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REGULAR ARTICLE

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# Identification of Reissner's fiber-like glycoproteins in two species of freshwater planarians (Tricladida), by use of specific polyclonal and monoclonal antibodies

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**Abstract** By using one polyclonal antiserum raised against bovine Reissner's fiber and seven monoclonal antibodies raised against bovine Reissner's fiber and against immunopurified bovine subcommissural organ glycoproteins, we have investigated two freshwater planarian species (*Girardia tigrina*, *Schmidtea mediterranea*) by light- and electron-microscopic immunocytochemistry. ELISA probes showed that the monoclonal antibodies recognized different, nonoverlapping, unrepeated, proteinaceous epitopes present in the same compounds of bovine Reissner's fiber. Cells immunoreactive to the polyclonal and monoclonal antibodies were found in the dorsal and ventral integument of both planarian species. Labeled cuboid epidermal cells bore cilia and displayed several types of secretory granules; they were covered by a film of immunoreactive material. Studies on adjacent thin and semithin sections revealed coexistence of label in the same regions and in the same cells when two different monoclonal antibodies were used. These results indicate that a secretory substance immunologically similar to the secretion of the vertebrate subcommissural organ is present in primitive tripoblasts such as planarians, suggesting that these secretions are ancient and well conserved in phylogeny.

**Key words** Subcommissural organ · Immunocytochemistry · Integument · Invertebrates · Phylogeny · ELISA · Immunoelectron microscopy · *Girardia tigrina*, *Schmidtea mediterranea* (Platyhelminthes, Turbellaria)

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## Introduction

The subcommissural organ (SCO) of vertebrates is an ependymal brain gland located in the roof region of the third ventricle. It secretes high-molecular weight, *N*-linked, sialylated glycoproteins into the ventricular cerebrospinal fluid. This secretion condenses into a fibrous structure named Reissner's fiber (RF), which runs along the third and fourth brain ventricles and the spinal central canal (Oksche et al. 1993; Rodríguez et al. 1998). Comparative immunocytochemical studies using polyclonal antibodies and monoclonal antibodies (MAbs) against SCO or RF glycoproteins have suggested the existence of conservative and nonconservative epitopes in chordate phylogeny (Sterba et al. 1982; Rodríguez et al. 1984; Grondona et al. 1994; Pérez et al. 1995). The SCO is not the only structure secreting RF-like glycoproteins; ependymal cells in the embryonic floor plate, especially in its rostral portion where they form the so-called flexural organ (Olsson 1956), secrete glycoproteins that are recognized by antibodies against SCO glycoproteins and that condense into a true RF (Rodríguez et al. 1996; López-Avalos et al. 1997; Yulis et al. 1998; Lichtenfeld et al. 1999). On the other hand, in the cephalochordate *Branchiostoma lanceolatum*, an immunocytochemically similar material forming RF was reported to be secreted by ependymal cells of the so-called infundibular organ, located in the ventral portion of the cephalic vesicle of the neural tube (Olsson et al. 1994). The simplest RF-secreting apparatus among chordates has been described in the nervous system of the urochordate *Oikopleura dioica* (Holmberg and Olsson 1984), where a single ependymal cell (fibrinogen cell) located in the rostral tip of the neural tube secretes a very thin RF. Southern blot analysis in several members of the chordate phylum including cephalochordates and urochordates have demonstrated that the gene for SCO secretion is evolutionarily conserved, thus suggesting that this secretory product is an ancient ependymal secretion in chordates (Gobron et al. 1999).

In order to investigate the phylogenetic age of RF glycoproteins, Viehweg et al. (1998) have studied the

nervous system of an echinoderm, the sea star *Asterias rubens*, with an antiserum against bovine Reissner's substance. They described immunoreactive bipolar cells in the ectoneural part of the radial nerve cord that were considered secretory radial glia sharing a common ancestral origin with the radial glia secreting RF in chordates, namely the parenchymal cells of the SCO. The echinoderms studied by Viehweg et al. are deuterostomians, as also holds true for the chordates. Interestingly, radial glia in the same species had been depicted at the ultrastructural level (Bargmann et al. 1962). Immunoreactivity to the specific antiserum used has also been reported to occur in the nervous system of an insect embryo (Lichtenfeld et al. 1998).

The aim of the present investigation was to study whether RF-immunoreactive proteins are also present in protostomians. For this purpose, we used Platyhelminthes, a phylogenetically old phylum considered to be quite primitive among Bilateralia.

In this paper we describe the presence of immunocytochemically related RF-like glycoproteins in the epidermal cells of two freshwater planarian species, *Girardia tigrina* and *Schmidtea mediterranea* (Platyhelminthes: Turbellaria, Seriata, Tricladida; Family: Dugesiidae) using a polyclonal antibody and seven MAbs raised against bovine RF and SCO glycoproteins.

