Identification of the epiplasmins, a new set of cortical proteins of the membrane cytoskeleton in *Paramecium*

Pierre Nahon*, Gérard Coffe, Hervé Le Guyader, Julienne Darmanaden-Delorme, Rachel Jeanmaire-Wolf, Jean-Claude Clérot and André Adoutte

Laboratoire de Biologie Cellulaire 4 (URA D 1134 du CNRS), Bâtiment 444, Université Paris-Sud, 91405 Orsay Cedex, France

*Author for correspondence

SUMMARY

In most ciliates, the epiplasm, a superficial cytoskeletal layer of variable thickness, both surrounds basal bodies and interacts tightly with adjacent membrane networks; it constitutes the predominant structure in *Paramecium* cell ghosts. Previous indirect data suggested several cortical proteins as potential constituents of the epiplasm. New sharp monoclonal antibodies presented in this paper, positive both on immunotransfers and in immunocytochemical tests carried out on permeabilized cells and ultrathin sections, definitively identify the epiplasmins: a set of about twenty protein bands ranging from 45 to 33 kDa and making up the bulk of the epiplasmic layer. The complete epiplasmin pattern characterized from gradient-purified cortex is also present in unfractionated whole cells, confirming that the pattern is not generated artifactualy. Comparative one-step extractions, performed either in 1 M KI or in 4 M urea, solubilize the epiplasmins as a whole, indicating that all of them share very similar biochemical properties. Two-dimensional electrophoresis shows the great complexity of this epiplasmin group. Epiplasmin solubilization properties are discussed with respect to other models of membrane-cytoskeleton interaction developed among protists and metazoans and also to intermediate filaments, specially lamins. Immunofluorescent labelling combined with confocal microscopy permits a more detailed study of epiplasm formation at the level of the fission furrow, with new insights into two successive steps of epiplasm growth. A first series of interspecific reactions has been carried out with one of the anti-epiplasmin antibodies, yielding results which are discussed in an evolutionary framework.

Key words: epiplasm, monoclonal antibody, cytoskeleton, intermediate filament, membrane-interaction, cell morphogenesis

INTRODUCTION

Many unicellular eukaryotes display an extensive development of their cytoskeleton and among them, ciliates probably correspond to the phylum in which cytoskeletal complexity has reached its maximum. Of the classical cytoskeletal elements recognized in “higher” eukaryotes, namely microtubules, microfilaments and intermediate filaments, the first two types have been extensively characterized in ciliates (for reviews see Cohen and Beisson, 1988; Grain, 1986). For both tubulins and actin, molecular data support the conclusion of an overall sequence conservation with those of other eukaryotes (Dupuis, 1992; Adoutte et al., 1991; Barahona et al., 1988; Cupples and Pearlman, 1987; Hirono et al., 1987; Cohen et al., 1984; Méténier, 1984; Kaine and Spear, 1982). As for intermediate filaments, direct characterizations have not yet been reported in protists. The two strongest pieces of evidence for intermediate-like filaments concern cortical networks in a heterotrichous ciliate (Mohr et al., 1990) and filaments in the *Paramecium bursaria* micronucleus during prophase (Lewis, 1989). Preliminary screening for intermediate filament-related proteins via immunological cross-reactions has also been carried out in *Paramecium tetraurelia* (Lai and Ng, 1991; Keryer et al., 1990) and in *Pseudomicrothorax dubius* (Peck et al., 1991); however, because of the broad evolutionary distance between these organisms and metazoans, the authors have pointed out that such cross-reactions remain questionable and no true evolutionary homology has been definitively established.

In parallel to these three classical elements of the cytoskeleton, ciliates have developed a number of filamentous networks of their own; for some of these, a certain similarity of their peptidic constituents with those of metazoans has begun to emerge, either structurally or functionally. For example, in many ciliates the oral apparatus is subtended by a class of filaments known as the oral filaments, which bear some resemblance to intermediate filaments, as shown in *Tetrahymena pyriformis* by Numata et al. (1991) and by Honts and Williams (1990). Another clear example of such similarity is provided in *Paramecium tetraurelia* by a set of calcium-binding proteins, making up
the cortical filamentous network known as the infraciliary lattice, and homologous to the centris and spasmins (Garreau de Loubresse et al., 1991; Vigues and Groliére, 1985). Molecular homologies remain unknown, however, for the other cytoskeletal systems of Paramecium cortex such as kinetodesmal fibers (Sperling et al., 1991), the outer lattice (Cohen et al., 1987), striated bands (Allen, 1971) and others reviewed by Keryer et al. (1990), except for a network tentatively related to spectrin through immunoprobeing (Kwiatowska and Sobota, 1992; Williams et al., 1989).

In addition to these cortical elements, almost all ciliates display a relatively thick and dense submembraneous layer, the epiplasm (Fauré-Fremiet, 1962). The epiplasm is either located immediately beneath the plasma membrane, as in a minority of ciliate species such as the entidiniomorphids (David et al., 1991) or, as in most classes of the phylum, is located beneath membrane vesicles themselves underlying the plasma membrane, and known as the cortical alveoli (Allen, 1971; Hufnagel, 1969; Pitelka, 1965). These alveoli, which are quite typical of the surface membrane architecture of ciliates, have recently been shown to constitute a vast calcium-storing compartment (Stelly et al., 1991). In all ciliates having alveoli, the epiplasm is located beneath the inner portion of the alveolar membrane and appears tightly adherent to it (see for example, Allen (1971) and Pitelka (1965) for Paramecium; Peck (1977) for Pseudomicrothorax; Satir and Wissig (1982) and Allen (1967) for Tetrahymena). In a few ciliates devoid of alveoli, the epiplasm is located directly beneath the plasma membrane (David et al., 1991). In both cases, the epiplasm surrounds basal bodies, which appear embedded in it at the level of their terminal plates (Peck, 1977). The epiplasm can either be continuous along the anterio-posterior axis of the cell (Tetrahymena, Pseudomicrothorax) or fragmented into adjacent scales, constituting the framework of the continuous cortical units forming the cortex (Paramecium). At division, the epiplasm appears to grow by an intussusceptive mode in Paramecium (Iftode et al., 1989) as originally described in Euglena gracilis (Hofman and Bouck, 1976). Two structural criteria now appear necessary and sufficient for a broad, interspecific definition of the epiplasm: (1) a strong interaction with the adjacent peripheral membrane system, either the plasma membrane or the inner alveolar membrane and (2) a role as a cement for basal bodies (Peck, 1977). The main function attributed to the epiplasm is that of stabilizing or guiding the organization of cortical units in Paramecium, Tetrahymena, and Pseudomicrothorax (Peck, 1977) and various entidiniomorphids (David et al., 1991), and probably also with other protists such as sporozoa and dinoflagellates (see Grain, 1986). Such a structural similarity may even extend to the much more distantly related euglenoids which lack alveoli and in which a thick, dense membrane skeleton is in direct contact with the plasma membrane (Bricheux and Brugerolle, 1986; Dubreuil and Bouck, 1985). This structural similarity is not reflected by similarity in molecular mass of their constituent proteins; nonetheless, some immunological similarities are observed among epiplasms of distantly related organisms such as a ciliate, a euglenoid and a dinoflagellate, as reported by Vigues et al. (1987).

