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Articulins and epiplasmins: two distinct classes of cytoskeletal proteins of the membrane skeleton in protists

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SUMMARY

The cortex of ciliates, dinoflagellates and euglenoids comprises a unique structure called the epiplasm, implicated in pattern-forming processes of the cell cortex and in maintaining cell shape. Despite significant variation in the structural organization of their epiplasm and cortex, a novel type of cytoskeletal protein named articulin is the principal constituent of the epiplasm in the euglenoid *Euglena* and the ciliate *Pseudomicrothorax*. For another ciliate, *Paramecium*, epiplasmins, a group of polypeptides with common biochemical properties, are the major constituents of the epiplasm. Using molecular tools and affinity purification we have selected polyclonal antibodies and identified epitopes of monoclonal antibodies that identify epitopes characteristic of articulins and epiplasmins. With these antibodies we have analysed the occurrence of the two types of cytoskeletal proteins in a dinoflagellate, a euglenoid and several ciliates. Our results indicate that both articulins and epiplasmins are present in these organisms, suggesting that both contribute to the organization of the membrane skeleton in protists. Articulins and epiplasmins represent two distinct classes of cytoskeletal proteins, since different polypeptides were labeled by articulin core domain-specific or epiplasmin epitope-specific antibodies in each organism studied. In one case, a polypeptide in *Pseudomicrothorax* was identified that reacts with both articulin core domain-specific and with anti-epiplasmin monoclonal antibodies; however, the epiplasmin monoclonal antibody epitope was mapped to the C terminus of the polypeptide, well outside the central VPV-repeat core domain that contains the articulin monoclonal antibody epitope and that is the hallmark of the articulins.

Key words: Articulin, Epiplasmin, Cytoskeleton, Membrane skeleton, Epiplasm

INTRODUCTION

Microfilaments, microtubules and intermediate filaments are the principal cytoskeletal elements in most eukaryotic cells. Each of these three filamentous systems is composed of a distinct class of cytoskeletal proteins and their associated proteins. Microfilaments are made of actin, microtubules of tubulins and the intermediate filaments of intermediate filament (IF) proteins. IF proteins show the greatest diversity, and they form a large family of related proteins (Fuchs and Weber, 1994). Microfilaments and microtubules have been extensively characterized in many protists. For both actins and tubulins, molecular data support the conclusion of an overall sequence conservation among eukaryotes (Baldauf and Palmer, 1993; Keeling and Doolittle, 1996; Ludueña, 1998). In contrast, the occurrence of IF proteins in protists is not yet settled, and many of the filamentous systems described in protists do not fall into either actin or IF categories (see, for example, Honts and Williams, 1990; Levy et al., 1996).

Protists are among the eukaryotes that possess the most elaborate cell shape and surface architecture. The latter is composed of precisely positioned cortical organelles, including cilia with their associated basal bodies, parasomal sacs, alveoli, mitochondria, and secretory structures such as trichocysts and mucocysts (for reviews see Grain, 1986; Bouck and Ngô, 1996). It is presently unknown how these cells maintain their specific cell shape and cortical organelle pattern, which are faithfully reproduced at each cell division; however, there is some evidence that the membrane skeleton is directly involved in these processes (Peck, 1977, 1986; Aufderheide, 1983; Dubreuil and Bouck, 1988; Williams and Honts, 1987; Kaczanowska et al., 1993).

In many ciliates, dinoflagellates and euglenoids, the membrane skeleton is a discrete cortical structure called the epiplasm (for a recent review, see Bouck and Ngô, 1996). It is generally a semi-rigid, prominent proteinaceous layer that is closely apposed to a cortical membrane. In most ciliates the epiplasm lies principally below, and in contact with, the inner membrane of the alveolus, a membrane system located beneath the plasma membrane. In dinoflagellates, the epiplasm may lie both within and below the amphiesmal vesicles, the equivalent of the ciliate alveolus, whereas in euglenoids such as *Euglena*, which lack an alveolus, the epiplasm lies directly below, and in contact with, the plasma membrane.

