (not shown). Therefore, we interpret the discrepancy between calculated and observed molecular mass as an instance of anomalous electrophoretic behavior, such as is occasionally encountered with small proteins.

Similarity with Stathmins—A BLITZ search (11) of the SwissProt data base revealed a similarity with proteins of the stathmin family, with about 1% probability that the similarity could occur by chance (Fig. 3). After sequence alignment, the 117 residues of SmSLP were 26% identical and 43% similar to chicken stathmin, whereas 33% of residues had identity and 50% had similarity to one or more of the proteins shown in Fig. 3. In addition, the position of mouse introns was found to coincide in one case with a SmSLP intron and in two other cases with the start and the end, respectively, of the SmSLP coding sequence (data not shown). A further element of similarity with stathmins was found at the biochemical level, because stathmins are known to be particularly heat-soluble (2, 12, 13), and SmSLP retained its solubility after 10 min of boiling, as shown in Fig. 1B, lane 4.

Stage Specificity of SmSLP—When schistosomula of various ages were labeled with the Bolton-Hunter reagent, the typical radioactive band of 19 kDa was only observed at 3, 9, and 24 h; at 48 h, it was very faint, and it totally disappeared thereafter (not shown). This was not the result of decreased protein exposure, but it reflected the actual disappearance of SmSLP, as shown by the parallel disappearance of the corresponding Coomassie Blue band (Fig. 4). Parasites of 14 days or adult worms of either sex were totally negative for the labeling of SmSLP, as well as for the presence of the Coomassie Blue band.

In order to obtain a precise assessment of the timing of SmSLP synthesis in the sporocyst, the hepatopancreas of infected snails was tested by Western blotting at various times after exposure of the molluscs to schistosome miracidia. It can be seen from Fig. 5 that no SmSLP band was detectable in eggs, in miracidia, or in snail hepatopancreas during the prepatent period (up to 27 days). Only on the same day (day 28) that a snail first showed the presence of emerging cercariae upon crushing of the shell did the SmSLP band appear in Western blots. The SmSLP band was present in cercariae and in 3-h schistosomula, but it had disappeared in 3-day-old schistosomula. It could thus be concluded that SmSLP is synthesized in the sporocyst just in the very few hours preceding and accompanying cercarial shedding and that the protein is present in cercariae (which usually have a life span of less than 24 h) and remains present during the first day or two after transformation into the mammalian form of the life cycle.

SmSLP Is Phosphorylated—Because the major functional property of stathmins is connected with their variable state of phosphorylation, it was of interest to determine whether SmSLP was also phosphorylated. A high salt schistosomulum extract was digested with alkaline phosphatase, separated by electrofocus, and analyzed by Western blotting with the specific anti-SmSLP serum. Fig. 6 shows that the undigested sample presents two major reactive bands (lane 1), whereas the phosphatase-digested sample has been essentially converted to a single band (lane 2).
Purified bovine tubulin (Cytoskeleton, catalog no. T238) was iodinated using the Bolton-Hunter reagent, yielding a single 55-kDa radioactive band in SDS-PAGE. Labeled tubulin was incubated at 4°C with SmSLP, anti-SmSLP serum was added, and an immunoprecipitate was obtained upon addition of protein A-Sepharose. The washed pellet contained 7550 cpm, whereas a control pellet from a tube without SmSLP contained 2070 cpm.

SmSLP Affects Tubulin Polymerization in Vitro—Native SmSLP, prepared from cercariae by high salt extraction followed by Sephadex purification, was mixed in vitro with bovine tubulin, in a standard polymerization assay (9), using a SmSLP-tubulin molar ratio of 0.8. As shown in Fig. 7A, SmSLP had a clear inhibitory effect on microtubule formation. In addition, SmSLP added to preassembled microtubules at a molar ratio of 0.5 caused a marked depolymerization (Fig. 7B). Purity of SmSLP used in these experiments could be estimated to be better than 97.5%, as shown by the absence of contaminants in a PAGE run of 40x the concentration, giving a detectable band of SmSLP (Fig. 7C).

