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Characterization and fine-structural localization of actin- and fibronectin-like proteins in planaria (*Dugesia lugubris s.l.*)

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Summary. Actin- and fibronectin-like proteins were characterized in the planarian, *Dugesia lugubris s.l.*, by sodium dodecyl sulphate polyacrylamide gel electrophoresis and immunoblotting analysis using antisera to vertebrate actin and fibronectin. These antisera recognized protein bands of 42 kDa and 220 kDa, respectively. In addition, the immunohistochemical distribution of both actin- and fibronectin-like material was examined by using immuno-electron microscopy. Actin-like protein was localized in myofibrils in various differentiation stages, and in the peripheral cytoplasm and lamellipodia of cells that were migrating. The fibronectin-like component was associated with the extracellular matrix in the fibrillar structures and with the surface of the migrating cells. Our data suggest that similar cellular and molecular mechanisms are involved in cell-matrix interactions and in the morphogenesis of living organisms at different evolutionary levels.

Key words: Actin-like protein – Fibronectin-like protein – Regeneration – Cell migration – Immunocytochemistry – *Dugesia lugubris* (Tricladida)

The interrelationships between fibronectin (FN; an extracellular matrix glycoprotein of 500 kDa) and some intracellular cytoskeletal proteins mediated by FN-receptors (integrins) have been studied in vivo and in vitro (Hynes 1987; Akiyama et al. 1989; Welch et al. 1990; Shaw et al. 1990). The interactions, involving transmembrane complexes, are characteristic of slowly moving or stationary cells. Normally migrating or malignant cells lack these complexes (Couchman and Rees 1979; Duband et al. 1988; Akiyama et al. 1989). Actin seems to be one of the intracellular protein molecules responsible for the phenotypic change that accompanies non-muscle cell movement (for references, see Small 1989) in embryonic development (Boucaut et al. 1984; Ekblom et al. 1986; Winklbauer 1990) wound healing (Grinnell et al. 1981; Clark et al. 1982; Welch et al. 1990; Gordon and Staley 1990) and in transformed cells (Roman et al. 1989; Aznavoorian et al. 1990).

Few data on invertebrate tissues have been reported (Akiyama and Johnson 1983; Bride et al. 1988; Hue et al. 1989). However, studies on the molecular evolution of FN and actin molecules indicate a marked evolutionary constancy as shown by amino acid sequence homology (Hue et al. 1989) and cross-reactivity to mammalian anti-FN and anti-actin antibodies (Akiyama and Johnson 1983; Hue et al. 1989).

Planarians may provide an excellent invertebrate model for analyzing migration events in vivo because the epidermis and perhaps all other somatic tissues are incapable of self-renewal (Elhers 1985, 1986; Baguña et al. 1990), and depend upon stem-like cells migrating from the underlying tissues (Skaer 1965; Pascolini et al. 1988a). This phenomenon seems to be responsible for the high regenerative capacity of planarians. The regenerative pattern in these animals consists of three partially overlapping stages: (1) wound healing in which the epidermal cells and their precursors migrate (Pascolini et al. 1988a), (2) an intermediate stage in which "programmed cell death" predominates (Bowen et al. 1982; Bowen 1984), and (3) a final stage in which the blastema develops (for a review, see Gremigni 1988).

In this paper, we report, for the first time, the characterization of actin- and FN-like proteins in planarians using vertebrate antibodies and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). In addition, the immunocytochemical localization of these proteins is described with particular reference to cell-matrix interactions during migratory events.

Materials and methods

Tissue preparation

Intact ($n=40$) and regenerating ($n=160$) freshwater planarians *Dugesia lugubris s.l.* were used. Head regeneration was induced by decapitating chloretone-anesthetized specimens immediately be-

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hind the auricles according to Hay and Coward (1975). The portion containing the transected area was processed at various times after dissection as follows.

SDS-PAGE, immunoblotting and analytical procedures

The extract for actin immunocharacterization was prepared by homogenization of the sample in PEM buffer (1 mM Pipes, 0.1 mM EGTA, 0.01 mM $MgCl_2$, pH 7). After centrifugation at 105000 *g* for 90 min, the supernatant was treated with a sample quenching solution containing 2% SDS, 50 mM β-mercaptoethanol, and boiled for 3 min. SDS-PAGE was carried out according to Laemmli (1970) in 10% acrylamide mini-slabs (Bio-Rad, USA) and protein was transferred onto nitrocellulose paper using a semi-dry transblot apparatus (Bio-Rad). After blotting, the proteins of the planarian extract and rat skeletal actin, prepared from acetone powder (Spudich and Watt 1971), were immunodecorated with a mouse monoclonal (mAb) anti-actin IgM antibody (Amersham, UK) as described by Towbin et al. (1979), and visualized using a secondary antibody coupled with alkaline phosphatase (Bio-Rad) for 60 min at 20° C.