The epiplasm of the ciliates *Tetrahymena* and *Pseudomicrothorax* is continuous throughout the cortex, while in the ciliate *Paramecium* it is separated into individual adjacent scales that are connected laterally by a continuous 'outer lattice' of fine filaments. In *Euglena*, the epiplasm is organized in a series of longitudinal, narrow stripes. Each stripe articulates with its neighbor, and sliding of the stripes relative to one another is involved in the rapid shape changes known as euglenoid movements (Suzaki and Williamson, 1985).

The development of various extraction and fractionation procedures for the isolation of pure epiplasm enabled the major components of this structure to be identified by SDS-PAGE for several species (Vigues et al., 1984; Dubreuil and Bouck, 1985; Bricheux and Brugerolle, 1986; Peck et al., 1991; Huttenlauch and Peck, 1991). In other cases, where the epiplasm has not been isolated in pure form, individual SDS-PAGE bands of cortical preparations have been identified by immunolabeling as constituents of the epiplasm (Williams et al., 1987; Vigues and David, 1989; Keryer et al., 1990; Jeanmaire-Wolf et al., 1993; Nahon et al., 1993). SDS-PAGE of the cortex or the epiplasm generally reveals many different molecular mass bands, with a pronounced interspecific variation in banding pattern, even among closely related species that are nearly identical morphologically (see, for example, Williams et al., 1984). Polyclonal and monoclonal antibodies (mAbs) directed against epiplasm proteins have been used to identify common epitopes on different epiplasm proteins, often from distantly related genera (Vigues et al., 1987; Lai and Ng, 1991; Nahon et al., 1993; Curtenaz et al., 1994; Sghir and David, 1995). The results suggest that there may be conservation of primary sequence or of secondary structure of specific protein domains, raising the question of common ancestry for several of these proteins.

The first epiplasm proteins to be characterized molecularly were the two principal constituents of the epiplasm of the euglenoid *Euglena gracilis*, which were given the name articulins (Marrs and Bouck, 1992). A clear demonstration of epiplasm protein conservation was brought to light when a cDNA for an epiplasm protein of the ciliate *Pseudomicrothorax dubius*, named p60, was sequenced, revealing striking similarity to the *Euglena* articulins (Huttenlauch et al., 1995). p60 is a quantitatively minor component of the epiplasm of *Pseudomicrothorax*, but more recently, the cDNA sequences of the two major proteins of the epiplasm of *Pseudomicrothorax* have been determined, and they too are very similar to the *Euglena* articulins (Huttenlauch et al., 1998). The similarities among the three ciliate proteins and the two euglenoid proteins clearly demonstrate that they all belong to a single protein family, the articulins.

All articulins have a tripartite organization, in which the central core domain is characterized by repetitive motifs of alternating valine and proline residues, the VPV-motif. The VPV-motif is unique to articulins, and is the hallmark of these proteins. Articulins do not show significant sequence similarity to other cytoskeletal proteins nor to other known proteins in general (for a review see Huttenlauch et al., 1998).

Molecular and immunological characterization of the epiplasm of some other species shows that, at least in part, it is composed of proteins that may be distinct from the articulins. The best characterized of these are three polypeptides of the ciliate *Tetrahymena* (Williams et al., 1995) and a group of polypeptides named epiplasmins of the ciliate *Paramecium* (Nahon et al., 1993). The three *Tetrahymena* proteins are not labeled by mAbs 4B5F3 or 1E11F5, both of which label nearly all of the epiplasm proteins of *Pseudomicrothorax* (Curtenaz et al., 1994), including the 78-80 kDa polypeptides identified as articulins (Huttenlauch et al., 1995, 1998). The major constituents of the epiplasm of *Paramecium* are the epiplasmins, a group of 30-50 kDa polypeptides with similar biochemical properties. Although the available short partial sequences of epiplasmins show that they are rich in valine and proline (Coffe et al., 1996), they do not display the characteristics of VPV-motifs, i.e. the alternation of valine and proline residues and the charge distribution typical of articulins.