**DISCUSSION**

We have described a protein of *S. mansoni* that is expressed in large amounts only during a very restricted period of the parasite life cycle. It can be estimated that at the early schistosomulum stage, SmSLP may represent a fraction of total proteins on the order of about 1%, whereas the protein becomes undetectable, by Western blots of total parasites, in mammalian stages older than a couple of days. The timing of SmSLP appearance is equally striking, because the protein is totally...
absent during the first 27 days of intramolluscan development—comprising sporocyst formation and active asexual multiplication of cercarial precursors—and it only appears to be first synthesized when it is time for the sporocyst to break open and release mature cercariae. The natural infective life span of cercariae is just a few hours, during which the next dramatic event occurs, i.e., penetration of the mammalian skin with passage from freshwater to the isotonic and isothermic environment of the host. Thus, it seems logical to assume that SmSLP has a function in the emergence of the larva from the snail into the water, the penetration from the water into the mammalian host, or both. Because no SmSLP is detectable at the egg/miracidium stage, it appears that the passage from the water and then to the mammalian environment. These events are accompanied by dramatic changes in the rate of cell division as well as in the overall size and shape of the organism. Although the functioning of signal transduction in these complex chains of events is still totally obscure, SmSLP is likely to belong to the set of schistosome regulatory molecules that are beginning to be identified as essential actors in the adventurous life of the parasite (22–25).

Acknowledgments—We are grateful to Rolando Moroni for competent maintenance of the schistosome life cycle and to Adalberto Di Luzio for technical assistance. We are also indebted to Prof. Donatella Barra (University of Rome La Sapienza) for protein sequencing.

REFERENCES

1. Sobel, A. (1991) Trends. Biochem. Sci. 16, 301–305
2. Belmont, L. D., and Mitchison, T. J. (1996) Cell 84, 623–631
3. Flurkey, W. H., Prentice, D. A., Fox, M. T., and Hughes, J. P. (1993) Biochem. Biophys. Res. Commun. 196, 589–595
4. Cioli, D. (1976) Int. J. Parasitol. 6, 349–354
5. Ramalho-Pinto, F. J., Gazzinelli, G., Hewells, R. E., Mota-Santos, T. A., and Pellegrino, J. (1974) Exp. Parasitol. 36, 360–372
6. Liberti, P., Festucci, A., Ruppel, A. Gigaite, S., and Cioli, D. (1986) Mol. Biochem. Parasitol. 18, 55–67
7. Laemmli, U. K. (1970) Nature 227, 680–685
8. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
9. Gaskin, F., Cantor, C. R., and Shelskani, M. L. (1974) J. Mol. Biol. 89, 737–755
10. Kusel, J. R., Gazzinelli, G., Colley, D. G., de Souza, C. P., and Cordeiro, M. N. (1984) Parasitology 89, 483–494
11. Pearson, W. R. (1996) Methods Enzymol. 266, 227–258
12. Sobel, A., Bouterin, M. C., Beretta, L., Chniewieisz, H., Doye, V., and Peyro-Saint-Paul, H. (1989) J. Biol. Chem. 264, 3765–3772
13. Maucler, A., Moreau, J., Mechain, M., and Sobel, A. (1993) J. Biol. Chem. 268, 16420–16429
14. Schubart, U. K., Alago, W. Jr., and Dannef, A. (1987) J. Biol. Chem. 262, 11871–11877
15. Hailat, N., Strahler, J., Melhem, R., Zhu, X. X., Brodeur, G., Seeger, R. C., Reynolds, C. P., Hanash, S. (1990) Oncogene 5, 1615–1618
16. Cooper, H. L., Pulidner, R., McDuffie, E., and Braverman, R. (1991) J. Immunol. 146, 3689–3696
17. Stein, R., Mori, N., Matthews, K., Lo, L. C., and Anderson, D. J. (1988) Neuron 1, 463–476
18. Okazaki, T., Yashida, B. N., Arrakab, K. B., Wang, H., Wueneschel, C. W., Jenkins, N. A., Copeland, N. G., Anderson, D. J., and Mori, N. (1993) Genomics 18, 360–373
19. Schubart, U. K., Banerjee, M. D., and Eng, J. (1989) DNA 8, 389–398

2 C. Valle, unpublished data.
20. Di Paolo, G., Lutjens, R., Pellier, V., Stimpson, S. A., Beuchat, M. H., Catsicas, S., and Grenningloh, G. (1997) *J. Biol. Chem.* **272**, 5175–5182
21. Larsson, N., Marklund, U., Grdin, H. M., Brattsand, G., and Gullberg, M. (1997) *Mol. Cell. Biol.* **17**, 5530–5539
22. Horwitz, S. B., Shen, H. J., He, L., Dittmar, P., Neef, R., Chen, J., and Schubart, U. K. (1997) *J. Biol. Chem.* **272**, 8129–8132
23. Schussler, P., Grevelding, C. G., and Kunz, W. (1997) *Parasitology* **115**, 629–634
24. Davies, S. J., Shoemaker, C. B., and Pearce, R. J. (1998) *J. Biol. Chem.* **273**, 11234–11240
25. Osman, A., Niles, E. G., and LeVerde, P. T. (1999) *Mol. Biochem. Parasitol.* **100**, 27–41