For immunocharacterization of actin-like proteins during regeneration, extracts were prepared as described above at various times after transection; the same amount of protein, 15–20 µg (Bradford 1976) was stratified on each gel.

The presence of large amounts of fats and polyphenol compounds made it difficult to extract FN-like molecule from planarian tissues. For this reason, the FN extraction suggested by Akyama and Johnson (1983) was unsuitable and gave a low yield. The best results were obtained as follows: in order to reduce proteolytic splicing of the FN-molecule, the tissue was homogenized in phosphate-buffered saline (PBS: 0.2 M NaCl, 0.081 M Na_2HPO_4, 0.0189 M NaH_2PO_4) (about 200 mg of fresh material/ml) containing 1 mM phenyl methyl sulfonyl fluoride (PMSF), 1 mM leupeptin, 0.1 mM pepstatin and 50 µg/ml aprotinin. After centrifugation at 23000 *g* for 15 min, the supernatant was collected and stored at 4° C. The pellet was re-extracted as described above in a PBS + antiprotease cocktail (1 mM PMSF, 1 mM leupeptin, 0.1 mM pepstatin, 50 µg/ml aprotinin) containing 4 M urea and 10 mg/ml heparin. It was then stirred for 1 h at 20° C after which 0.2 vol of cold carbon tetrachloride was added and the suspension stirred vigorously at 4° C for 5 min. The aqueous phase, obtained after centrifugation at 5000 *g* for 10 min, was aspirated, and the proteins concentrated by adding $(NH_4)_2SO_4$ at 70% saturation. The $(NH_4)_2SO_4$ pellet was solubilized in the sample quenching solution of Laemmli (1970) and used for electrophoresis.

Human plasma FN was purified as described by Akiyama and Johnson (1983) and used as a marker in SDS-PAGE. Electrophoretic analysis (5% acrylamide) and Western blotting were carried out as described above and planarian FN was detected by overnight incubation with a rabbit polyclonal antiserum (pAb) anti-human plasma FN diluted 1:100 (a generous gift of Prof. L. Zardi, IST Genova). ^{125}I-labelled protein A (Amersham) was used to detect the antisera and visualization was by autoradiography (Burnette 1981). Alternatively the nitrocellulose replicas were labelled with rabbit or mouse anti-human plasma FN (ICN, USA). Antibody detection was accomplished with an appropriate secondary antibody labelled with alkaline phosphatase.

The antiserum characterizations were carried out by preabsorption with the corresponding purified antigen as reported by Camatini et al. (1986) and by assaying the residual activity by Western blotting as described above.

For quantitative determination of FN-like protein in planarian tissues, the extract obtained from 5 animals, containing about 0.2 mg protein, was adsorbed on nitrocellulose membrane by a dot-blot apparatus (Bio-Rad) and labelled with pAb or mAb anti-human plasma FN. The antibody detection was accomplished with the appropriate secondary antibody labelled with alkaline phosphatase. Purified human plasma FN was also adsorbed at various concentrations and stained in the same way. The strips containing stained spots were scanned with a GS 300 dual speed scanning densitometer (Hoefer, USA) operated with reflectance mode. When the area of the peak relative to each spot was plotted against the relative human FN concentration, a straight line was obtained in the range 10–100 ng FN.

Controls with rabbit or mouse pre-immune sera were carried out in all experiments.

Immuno-electron microscopy

For immuno-electron microscopy, the samples were fixed for 90 min at 20° C by immersion in 2% paraformaldehyde and 0.2% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.2). After fixation, they were rinsed overnight in the same buffer. Tissue samples were then dehydrated in graded ethanols at low temperature and embedded at −35° C in Lowicryl 4KM (Agar Scientific, UK).

Ultrathin sections were cut, mounted on uncoated 400-mesh nickel grids, and processed for the immunogold staining according to Camatini et al. (1986). They were rehydrated for 5 min on drops of TRIS-buffered saline (TBS: 0.02 M TRIS, 0.9% NaCl, pH 8.2) containing 0.1% bovine serum albumin (BSA), labelled for 2 h with the anti-actin mAb diluted 1:1000 in TBS-BSA, and after several washes in TBS-BSA, stained for 90 min with gold-labelled goat anti-mouse antibody (GAM) IgM 10 and 15 nm (Janssen, Beerse/Belgium). Immunostaining was also performed with the