We have analysed the occurrence of articulins and epiplasmins in the dinoflagellate *Amphidinium carterae*, the euglenoid *Euglena gracilis* and in the ciliates *Paramecium caudatum*, *Paramecium tetraurelia*, *Euplotes aediculatus* and *Pseudomicrothorax dubius*. The two types of proteins were detected by immunoblotting of total cellular proteins or of purified cortical or epiplasm preparations. Articulins were detected by a mAb that recognizes an epitope within the central VPV-repeat domain of articulins, and by polyclonal antibodies that were affinity selected against the bacterially expressed central VPV-repeat domain of *Pseudomicrothorax* articulin p60. Epiplasmins were detected by a mAb raised against *Paramecium* epiplasmins and by polyclonal antibodies that were affinity selected against epiplasmins of *Paramecium tetraurelia*. In most species, articulin-reactive polypeptides have relative molecular masses of 60 to >130 kDa, and epiplasmin-reactive polypeptides have relative molecular masses of 30-60 kDa. In all species that we analysed, both types of proteins were detected. In all species, except for *Pseudomicrothorax*, articulin- and epiplasmin-reactive bands are different, indicating that the epitopes reside on different polypeptides. Notably one of the *Pseudomicrothorax* articulins, articulin 1, reacts with both anti-articulin and anti-epiplasmin mAbs. The epitope that is recognized by the anti-epiplasmin mAb maps, however, to the C-terminal domain of articulin 1 rather than to the central VPV-repeat domain in which the articulin-specific mAb epitope resides. Our results demonstrate the widespread occurrence of both articulins and epiplasmins in protists, suggesting that both types of proteins contribute to the organization of the membrane skeleton in these unicellular organisms.

MATERIALS AND METHODS

Cells, culture conditions and cortical or whole cell preparations

Amphidinium carterae strain B 37.80 from the Sammlung von Algenkulturen, Universität Göttingen, was cultivated in Brackish Water Medium (Schlösser, 1982) at 22°C and illuminated with Osram L-Fluora lights at 400 lux for 12 hours/day. 10 ml of cells were harvested by centrifugation at 150 ***g*** for 10 minutes in bulb tubes (Peck, 1992), the contents of the bulb, which included the cells, were transferred to a Nissel tube and centrifuged for 1 minute at 150 ***g***. The pellet was dissolved in the Nissel tube with mixing in 2× concentrated SDS-PAGE sample buffer in a boiling water bath for 5 minutes. During boiling, 7 μl of EDTA (100 mM) and 40 μl of water were added. Immediately after boiling, 7 μl 10 mM p-chloromercuriphenyl-

sulfonic acid and 7 μl 100 mM PMSF were added with mixing and the sample was frozen at –30°C until use.

Euglena gracilis was cultivated in an organic *Chlorogonium* medium (Peck et al., 1975) at 22°C with 400 lux illumination for 12 hours/day. 5 ml of cells were harvested by centrifugation for 1 minute at 150 ***g*** in bulb tubes and the pellet was resuspended in 10 ml of an inorganic medium (0.5 mM $CaCl_2$, 0.05 mM $MgSO_4$, 0.11 mM KH_2PO_4 and 0.16 mM K_2HPO_4) and maintained under temperature and light conditions as for growth in the organic medium. After 12-24 hours cells became elongated with a typical *Euglena gracilis* morphology and movement. These cells were then harvested by centrifugation at 150 ***g*** for 1 minute in bulb tubes and the pellet was prepared for SDS-PAGE as described for *A. carterae*.

Pseudomicrothorax dubius strain N5b was cultivated, harvested and the epiplasm isolated as described (Peck et al., 1991).

Euplotes aediculatus was cultivated in the phosphate-buffered inorganic medium employed for *Pseudomicrothorax dubius* (Peck, 1977) and fed *Chlorogonium elongatum*. The latter was cultivated as described for *E. gracilis*. For *Euplotes* cortical preparations, the Triton high-salt method of Williams et al. (1989) was modified as follows. Stationary-phase cells were harvested by continuous-flow centrifugation and then further concentrated by centrifugation in bulb tubes at 500 ***g*** for 3 minutes. 10 ml of cold (6-8°C) modified Triton high-salt buffer (1.5 M KCl, 1% (w/v) Triton X-100, 2 mM EDTA, 0.2 mM PMSF) were added to the pellet. Phase-contrast observation revealed well-preserved cortexes with minimal cytoplasmic contamination. The cortexes were harvested by centrifugation at 2000 ***g*** for 10 minutes and the pellet was resuspended in 2 ml cold buffer containing 20 mM NaH_2PO_4-Na_2HPO_4, 2 mM EDTA, pH 6.4. The cortexes were centrifuged in a microfuge at 6000 ***g*** for 3 minutes and the pellet was dissolved in SDS-PAGE sample buffer in a boiling water bath for 3 minutes.