## Materials and methods

### Species

Ten specimens of the planarian *Girardia tigrina* (Girard 1850) used in this study belong to an asexual race (class A; Ribas et al. 1989). They were captured near Barcelona (Spain) and were kindly supplied by Professor R. Romero (see Bueno et al. 1997 for culture conditions). Ten specimens of the planarian *Schmidtea mediterranea* (Benazzi et al. 1975) were collected near Malaga (Spain) and kindly supplied by Professor M. Marí-Beffa. They were maintained in spring water at room temperature.

### Light-microscopic immunocytochemistry

Planarians *G. tigrina* and *S. mediterranea* were fixed by immersion in 4% paraformaldehyde in 100 mM phosphate-buffered saline (PBS), pH 7.4, for 3 h or in Bouin's fluid overnight. Then they were dehydrated, embedded in paraffin, and cut in sagittal or transverse sections (10 µm thick). In order to study the coexistence of immunolabeling in the same cells using different antibodies, two specimens of *S. mediterranea* were embedded in Araldite, and semithin sections (1 µm thick) were cut with an ultramicrotome. Resin was removed from semithin sections by incubation with sodium hydroxide 2% in 100% ethanol for 10 min and successive rinses in ethanol and PBS. Thin and semithin sections were treated with hydrogen peroxide to block endogenous peroxidase and processed for immunostaining according to Sternberger's peroxidase-antiperoxidase method (Sternberger et al. 1970). Sections were sequentially incubated in: (1) the primary antibody for 18 h at room temperature; (2) the secondary antibody: anti-rabbit IgG developed in goat for polyclonal antibody, and anti-mouse IgG developed in rabbit for MAbs (both raised in our laboratory, dilution 1:50) for 45 min; and (3) a monoclonal rabbit or mouse peroxidase-antiperoxidase (PAP, dilution 1:400; Sigma, Madrid, Spain) for 45 min. Finally 3,3′-diaminobenzidine tetrahydrochloride (DAB; Sigma, Madrid, Spain) was used as the electron donor. All antibodies were diluted in a solution composed of 100 mM TRIS-buffer, pH 7.8, containing 0.7% (w/v) lambda carrageenan as blocking agent and 0.5% (v/v) Triton X-100 (Sigma, Madrid, Spain, for both). Incubations were performed at room temperature. Bovine paraffin SCO sections were taken from our laboratory collection.

### Electron-microscopic and whole-mount immunocytochemistry

Two specimens of *G. tigrina* were fixed by immersion in PLP fixative (Hall et al. 1987) for 2 h at 4°C. Then they were washed in phosphate buffer and cryoprotected by incubation in increasing sucrose gradients. After washing with the same buffer, they were processed for whole-mount immunostaining using AFRU and 2A5, following essentially the same protocol as for sections. Antibodies were diluted in phosphate buffer containing 1% (w/v) bovine serum albumin (Sigma) as blocking agent, and the PAP complex was dissolved in phosphate buffer. Once revealed with DAB solution and washed in phosphate buffer, planarians were photographed and postfixed for 2 h at 4°C with 1% (v/v) osmium tetroxide (Sigma) dissolved in phosphate buffer. Then they were dehydrated, embedded in Araldite 502 (Sigma), and cut. Semithin sections were stained with toluidine blue. Some ultrathin sections were counterstained with uranyl acetate and lead citrate.

### Primary antibodies

#### *Polyclonal antibody*

AFRU is a rabbit antiserum raised in our laboratory against bovine Reissner's fiber dissolved with urea according to Rodríguez et al. (1984). For immunocytochemistry this antiserum was used at a dilution of 1:1000.

#### *Monoclonal antibodies*

We used three MAbs raised against bovine RF (2A5, 2A8, 1H3; Pérez et al. 1995, 1996) and four MAbs raised against bovine SCO secretory glycoproteins partially purified by immunoabsorption using 2A5 (4H6, 4A6, 5B9, 2B12). These MAbs were raised following the protocol of Pérez et al. (1996). All MAbs (anti-RF and anti-SCO) were IgG and selectively recognized the bovine SCO and RF in the immunocytochemical procedure (Fig. 1; Pérez et al. 1995). All MAbs used proceeded from hybridoma culture media purified by affinity chromatography through a protein G-sepharose column (Sigma). The working solution for immunocytochemistry was 50 µg/ml.