The cortex of Paramecium tetraurelia is a biochemically purifiable entity (Keryer et al., 1990). Its electrophoretic pattern of approximately 200 polypeptides extends from about 300 kDa to 18 kDa, and very few major bands are detected. Many of the proteins making up the different cortical elements have been identified, either directly from purified organelles or by immunological probes. One of the major cytoskeletal structures for which cortical proteins have not been identified is the epiplasm. Bands in the zone ranging from 45 to 30 kDa were tentatively attributed to the epiplasm, since this zone corresponded to abundant proteins not assigned to any other structure (Keryer et al., 1990). Labelling of this structure in permeabilized cells by immunofluorescence microscopy remained inconclusive, both with an anti-actin antibody (Lai and Ng, 1991) and with Lin’s monoclonal anti-actin antibody (Cohen and Beisson, 1988), since no bands were labelled on transfers. Other results appeared paradoxical with respect to the number of peptides detected on transfers, as for example the monoclonal antibody CTR211 and the anti-Pseudomicrothorax dubius epiplasm serum 18, both of which decorated by indirect immunofluorescence the epiplasm or permeabilized cells. The CTR211, initially raised against purified human centrosomes, reacted against a single cortical protein of 43 kDa on immunoblots (Keryer et al., 1990), whereas the anti-Pseudomicrothorax dubius epiplasm serum 18 identified numerous bands on immunotransfers of Paramecium cell ghosts (Peck et al., 1991). More recently, a first generation of monoclonal antibodies (mAbs) raised against the cortex of this cell produced further, albeit still indirect, evidence for identifying the component(s) of the epiplasm, since detection on immunoblots required non-reduced conditions (Jeannaire-Wolf et al., 1992).

The present paper describes the first extensive set of monoclonal antibodies directed against the epiplasm of Paramecium. These mAbs have allowed the identification of a set of closely related proteins making up the core of the epiplasm and have been used in confocal microscopy to follow the dynamics of the epiplasm during division, since data were scarce concerning the earliest stages of this process. Probing at different antibody dilutions has identified one set of proteins of higher affinity, and two-dimensional electrophoretic analysis has revealed the extreme molecular complexity of epiplasmic constituents. The likelihood of an intermediate filament-like nature of the epiplasm is discussed, following comparative one-step solubilization procedures. Finally, preliminary cross-reactions with other ciliates have been investigated using one of these new mAbs as a probe.

**MATERIALS AND METHODS**

**Strains and growth conditions**

Paramecium tetraurelia d4-2 stock strain, a derivative of the wild-type strain 51S (Sonneborn, 1975), were grown at 27°C in a wheat grass medium (Pines International Co., Lawrence, KS, USA) incubation. This medium, buffered with 0.75 g/l Tri-HCl, pH 7.1, 0.2 g/l NaH₂PO₄ and 0.75 g/l Na₂HPO₄, was bacterized the day before
use with *Aerobacter aerogenes* and complemented just before cell inoculation with 0.5 μl/ml of β-sitosterol prepared in ethanol at 10 mg/ml. Cells were harvested from early stationary-phase cultures for cortical preparation and, when dividers were required for immunofluorescence, from exponential growth-phase cultures. Other species were grown according to the method of Dragesco and Dragesco-Kernéis (1986).

**Production of antibodies to cortical Triton X-100 supernatant, the CTS fraction**

The procedures for cortex (C) purification on Percoll gradients were exactly those described by Stelly et al. (1991), yielding material at –4 mg/ml for a further solubilization carried out as follows. In Corex tubes, 1 volume of purified cortex resuspended in 200 mM Tris-maleate, 30 mM EDTA (or TME), pH 7.4, was suspended in 5 volumes of 1% Triton X-100 (TX-100: Sigma) in TME, pH 7.4, in the presence of protease inhibitors (1 mM phenylmethyl-sulfonyl fluoride (PMSF) and leupeptine at 20 μg/ml) for 15 to 30 min on ice with gentle Pasteur pipet flushing. The Triton X-100 cortical pellet (CP) was collected after 15 min at 4500 g on a RC2B Sorvall centrifuge, at 4°C. The cortical Triton X-100 supernatant (CTS), enriched in membrane proteins, also contained a residual fraction of other cortical proteins that were solubilized in Triton; CTS-preparations have regularly yielded this residual fraction for this set of CP peptides. The supernatant, representing about 5% of integral cortex proteins, was concentrated by precipitation in Eppendorf tubes, precipitated pellets were resuspended in sterile PBS, pH 7.4, for either immunization or immunodetection tests and electrophoretic analysis.

A library of mAbs was raised against this precipitated fraction. This CTS fraction, used as an immunogen, was prepared in Freund’s adjuvant for immunization. After pre-immune sera were collected from several mice, mAbs were generated following the methods for immunization described by Jeanmaire-Wolf et al. (1992) with 4 to 5 injections of CTS-antigens, of 50 μg each, per mouse.

Positive clones, issued from a single mouse, were initially screened by positive ELISA-detection against CTS samples, then identified by immunofluorescence and characterized on immunoblots. Immunoglobulin characterization was done using a typing kit (RPN 29, Amersham); four of the main monoclonals to be described, namely CTS-32, CTS-43, CTS-412 and CTS-510, have respectively been determined as being IgG of the following types: 2b, 1, 1 and 2b, each with κ light chains.

**Gel electrophoresis and immunoblotting**

SDS-PAGE was carried out according to the method of Laemmli (1970), without β-mercaptoethanol, for the first series of immunoblots. The gels were stained in 50% ethanol, 7.5% acetic acid containing 0.1% Coomassie Blue and destained in 7.5% acetic acid; the apparent molecular masses were determined by comparison with standard molecular mass markers (Amersham), following the method of Weber and Osborn (1966).