Paramecium caudatum and *Paramecium tetraurelia* were cultivated in lettuce medium (Hiwatashi and Watanabe, 1968) bacterized with a non-pathogenic strain of *Klebsiella pneumoniae*. Stationary-phase cells were harvested by continuous flow centrifugation, briefly rinsed twice with distilled water in the continuous flow rotor and resuspended to a total volume of 10 ml in distilled water. 50 ml of cold (8-10°C) isolation medium (34.2% (w/v) sucrose, 2 mM EDTA, 40 mM Tris, 0.1% (v/v) 2-mercaptoethanol) was added. Following mixing on a magnetic stirrer for 2 minutes, 1 ml of 2% (w/v) Triton X-100 was added and stirring was continued for 1 minute more. 50 ml of cold buffer (20 mM NaH_2PO_4-Na_2HPO_4, 2 mM EDTA, pH 6.4) was added and stirring was continued for 1 minute more. Cortexes were harvested by centrifugation at 2000 ***g*** for 10 minutes at room temperature. The cortical pellet was resuspended in the above buffer containing 12% (w/v) sucrose and centrifuged again. This rinse step was repeated once more in a microfuge tube, the cortexes were centrifuged at 5000 ***g*** for 3 minutes, and the pellet was dissolved in SDS-PAGE sample buffer in a boiling water bath for 3 minutes.

Tetrahymena pyriformis GL was cultivated at 22°C in 1.5% (w/v) proteose-peptone and 0.25% (w/v) yeast extract. Early stationary-phase cells were harvested and cortical preparations were made as described above for *Paramecium*.

Electrophoretic procedures and immunoblotting

SDS-PAGE using 8-12% (w/v) polyacrylamide slab gels and two-dimensional gel electrophoresis was performed as described (Huttenlauch and Peck, 1991). Electrophoretic transfer and immunolabeling of gel blots was carried out as described (Stick, 1988). Secondary antibodies were peroxidase-conjugated goat anti-mouse IgG or goat anti-rabbit IgG (Dianova, Germany) diluted 1:2500. The immunocomplexes were detected by chemiluminescence using the Super signal CL-HRP substrate system (Pierce, USA) according to the instructions of the manufacturer, and ECL Hyperfilm (Amersham, UK).

Antibodies and affinity selection

The mAb 4B5F3 raised against a subfraction of the epiplasm of *Pseudomicrothorax dubius* has been described (Curtenaz and Peck, 1992) and was kindly provided by S. Curtenaz. The mAb CTS-32 raised against the Triton X-100 supernatant of cortical preparations of *Paramecium tetraurelia*, was kindly provided by A. Adoutte and G. Coffe, and has been described (Nahon et al., 1993). This antibody recognizes a set of epiplasm proteins of *Paramecium* called epiplasmins.