### Characterization of the monoclonal antibodies

Anti-RF MAbs have been characterized previously (Pérez et al. 1996). Four different ELISA probes and a control test were designed (for methodological details, see Pérez et al. 1996) to characterize anti-SCO MAbs. In general, anti-SCO MAbs behaved in ELISA as anti-RF MAbs:

1. *Antigen-mediated ELISA*. The microtiter plates were coated with RF glycoproteins and peroxidase-labeled MAbs used as binding antibodies. Anti-RF MAbs (Pérez et al. 1996) and anti-SCO MAbs (Fig. 2) selectively bound to RF glycoproteins, thus indicating that all epitopes were present in RF glycoproteins.
2. *Double-antibody sandwich ELISA*. One MAb was used as the capture antibody, RF glycoproteins as antigen, and the same or other HRP-labeled MAbs as binding antibody. In all cases, for any pair of different MAbs, a signal was always detected. This indicated that the epitopes for any pair of MAbs (either anti-RF or anti-SCO) were different and resided in the same compound of the bovine RF and, therefore, all epitopes detected

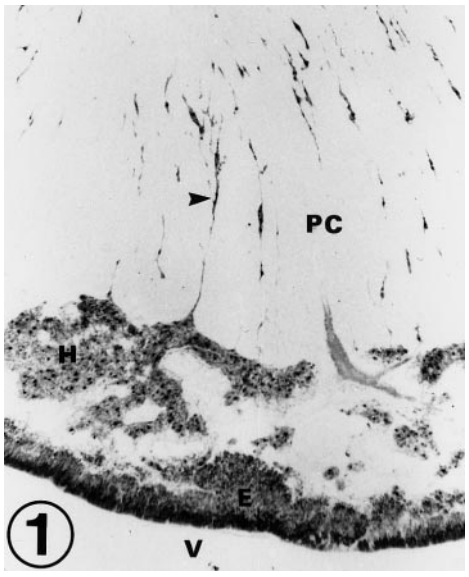



**Fig. 1** Bovine subcommissural organ (SCO) immunostained with MAb 5B9 raised against SCO glycoproteins. Note a strong staining of ependymal (*E*) and hypendymal (*H*) cells, including scattered elements (*arrowhead*) among the fibers of the posterior commissure (*PC*) (*V* third ventricle). ×100

with MAbs very probably coexisted in the same compound. When the same MAb was used, no signal was detected, thus suggesting that the epitopes are not repeated in the molecule(s) of the bovine RF.

3. *Competitive ELISA*. Wells were coated with RF glycoproteins and subsequently incubated with an increasing concentration of unlabeled MAb (competing MAb) and then with the labeled MAb. As a control of the assay, the same MAb was used as competitive and labeled MAb. Each MAb only competed with itself but not with the other MAbs, thus indicating that the epitopes did not overlap (Pérez et al. 1996; Fig. 3).
4. *ELISA on deglycosylated RF*. In order to study whether the epitopes of anti-RF MAbs reside in the proteinaceous or the glycosidic components of the bovine RF glycoproteins, we designed an antigen-mediated ELISA in which the wells of a microtiter plate were covered with deglycosylated RF glycoproteins and incubated with labeled MAbs as described previously (Pérez et al. 1996). Deglycosylation of bovine RF extracts was performed with *N*-glycosidase F, from *Flavobacterium meningosepticum* (Boehringer-Mannheim). This enzyme removes *N*-linked glycosidic chains from mammalian glycoproteins (Tarentino and Plummer 1994). An extract of 4 µg/ml of bovine RF in 0.1 M PBS was incubated with the enzyme, at a fi-

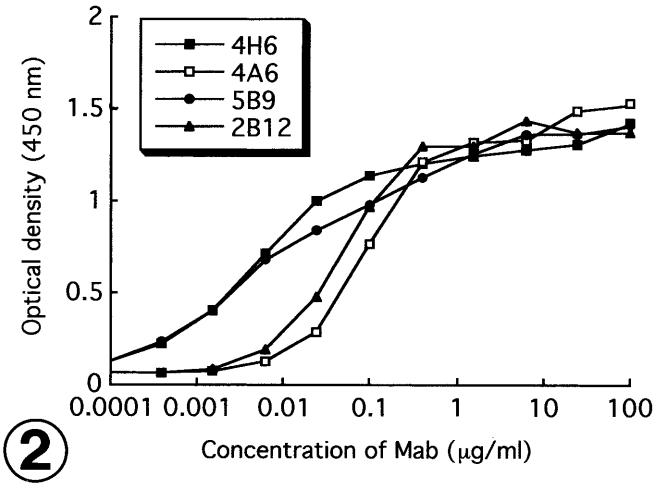



**Fig. 2** Antigen-mediated ELISA. The wells were coated with Reissner's fiber (RF) glycoproteins; MAbs 4H6, 4A6, 5B9, 2B12 were then used at increasing concentrations. All MAbs showed a high affinity for RF glycoproteins