Initially, immunoblotting was carried out essentially as described by Towbin et al. (1979), using ethanol instead of methanol during liquid electrophorsetransfer. Eventually, this was replaced by semi-dry procedures: no qualitative alteration of reactivity on filters was observed. After western blotting onto 0.45 μm nitrocellulose membranes (Schleicher and Schuell), the latter were briefly stained with 0.2% Ponceau salt (Sigma) in 0.3% trichloracetic acid. After destaining in PBS, pH 7.4, transfers were blocked for 1 h at 34°C or overnight at 4°C in PBS, pH 7.4, containing 3% bovine serum albumin (BSA, Albumin Bovine Fraction V, pH 5.2; IBF). They were then incubated for 1 h at 34°C or overnight at room temperature with the mAbs in the hybridoma cell supernatants, washed 5 times in PBT (PBS, 0.3% BSA, 0.1% Tween, pH 7.4), and then incubated with anti-mouse immunoglobulins conjugated to alkaline phosphatase diluted 1: 7500 in PBT (Promega, Birtech, Madison, USA) for 30 min at 34°C. After 5 washes in PBT, colour revelation in alkaline phosphatase buffer (100 mM Tris HCl, 100 mM NaCl, 5 mM MgCl₂, pH 9.5) (APB) was obtained by successive addition of 66 μl of Nitroblue Tetrazolium (NBT, Sigma) at 50 mg/ml in 70% dimethylformamide (DMF) (BDH), plus 66 μl of 5-bromo-4-chloro-3-indolyl phosphate (BCIP, Boehringer) at 25 mg/ml in 100% DMF per 10 ml of APB. Colour development was stopped by rinsing blots in distilled water. Alternatively, anti-mouse immunoglobulins conjugated to peroxidase (Pasteur-Diagnostics), diluted 1:200 in PBT, were used; revelation was done in 100 mM Tris, pH 7.6, with 0.5 mg diamino benzidine (DAB, Sigma) and 3 μl H₂O₂ (30 vol.) per ml of Trius buffer.

The presence of post-translational modifications was also investigated. Identification of phosphoproteins was attempted by enzymatic digestion with an alkaline phosphatase according to the method of Keryer et al. (1987). The presence of glycoproteins was tested for with the use of a kit for the detection of sugars in glycoconjugates by an enzyme immunoassay involving digoxigenin labelling (DIG Glycan Detection Kit, Boehringer) and, alternatively, after 10 mM HCl extraction (Huppenlauch and Peck, 1991). Further characterizations of phosphorylated proteins were carried out thanks to generous gifts of monoclonal MPM-antibodies from P. N. Rao (Houston) and of glycosylated proteins using mAbs generously given by S. Curtenaz (Geneva). MPM-antibodies are directed against mitosis-specific phosphoproteins (Davis and Rao, 1987); the monoclonals raised against glycoproteins from the plasm of the ciliate *Pseudomicrothorax* were the 2D4, plus the 5C2 of similar spectrum, and the 2F11 (Curtenaz and Peck, 1992).

**Immunocytochemistry**

Indirect immunofluorescence microscopy on cells

Indirect immunofluorescence labelling with the anti-epiplasmin antibodies, or with MPM-3 (1:50), was performed on permeabilized *Paramecium* and *Euplotes* cells as described by Cohen and Beisson (1988). To produce ghosts, cells were treated in PHEM buffer (60 mM PIPES, 25 mM HEPES, 10 mM EGTA, 2 mM MgCl₂ at pH 6.9) containing 1% Triton X-100 for 5 min in the presence of protease inhibitors, 20 μg/ml leupeptin and 1 mM PMSF. Cell ghosts were fixed for 1 h in 2% paraformaldehyde in PHEM buffer at room temperature, washed in PBS, 10 mM EGTA, 2 mM MgCl₂, pH 7.4, then incubated (10 min) in the same buffer containing 2.5% BSA, 0.1% Tween (PB), with 3×10 min PB washes before 1 h incubation in the mAb, either undiluted or diluted in 0.3% BSA-PB buffer. After four more rinses in 0.3% BSA-PB, cells were incubated 0.5 to 1 h in the secondary antibody (FITC-labelled, goat anti-mouse Ig antibody, Pasteur-Diagnostics) diluted 1:200 in 0.3% BSA-PB buffer, and washed four times in the latter buffer before mounting in Citifluor. They were observed with a Leitz epifluorescence microscope and photographed with Agfapan 100 or Kodak Plus X 125 films.

For confocal microscopy, *Paramecium* cells were prepared as described for conventional fluorescence microscopy and observed on a Bio-Rad MRC 600 confocal laser scanning microscope (Service Commun d’Imagerie Cellulaire; Université de Paris-Sud, Centre d’Orsay). After acquisition of the series of optical sections in the z dimension, the final images were obtained by linear projection of the required number of successive horizontal sections (Shotton, 1989).

**Indirect immunolabelling on cryosections and cryoultramicrotomy**

Cells were harvested at 250 g and fixed for 2 h in 3%
parafomaldehyde, 0.05% glutaraldehyde, 50 mM sodium cacodylate buffer, pH 7.4, at room temperature before embedding in fibrinogen pellets (Charret and Fauré-Fremiet, 1967). Pellets were infiltrated with 1.8 M sucrose, 20% polyvinylpyrrolidone (PVP10,000, Sigma), PBS for cryoprotection and applied to pegs before immersion in liquid nitrogen. For semi-thin cryotomography, sections (~1 µm) were obtained with glass knives on a Cryotome (LKB) ultracryotome. They were transferred to glass slides stained previously in 0.2% Alcian Blue, where they were quenched in 50 mM NH4Cl, PBS and saturated in 1% ovalbumin, 10 mM glycine, PBS in order to reduce background, before primary incubation with polyclonal anti-surface antigen antibodies (Capdeville et al., 1992) or monoclonal anti-epiplasmins. Consecutive steps were carried out in PBS, glycine; appropriate secondary antibodies were FITC-conjugated (Pasteur-Diagnostics). For ultrathin localization and immunogold detection, frozen sections (~0.3 to 0.1 µm) were directly transferred to grids for a first ovalbumin saturation step and a second fixation step in 0.1% glutaraldehyde before the primary antibody incubation; following a second saturation step, grids were incubated with 10 nm gold-conjugated secondary antibodies at 1:100 (Auoprobe, Amersham) according to the method of R. Charret (personal communication) and to Tokuyasu (1986). They were stabilized in neutralized uranyl acetate and finally embedded in polyvinyl alcohol (PVA10,000, Sigma), uranyl acetate. Grids were observed on an Elmiskop 102 electronic microscope (Siemens) at 80 kV.