For affinity selection, a rabbit antiserum (serum 018) raised against SDS-denatured epiplasm proteins of *P. dubius* was used (Peck et al., 1991). For the affinity selection of articulin VPV-repeat motif-specific antibodies the central VPV-repeat motif domain of *P. dubius* articulin p60 was expressed as a fusion protein in *E. coli* BL 21 cells (see below). 8-9 slots (slot width 8 mm) of a 12% polyacrylamide gel were charged with a lysate of entire bacterial cells. Proteins were separated and transferred to nitrocellulose sheets. The position of the fusion protein was identified by immunostaining of small strips from the sides of the blot with serum 018 and alkaline phosphatase-conjugated goat anti-rabbit IgG (Dianova) as secondary antibody. Immunocomplexes were detected with a color reaction with bromo-chloro-indolylphosphate and nitro-blue tetrazolium. A horizontal strip (4-5 mm wide) corresponding to the position of the fusion protein was cut out of the unlabeled blot and used as an affinity matrix. Nitrocellulose strips were blocked for 15 minutes in 0.05% Tween-20 in PBS (137 mM NaCl, 3 mM KCl, 1 mM KH_2PO_4, 6 mM Na_2HPO_4, pH 7.1) and for 90 minutes in 5% nonfat dry milk in Tween-PBS, and then incubated overnight at 4°C in serum 018 diluted 1:1000 in Tween-PBS. The strip was then washed for 30 minutes in Tween-PBS with several changes of buffer. For elution of the bound antibodies the strip was incubated at room temperature for 5 minutes in 3.0 M KSCN in PBS (final pH 6.0-6.5). The eluted antibodies were immediately diluted to 1.0 M KSCN with 0.1% BSA in PBS-Tween, and dialyzed overnight against PBS. In some experiments additional washes of the strips with Tween-PBS containing 0.5 M NaCl followed by Tween-PBS containing 0.5% Triton X-100 were done to select for high affinity antibodies. Each nitrocellulose strip was used at least three times for antibody purification without recognizable reduction of binding capacity. The dialyzed antibodies were used diluted 1:1 with 0.1% BSA in Tween-PBS or used undiluted. Control preparations were done with strips cut from the corresponding region of gel blots obtained after separation of lysates from non-transformed bacteria.

Affinity selection of epiplasmin epitope-specific antibodies was also done with serum 018. Strips were cut from gel blots (14-16 slots, slot width 4 mm) of *Paramecium tetraurelia* cortical preparations, and the remainder of the affinity absorption procedure was as described above for articulin p60.

Plasmid construction and protein expression

To express the central VPV-repeat motif domain of *P. dubius* articulin p60 in *E. coli*, the region between amino acids 152 and 487 of p60 (Huttenlauch et al., 1995) was amplified from a plasmid containing the full-length cDNA by PCR using a sense primer (0.25 mM) that contained a *Bam*HI recognition site at its 5′ end (5′-CGGGATCC-GTTCCTCGCGAGGTCGAAAAGCC-3′) and an antisense primer (0.25 mM) that contained a *Hin*dIII recognition site at its 3′ end (5′-CCCAAGCTTATTGGACGTATCCTGACTGCTGC-3′). Cycling parameters were: initial denaturation (2 minutes, 94°C) followed by 40 cycles (1 minute, 94°C; 1 minute, 60°C; 1.5 minutes, 72°C) and a final polymerization step (10 minutes, 72°C). The PCR product was purified using a spin column (Qiagen, FRG), double-digested overnight with *Bam*HI and *Hin*dIII, again purified using a spin column and concentrated by ethanol precipitation. The fragment was cloned into *Bam*HI/*Hin*dIII double-digested pINDU vector (Bujard et al., 1987) and used to transform *E. coli* BL 21 (pINDU-p60-152/487). Transformed cells were grown overnight in rich medium (Qiagen) in the presence of 100 mg/ml ampicillin. Positive clones were identified

by SDS-PAGE and immunoblotting of lysates of entire cells with antiserum 018.

Cloning of an N-terminally Flag epitope-tagged version of articulin 1 and its translation in vitro in a coupled transcription-translation system has been described (Huttenlauch et al., 1998). For the expression of the C-terminally truncated articulin 1, the plasmid encoding Flag-articulin 1 was first linearized with restriction enzyme *Psh*AI that cuts at position 1588 of the coding region, which corresponds precisely to the end of the VPV-motif domain of articulin 1 (Huttenlauch et al., 1998). RNA was synthesized with Sp6 RNA polymerase using an Ambion mMessage mMachine kit (Ambion, USA). The RNA was purified with a Qiagen RNeasy kit and 1 μg RNA was translated in a rabbit reticulocyte lysate (Promega, USA) in a 50 μl standard reaction. About 1/20 of the translation reaction was used per lane. Flag-tagged articulins were detected with mAb M2 (Eastman Kodak, USA) at 0.7 μg/ml.

