Expression and immunolocalization of the 14-3-3 protein of *Schistosoma mansoni*

D. SCHECHTMAN[1], R. WINNEN[2], R. TARRAB-HAZDAI[1], D. RAM[1], V. SHINDER[1], C. G. GREVELDING[2], W. KUNZ[2] *and* R. ARNON[1]*

[1] *Department of Immunology, Weizmann Institute of Science, Rehovot, Israel*
[2] *Institute for Genetics, Heinrich-Heine University, Duesseldorf, Germany*

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SUMMARY

The 14-3-3 protein is a key player in signal transduction processes in various species. We have previously cloned and expressed the 14-3-3 of *Schistosoma mansoni*. Using the purified protein we have now raised antibodies against it. A highly specific, affinity-purified antibody preparation was employed for the localization of the 14-3-3 protein in the parasite, by both immunohistochemistry and immunoelectron microscopy. The results demonstrate wide distribution of this protein. It was observed in the female excretory system, the nephridia as well as in the genital systems of both sexes, namely in the vitelline gland of female and in the testis of the male. It is also present in the parenchyma and muscle of both male and female worms. Immunoelectron microscopy demonstrated the presence of immunogold-labelled protein in the tegument, subtegument, muscle, parenchyma and in the female reproductive system, in both the cytoplasm and nucleus of vitelline cells, and oocytes. The possible role of the 14-3-3 protein in the genital organs is discussed.

Key words: 14-3-3 protein, immunolocalization, *Schistosoma mansoni*, signal transduction.

INTRODUCTION

Schistosomiasis is an endemic disease in over 70 countries affecting more than 200 million people (Savioli *et al*. 1997). The causative agent is a trematode with a complex life-cycle, involving 2 hosts, human and a fresh water snail (Warren & Bowers, 1983). Upon penetration of the human skin, the parasite larvae, the cercariae, lose their tails, discharge the contents of the pre- and post-acetabular glands (Marikovsky *et al*. 1990), shed their glycocalyx, become water intolerant and are rendered resistant to complement-mediated lysis (Stirewalt, 1974). The trilaminar surface membrane transforms into an heptalaminar membrane limiting a syncytial cell layer called the tegument (Hockley & McLaren, 1973). Signal transduction processes are involved in the transformation of the cercariae to schistosomula; phosphorylation of specific proteins is observed as well as a redistribution of protein kinase C which translocates to the surface membrane (Wiest *et al*. 1992).

Recently we have cloned and expressed a *Schistosoma mansoni* 14-3-3 protein (Sma1 14-3-3), a key player in signal transduction processes in various species. As of now at least 3 isoforms have been identified in schistosomes: Sma1 14-3-3 isolated from *S. mansoni* described in our laboratory (Schechtman *et al*. 1995) and used in this paper, Sma2 14-3-3 documented in the EMBL gene data base accession number L7844, and Sj 14-3-3-2 isolated from *S. japonicum* (Zhang *et al*. 1999). Amino acid sequence identity between Sma1 and Sj 14-3-3 is 69%. The similarity of schistosome Sma1 14-3-3 with that of other species also range from 54% (with barley) to 65% with (*Drosophila melanogaster*) (Schechtman *et al*. 1995); 14-3-3 is an acidic protein with a relative molecular mass of 29–30 kDa which may form mono- and heterodimers (Boston *et al*. 1982). In mammals there are 7–8 isoforms, which have been isolated according to their elution position after HPLC (Martin *et al*. 1993; Toker *et al*. 1992). Five of these different isoforms are derived from different genes and are not due to alternative splicing, while other isoforms derive from post-translational modifications (Ichimura *et al*. 1992).

In mammals, the 14-3-3 family of proteins is preferentially localized in neurons (Boston *et al*. 1982; Brandt *et al*. 1992) but other tissue specific isoforms have been found (Ichimura *et al*. 1991; Swanson, Dhar & Joshi, 1993), and are present at low levels in the brain. These include an isoform found in T-cells (t) (Nielsen, 1991), and another found in epithelial cells (stratifin, or HME-1 (q)). This latter isoform is down-regulated in neoplastic epithelial cells and could probably be used as a marker for detection of neoplasia (Prasad *et al*. 1992).

The 14-3-3 protein plays a central role in the MAP kinase signalling cascade (activating and inhibiting various enzymes involved in this cascade) (Ferl,

* Corresponding author: Department of Immunology, Weizmann Institute of Science, Rehovot, 76100, Israel. Tel: +972 8 9344017. Fax: +972 8 9469712. E-mail: ruth.arnon@weizmann.ac.il

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1996). Other members of the MAP kinase signalling cascade have been identified in schistosomes (Schussler, Grevelding & Kunz, 1997) and apparently are developmentally regulated during transformation from cercaria to schistosomulum, and then to adult worm (Wiest *et al*. 1992; McGonigle *et al*. 2001). However, the biological functions of the 14-3-3 proteins, in schistosomes have not been described as of yet.