RESULTS

In Paramecium tetraurelia cortical units, a tight interaction appears to exist between the inner alveolar membrane and the cytoskeletal epiplasmic layer which subtends it. Proteins involved in membrane-cytoskeletal interaction might therefore belong to either of these two structures. In order to identify such proteins, either membraneous or cytoskeletal, a Triton X-100 solubilization was carried out on purified fragments of cortex with the aim of using the Triton-soluble fraction as an immunogen for the production of mAbs. Gradient-purified cortical fragments (C) (Fig. 1, lane A3) yielded, after a Triton X-100 extraction, the cortical pellet (lane A4) and a TX-100 supernatant, the CTS, with contaminating tubulins indicated by circles (lane A2). This supernatant is noticeably enriched in SAg and 40 kDa proteins but contains also a faint peptide triplet signalled by dots beneath 46 kDa (lane A2). The cortical pellet (lane A4) was further solubilized in 1 M KI, yielding (1) a supernatant (SKI, lane A5) enriched for the peptide triplet and the successive bands and (2) a pellet containing 40 kDa proteins resistant to the Triton X-100 treatment (lane 6). Samples of lanes A2 and A4 to 6 were probed, respectively, in lanes B2’ and B4’ to 6’ with CTS-32, one of the mAbs raised against the CTS fraction (lane A2). In each B lane, CTS-32 identifies the same major group of cortical proteins, the epiplasmins, whose segregation in KI is further analyzed in Fig. 7. Each of the three other monoclonals, CTS-43, CTS-412 or CTS-510, revealed exactly the same pattern on identical transfers. These epiplasmins are indicated by dots in lanes A2 to A5, either extensively in lane A3 or for the triplet of higher molecular mass epiplasmins in lanes A2, 4 and 5.

Identification of the epiplasmin set

Electrophoretic analysis of the immunogen used

Since the CTS represented about 5% of the total cortical proteins, it was concentrated 20-fold by precipitation in cold acetone. The efficiency of this procedure, as well as the
safety of the solubilization, were measured by the enrichment in surface antigens (SAG), large glycosylphosphatidylinositol-linked membrane proteins (Capdeville et al., 1992), which are prone to proteolysis (compare Fig. 1, lanes A2 and 3). After one or even two rounds of Triton X-100 treatment, the CTS fraction was enriched in proteins which are well-known components of the membrane, such as the SAG and the 40 kDa group (Adoutte et al., 1980; Keryer et al., 1990). It also contained a few other minor bands as seen on SDS-PAGE; one can identify the high molecular weight cytoskeletal bands 3 and 4 beneath the SAG, as well as remaining tubulins which were detected in immunoblotting (data not shown); but most of the bands, such as those present between 45 and 33 kDa (outlined by dots; Fig. 1, extensively in lane 3 and limited to the higher molecular mass bands in lanes A2, 4 and 5), were unknown based upon the reference pattern for cortical bands previously established by Keryer et al. (1990). As shown below, these bands in fact belong to the epiplasm, i.e. they correspond to a structure that is operationally defined as cytoskeletal and mostly Triton-insoluble, a small part of which is recovered in the Triton supernatant of the whole cortex.

Immunocaracterization

On immunoblots, four monoclonal antibodies reacted against the same series of bands ranging from 45 to 33 kDa from the initial immunogenic fraction, the CTS (Fig. 1, lane B2'). As expected, they yielded the same results on the integral cortex fraction (Fig. 2, lane B5), but also reacted powerfully on the CP fraction, exactly at the same molecular level (Fig. 1, lane B4'); KI treatment of the CP sample provided further indication that the antigens were corresponding to cytoskeletal constituents (Fig. 1, lanes B5' and B6').

The possibility of an artifactual molecular diversity generated by proteolysis was first investigated (Fig. 2); the four antibodies positive on CTS-immunoblots (Fig. 1) were positive as well on cryosections (see below in Fig. 3). Each yielded the same pattern of immunoreactive bands on transfers of both C and CP samples in the presence or absence of β-mercaptoethanol as a reducing agent (Fig. 2, lanes 4 to 7). In addition, the same pattern was observed on direct transfers of whole cells in the presence or absence of protease inhibitors and of detergent (Fig. 2, lanes 1 to 3); this last experiment considerably lowered the possibility of diversity of molecular mass generated by proteolysis.

Immunolocalization

In order not only to confirm that these 20 consecutive proteins are cytoskeletal but also to determine whether they make up the same cortical structure, immunocytochemical tests were carried out. Since the CTS fraction was mainly enriched in membrane proteins, immunodetection was first carried out on semi-thin cryosections of whole cells, i.e. on material with preserved membranes (Fig. 3A). With each of the four mAbs CTS-32, CTS-43, CTS-412 and CTS-510, the same labelling pattern was observed at the cell periphery; each cortical unit was specifically revealed whereas neither the cytoplasm nor cilia were decorated (Fig. 3B and C). Whether performed on 0.15% saponin- (data not shown) or 1% Triton X-100-permeabilized cells, immunofluorescence tests revealed exactly the same cortical structure, i.e. the epiplasm of an interphasic cell (Fig. 3D) and a dividing cell (Fig. 3E, under confocal microscopy). In agreement with the two criteria for defining the epiplasm, basal bodies were unstained within each cortical unit (Fig. 3G), while depressions corresponding precisely to successive epiplasmic scales were brightly decorated (Fig. 3F and H). Within

Fig. 2. Preservation of the epiplasmin pattern. CB-stained samples run on 10% SDS-PAGE (A) and corresponding immunotransfers probed with CTS-32 (B). Samples are reduced in lanes 1 to 5 and non-reduced in lanes 6 and 7, with non-reduced protein standards (Mr) in between, preventing β-mercaptoethanol diffusion. Whole cells (~400 µg per lane) have been boiled directly in sample buffer in the absence of protease inhibitors (lane 1); Triton X-100 cell ghosts have been prepared in the absence (lane 2) or presence of protease inhibitors (lane 3). Both reference cortical TX-100 pellet (lane 4) and gradient-purified cortex (lane 5) are identical to those presented in Fig. 1. In addition to the possible peptide cleavage generated during purification by TX-100 solubilization or proteases, the intensity and the effects of reduction by β-mercaptoethanol were checked by loading non-reduced fractions of purified cortex (lane 6) and TX-100 cortical pellet (lane 7). In part B, whatever the conditions of preparation, each sample reveals the same pattern as that of the reference for epiplasmins (lane 5).
the same cell, some heterogeneity of labelling is often observed, the whole epiplasmic “scale” being decorated in some areas while only the ridges of the epiplasm are decorated in others (Fig. 3D); this heterogeneity does not reflect real differences of labelling but simply results from the fact that neither the cell nor the individual units are perfectly flat; therefore, different focal levels are observed simultaneously. The curvature of each epiplasmic scale in the outermost kinety is clearly seen in sections (Fig. 3C) as well as in magnified views (Fig. 3G).