Epitope mapping

To map the epitope recognized by mAb 4B5F3 on the central domain of articulin p60, we used a NovaTope Library Construction kit (Novagen, USA), following the manufacturer's instructions. As DNA for expression library construction, we used the purified *Bam*HI/*Hin*dIII insert of the pINDU-p60-152/487 vector encoding the central domain of articulin p60 (see above). Immunodetection on colony lifts was done by chemiluminescence using the Super signal CL-HRP substrate system (Pierce). Positive clones were purified by replating until single colonies could be picked. Plasmid DNA was isolated and inserts were sequenced by double-stranded sequencing with flanking primers using a Prism Dye Deoxy Terminator Cycle Sequencing kit (Applied Biosystems, USA) and an ABI 392 DNA Sequencer.

To verify the results of the colony screening by an independent method, 5 ml cultures of relevant clones were grown to an OD_{600} of 0.2-0.4, induced with isopropyl-thio-galactopyranosid and grown for another 4-5 hours to allow for production of fusion protein. Lysates of entire cells were separated by SDS-PAGE and processed for immunoblotting with mAb 4B5F3. Only those clones that showed reaction of the fusion protein with 4B5F3 on gel blots were considered further.

Synthetic peptides

Synthesis and coupling of peptides was essentially as described (Harborth et al., 1995). The additional cysteine at the N-terminal end was used to couple the peptides with Sulfo-MBS (Pierce) to ovalbumin. The following peptides were used: P1 (CVDVPYVVTRDVEVPRYVDK); P2 (CYVVTRDVEVPRYVDK); P3 (CTRDVEVPRYVDK); a single amino acid replacement (bold) was introduced in the following peptides: P4 (CVDVPYVVT**K**-DVEVPRYVDK); P5 (CVDVPYVVTR**A**VEVPRYVDK); P6 (CVDVPYVVTRDV**A**VPRYVDK); P7 (CVDVPYVVTRDVEVP-**K**YVDK).

2-10 μl of the coupled peptides were spotted on a sheet of nitrocellulose, dried and then processed for immunoblotting (see above). Immunocomplexes were detected by chemiluminescence using the Super signal CL-HRP substrate system (Pierce).

RESULTS

Identification of antibodies recognizing either articulin or epiplasmin-specific epitopes

A polyclonal serum raised against an entire epiplasm preparation of *Pseudomicrothorax* shows broad interspecific cross-reaction even with distantly related protists (Vigues et al., 1987; Peck et al., 1991). This rabbit serum (serum 018) reacts with multiple bands of epiplasm preparations of

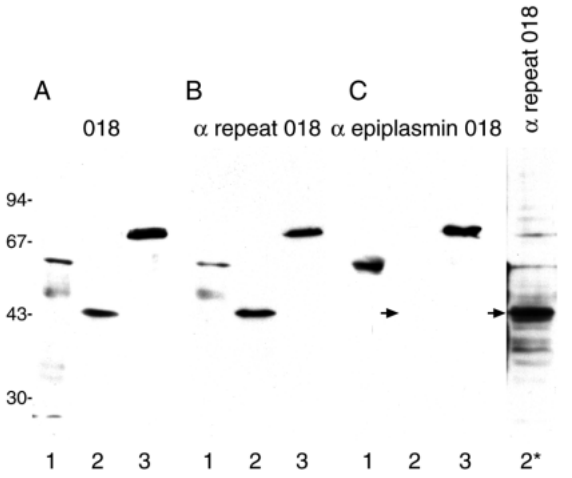

Fig. 1. Characterization of affinity-selected polyclonal antibodies by immunoblotting. Lysates of bacteria expressing fusion proteins of *Pseudomicrothorax dubius* articulin p60 (lanes 1) or the p60 central VPV-motif core domain (lanes 2) and polypeptides of the isolated epiplasm of *P. dubius* (lanes 3) were separated by 10% SDS-PAGE. Polypeptides were transferred to nitrocellulose membranes and probed with (A) unfractionated serum 018, raised against total epiplasm proteins of *P. dubius* (A; 018); (B) 018 antibodies affinity selected against a fusion protein containing the central VPV-motif domain of *P. dubius* articulin p60 (B; α repeat 018); or (C) 018 antibodies affinity selected against *Paramecium tetraurelia* epiplasmins (C; α epiplasmin 018). To control for the presence of the VPV-repeat motif domain fusion protein in lane 2 of C, the membrane was reprobed with antibodies affinity selected against the fusion protein containing the central VPV-motif core domain (C, lane 2*; α repeat 018). Due to the multiple antibody incubations, the background in this detection is high. Arrows point to the position of the VPV-repeat motif domain fusion protein in C, lanes 2 and 2*. The relative mobilities of marker proteins phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa) and catalase (30 kDa) are indicated.