Studies on the characterization and functions of 14-3-3 in schistosomes present some difficulties due to the complexity of the life-cycle, lack of parasite cell lines and because mutants and other genetic tools are not yet available. Nonetheless, it is possible to approach this issue by determining cellular and subcellular localization of the protein which may provide clues to its function. Here, we report the preparation of antibodies to Sma1 14-3-3 and application of these antibodies to localize the protein in *S. mansoni* by immunohistochemistry and immunoelectron microscopy.

MATERIALS AND METHODS

Parasites

A Puerto Rican strain of *S. mansoni* was maintained in outbred CD1 mice and *Biomphalaria glabrata* snails. Cercariae were collected from the infected snails after a 90 min shedding period in bright light. Adult worms (Aw), were obtained by liver perfusion from chronically infected mice 6–8 weeks post-infection (Smithers & Terry, 1965). Perfusion of the mice was performed in RPMI medium, supplemented with 50 u/ml heparin (Sigma Chemical Co. St Louis, Missouri).

Synthesis of free 14-3-3 protein

The cDNA encoding Sma1 14-3-3 protein in the KS plasmid (Schechtman *et al*. 1995) was amplified by PCR using as a 5′ an oligonucleotide containing the *Nco*I restriction site for the 5′-GGCCATGG-CTACGTCGTGGG-3′ and as 3′ primer an oligonucleotide containing the *Bam*H1 site (5′-CA-ACTTTTACTGTCGATCCTAGGGG-3′). The PCR product was cleaved and cloned in the pET8c expression vector (Studier & Moffatt, 1986), which in turn was expressed in HB101 bacteria (Schechtman *et al*., manuscript in preparation).

Preparation of antibodies

To obtain antibodies against the 14-3-3 protein, free recombinant 14-3-3 protein was used for immunization. New Zealand rabbits were immunized subcutaneously with 100 μg protein once with complete Freund's adjuvant and twice with incomplete adjuvant, at 3-week intervals. The rabbits were bled 2 weeks after the second and third immunizations, and the antibody titres in the sera were tested by ELISA and Western blots. Antibodies against 14-3-3 were affinity purified by successive passage through 3 columns, as follows: firstly, a column comprising lysate of HB101 bacteria coupled to Sepharose; secondly, through GST-Sepharose column (both proteins were coupled using cyanogen bromide (Wilchek, Miron & Kohn, 1984) and thirdly, passage through 14-3-3-GST Affigel 10 column (Bio-Rad), (Smith & Johnson, 1988). Coupling of 14-3-3-GST to Affigel was performed in PBS following manufacturer's instructions. Antibodies were eluted with 0·1 M acetic acid and then with 0·14 M NH_3, and dialysed against PBS.

Radioactive labelling of proteins

The proteins (recombinant or goat anti-rabbit purified antibodies) were quantitated according to the method of Bradford (1976) and radio-iodinated with ^{125}I by the Chloramine-T method as described by Hunter & Greenwood (1962).

Western blot analysis

Western blotting of electrophoresed proteins was performed according to the method described by Towbin, Staehelin & Gordon (1979). Briefly, proteins were electroblotted from SDS–PAGE to nitrocellulose in a 50 mM Tris-glycine buffer, pH 8·3 at 40 mAmps overnight at room temperature. After protein transfer, blots were either stained with Ponceau S and destained with water or used for antibody detection. For antibody detection, blots were first blocked for 2 h at room temperature in a blocking solution (PBS 0·05 % Tween 20 containing 10 % low fat milk), to avoid non-specific binding. Filters were then incubated with affinity-purified anti-14-3-3 (0·5 mg/ml) antibody in PBS either overnight at 4 °C or for 2 h at room temperature, and washed 3 times for 15 min in PBS 0·05 % Tween 20 at room temperature. Addition of the second antibody, ^{125}I-purified goat anti-rabbit Ig (3–5 × 10^5 cpm/ml) solution, was followed by incubation for 2 h at room temperature. Following extensive washing as above the ^{125}I was detected by autoradiography.

Immunohistochemistry

Adult parasites were fixed in Bouin's solution (picric acid/acetic acid/formaldehyde 15:1:5) and embedded in paraplast. Sections for immunohistology were deparaffinized and then hydrated in a descending ethanol row. Non-specific antibody binding sites were blocked by incubation with 5 % BSA (in PBS: 150 mM NaCl, 10 mM NaH_2PO_4, pH 7·2) for 30 min at room temperature. The sections were incubated overnight at 4 °C with purified anti-14-3-3 antibody (50 ng/ml in PBS) or normal purified rabbit serum.