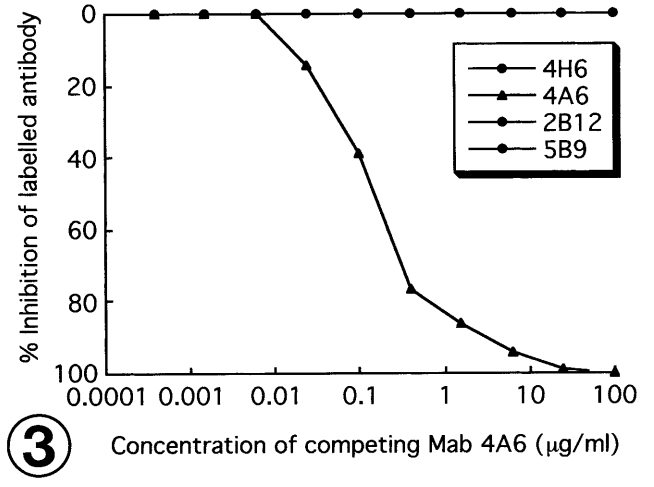



**Fig. 3** Competitive ELISA. The wells were coated with RF glycoproteins and incubated first with increasing concentrations of unlabeled MAb 4A6, and then with a fixed concentration (1 µg/ml) of labeled MAbs 4H6, 2B12, or 5B9. MAb 4A6 does not compete with the other three MAbs for binding to RF glycoproteins

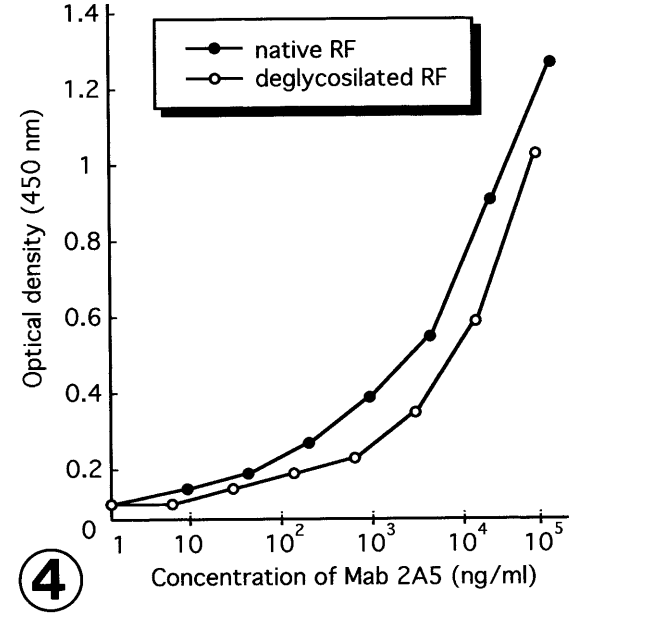



**Fig. 4** ELISA on deglycosylated RF. Antigen-mediated ELISA. The wells were coated with native or deglycosylated RF glycoproteins; MAb 2A5 raised against bovine RF was then used at increasing concentrations. There is no significant difference between the two affinity curves

nal concentration of 0.5 activity units/μg of RF glycoprotein, for 30 min at 37°C. Figure 4 shows the affinity curves obtained in direct ELISA using native and deglycosylated RF glycoprotein extract and MAb 2A5. No significant differences exist between the signals obtained with native and deglycosylated RF. Similar curves were obtained for the other anti-RF MAbs (Estivill-Torrús 1997). This result indicates that the epitopes for anti-RF MAbs reside in the proteinaceous moiety of the RF glycoprotein(s).

Control tests

For positive controls, paraffin sections of bovine SCO and RF from our collection were immunostained in the same session. For negative control, consecutive planarian sections were incubated in nonimmune rabbit serum or in mouse IgG (Sigma) as primary antibodies. AFRU and MAbs preabsorbed with a excess of bovine RF extracts were also used as primary antibodies. As controls we also used other anti-RF MAbs (described in Pérez et al. 1996). None of the negative controls rendered any labeling in the planarian epidermis. However, unspecific staining occurred in certain subepidermal glands, especially in two bilateral glands opening in the rostral region. Subepidermal pigmented cells were also visualized in some sections of *S. mediterranea*.

## Results

Polyclonal antibodies and MAbs used in the present study selectively stained the bovine SCO and RF; they also strongly stained some epidermal cells in the planarian integument. Immunoreactivity was similar in specimens fixed in Bouin's fluid or in paraformaldehyde.