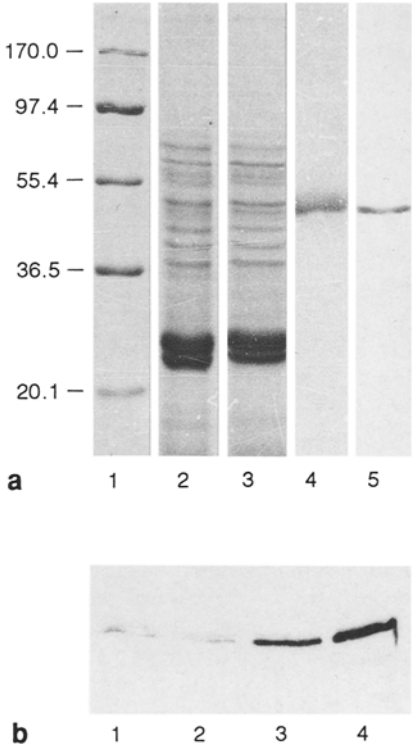

Fig. 1a, b. Immunoblots of planarian extracts after 10% SDS-PAGE. **a** *Lane 1* Coomassie-blue-stained protein markers; *lanes 2–3* Coomassie-blue-stained planarian extract, carried out on 0 h and 4-day regenerants; *lane 4* Coomassie-blue-stained purified rat skeletal muscle actin; *lane 5* Western blotting of extract from non-injured planarians decorated with anti-actin mouse mAb. **b** Western blotting of extracts from planarian at 0 h (*lane 1*), 4 h (*lane 2*), 24 h (*lane 3*) and 4-day regenerants (*lane 4*), decorated with anti-actin mouse mAb. The same amount of protein was stratified on each slab hole. The molecular weights of protein markers are reported in kDa

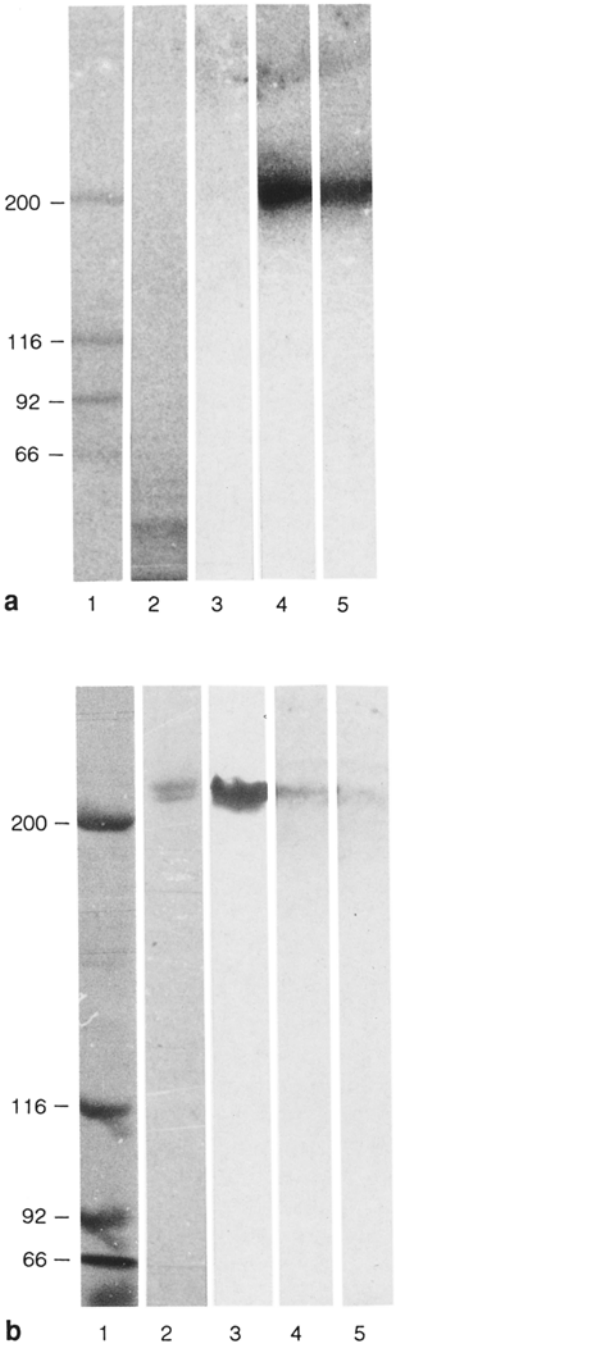

Fig. 2a, b. Immunoblots of planarian extract after 5% SDS-PAGE. **a** Mini-slab gel electrophoresis of whole extract (*lane 2*) and protein markers (*lane 1*) stained with Coomassie blue. Whole extract probed with pAb anti-human FN radiolabelled with ^{125}I-Protein A (*lane 5*), *lane 3* control in which preimmune serum was used instead of the first antibody; *lane 4* purified (about 5 ng) human plasma FN stained as in *lane 5*. **b** Slab-gel (16 × 14 cm) electrophoresis and immunoblots of planarian extract. Coomassie-blue-stained protein markers (*lane 1*) and 5 µg purified human plasma FN (*lane 2*). *Lane 3* Western blotting of purified human plasma FN decorated with pAb and of planarian extract decorated with pAb (*lane 4*) and mAb (*lane 5*). The molecular weight of protein markers are reported in kDa