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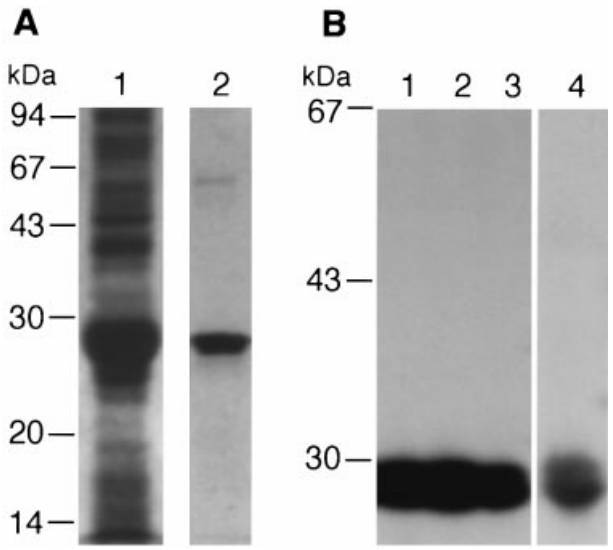

Fig. 1. Purified recombinant Sma1 14-3-3 protein and anti-Sma1 14-3-3 antibody. (A) Pattern of polyacrylamide gel electrophoresis (SDS–PAGE) of: total lysate of bacteria expressing 14-3-3 (lane 1); protein purified from the bacterial pellet solubilized with 1% SDS and chromatographed on DE–52 (lane 2), Coomassie blue staining. (B) Western blot of parasite lysates developed with affinity-purified antibodies to Sma1 14-3-3 protein followed by ^{125}I-labelled secondary antibody and autoradiography. Lysates (30 μg/slot) are of: cercariae (lane 1), 6 h mechanically transformed schistosomula (lane 2), adult worm (lane 3). Lane 4 depicts the blot of the purified free recombinant Sma1 14-3-3. Molecular weight markers (kDa) are indicated.

The slides were then washed in PBS, incubated for 2 h at room temperature with alkaline phosphatase-conjugated goat anti-rabbit IgG (1:2500 in PBS, Dianova). Detection was performed using naphthol-AS-MX phosphate and Fast Red TR (Sigma) as substrate (Finken *et al*. 1994).

Immunoelectron microscopy

Adult worms were fixed in 3% paraformaldehyde and 1% glutaraldehyde in 0·1 M cacodylate buffer, pH 7·4, initially for 1 h at room temperature, then cut into 3 mm pieces and further immersed in the same fixative for 48 h at 4 °C. The parasites were then treated with 1% OsO_4, dehydrated in a series of graded alcohol solutions, and infiltrated with Epon resin at 60 °C. Ultrathin sections (70–90 nm) were cut on a Rechter ultramicrotome. The sections were collected on nickel 200 mesh grids coated with Formvar. Grids were floated on droplets of the desired solutions treated with fresh 1% meta-periodate for 30–60 min, washed in PBS and blocked in a solution which contained 3% normal goat serum, 0·1% Tween 20, 0·1% gelatin, 1% BSA in PBS. The affinity-purified anti-14-3-3 antibodies were diluted in blocking solution (50 ng/ml) and normal rabbit immunoglobulin was used as control, and both were incubated with the grids overnight at 4 °C. The grids were then washed 6 × 5 min in PBS and placed for 30 min on a drop of goat anti-rabbit Ig conjugated to 10 or 15 nm gold particles Auro-probe EM (Janssen, Belgium), diluted 1:20 in PBS. This was followed by 3 washes in PBS and then distilled water. Finally, the grids were stained with 2% uranyl acetate in 50% alcohol for 5 min in the dark and with lead solution for 5 min. Alternatively, cryo-sections of cercariae were fixed in a mixture of 3% paraformaldehyde and 0·1% glutaraldehyde in 0·1 M cacodylate buffer, pH 7·4, overnight at 4 °C. Fixed parasites were infiltrated with 2·3 M sucrose in PBS at 4 °C overnight and frozen in liquid nitrogen. Sections (70–90 nm) were cut with a Reichert-Jung Ultracut E equipped with a FC4D Cryo unit at −100 °C using glass knives. The sections were collected on grids, incubated with antibodies and embedded in 2% uranyl-acetate with methyl-cellulose. Electron micrographs were obtained using a Phillips EM410 electron microscope at 100 kV.

RESULTS

14-3-3 protein expression and purification

In order to characterize the *S. mansoni* 14-3-3 protein it was necessary to express the Sma1 14-3-3 cDNA. We expressed and purified the 14-3-3 protein both as a fusion protein to GST and as a free protein. The cDNA insert of clone 14-3-3 was amplified by PCR using oligonucleotides with added restriction sites required for cloning into the appropriate expression vector. The cDNA insert was cloned into the *Bam*HI and *Eco*RI sites of the pGEX plasmid in order to produce a fusion protein consisting of 14-3-3 coupled to *S. japonicum* GST (to be published). The cDNA 14-3-3 insert was also amplified with other oligonucleotides for cloning into the *Nco*I and *Bam*HI sites of pET8C expression vector. The free 14-3-3 protein synthesized by the pET8C vector was purified by DE-52 ion-exchange chromatography to 95% purity (Fig. 1A, lane 2). This purified preparation was used for the induction of antibodies against the 14-3-3 protein.