The ultimate confirmation, both for the specificity of these antibodies and the localization of their epitopes, was provided by immunogold cytochemistry on ultrathin cryosections (Fig. 4). In cross-sections, the immunostaining was specifically located over the thickness of the epiplasmic scale, beneath the inner alveolar membrane (Fig. 4A), and was interrupted both where trichocysts were docked between two successive scales (Fig. 4B) and where basal bodies were inserted at the center of a scale (Fig. 4C). Other parts of these ultrathin cryosections were totally

Fig. 3. Immunofluorescent detection. Detection was first done on cryosections (A to C) and then on permeabilized cells (D and E), including a series of confocal enlargements (F to H). A positive control of SAg labelling (A) decorates the continuous plasma and ciliary membrane with a slight cytoplasmic background, whereas the two first anti-epiplasmin antibodies presented, CTS-510 (B) and CTS-412 (C, probably on a dividing cell), label uniquely the cellular periphery and none of the cilia. On permeabilized cells, the two other anti-epiplasmin antibodies reveal the same cortical localization for both an interphasic cell with CTS-32 (D) and a dividing cell with CTS-43 (E). Confocal microscopy on cells (E) enables high magnification views of units in the anterior-ventral region (F) and along the outer kineties or rows of units (G) from interphasic cells with CTS-32, or at the level of the division furrow with CTS-412 (H). Bars, 15 µm in D and E, 5 µm in both F and G, and 10 µm in H.
devoid of background, e.g. mitochondria (Fig. 4A) or cilia (Fig. 4C).

All these results indicate that the 20 consecutive cortical protein bands identified on transfers are constitutive of the epiplasm and therefore could be related, as members of a new set of cortical proteins which we have named the epiplasmins. The *Paramecium tetraurelia* cortical pellet after Triton treatment, the CP fraction, is composed of the following cytoskeletal elements according to the numbering of Keryer et al. (1990): the striated bands corresponding to cortex bands 3 and 4, oral filaments corresponding to bands 10 and 11, kinetodesmal fibers and trichocyst bands of low molecular weight. To this list, we can now add the epiplasm corresponding to cortical bands 15 to at least 25.

**Dynamics of the epiplasm**

The new antibodies have allowed a preliminary analysis of the dynamics of the epiplasm by immunofluorescence microscopy, both at the whole cell level and that of individual cortical units.

It was striking to observe that the intensity of fluorescence was clearly not homogeneous at two different cellular levels. First, interphasic cells were frequently more strongly labelled than dividers among cells treated simultaneously (data not shown). Second, each dividing cell was not regularly decorated by the anti-epiplasmin antibodies; this cellular difference was especially evident after antibody dilution. In the two daughter cells emanating from a divider, the anterior half of the dividing cell appears to be more...
weakly labelled by antibodies, as if less epiplasmic material is present there (Fig. 5, compare A to C and B to D). Units located posteriorly to the furrow were more intensely labelled, especially at early stages of division (Fig. 5C and D). This local difference was employed to locate cells starting cortical division. Later, during cell elongation and subsequent unit and epiplasm duplication, the fluorescent signal was regular all along the cell (Fig. 3E and H).

Fig. 5. Confocal microscopic study of epiplasmic formation. Immunofluorescent images of interphasic cells (A, B, E and H) have been compared with those of dividing cells (C, D, F, G, I and J). The same antibody at the same dilution, CTS-32 at 1:100, labels homogeneously an interphasic cell both dorsally (A) and ventrally (B), whereas a dividing cell is asymmetrically decorated dorsally (C) and ventrally (D). Confocal microscopy allows the study of a single kinety (see F, which shows the optical section of a peripheral kinety). A comparison between interphasic and dividing cells has been carried out along peripherical kineties where epiplasmic scales are better visualized. For a non-dividing cell, each epiplasmic scale presents ridges clearly labelled (E and H); at early stages of division (I) and during division (G and J), new units are devoid of labelled ridges, displaying only a flat and stretched shape. Basal body insertion points are negatively stained in both cases (indicated by arrowheads, H and I). Bars, 15 µm (A to D); 4-5 µm (E, G to J); 10 µm (F).
At the cortical level and during interphase, units appeared as regular rows of depressed scales with uniform and continuous labelling both on their ridges and bottom, with an interruption only where the basal body was inserted (Fig. 5E and H). During division, it is noteworthy that at the level of the division furrow, insertion points for basal bodies were already present in the emerging unit (Fig. 5I, arrowheads), indicating that formation of these sites and implantation of the basal body within them were very early morphogenetic events in scale duplication. Following continuous incorporation, new units grew in length but remained flat (Fig. 5J). Furthermore, ridge completion took place only after these two steps of elongation and of numerical increase (Fig. 5G and J). This result confirms that a restricted number of units are formed before partitioning. In this region of the divider, the bottom remained clearly labelled while the ridges of the scale were not visible (Fig. 5G). Concomitantly, the bottom of the unit was more developed in length (Fig. 5I), indicating that the ridges of the very new unit were not yet formed at this stage of cortical morphogenesis (Fig. 5G, I and J) and suggesting that they appear later.

Biochemical properties of the epiplasmins

Differential affinity for antibodies

Identical detections have been observed on immunoblots using any of the four anti-epiplasm antibodies. In order to distinguish different degrees of affinity among the epiplasmins, both dilutions in immunoglobulins and in CP-loads were probed on transfers (Fig. 6). The intensity of detection decreased linearly for successive dilutions of the cortical loadings, i.e. under conditions in which immunoglobulins were saturating (Fig. 6A). The pattern followed the quantitative pattern observed on a Coomassie Blue-stained gel, as in Fig. 1. On the other hand, after an initial regular signal decrease, antibody dilutions revealed a higher affinity towards the first triplet of epiplasmins (Fig. 6B); after the 6th dilution, a gap appeared in the signal decrease. This was revealed particularly in the two main sets of epiplasmins, the first triplet of higher molecular mass circa 45 kDa and the last doublet circa 32 kDa (Compare for instance in Fig. 6 lanes A6 and A8, and those lanes with lanes B2 and B6).