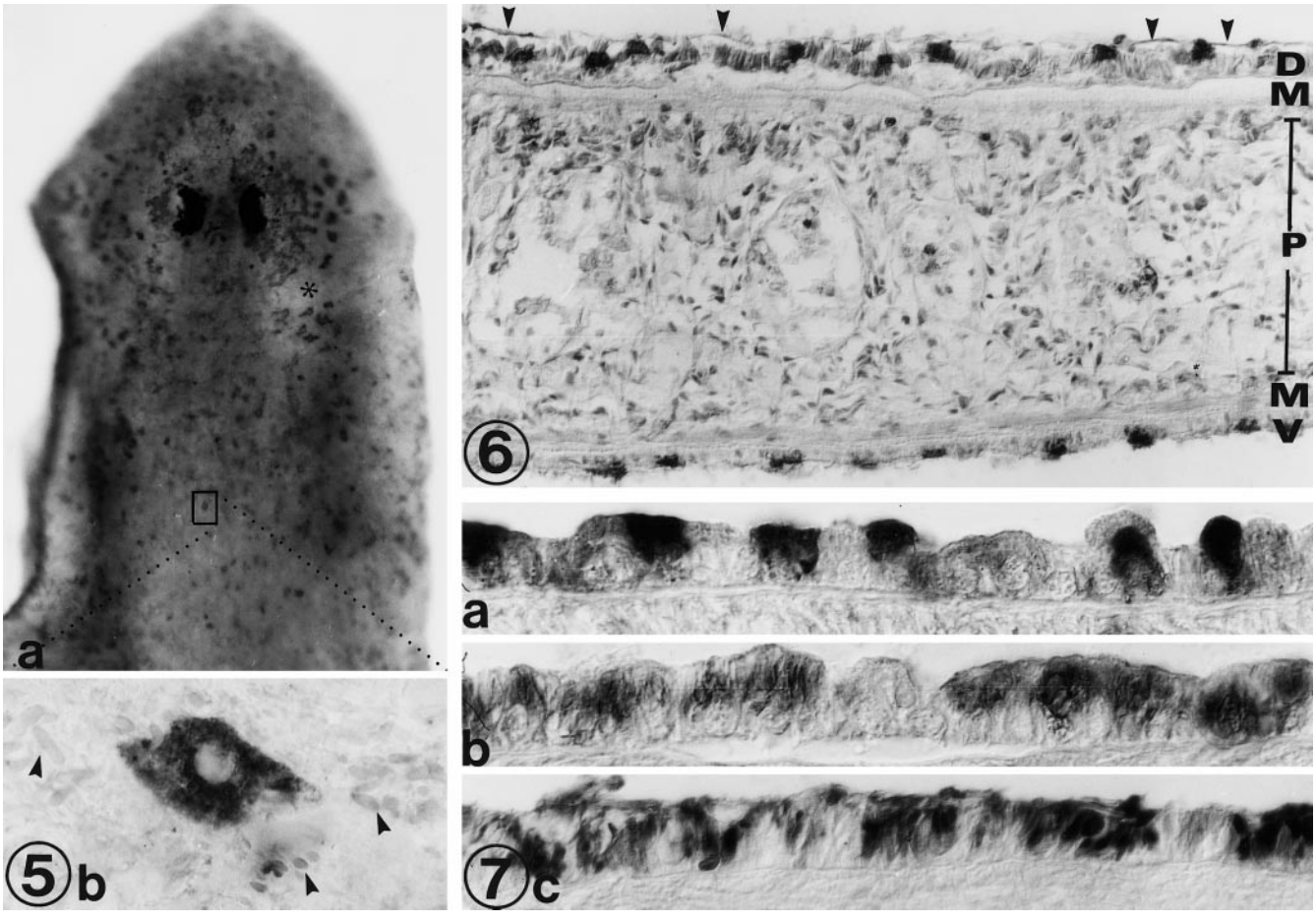



**Fig. 5** **a** Dorsal view of the rostral half of a whole-mount preparation of the planarian *Girardia tigrina* immunostained with an antiserum against bovine Reissner's fiber (AFRU). Immunopositive epidermal cells decorate the integument in a mosaic-like pattern. Numerous reactive cells clustered around the eyes. The neck region is almost devoid of immunoreactive cells (*asterisk*). The *framed* immunopositive cell is shown in detail in **b**, where immunonegative profiles of rabdhites were found elsewhere (*arrowheads*) in the integument but not inside the immunoreactive cell. **a** ×250; **b** ×1250

**Fig. 6** Overview of a sagittal section through the a planarian *Schmidtea mediterranea*, immunostained with AFRU and counterstained with hematoxylin. Immunopositive cells were found in the dorsal (*D*) as well as the ventral (*V*) epidermis. In some regions, a film of immunoreactive material is visible on the epidermal surface (*arrowheads*) (*M* muscle layer, *P* body parenchyma). ×500

