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The identification and localization of two intermediate filament proteins in the tunic of *Styela plicata* (Tunicata, Styelidae)

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ABSTRACT

The intermediate filament (IF) proteins *Styela* C and *Styela* D from the tunicate *Styela* (Urochordata) are co-expressed in all epidermal cells and they are thought to behave as type I and type II keratins. These two IF proteins, *Styela* C and *Styela* D, were identified in immunoblots of proteins isolated from the tunic of *Styela plicata*. The occurrence and distribution of these proteins within the tunic of this ascidian was examined by means of immunofluorescence and immunoperoxidase techniques, using anti-*Styela* C and anti-*Styela* D antibodies. In addition, immuno-electron microscopy of the tunic showed that the two proteins are located in the cuticle layer and in the tunic matrix. These results represent the first data about the presence of IF proteins in the tunic of adult ascidian *S. plicata*. The possible involvement of these IF proteins in reinforcing the integrity of the tunic, that represents the interface between the animal body and the external environment, is discussed.

1. Introduction

Intermediate filaments (IFs) together with microtubules and actin microfilaments constitute the integrated dynamic filamentous network or cytoskeleton, present in the cytoplasm of metazoan cells (Schliwa, 1986; Aebi et al., 1988; Fuchs and Weber, 1994; Herrmann and Aebi, 2004).

The IFs are composed of protein polymers assembled into fibers 10 nm in diameter. All IF proteins exhibit a common secondary structure, but according to their primary and gene structure, five major IF protein types have been characterized (Strelkov et al., 2003). Cytoplasmic IF proteins can be classified into four major types while the fifth type represents the nuclear lamins found within the nucleus. The nuclear IFs differ from the cytoplasmic IF proteins for the presence of extra 42 residues in their coil 1b subdomain (for review see Fuchs and Weber, 1994); they also form a meshwork rather than filaments.

The various types of IF proteins are differentially expressed in most tissues and cell types. Types I and II are the largest subfamilies and represent keratins of epithelia and epidermal appendages. Type III proteins belong to the mesenchymally derived IF proteins: desmin, vimentin, GFAP, peripherin and plasticin, syncoilin. Type IV includes the various neurofilament proteins NF-L, NF-M, NF-H and α-internexin that are found in the neurons of the central system and also the closely homologous gefiltin and xefiltin. In addition to types I–V there are nestin, synemin, desmuslin, tanabin, transitin, paranemin which are occasionally grouped together as type VI IFs, and two more distantly related beaded filament proteins, phakinin (CP49) and filensin (CP115) components of the ocular lens (Moll et al., 1982; Herrmann and Aebi, 1998, 2000; Schweizer et al., 2006; Guérette et al., 2007; Oshima, 2007).

Most of IF proteins assemble into homodimers but keratins are obligatory heterodimers (Hatzfeld and Weber, 1990; Strelkov et al., 2003).

Genetic studies relating IFs to skin diseases have demonstrated that they are essential for maintaining the mechanical integrity of the cells (McLean and Lane, 1995; Fuchs and Cleveland, 1998; Omary et al., 2004; Pekny and Lane, 2007); the epidermal keratinocytes, with their dense, anastomosing meshwork of IFs, form the outermost physical barrier of the body.

In higher vertebrates such as man, more than 60 genes for cytoplasmic IF proteins have been identified, most of which are coding for keratins (Herrmann et al., 2003; Hesse et al., 2004); ortholog genes have been identified and sequenced in several other genera (Hesse et al., 2001) and genome-wide analysis has revealed that keratin genes have expanded during chordate evolution (Padhi et al., 2006). Intermediate filaments were recognized in vertebrates, such as lamprey, trout, shark, zebrafish, in molluscs and other invertebrates (Bartnik and Weber, 1989; Karabinos et al., 2001; Schaffeld et al., 2002a,b). IF proteins resembling types I, II, and III have also been detected in lower chordates such as ascidians and two lancelets (Erber et al., 1998; Riemer and Weber, 1998; Riemer et al., 1998;

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Luke and Holland, 1999; Karabinos et al., 1998, 2000, 2002a,b, 2004; Wang et al., 2000, 2002). However, the orthologous relationships for all these IF proteins found in lower chordates cannot unequivocally be assigned and it is still difficult to clearly classify IF proteins exclusively on the basis of the gene structure and/or aminoacid sequences (Schaffeld and Schultess, 2006).

In particular, several studies (Riemer and Weber, 1998; Wang et al., 2000) document that the urochordates *Styela plicata* and *Styela clava* (Stolidobranchiata, Styelidae) have among their cytoplasmic IF proteins: (a) a muscle-specific, homopolymeric filament protein called *Styela* A, clearly belonging to the group of the vertebrate type III IF proteins; (b) an IF protein *Styela* B found in interior epithelia; (c) the IF proteins *Styela* C (St-C) and *Styela* D (St-D), co-expressed in the epidermis which is a simple epithelium. Due to the obligatory heteropolymeric IF formation and level of sequence identity, the latter two proteins have been assumed to represent type II and type I keratins, respectively (Wang et al., 2000). The expression of these simple epithelial keratins was also observed in *Ciona intestinalis* (Wang et al., 2002) and it is strikingly conserved in a wide variety of species up to mammals including teleosts (Schaffeld et al., 2003) that it should be noted, however, express keratins also in mesenchymal cells (Markl and Franke, 1988).