Cytoskeletal characteristics

Cytoskeletal and membrane proteins of the whole cortex (C) displayed comparable partitioning in urea as well as in KI (Fig. 7). After 4 M urea extraction from C (Fig. 7, lane 3), two fractions were obtained: the urea-soluble one (Fig. 7, lane 2) displayed a pattern very similar to that of the Triton-treated cortex (CP), i.e. corresponding basically to all the cytoskeletal proteins, and the urea-insoluble fraction essentially contained membrane proteins, as shown by the presence of the 40 kDa proteins (arrowheads, Fig. 7, lane 1). Since SAg are very large, disulfide-bonded molecules, they penetrated into the running gel with great difficulty and the presence of material at the top of the corresponding lanes probably represent these proteins (Fig. 7, lane 1 particularly). Conversely, the membrane protein markers were absent from the urea-soluble fraction (Fig. 7, lane 2). Extractions with 1 M KI were performed directly on CP.

The KI supernatant (Fig. 7, lane 5) resembled the CP fraction, apart from a pair of bands present in the CP fraction, referred to as numbers 10 and 11, which were insolubilized (indicated by squares in Fig. 7, lane 4). In addition, the KI pellet revealed the presence of residual 40 kDa proteins (Fig. 7, triangles lane 4). It is important to note that in both cases there was no differential segregation for any of the epiplasmins which were homogeneously solubilized by urea and KI (Fig. 7, lanes 2 and 5, respectively).

Putative post-translational modifications

Search for glycosylation and phosphorylation

Cells were extracted with 10 mM HCl; this yielded a supernatant enriched with membrane proteins but none of the usual CP components including the epiplasmins (data not shown). A parallel test with an anti-DIG for the detection of glycoproteins remained negative on the epiplasmins. In addition, cross-reactions with mAbs kindly provided by Drs S. Curtenaz and R. K. Peck were assayed; those antibodies specifically directed to glycosylated epitopes from the epiplasm of *Pseudomicrothorax dubius* were negative on transfers of *P. tetraurelia* cortices (data not shown).
The three mAbs raised against mitosis-specific phosphoproteins, MPM-1, -2 and -3 (Davis and Rao, 1987), were tested in the present work with special emphasis on the epiplasmins. Kinetodesmal fiber proteins and oral filament proteins served as positive internal controls (Sperling et al., 1991; Keryer et al., 1987). MPM-2 yielded an orthodox immunofluorescent decoration on dividing as well as non-dividing cells, whereas our MPM-1 tests remained negative with regard to the epiplasm both on cells and transfers (data not shown). In the presence of a phosphatase inhibitor (20 mM NaF), MPM-3 primarily reacted with the ciliary rootlets during division and gave only a weak cortical decoration at interphase on permeabilized cells (Fig. 8A and B). When immunotransfers of CP samples were treated with an alkaline phosphatase, all epiplasmins were detected by antibodies raised against them (data not shown), and within the zone of molecular size comprising the epiplasmins, only

MPM-3 revealed that a faint band had disappeared after alkaline phosphatase digestion (data not shown).

**IEF analysis and two-dimensional immunoblotting**

The CP fraction was submitted to two-dimensional analy-
Monoclonals to Paramecium epiplasm, according to the method of O’Farrell (1975), and gave, as expected from the one-dimensional electrophoretic results, a highly complex pattern of 60 or more spots (Fig. 9A), including putative isoforms. The epiplasmins of higher molecular mass were the richest in spots and the lower molecular mass epiplasmins were scattered in fewer, less basic spots (Fig. 9B). Antibody dilution revealed a linear distribution for the set of higher molecular mass epiplasmins, for which the corresponding spots covered a range of several pH units (Fig. 9C).

Interspecific reactivity and evolutionary comparisons

Preliminary cross-reactions were assayed with the CTS-32 mAb, mainly on immunotransfers (Fig. 10), but also on permeabilized cell ghosts of several other ciliates. For immunotransfers, the positive control was performed against a CP sample (Fig. 10, lane 4). CTS-32-labelled Paramecium caudatum, a species of the same genus as Paramecium tetraurelia, was assayed in an immunofluorescent test (Fig. 11A).

Going to increasingly distant ciliates (Baroin et al., 1988; Baroin-Tourancheau et al., 1992), there was no significant cross-reaction with the oligohymenophoran Tetrahymena pyriformis, a species belonging to the same class as Paramecium tetraurelia, either on blots (Fig. 10, lane 1) or on cell ghosts (data not shown). Cross-reactivity on immunoblots was also negative using extracts of Colpidium campylum, another representative of the oligohymenophoran ciliates (Fig. 10, lane 2), and extracts of the hypotrich Paraurostyla weissei (Fig. 10, lane 6).

Positive cross-reactions were obtained, however, with the nassophorean ciliate Pseudomicrothorax dubius on transfers (Fig. 10, lane 3); this was expected, since antibodies...
from polyclonal sera (Peck et al., 1991), as well as mAbs raised against its epiplasm (S. Curtenaz, personal communication), were positive on *P. tetraurelia*. Immunoblots were positive after a five-fold dilution of the sample on transfers, with or without β-mercaptoethanol (data not shown). But these detections were not corroborated by labelling on cells.

Positive cross-reactions on transfers were also obtained with the hypotrich ciliate *Euplotes aediculatus* and might be one of the most significant (Fig. 10, lane 5), since the protein load was one of the lowest. The complementary reaction on *Euplotes* cells, however faint, was precisely located within alveoli, suggesting a reaction with alveolar plates (Fig. 11B), the main cytoskeletal structure within the alveoli of this ciliate otherwise devoid of epiplasm; thus, it is tempting to speculate that the bands detected might correspond to the plateins, the proteins making up the plates (Kloetzel, 1991). Finally, a positive signal was surprisingly obtained with the very distant euglenoid flagellate *Euglena acus* (Fig. 10, lane 7).

In summary, some cross-reactions were observed within ciliates, but they do not follow a simple pattern compared to the known genetic distances between the species considered, and a positive reaction with a very distant flagellate was also noted.