Pseudomicrothorax, including the major band at 78-80 kDa (Peck et al., 1991) that contains several isoelectric variants, some of which were identified recently as articulins 1 and 4 (Huttenlauch et al., 1998). It also recognizes bacterially expressed *Pseudomicrothorax* articulin p60 (Fig. 1A, lane 1; Huttenlauch et al., 1995). In order to isolate articulin core domain-specific antibodies, the central core domain of p60 was cloned into a bacterial expression vector and the fusion protein was expressed in *E. coli* and used as an affinity matrix to select those antibodies of serum 018 that specifically react with the core domain of articulin p60 (see Materials and methods for details).

The affinity-purified antibodies reacted with bacterially expressed p60 and the p60 core domain, as expected (Fig. 1B, lanes 1, 2), as well as with the major articulin(s) of epiplasm preparations (Fig. 1B, lane 3), although reactivity of the affinity-purified antibodies was lower than the complete serum.

Serum 018 cross-reacts with polypeptides of cortical preparations of *Paramecium tetraurelia* (see below). Some of these cross-reacting polypeptides were identified as epiplasmins, the major constituents of the epiplasm of *Paramecium tetraurelia* (Nahon et al., 1993; Coffe et al., 1996). The identification was based on the mobility of the

polypeptides subjected to SDS-PAGE and their reaction with mAb CTS-32, raised against *Paramecium* epiplasmins (Nahon et al., 1993; see below). We therefore carried out antibody selection against *P. tetraurelia* epiplasmins with serum 018. Gel blots of cortical preparations of *P. tetraurelia* were prepared and strips were cut from regions of the epiplasmins (ca. 45-50 kDa). Antibodies selected in this manner for the epiplasmins (anti-epiplasmin 018) react with epiplasmins of *P. tetraurelia*, as expected, but do not react with a higher molecular mass component of cortical preparations that is recognized by the unfractionated serum (see below). Surprisingly, these antibodies recognize recombinant articulin p60 (Fig. 1C, lane 1) as well as the major articulin band of *P. dubius* epiplasm preparations (Fig. 1C, lane 3; see also Discussion). The affinity-selected anti-epiplasmin antibodies do not, however, react with the bacterially expressed p60 core domain (Fig. 1C, lane 2). The blot in Fig. 1C was reprobed with antibodies affinity-selected against the p60 core domain to prove that the p60 core domain fusion protein was present in lane 2 (Fig. 1C lane 2*). Taken together, these results indicate that this affinity-selection method for serum 018 selects for antibodies that specifically recognize either articulin core domains or epitopes found on epiplasmins, and this specificity is further confirmed below.

Epitope mapping of the articulin-specific antibody 4B5F3

Since mAb 4B5F3 reacts with several of the epiplasm polypeptides of *Pseudomicrothorax* (Fig. 2, lane 3) as well as with the bacterially expressed articulin p60 of *Pseudomicrothorax* (Fig. 2, lane 1; Huttenlauch et al., 1995), and shows cross-reaction with epiplasm proteins of other ciliates (Curtenaz et al., 1994), it was of particular interest to localize the 4B5F3 epitope within the p60 polypeptide. In a first step, the central VPV-repeat domain of articulin p60 (Fig. 3A; for details see Materials and methods) was expressed as a bacterial fusion protein and probed by immunoblotting with mAb 4B5F3. The antibody reacts strongly with the central core domain of p60 (Fig. 2, lane 2). The strategy to further delineate the epitope involved the analysis of fusion proteins and of synthetic peptides. An expression library was generated by cloning into the pTOPE vector small randomly cut fragments of the DNA encoding the core domain of p60 downstream of the T7 gene 10. Clones expressing fusion protein that reacted with the 4B5F3 antibody were selected by immunoscreening of bacterial colonies and positive clones were verified by immunoblotting of the fusion protein after SDS-PAGE of bacterial lysates (not shown). Positive clones were sequenced to determine the positions of their inserts with respect to the p60 sequence (Fig. 3B). Inserts of all positive clones contain the region between amino acids 183 and 201. Therefore, the epitope must reside within this region. This was further substantiated with synthetic peptides. In a first set of experiments, three peptides were synthesized: peptide P1 (residues 182-200), peptide P2 (residues 186-200) and peptide P3 (residues 189-200) (Fig. 3C). In dot blots of these peptides