Antibody preparation and biochemical characterization of 14-3-3 protein

Despite the conserved nature of the 14-3-3 protein, high antibody titres were obtained in rabbits immunized with the purified 14-3-3 protein. Sera from immunized rabbits were affinity purified by consecutive passages on 3 columns: HB101 bacteria bound to Sepharose, GST bound to Sepharose and 14-3-3-GST bound to Affigel 10 (as described in the Materials and Methods section). The resulting anti-Sma1 14-3-3 antibodies, were purified by affinity chromatography and used for immunocharacterization. The specificity of these antibodies was checked by reacting them with the parasite lysate on

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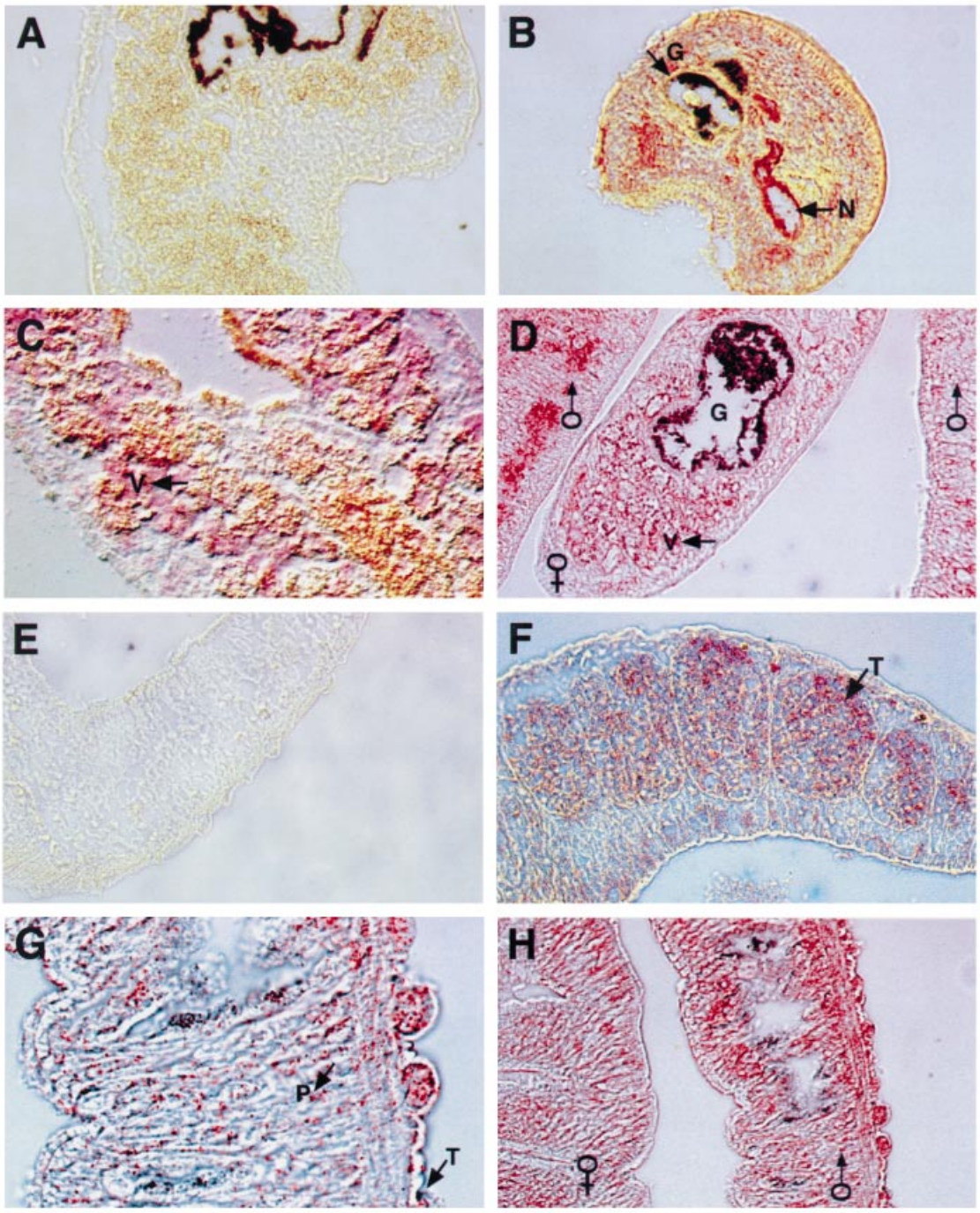

Fig. 2. Immunohistochemistry of adult worms. Sections were developed with affinity-purified anti-14-3-3 antibody followed by alkaline phosphatase conjugates of the secondary antibody (goat anti-rabbit Ig). (A) Female negative control; (B) female gut (G) and nephridia (N); (C), female vitellaria (V); (D), female in the gynaecophoric canal, showing mainly gut (G); and vitellaria (V); surrounded by the male; (E), male control; (F), male testes (T); (G), male tegument and parenchyma; (H), male and female tegument and parenchyma.

a Western Blot. A clear recognition of only 1 band (Fig. 1B) from parasite lysates was observed, indicating the specificity and purity of the antibodies. Moreover, as seen in Fig. 1B, the antibodies recognized both the recombinant free 14-3-3 protein (lane 4), as well as a band of approximately 28 kDa (the expected molecular weight for 14-3-3 proteins), and the native 14-3-3 protein in cercaria, schistosomula and adult worms (lanes 1–3).