**DISCUSSION**

**Multiplicity of the epiplasmin bands**

Biochemical unity of the epiplasmin set

Ciliates are very highly differentiated in their cytoskeletal structures (Grain, 1986). It appears that, at least in *Paramecium*, they have frequently resorted to the use of multiple genes encoding for the structural proteins of major organelles present in the cortex of the cell. Thus, the crystalline matrix of trichocysts could be made up of more than 100 distinct peptides (reviewed by Adoutte, 1988 and discussed by Shih and Nelson, 1991); the kinetodesmal fibers are composed of a group of 36–30 kDa polypeptides (Sperling et al., 1991), and the infraciliary lattice of a major doublet of 24-23 kDa polypeptides (Garreau de Loubresse et al., 1988). In the present paper, we provide evidence that the same situation holds for the epiplasm, an important cytoskeletal element of the cortex not characterized biochemically in previous reports.

Within the electrophoretic pattern of the whole cortex, the epiplasmic proteins represent a vast fraction in terms of range of molecular mass, i.e. from 45 kDa to 33 kDa. In the presence or absence of proteolytic inhibitors, or of a reducing agent, remarkably identical electrophoretic and immunotransfer patterns are obtained; these results considerably lower the possibilities of artifactual peptide-severing during cortical purification on gradients, or simply of incomplete SDS-PAGE separation. Detection on immunoblots, using either whole cells boiled directly in SDS or permeabilized ghosts, in the presence or absence of protease inhibitors, also suggests an unusual resistance to proteolysis. Parallel urea- and KI-solubilization reveals, in each case, the same epiplasmic pattern of twenty peptides ranging from 45 to 33 kDa. Not only do these results strongly suggest that the epiplasmin molecular heterogeneity is not artifactual, but they also illustrate a remarkable biochemical homogeneity.

The twenty or so epiplasmins comprise a single cortical structure, perhaps due only to intrinsic molecular properties which permit self-assembly. This biochemical unity accompanies similar immunoreactive behaviour on transfers. The striking result yielded by several mAbs is that each of them labels identically the vast set of polypeptides biochemically corresponding to the epiplasm. Since within this family all polypeptides are characterized by at least (1) the presence of a common determinant and (2) similar solubilization behaviour, the question arises whether the epiplasmins constitute a family of genetically related proteins. One extreme hypothesis is that epiplasmin molecular diversity represents the unmodified products of numerous related, but nonetheless different, genes. According to this hypothesis, the presence of a common epitope on each peptide of the epiplasmin set would be the mark of an evolutionary conservation among the genes encoding these proteins; variations in the number of copies of a common peptide motif would then partially account for the molecular mass variations displayed within this set.

The only heterogeneity among this otherwise extremely homogeneous set is revealed by antibody dilution experiments. When diluted, all antibodies used show a greater...
apparent affinity for the three epiplasmins of higher molecular mass, either because these protein bands are the most concentrated or, more probably, because each of them bears several copies of the epitope for which the anti-epiplasmin antibodies are specific (Figs 6 and 9). Both interpretations could fit with a common origin for the diversity characterizing this set.

Putative post-translational modifications

Nonetheless, in view of the molecular variation among the epiplasmins and also of functional examples within *P. tetraurelia* or other cell types, the alternative hypothesis of post-transcriptional modifications was considered. Direct 2D analyses on a CP sample or on a SKI sample after antibody dilution suggest the presence of post-translationally modified forms among the epiplasmins of higher molecular mass in the form of a series of spots of decreasing intensity.

Phosphorylation is the first type of modification we analyzed; a parallel is provided by protein constituents of the lamina or of neurofilaments. Biochemically, neurofilament proteins constitute a spectacular example of molecular mass shifts due to extreme phosphorylation (Carden et al., 1987); this situation could account, for instance, for the continuous size distribution of the epiplasmins from 45 to 33 kDa. Furthermore, epiplasmic scales present a structural resemblance with a metazoan model of membrane-cytoskeleton interaction, which is the nuclear lamina (Gerace and Blobel, 1980). A well-studied precedent for the generation of electrophoretic diversity by phosphorylation is found in *Paramecium* itself with the ciliary rootlets, the components of which undergo a wave of intensive phosphorylation during division for their disassembly and reassembly, as revealed by their cross-reactivity towards the MPM-2 antibody (Sperling et al., 1991).

Direct probing with the MPM-3 antibody did not demonstrate phosphorylation of the epiplasm; similarly, single digestion with an alkaline phosphatase yielded no positive evidence (data not shown). The occurrence of phosphorylation of the epiplasmins therefore remains doubtful at the moment; the only evidence obtained so far is the immunofluorescent decoration with the MPM-1 antibody, corroborated by a detection on transfers (Keryer et al., 1987), possibly corresponding to the epiplasmins of higher molecular mass.

As for the possibility of glycosylation, unusual types of such modifications have been described for nuclear and cytoplasmic proteins (for a review, see Hart et al., 1988) and for cytkeratins, involving O-linked sugars (King and Hounsell, 1989); the presence of glycoproteins in the epiplasm of a protist has been recently reported (Huttenlauch and Peck, 1991). Direct evidence demonstrates, however, that these glycoproteins are located at the outer surface of the epiplasm and most probably correspond to membrane proteins tightly bound to the epiplasm (Curtenaz and Peck, 1992). Evidence for glycoproteins among epiplasmins was negative in *Paramecium*.

In summary, the search for post-translational modifications within the epiplasmins has so far been essentially negative. This favours, provisionally, the hypothesis of a direct, genetic origin for the epiplasmin diversity.

**Cortical morphogenesis and membrane interaction**

The confocal study of cortical unit formation gave a detailed picture of the two stages of elongation and partitioning, previously described by Sonneborn (1970). Immunofluorescent labelling revealed that epiplasmic ridges are formed sequentially, since a restricted number of ridgeless units were visible, and also provided indications that not all ridges are completed simultaneously. Within growing units, epiplasmic ridges next to the furrow seemed to appear later than those on the other side of the epiplasmic scale, indicating a possible differentiation gradient.