In the ascidians the epidermis is attached to the tunic that constitutes a supporting integument covering this simple epithelium like a mantle; the tunic is considered an unusual tissue because of its extracellular components (cellulose-like structures associated with collagen–elastin-like proteins) and the presence of free-living cells (c.f. Burighel and Cloney, 1997, and references therein).

The present study describes the presence of IFs within the tunic of the solitary ascidian, *S. plicata*. The identification and distribution of two types of IFs was investigated by immunoblotting, immuno-light and immuno-electron microscopy.

This was designed to examine their possible role in the structure and function of tunic, a complex and active tissue which provides an outer protective covering, a supporting exoskeleton and owing to its cellular component, is also involved in various biological functions (reviewed by Burighel and Cloney, 1997). Thus the present study aims at extending the understanding of the main tunic functions and to gain important insight into cytoplasmic IF function in invertebrates on account of the insufficient and inconsistent data.

2. Materials and methods

2.1. Animals

Adult specimens of the ascidian *S. plicata* were collected from the coast lake of Ganzirri, Messina (Italy). They were carried in plastic containers with natural seawater and kept in aerated aquaria at 15–18 °C until used.

Animals were fed daily with various food types including freeze-dried rotifers, green unicellular algae and artificial diet and were cleaned once a week by gentle brushing to remove debris and fouling organism. Pieces of tunic were dissected from different regions of the body and used in subsequent experiments. To avoid extensive contractions of the animals, the specimens were anaesthetized by adding of MS222 to the medium (final concentration 0.2%).

2.2. Protein gel electrophoresis and Western-blotting

Fresh and frozen tunic fragments of *S. plicata* (100 g wet weight) were cut into small cubes and washed in a Free Herbst's artificial seawater (FHASW) at 4 °C. To avoid contaminations of the tunic protein preparation, fragments were carefully separated from the epidermal cells attached to the tunic, the procedure being monitored under a binocular microscope. Moreover to avoid contamination with the human keratins, gloves were used in each step; equipment was autoclaved and protected from dust.

To isolate the proteins, the tissue samples were first homogenized in a blender at 4 °C in Tris/HCl. Homogenates were dialyzed in Tris/HCl o.n. and then centrifuged. The pellets were resuspended in lysis buffer (300 mM NaCl, 50 mM Tris/HCl pH 7.6, 0.1% Triton, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml apoprotinin) on ice and kept o.n. at 4 °C.

For sodium dodecyl-sulfate polyacrylamide gel electrophoresis, samples of proteins of the tunic were run through an 8% gel and stained with Coomassie brilliant blue; protein standards (Prestained molecular weight marker, Sigma) were run in adjacent lane. Aliquots of the same samples were separated on 4–12% SDS-polyacrylamide gel electrophoresis (NuPAGE 4–12% Bis–Tris gel, protein standard SeeBlue Plus2, Invitrogen) and transferred on to nitrocellulose membranes. Membranes were blocked with a solution containing 1 M glycine, 1.25% dry milk, 0.1% BSA and then probed o.n. with anti-*Styela*-C or anti-*Styela*-D polyclonal antibodies raised in rabbit (preabsorbed and affinity-purified; dilution 1:10 and 1:20, respectively) given by Prof. Klaus Weber (Max Planck Institute for Biophysical Chemistry, German) and with mouse monoclonal anti-pan cytokeratin (1:400; Sigma–Aldrich, St. Louis, USA; C-2562).

The blots were washed three times and proteins were detected after incubation with goat anti-rabbit IgG HRP (1:10,000; Santa Cruz Biotechnology, Inc.) and goat anti-mouse IgG HRP (1:20,000; Santa Cruz Biotechnology, Inc.) secondary antibodies by using an enhanced chemiluminescence detection system (Super-Signal, Pierce, Rockford, IL, USA).

2.3. Immuno-light microscopy

For both immunofluorescence and immunoperoxidase staining, the tunic fragments taken from different regions of *S. plicata* adult body and a sample of the oral siphon were fixed in 4% paraformaldehyde in FHASW and processed for wax histology using standard techniques. Five-micrometer sections were collected on to 0.1% poly-L-lysine-coated slides (Sigma Diagnostic Inc.). After removing the wax with absolute ethanol, they were washed in phosphate buffer saline (PBS). In sections for immunoperoxidase staining endogenous peroxidase was blocked with H_2O_2. For both techniques, sections were blocked with 1% bovine serum albumin in PBS to avoid non-specific binding of immunoglobulins and then exposed to the following primary antibodies: rabbit anti-*Styela*-C, anti-*Styela*-D, mouse monoclonal anti-pan cytokeratin and mouse monoclonal anti-cytokeratin peptide 18 (Sigma–Aldrich, St. Louis, USA; C-1399). For examination by immunofluorescence, after washing the bound antibodies were detected using the appropriate secondary antibody conjugated to FITC, while for the immunoperoxidase stained sections, after washing the sections were exposed to the secondary antibody conjugated to peroxidase. The location of the secondary antibody was visualised using DAB/H_2O_2 as chromogen. Sections were mounted and examined with a fluorescence microscope (Olympus BX50) or an optical microscope and illustrations were produced using an Olympus PM-20 automatic photomicrographic system. Control sections were stained as above with the omission of the primary antibodies or substitution of them with inappropriate ones: a rabbit anti-*Ciona*-peptide Ci-MAM-A; anti-bcl-xl (Santa Cruz Biotechnology, Inc.); anti-mouse IgG whole molecule, and anti-human cK 14 keratin (Sigma–Aldrich, St. Louis, USA).

2.4. Electron microscopy

For routine microscopy, samples were processed by standard techniques which can be summarised as follows: fragments