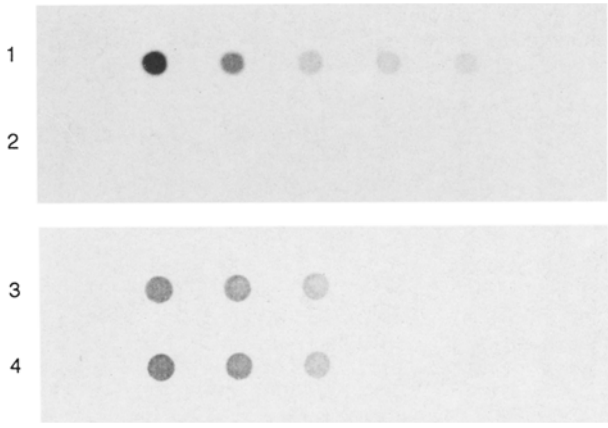

Fig. 3. Dot-blot analysis of FN-like material from planarian extract. *Line 1* Purified human plasma FN at various concentrations (from left: 100 ng, 50 ng, 25 ng, 15 ng, 10 ng) labelled with anti-human plasma FN mAb. *Line 2* Control in which pre-immune serum was used instead of the first antibody. *Lines 3, 4* Planarian extract was adsorbed at three different protein concentrations (from left: 1.5 mg, 0.75 mg, 0.4 mg) and labelled with anti-human plasma FN pAb (*line 3*) and anti-human plasma FN mAb (*line 4*)

anti-FN mAb diluted 1:300 in TBS-BSA. This antibody was recognized with GAM IgG 5 and 10 nm (Janssen). In controls, the primary antiserum was replaced by non-immune sera.

Sections were counterstained with uranyl acetate and lead citrate, and examined with a Philips 400T electron microscope. Some specimens were processed for conventional electron microscopy and some for ruthenium red staining as previously reported (Pascolini et al. 1988b).

Results

Immunological characterization of actin and FN-like proteins

Fig. 1 shows the results of a Western blot analysis carried out on extracts from planarian tissues. The anti-actin mAb used reacts with various actin isoforms of vertebrate cells, and recognizes a single electrophoretic band of 42 kDa (Fig. 1a, lane 5). Purified actin from rat skeletal muscle co-migrated with the protein band that was immunostained in the planarian extract (Fig. 1a, lane 4). When the extract was carried out at different regeneration stages and processed for Western blotting, there was a progressive increase in actin immunostaining (Fig. 1b) with a maximum in 4–5 day regenerants (Fig. 1b, lane 4). After this time, the actin immunostaining returned to the same values as in the control animals (not shown).

SDS-PAGE and Western blot analyses of planarian extracts decorated with pAb labelled with iodinated protein A indicated that a 220-kDa radioactive protein band was present (Fig. 2a, lane 5) with the same electrophoretic mobility as purified human plasma FN (Fig. 2a, lane 4). The same results were also obtained with anti-FN mAb although the band obtained was faint and only appeared after a prolonged exposure.