Localization of the 14-3-3 protein in adult Schistosoma mansoni *worms by immunohistochemistry*

Localization of the 14-3-3 in the parasite was performed by immunohistochemical analysis, using the affinity-purified anti-14-3-3 antibodies and staining with alkaline phosphatase antibody conjugates. Positive staining of 14-3-3 protein was

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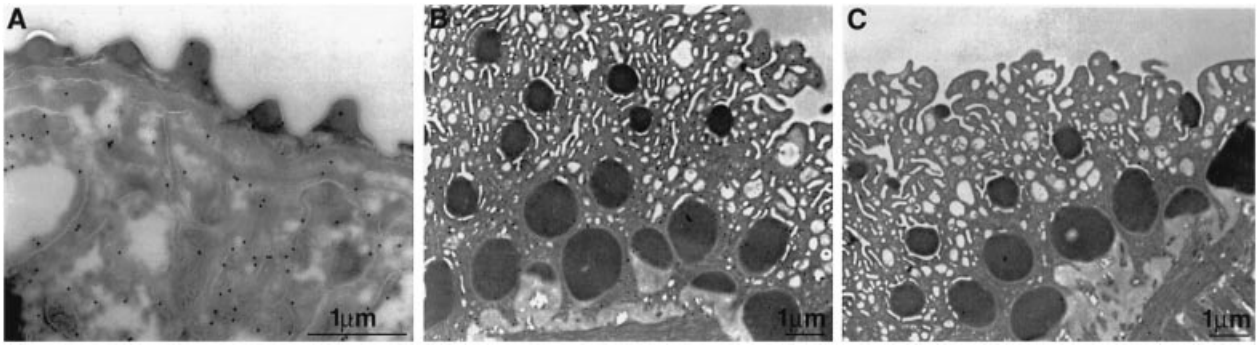

Fig. 3. Immunogold labelling of Sma1 14-3-3 in the tegument and subtegument. Electron micrographs of cercariae showing mainly subtegument labelling (A); tegument and subtegument of adult worms reacted with purified antibodies to Sma1 14-3-3 protein (B) and the negative control reacted with normal immunoglobulin (C).

observed with particular intensity in the epithelium surrounding the female excretory system, the nephridia (Fig. 2B). These organs, in schistosomes, are suggested to function as protein excretory organs (Finken *et al*. 1994; Finken-Eigen & Kunz, 1997). Positive staining of 14-3-3 protein was also observed in the genital systems of both sexes, namely in the vitelline gland, in the parenchyma and the muscles of both male and female worms (Fig. 2C, G and H). There was a particular concentration of label in the tubercles of males (Fig. 2F), whereas the tegument (Fig. 2G), as well as the surrounding epithelium of the gut, the gastrodermis, were not positively stained. No immune reactivity was found in control sections using normal rabbit immunoglobulin with either female (Fig. 2A) or male (Fig. 2E) worms.

Localization of the 14-3-3 protein in cercaria and adult worms by immunoelectron microscopy

Immunolabelling, using gold-labelled secondary antibodies, was demonstrated in both sexes of schistosomes after interaction with purified anti-14-3-3 antibodies, with results quite similar to those obtained by light microscopy. In cercariae, gold particles were localized in the subtegument (Fig. 3A), in muscle and in the parenchyma (not shown). In adult worms the tegument had the same basic organization in males and females. It is composed of 2 lipid bilayers and it is deeply invaginated. Staining in the tegument was slight and equally distributed (Fig. 3B). The tegument contained membraneous bodies (Hockley & McLaren, 1973) that were free of labelling. Controls with normal rabbit immunoglobulin did not show any staining (Fig. 3C).

Immunogold labelling was also found in the female reproductive system. We clearly detected staining in both the cytoplasm and the nuclei of vitelline cells (Fig. 4A). Mature vitelline cells were easily recognized by their large vacuoles that contained the precursor proteins for the egg-shell (Köster *et al*. 1988). Right of the centre in Fig. 4A is a younger vitelline cell whose nucleus and cytoplasm are strongly labelled. Intense labelling was also found in the parenchyma (right in Fig. 4A, arrowhead). Fig. 4B is a close-up of a female germ cell that probably is an oocyte, showing some labelling in the nucleus and in the big nucleolus. Fig. 4C is a close up of 4 egg-shell precursor protein storage vesicles in the cytoplasm of a mature vitellocyte, showing gold particles in these vesicles. Controls of normal rabbit immunoglobulin showed almost no labelling of this region (not shown). Intense labelling of 14-3-3 protein was also found in the parenchyma, a region of intense protein synthesis (Fig. 5A), and delicate labelling of the striated muscles (Fig. 5B).