A difference of labelling was observed between the two halves of a dividing cell (Fig. 5C or D). The anti-epiplasmin antibodies which constitute the first direct probe available against the epiplasm thus revealed a variation in the distribution of epiplasmic material at an early stage of cortical morphogenesis. This asymmetry possibly illustrates an early phase of the mosaic mode of duplication typical of the cortical elements in *P. tetraurelia*, where successive waves of duplication are initiated from the fission furrow (Iftode et al., 1989). First, a qualitative variation is conceivable; epiplasmins could be integrated within units, for instance under a biochemical form of high “immunoreactivity”; this qualitative option postulates that epiplasmins are partially modified once duplication occurs. Second, in situ interactions might be involved as cortical growth progresses; one expects a rapid entry of epiplasmins into stable molecular interactions, and therefore a corresponding decrease in the accessibility of these proteins to antibodies in units no longer duplicating. Such a process could reproduce the model of intermediate filament associations (discussed later), or the model of membrane-cytoskeletal associations (see below).

Within each cortical unit, the membrane-interaction developed by the epiplasmic scales resembles some models elaborated for metazoans. At first sight, it evokes the cytoskeletal organization of spectrin-networks beneath the erythrocyte membrane (Lazarides, 1987), as first suggested by Williams et al. (1979). The epiplasm is a direct support as a true cytoskeleton, but also constitutes an indirect support as a matrix integrating cortical elements into a structurally coherent ensemble (Peck, 1977). Spectrin is involved in stabilizing the infrastructure of cell membranes by its supportive role in the submembraneous skeleton (Marchesi, 1985), and cross-reactions of a 70 kDa cortical protein have been reported with an anti-spectrin antibody (Williams et al., 1989). Recently, the presence of another 240 kDa immunoanologue of vertebrate spectrin has been reported in *Paramecium* cells, with immugold detection, beneath the inner alveolar membrane and on the surface of trichocyst tips (Kwitowska and Sobotka, 1992), but not along the epiplasmic rail.

Epiplasmins behave as proper cytoskeletal constituents, in view of their resistance to non-ionic detergent on the one hand and of their homogeneous solubilization, both in urea and in KI, on the other hand. Such urea- and KI-supernatants of *Paramecium* precipitated when dialyzed against low ionic strength buffers (data not shown), as tettrins of *Tetrahymena* (Hunts and Williams, 1990) or articulins, the
Euglena epiplasmic proteins (Marrs and Bouck, 1992; Dubreuil and Bouck, 1988; Bricheux and Brugerolle, 1986), do in such ionic strength conditions. In addition, selective separation of the complete epiplasmic series was obtained by SDS-free PAGE (G. Coffe, unpublished observation). Epiplassins represent the major structural components of the epiplasm; the other putative components of the epiplasm, i.e. those closely interacting with the inner alveolar membrane, have not yet been identified. Possible candidates for this interaction have however been strongly suggested by immunogold localization with some mAbs. For example, the monoclonal antibody I4B7 predominantly labels the outer surface of the epiplasm, i.e. a “fluffy layer located immediately on top of it”, precisely where the inner alveolar membrane is found (Jeannaire-Wolf et al., 1992). It should be recalled that I4B7 and CTS-32 cross-react with Paramecium caudatum. In Pseudomicrothorax dubius, whose complex epiplasm includes glycoproteins (Peck et al., 1991; Huttenlauch and Peck, 1991), mAbs have recently localized these glycoproteins precisely as “confined to the outer part of the epiplasm, which underlies the inner alveolar membrane” (Curtenaz and Peck, 1992). The situation with the I4B7 epitope and the epiplassins within P. tetraurelia presents a strong parallel with this example.

The question of homology

Among various ciliates and some other protists, the epiplasmic layer shows a striking structural resemblance, since it is systematically adjacent to a cortical membrane, either the plasma membrane for flagellates and entidiniomorphid ciliates or the inner alveolar membrane for all other ciliates (Peck et al., 1991); it might be functionally related in all these different organisms devoid of a cell wall. These apparent similarities have raised the question of whether the proteins involved are truly homologous, being derived from a common ancestral peptide (or even a few related ones) or whether, during evolution and speciation, the same strategy could have been adopted independently several times, thus leading to analogous structures.

Viguès et al. (1987) established the existence of common epitopes among proteins of the membrane-skeleton of the ciliate Pseudomicrothorax dubius, the euglenoid flagellate Euglena acus and the dinoflagellate Nocitula scintillans, in spite of the heterogeneity in molecular mass of the epiplassmic proteins of these three species. This concept of similar structures made up of different molecules, at least in terms of their molecular mass, is also confirmed for more closely related ciliates (Williams, 1986; Peck et al., 1991). Numerous immunological screenings against epiplassmic structures or similar structures have been reported among protists (for a review, see Cohen and Beisson, 1988; David et al., 1991; Grain, 1986; Peck et al., 1991), including Paramecium (Lai and Ng, 1991). In spite of a generalized molecular mass diversity, a common feature for the proteins involved is their insolubility, and it is postulated that some peptidic domains have been highly conserved (Viguès et al., 1987). Putative duplications in multiple copies of these domains would then account for a part of the molecular mass heterogeneity; it is noteworthy that the occurrence of such duplications has now been demonstrated in the gene coding for the major constituent of Euglena gracilis epi-

plasm, in the form of 30 repeats of a 12-amino acid motif (Marrs and Bouck, 1992).

In spite of the reported immunological cross-reactions, however, the homology of epiplassm proteins remains incompletely determined for the moment, because the cross-reactions display unexpected patterns with respect to the known genetic distances separating the organisms probed (Baroin et al., 1988; Baroin-Tourancheau et al., 1992). It is surprising, for example, that the anti-Paramecium antibody reacts with Euglena acus but fails to do so with much closer species, especially Tetrahymena and Colpidium. Further direct sequencing may determine to what degree these cross-reactions are due to the possible evolutionary conservation of common epitopes or to convergence towards similar amino acid motifs.

Molecular mass diversity is no longer in contradiction with structural and even functional similarities in the case of intermediate filaments (Skally and Goldman, 1991). Epiplassms behave as intermediate filament-like proteins, with respect mainly to their biochemical properties and to their possible interspecific distribution. One additional point may be mentioned; their in situ association with adjacent membrane systems is reminiscent of other networks of membrane-associated intermediate filaments within metazoans, the most striking case being that of lamins (Ulitzur et al., 1992; reviewed by Steinert and Roop, 1988). However, the test with the anti-IFA (Pruss et al., 1981) indicates that one consensus region of intermediate filaments is absent from Paramecium (Keryer et al., 1990) and the sequence data on articulins do not reveal IF-like patterns. Again, further biochemical and sequencing work is required to establish whether these similarities between IF and epiplassm protein correspond to convergence or whether the evolutionary homologues of metazoan IFs have evolved in the form of epiplassins in protists.

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