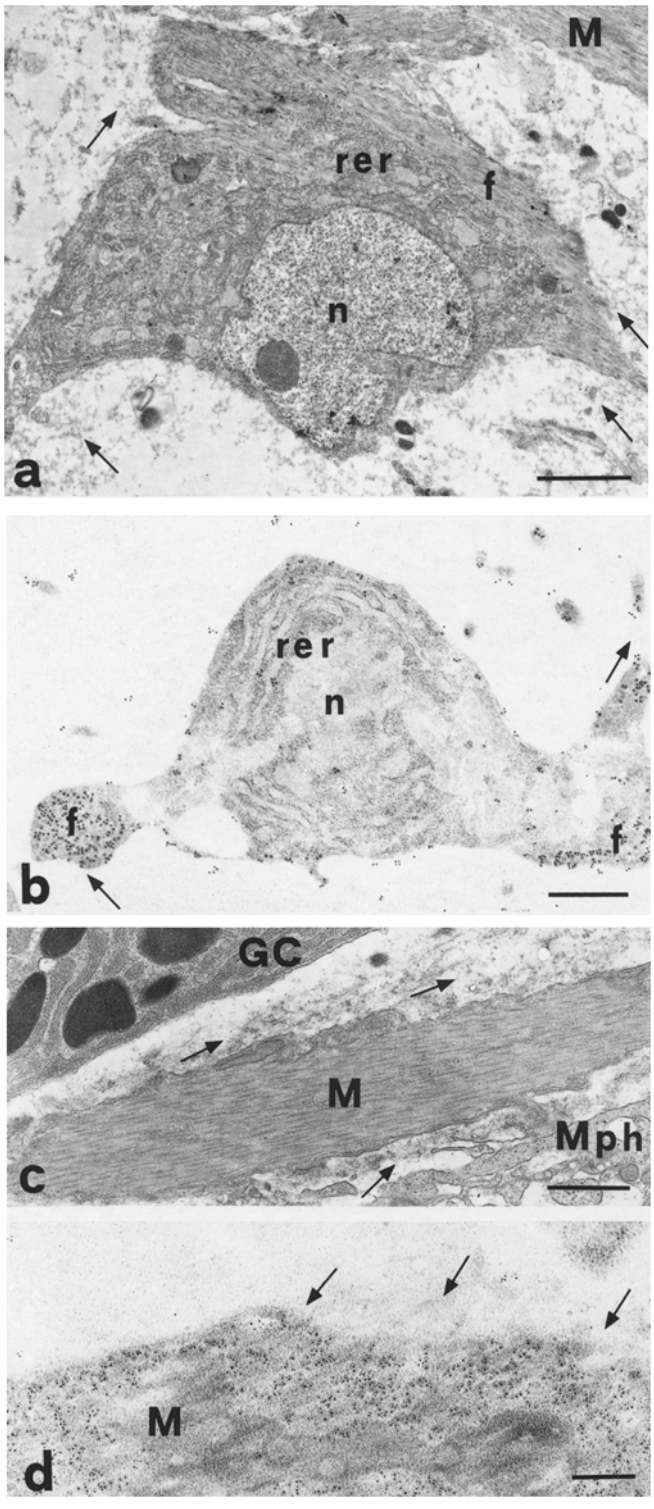

Fig. 4. **a** Electron micrograph of myoblast in the subepidermal region 2 days after amputation. *Bar*: 2.5 μm, ×5000. **b** Immuno-electron micrograph. Decoration with anti-actin mAb; 10 nm gold particles mainly label filaments of the organizing myofiber. *Bar*: 0.5 μm, ×20000. **c** Electron micrograph of longitudinally sectioned differentiated myofibril. *Bar*: 1 μm, ×10000. **d** Immuno-electron micrograph. Decoration with anti-actin mAb; 5-nm gold particle labelling. *Bar*: 0.25 μm, ×37000. *f* Filaments; *GC* gland cell; *M* myofiber; *Mph* macrophage-like cell; *n* nucleus; *rer* rough endoplasmic reticulum. *Arrows* indicate the ECM fibrils closely associated with myofibril filaments

No apparent changes in FN-like protein immunostaining were observed during regeneration. In order to compare the radioactive bands of human and planarian material, we studied 5 ng or less of human FN; this did not give a clearly separated 220-kDa doublet.

FN-like protein in planarians was also decorated with both pAb and mAb anti-human FN, and could be demonstrated with the appropriate secondary antiserum labelled with alkaline phosphatase (Fig. 2b). The staining observed with mAb (Fig. 2b, lane 5) was less intense than that obtained with anti-human FN pAb (Fig. 2b, lane 4), but a double 220-kDa subunit was clearly visible in both cases.

The relative amount of FN-like material in planarian tissue was tested using a dot-blot immunolabelling method. The results indicated that both mAb and pAb detected approximately the same amount of antigen in planarian extract (30–40 ng FN/mg protein) as determined by the calibration curve obtained by varying the concentrations of purified human plasma FN (Fig. 3). The control with preimmune serum did not show a positive reaction.

A series of anti-actin and anti-FN antibodies, both polyclonal and monoclonal, from commercial and laboratory preparations were tested. However, the best reproducible results were obtained with those used in the experiments described here.

Immuno-electron microscopy

Immuno-electron microscopy of control (non-injured) planarians indicates that actin-like protein occurs mainly in muscle cells, at various stages of differentiation. Fig. 4a, b shows a myoblast that is characterized by a well-developed rough endoplasmic reticulum with dilated cisternae and by numerous mitochondria. The myofilaments occur at the cell periphery. The cytoplasm of the differentiated myofibril is mainly occupied by myofilaments (Fig. 4c). A close association between these myofilaments and matrix fibrils was detectable (Fig. 4a–d). A strongly positive gold label in muscle cell filaments could be seen in specimens embedded with Lowicryl KM4 and processed for immuno-electron microscopy (Fig. 4d).

In order to study the fine-structural localization of actin-like protein during cell migration, specimens of regenerating planarians were used. Fig. 5a shows a basal portion of this epidermis a few hours after cutting; a precursor cell is detectable. The presence of a microfilament meshwork suggests that it is passing from being a migratory to a stationary phenotype, and that it is being integrated between old epidermal cells. The presumptive sites of adhesion between the precursors and basal lamina were shown with ruthenium red staining (Fig. 5b). Anti-actin mAb specifically decorated the peripheral cytoplasm of a migrating precursor extension (Fig. 5c) and the organizing cytoskeleton of a newly integrating epidermal cell (Fig. 5d).