**Fig. 7a–c** Details of immunoreactive cells in the dorsal epidermis of planarians. Immunostaining was with AFRU in *Schmidtea mediterranea* (**a**), the monoclonal antibody against bovine Reissner's fiber 2A5 in *Schmidtea mediterranea* (**b**), and the monoclonal antibody against bovine SCO secretory glycoproteins 5B9 in *Girardia tigrina* (**c**). ×1375

Structures appearing immunoreactive inside the planarian body (parenchyma) corresponded to unspecific staining or to pigmented cells that were also present in negative controls in which labeling of epidermal cells were completely absent. The planarian *S. mediterranea* exhibited a greater number of stained cells in comparison with *G. tigrina*, although the location and labeling pattern were quite similar.

Whole-mount immunocytochemistry of *G. tigrina* using AFRU showed many stained epidermal cells, with ir-

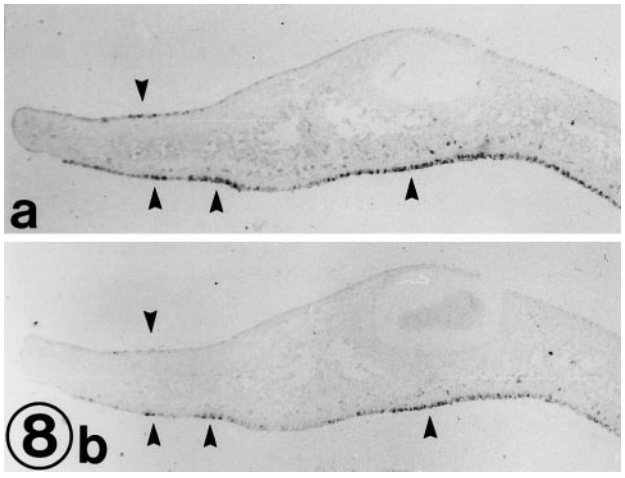



**Fig. 8a,b** Overview of two consecutive sagittal paraffin sections of *Girardia tigrina* immunostained with MAbs: **a** 2A5 (raised against RF); **b** 5B9 (raised against SCO). Immunoreactivity is very patent in the same epidermal regions for both antibodies (*arrowheads*). ×75

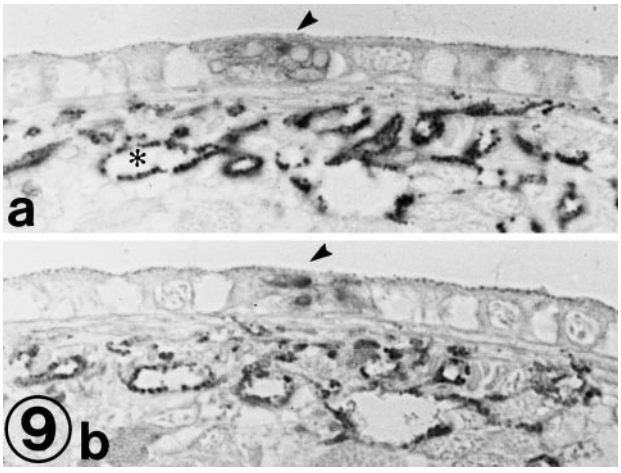
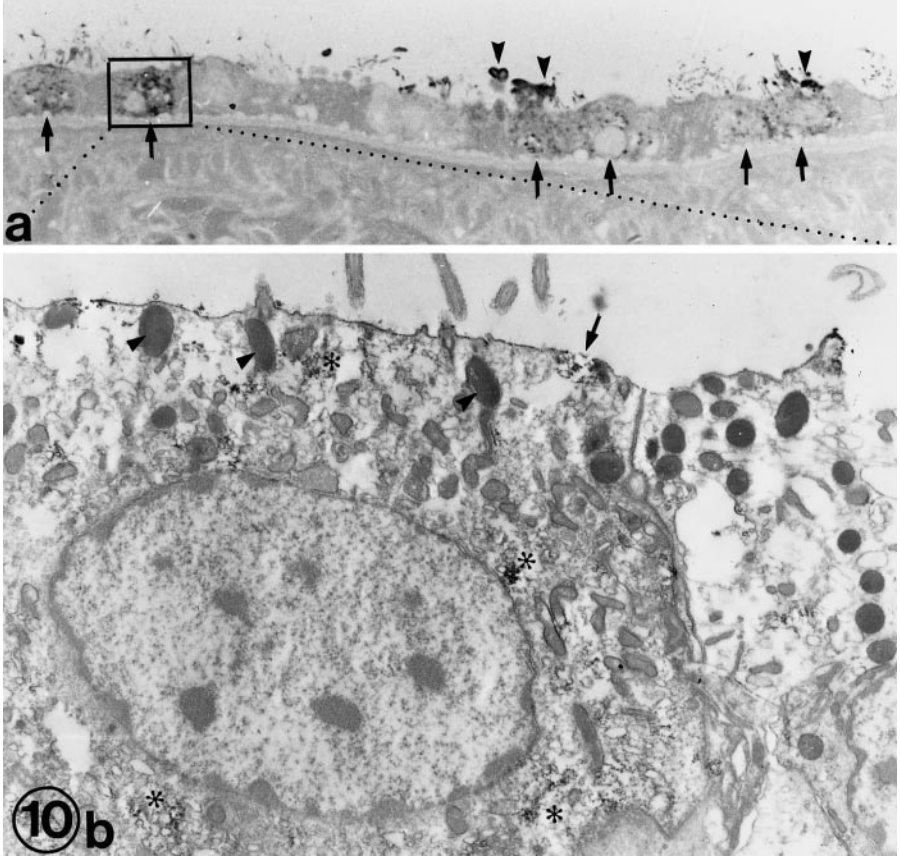