DISCUSSION

The 14-3-3 proteins (various isoforms) of *S. mansoni*, as those of other organisms, seem to be essential for the parasite viability. Thus, for example, *Caenorhabditis elegans* knockouts of 14-3-3 protein are not viable. Little is known about the function of 14-3-3 protein in schistosomes, yet we have found indirect evidence for its interaction with different proteins, including itself, by forming homo- and hetero-oligomers (Boston *et al*. 1982). Furthermore, these proteins have a putative phosphorylation site which undergoes *in vitro* phosphorylation, which may be a mechanism of regulation of function of the 14-3-3 proteins. The localization of the 14-3-3 protein in *S. mansoni* could help elucidate the function. It is of interest that even though the mRNA is absent in the short-lived cercariae, the protein itself is present at all of the life-cycle stages of the parasite (Schechtman *et al*. 1995). Its presence in the tegument could indicate a role at the interface between the host and parasite or between male and female worms. The nuclear localization of 14-3-3 proteins could implicate other putative functions: in schistosomes, as in other systems, they can be essential in the signal transduction pathways responsible for interaction between proteins (Ferl, 1996).

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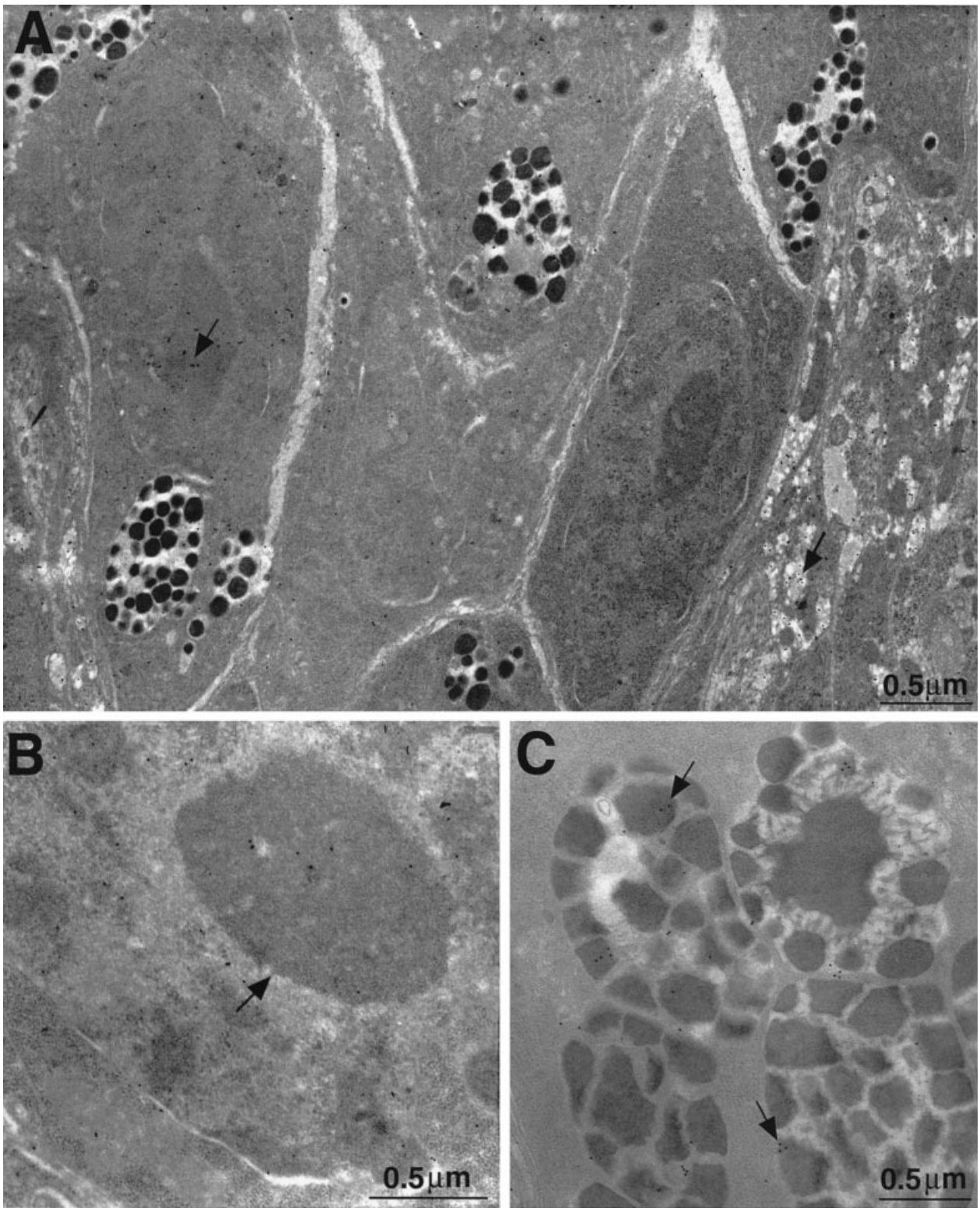

Fig. 4. Immunogold labelling in the female reproductive system Stage 4 vitellocytes labelled both in the cytoplasm and in the nucleus (arrow); stronger labelling was found in the parenchyma at the right (arrowhead) (A). Maturing female germ cell nucleus that probably is an oocyte nucleus, showing labelling in the nucleus and in the nucleolus (arrow) (B). Four egg-shell precursor protein storage vesicles in the cytoplasm of a mature (stage 4) vitellocyte (C).