Blastema-forming cells could be observed when the blastema developed (Fig. 6a, b). They were character-

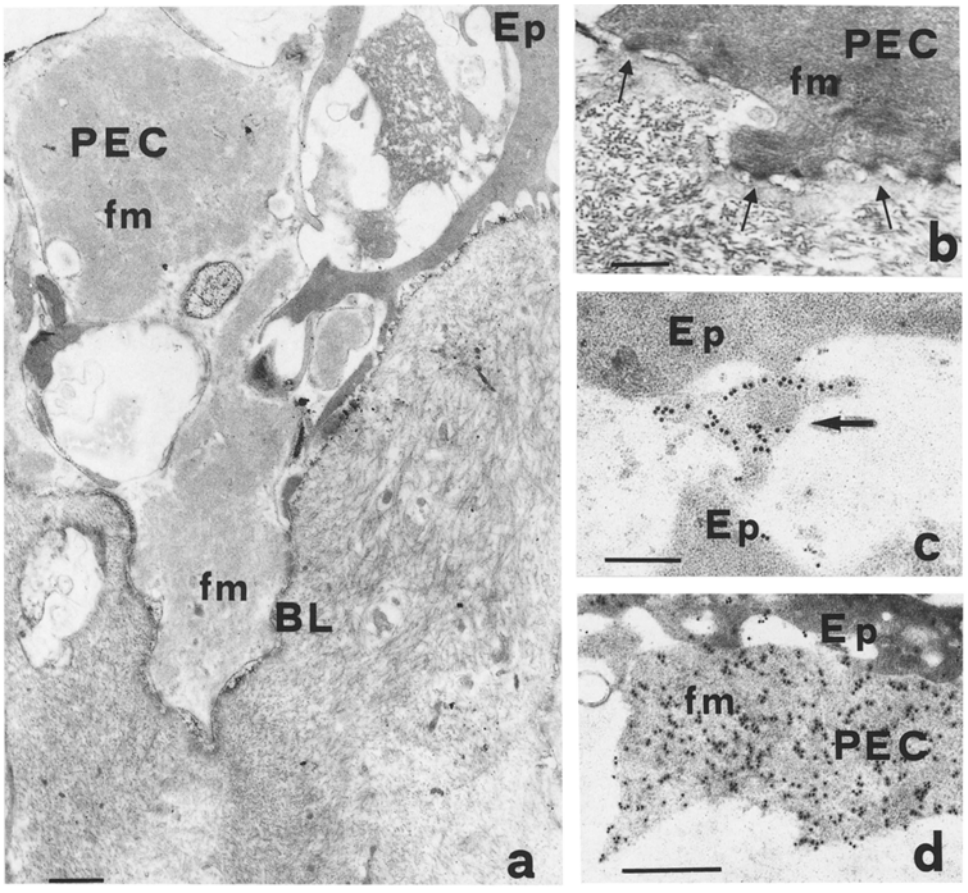

Fig. 5a–d. Basal portion of the epidermis adjacent to the wound, 7 h after wounding. **a** Electron micrograph showing a rich filament meshwork in a precursor process. *Bar*: 1 μm, ×7000. **b** Presumptive sites of adhesion (*thin arrows*), shown by ruthenium red staining, between a precursor cytoplasmic process and the basal lamina. *Bar*: 0.25 μm, ×33000. **c**, **d** Immunocytochemical decoration with anti-actin mAb, 15-nm gold particle labelling of the peripheral cytoplasm of migrating precursor extension (*wide arrows*) (**c**) and of the organizing cytoskeleton of epidermal cell precursor (**d**). **c** *Bar*: 0.5 μm, ×20000. **d** *Bar*: 1 μm, ×13000. *BL* Basal lamina; *Ep* epidermal cell; *fm* filament meshwork; *PEC* epidermal cell precursor