**Fig. 9a,b** Details of epidermal cells in two consecutive semithin sections of *Girardia tigrina* immunostained with MAbs: **a** 2A5 (raised against RF); **b** 1H3 (raised against SCO). Immunoreactivity is very patent in the same epidermal cell for both antibodies (*arrowheads*). Dark dots beneath the epidermis (*asterisk*) represent pigment cells. ×800

**Fig. 10** **a** Semithin section of the ventral epidermis of a planarian *Girardia tigrina* immunostained with AFRU. Immunoreactive cuboid ciliated cells are readily distinguishable (*arrows*). Labeled material is present on the surface of the cells (*arrowheads*). The *framed* cell is shown in detail in an ultrastructural overview (**b**) depicting this epidermal element of *Girardia tigrina* after immunostaining with AFRU. The label is localized around membranous structures in the cytoplasm (*asterisks*) and in vesicles that appear to open at the outer surface (*arrow*). Other dense secretory granules are devoid of label (*arrowheads*). **a** ×1000; **b** ×8500



regular polygonal shapes, randomly distributed along the entire dorsal and ventral body surfaces (Fig. 5). Positive cells appeared isolated or clustered and were especially abundant around the eyes, identified as two typical, highly pigmented rostrodorsal spots. Characteristically, the neck region was almost devoid of AFRU-immunoreactive cells. Whole-mount immunocytochemistry using the MAb 2A5 showed essentially the same staining pattern, but the intensity of the staining was lower.

Immunocytochemistry of paraffin sections revealed labeled cells in the dorsal and ventral epidermis (Figs. 6, 7). Immunoreactivity was quite intense in the apical cytoplasm of reactive cells. In some regions, an immunoreactive film was quite apparent on the surface of labeled and nonlabeled cells (Fig. 6). The study of adjacent paraffin sections stained with different MAbs showed that cells immunoreactive to two different MAbs were localized in identical regions of the planarian integument (Fig. 8). The study of consecutive semithin sections stained with different MAbs demonstrate that the labeling occurred in the same epidermal cells (Fig. 9). A coexistence study was performed with different pairs of MAbs. In semithin sections of planarians stained by use of the preembedding method, the integument appeared as an epithelial monolayer over a thick basement membrane. The ventral epidermis (Fig. 10a) was cuboidal and most cells exhibited cilia; the dorsal surface was more squamous and displayed less ciliated cells. Immunoreactive cells showed the label in their cytoplasm, and extracellular labeling was evident on the epithelial surface.

Under the electron microscope, the integument of planarians appeared as a highly secretory epithelial monolayer containing a heterogeneous population of cells. Many of them displayed characteristic giant, secretory organelles called rabdhites that were already visible by light microscopy, even in whole-mount preparations (Fig. 5b). The rabdhites were elongated and very electron-dense. In addition to rabdhites, secretory granules of different size, shape, and electron density were common in all epidermal cells. AFRU-immunoreactive cells displayed cilia, several types of medium-sized secretory granules, vesicles, and cisternae of endoplasmic reticulum; however, typical rabdhites were absent (Fig. 10b). The antibody labeled cisternae and vesicles, but the dense granules were immunonegative (Fig. 10b). Some labeled vesicles appeared to open at the outer surface of the cell (Fig. 10b). On the surface of immunopositive and immunonegative cells, a film of immunoreactive label was evident.