Many functions have been attributed to the 14-3-3 family of proteins, some of which are associated with kinases and with other members of the MAP kinase signalling cascade (Braselmann & McCormick, 1995). The 14-3-3 protein has also been associated with inhibition (Toker *et al*. 1990) or activation of calcium and phospholipid-dependent protein kinase C (Acs *et al*. 1995), as activator of Raf (Fantl *et al*. 1994), as activator of tyrosine and tryptophan hydroxylases (Ichimura *et al*. 1995), as a

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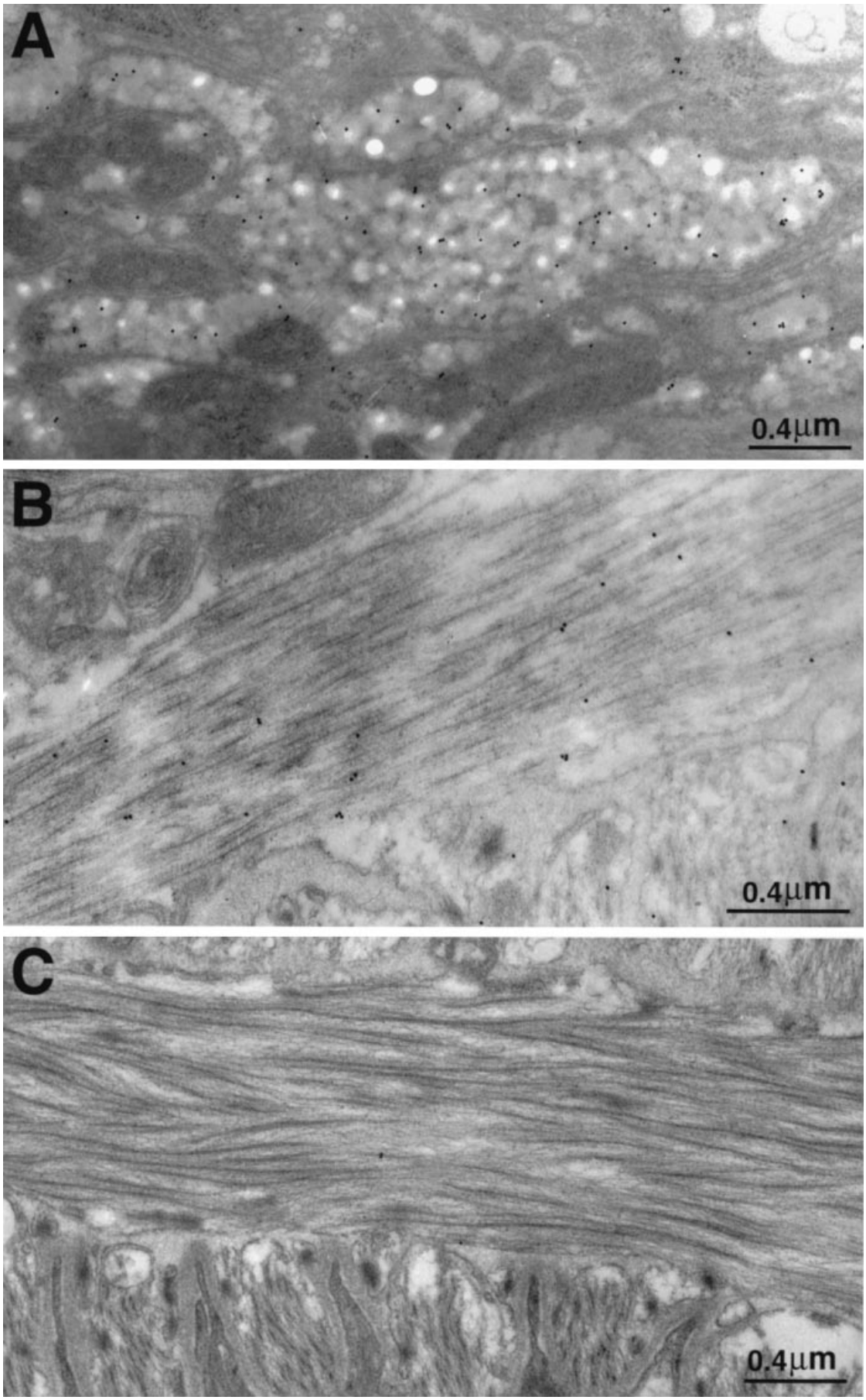

Fig. 5. Immunogold labelling in the parenchyma and muscle of adult worms. Gold particles showing the localization of Sma1 14-3-3 in the parenchyma (A), the muscle (B) and negative control of muscle developed with normal immunoglobulin (C).