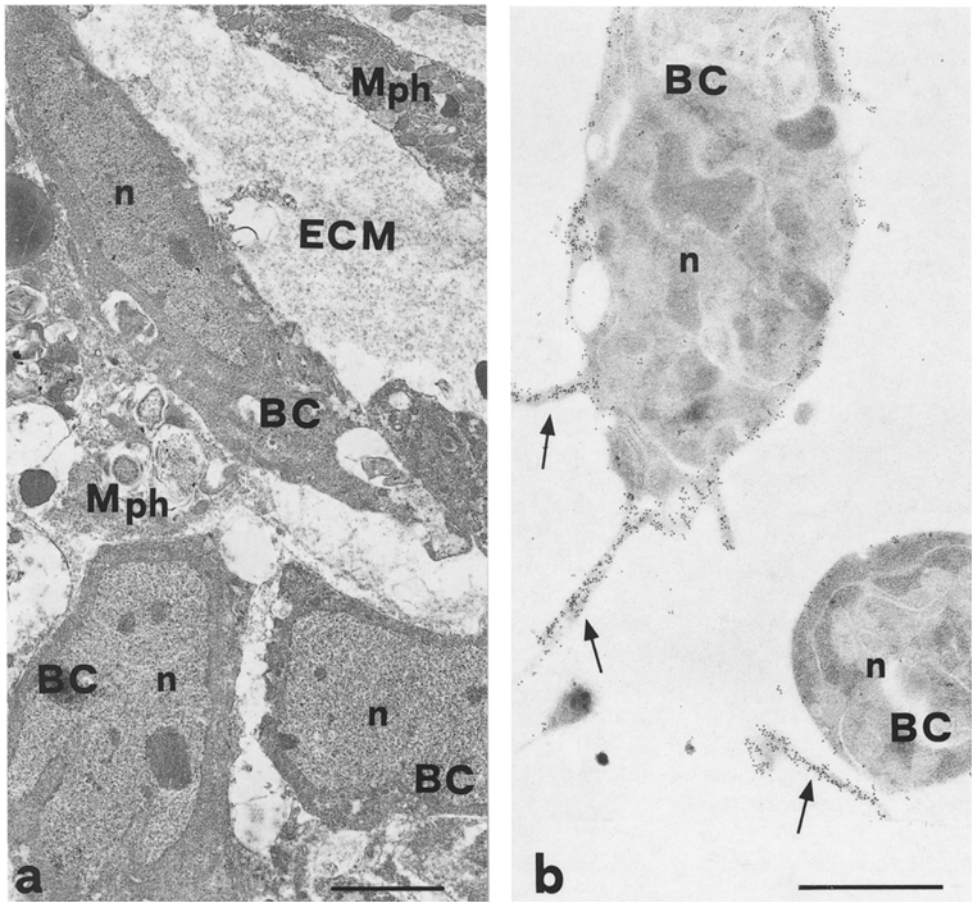

Fig. 6. a Electron micrograph. Blastema-forming cells in 4-day-old regenerants. *Bar*: 2.5 μm, ×5600. **b** Immuno-electron micrograph showing blastema cell lamellipodia (*arrows*) stained with anti-actin mAb labelled with 10 nm gold particles. *Bar*: 1 μm, ×19000. *BC* Blastema cell; *ECM* extracellular matrix; *Mph* macrophage-like cell; *n* nucleus

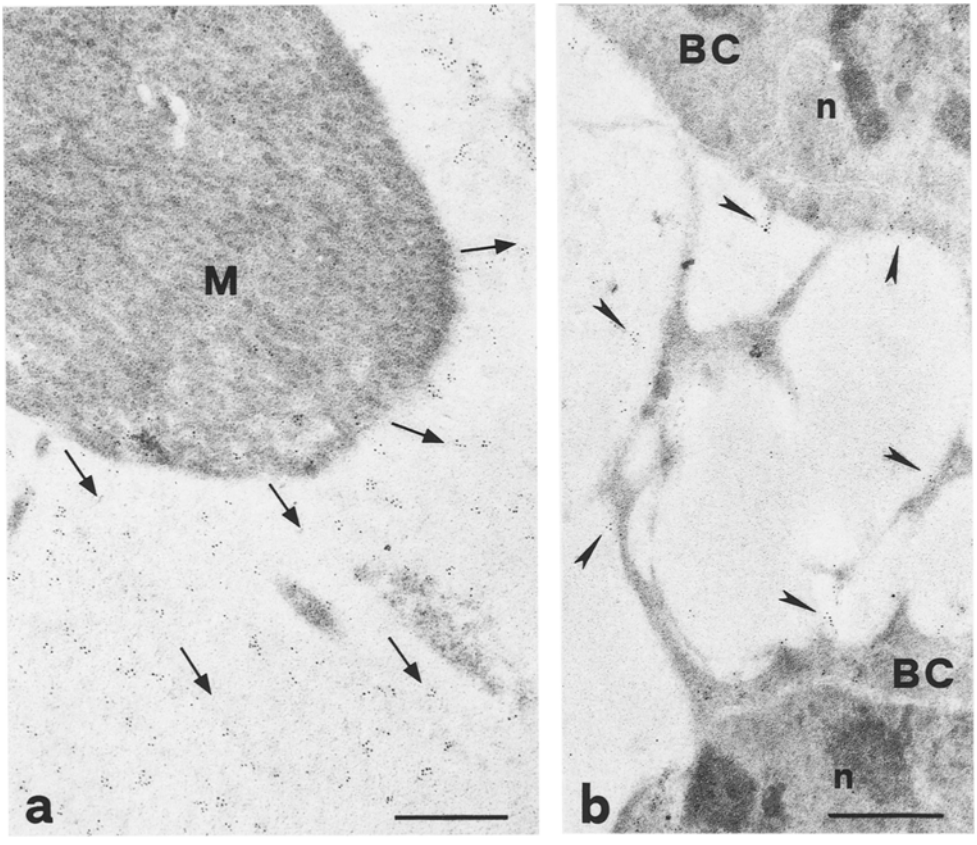

Fig. 7a, b. Immuno-electron micrographs. Decoration with anti-FN mAb; 5-nm gold particle labelling of extracellular matrix fibrils closely associated with a myofibril (**a**) and lamellipodium surface of blastema cells (**b**). *Bar*: 0.5 μm, ×30000. *BC* Blastema cell; *M* myofiber; *n* nucleus; *arrows* labelled fibrils; *arrowheads* lamellipodia labelling