## Discussion

Freshwater planarians are acoelomate organisms, with a three-layered structure composed of a single layer of ciliated epidermis, a usually thin muscle layer, and a sacklike gut tube divided into three branches. The integument of planarians is characteristically highly glandular, bearing secretory epidermal cells and subepidermal mucous glands (see Rieger et al. 1991 for a review). These glands permanently release secretions that wrap the planarian body surface, acting as a protective barrier and allowing locomotion. Neither the biochemical nature of the secretions nor their exact functions are presently known.

This study shows that one polyclonal antibody and seven MAbs raised against bovine RF and SCO secretory glycoproteins bind to products present in certain epidermal cells of the two planarian species studied. The following observations indicate that the immunolabeled material(s) in planarians is(are) secretory: (1) the presence of an extracellular immunoreactive film covering immunopositive and immunonegative epidermal cells; (2) the ultrastructural features of the labeled cells, showing a highly developed secretory apparatus; and (3) the occasional finding of secretory vesicles filled with labeled material and opening at the apical plasma membrane.

As has been shown previously (Pérez et al. 1996) and in the present work (see Materials and methods), MAbs identified different, nonoverlapping, nonrepetitive, proteinaceous epitopes present in the same compounds of the bovine RF. Thus, at least seven distinct epitopes, probably belonging to the same molecule(s) of the bovine RF, are present in planarian integument. Moreover, the study of adjacent sections labeled with different pairs of MAbs have shown that these epitopes coexist in the same planarian tegumentary cell. This evidence strongly suggests a partial homology between bovine SCO-secretory glycoproteins and some secretory proteins of the planarian integument. However, immunocytochemistry alone does not imply molecular homologies, since the epitopes recognized by the antibodies could be conformational instead of sequential. In this sense, a study using antisera against native and denaturalized bovine RF demonstrated that antibodies present in AFRU recognize mostly conformational epitopes related to the presence of disulfide bonds (Nualart and Rodríguez 1996). At present, however, our results on the sequential or conformational nature of the RF epitopes recognized by MAbs are contradictory (cf. Estivill-Torrús 1997).

It has been shown that bovine SCO and RF proteins contain repeated domains, e.g., thrombospondin-like, that are highly conserved in phylogeny and present in a great number of proteins such as human mucins (Gobron et al. 1996; Nualart et al. 1998). Thus, the possibility exists that the epitopes in the planarian integument that cross-react with our antibodies reside in similar repeated domains of mucin-like proteins. In this case one has to assume that all the reacting antibodies (seven MAbs and AFRU) recognize repeated domains. As we have shown in Materials and methods, in double-antibody sandwich ELISA using the same MAb as capture and binding antibody, no signal was ever found. If some MAbs were directed against a repeated motif one should expect that not all sites were occupied by the capture antibody and that some signal would persist (cf. Harlow and Lane 1988). Thus, very probably, MAbs used in the present

study recognize epitopes occurring in nonrepeated domains. Therefore, in addition to conserved repeated domains, there should exist other conserved domains that do not repeat in RF glycoproteins and that are present in the planarian integument. In conclusion, planarian epidermal secretory cells synthesize compounds that share several proteinaceous, nonoverlapping, nonrepetitive epitopes with bovine SCO and RF glycoproteins, thus indicating that these proteins may be phylogenetically conserved.

At present, proteins immunologically related to vertebrate SCO glycoproteins have been found in echinoderms (Viehweg et al. 1998), insect embryos (Lichtenfeld et al. 1998), and in Platyhelminthes (present results). In the sea star *Asterias rubens*, cells immunoreactive to an antiserum against Reissner's substance have been described in the ectoneural part of the radial nerve cord and were considered to represent secretory radial glia cells related to vertebrate SCO (Viehweg and Naumann 1996). The ectoneural system of the sea star is actually a part of the epidermis, and neurons and glial cells are separated from the sea water by an immunoreactive hyaline layer that appears to be a secretory product of the radial glia secretory cells. In planarians, immunoreactive cells are located in the epidermis. However, planarian immunoreactive cells are dispersed throughout the skin and do not constitute an organ. We do not know the exact nature of the immunoreactive cells in the planarian epidermis. In this connection, we have also observed that certain vertebrate epidermal cells (fish gills, tadpole skin) are immunoreactive to AFRU (P. Fernández-Llebrez, unpublished results).

The three systems (vertebrate SCO and its ontogenetic and phylogenetic relatives, secretory radial glia of sea star, and planarian epidermal cells) share some interesting features: (1) they are composed of ciliated secretory cells; (2) they secrete glycoproteins possessing similar epitopes; (3) their secretion is released via the apical surface of the cells and forms fiber- or layer-like structures by polymerization. The function of these secretions is not known but their ancient phylogenetic character suggests an important biological role for all three systems.

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