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stimulator or calcium-dependent exocytosis in adrenal chromaffin cells, or as a chaperone (Morgan & Burgoyne, 1992*a*, *b*). A peptide derived from 14-3-3, homologous to annexins, was shown to inhibit translocation of activated PKC from cytosol to cytoskeleton by receptors of activated kinase C (RACKS) (Mochly *et al*. 1991).

The antibodies used for the localization studies were prepared by immunization with a highly purified (> 95 % pure) preparation of Sma1 14-3-3. The antibodies themselves were purified by 3-step affinity chromatography and they recognize specifically the 14-3-3 in all life-cycle stages of *S. mansoni*, as well as the recombinant 14-3-3 protein. Until now three 14-3-3 isoforms have been found in schistosomes and the anti-14-3-3 antibodies employed in this study recognizes all of them (not shown).

The 14-3-3 protein is ubiquitous in its nature and has a key role in many signal transduction processes and therefore we expected to find it dispersed throughout the whole parasite. Indeed, we demonstrated that the 14-3-3 protein was present in many organs of the parasite, although it was more concentrated in specific areas such as, subtegument, parenchyma, muscle, reproductive organs and excretory system. The 14-3-3 is concentrated in the subtegument of adult worms, which would be expected for its participation in the MAP kinase signalling cascade. This cascade is activated by external signals such as stress, or activation of growth factor receptors (Seger & Krebs, 1995). Therefore, it is conceivable that 14-3-3 is located in the tegument of the parasite, and is involved in transfer of signals from host environment to the parasite. Signalling between the two sexes has been shown to activate the MAP kinase signalling cascade (Schussler *et al*. 1997, 1998), suggesting that a male stimulus induces vitelline cell proliferation in female schistosomes propagated via a signal transduction cascade.

The expression of the 14-3-3 protein in genital organs such as the female vitelline glands and oocytes where protein is detected both in the nucleus and the cytoplasm and male testis, indicates a role of this protein in sexual maturation of both genders. Vitelline cells are easily recognized by their large vacuoles that contain the precursor proteins of the egg-shell (Köster *et al*. 1988). Indeed in sexual maturation many signal transduction processes are involved. The staining of 14-3-3 in the nephridia is of particular interest, since this part in the schistosome is suggested to function as protein excretory organ. It is conceivable that the molecular processes occurring in the ER lumen of the nephridial wall cells (Finken *et al*. 1994; Finken-Eigen & Kunz, 1997) destined to sort and structure proteins for their task to become secreted, require the activity of signalling molecules for this regulation. The detection of 14-3-3 in nuclei of parenchyma and oocytes is very interesting. This nuclear localization has also been found in plants where specific 14-3-3 isoforms have been localized to the nucleus (Bihn *et al*. 1997). As suggested by Bihn *et al*. (1977) the 14-3-3 protein monomers could be small enough to enter the nuclear pores so that the 14-3-3 proteins could 'piggyback' on other proteins that are localized to the nucleus (consistent with the 14-3-3 properties of binding to other proteins). One other possibility could be simple diffusion (Bihn *et al*. 1997).

In animals so far, no work has associated the 14-3-3 with transcription factors, although in yeast there may be a potential nuclear role since 14-3-3 proteins serve as a checkpoint regulation of damaged DNA prior to mitosis (Ford *et al*. 1994). In schistosomes there could also be a putative role for 14-3-3 related to processes involved with DNA, as suggested by its nuclear localization. The localization of the 14-3-3 protein on muscle of adult worms is also interesting. As homologue of the epidermal growth factor receptor, SER, another member of the MAP kinase signal transduction cascade, has also been localized at the schistosome muscle (Ramachandran, Skelly & Shoemaker, 1996). The role that either the 14-3-3 protein or SER may play in the muscle tissue is in accord with the finding that EGF has been shown to induce mitogenic responses and to regulate contractile responses in muscle (Ramachandran, Skelly & Shoemaker, 1996; McGonigle *et al*. 2001).

Recently, the molecular cloning and characterization of *S. japonicum* 14-3-3 protein (Sj14-3-3) was published (Zhang *et al*. 1999). The deduced protein sequence of 254 amino acids is 2 amino acids longer than that reported from our laboratory for Sma1 14-3-3 (Schechtman *et al*. 1995). The Sj14-3-3 has a 69 % amino acids homology with the *S. mansoni* protein and represents a high homology between these two species. It is interesting that the cloning of the Sj14-3-3 protein was obtained using an antibody derived from mice vaccinated with UV-attenuated cercaria which are capable of transferring high levels of passive immunity to the parasite (Zhang *et al*. 1999).

In conclusion, immunolocalization of the 14-3-3 protein in schistosomes, shows that this protein is relatively abundant in organs such as the genital organs of both male and female, the parenchyma, muscle, subtegument as well as the in the tegument of adult worms. This may indicate that these are sites where signal transduction is more intense, and that the 14-3-3 protein could be involved in various processes, such as interaction between sexes and sexual maturation, interaction with the host, excretion and movement.

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