ized by a large nucleus, which had one or more prominent nucleoli, and which constituted the bulk of the cell volume (Fig. 6a). The cell surface and lamellipodia were strongly stained with anti-actin mAb (Fig. 6b).

The anti-FN mAb used stained extracellular matrix fibrils that were mainly associated with muscle cell filaments (Fig. 7a). FN-like material was also present at the surface of the migrating cells, such as the blastema-forming cells (Fig. 7b), and seemed to be associated with actin-like material. Similar results were also obtained when the pAb anti-human FN was used.

Discussion

The involvement of actin in non-muscle cell movement is well established, and morphological cell changes have been related to various cytoskeletal components (Small 1989). Numerous studies have also shown that actin is involved in the formation of contacts with extracellular matrix fibrils at specific sites, namely at focal contacts (Burridge et al. 1988). FN, a multifunctional glycoprotein that mediates interactions between cells and extracellular matrix (Ruoslahti 1988) seems to be involved in the organization of intracellular actin filaments (Welch et al. 1990). Studies of the wound-healing process in most vertebrates have indicated the importance of actin-FN interrelationships and their relevance in wound contraction and repair (Gabbiani et al. 1971; Singer et al. 1984; Welch et al. 1990).

We propose the use of the lower metazoan planarian as another model for studying morphogenesis in living organisms because cell migration and cell differentiation constitute the cellular basis of both the physiological adaptation and the high regenerative power of planarians (Bowen et al. 1982; Gremigni 1988; Pascolini et al. 1989; Baguña et al. 1990). As a first step in studying the molecular interactions that occur during cell migration in planarians, we characterized actin- and FN-like components, by SDS-PAGE analysis and immunolabelling of nitrocellulose replicas with anti-actin and anti-FN antibodies raised in higher animals.

In planarians, the anti-actin mouse mAb stained muscle cells and migrating cells, such as epidermal cell precursors, and blastema-forming cells. The immuno-electron-microscopic distribution of actin-like proteins in muscle cells and migrating cells seems to be different and this may reflect the presence of different actin isoforms. The use of antibodies that recognize specific actin isoforms will be necessary to elucidate this point.

Our biochemical data strongly suggest the involvement of an actin-like protein in the regeneration process. It is unclear whether the neosynthesis of actin-like material or a different conformational configuration of the protein (e.g., polymerization-depolymerization) is the basis of the transient changes in the amount of antigen detected. However, the changes in actin-staining intensity during regeneration and wound repair, as revealed by Western-blot analysis, could be related to the increase in cells that actively migrate from the parenchyma to the newly forming epidermis and developing blastema.

The association of matrix fibrils with muscle cell filaments, giving a network that resembles the fibronexus-like structure (Singer et al. 1984), is also of interest because it may function as a primitive skeleton in planarians. The slight difference in FN-like protein staining after electroblotting experiments with pAb and mAb seemed to be caused by the SDS procedure. Indeed, dot-blot experiments carried out with the native protein indicate that both antibodies detect about the same amount of antigen in planarian extract, whereas when the extract is treated with 0.1% SDS, the protein staining with mAb is less intense with respect to that obtained with pAb.

The doublet of the FN-like molecule at 200-kDa, detected in planarians with both mAb and pAb, shows the same subunit molecular weight as the human FN used as a reference (reported here) and of the vertebrate FN previously studied (Hynes 1985; Yamada 1989). In addition, similar molecular weight values and subunit composition have been published for FN-like material isolated from *Microciona prolifera* (Akiyama and Johnson 1983) and from the hemolymph of *Helix aspersa* (Bride et al. 1988) and *Drosophila* (Gratecos et al. 1988).

These data suggest that, in planarians as in vertebrate models, (1) the FN-like protein constitutes a fundamental extracellular matrix component, (2) it may provide an essential substratum for cell migration, as is found in many other systems (Ali and Hynes 1978; Newgreen and Thiery 1980; Heasman et al. 1981; Dufour et al. 1988), and (3) there is evolutionary conservation of this process. Moreover, our results provide indirect evidence of the similarity in the cell-matrix interactions at lower and higher evolutionary levels in the animal kingdom.

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