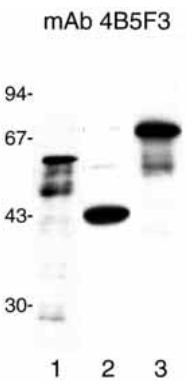

Fig. 2. Characterization of articulin-specific mAb 4B5F3 by immunoblotting. Lysates of bacteria expressing fusion proteins of *Pseudomicrothorax dubius* articulin p60 (lane 1) or the central VPV-motif core domain of articulin p60 (lane 2) or polypeptides of the isolated epiplasm of *P. dubius* (lane 3) were separated by 10% SDS-PAGE. Polypeptides were transferred to nitrocellulose membranes and probed with mAB 4B5F3. The relative mobilities of marker proteins phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa) and catalase (30 kDa) are indicated.

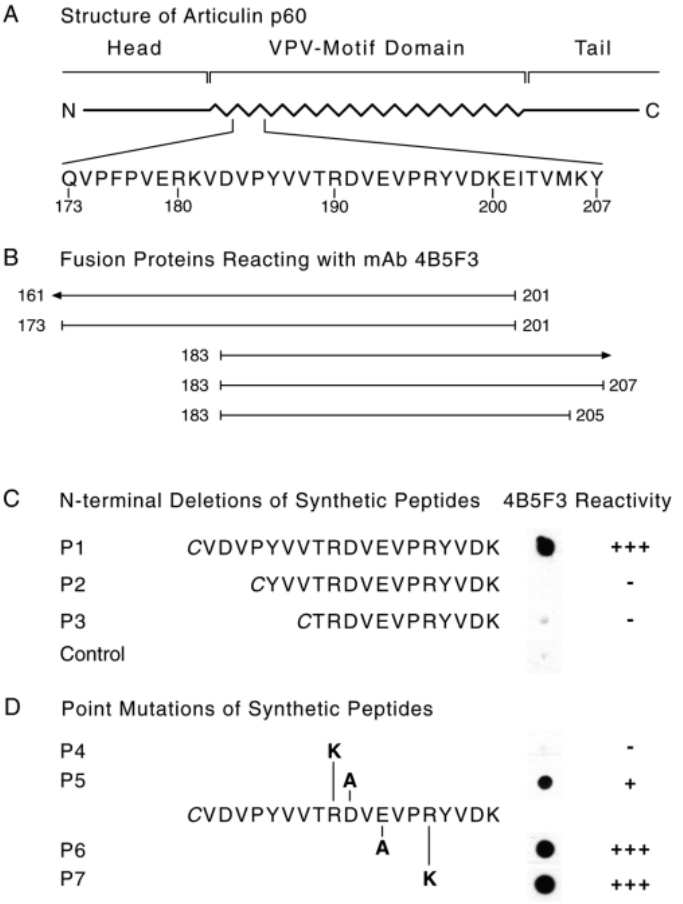

Fig. 3. Mapping of the mAb 4B5F3 epitope of *Pseudomicrothorax dubius* articulin p60. (A) Schematic representation of the three-domain structure of articulin p60 and the amino acid sequence of the region to which the mAB 4B5F3 epitope was mapped. The central VPV-motif domain is drawn as a zig-zag line. Amino acid residues are numbered according to Fig. 4 in Huttenlauch et al. (1995). (B) Peptide domains of articulin p60 present in T7 gene 10 fusion proteins that were positive following screening with mAb 4B5F3. (C,D) Reactivity of synthetic peptides with mAb 4B5F3. P1-P3, N-terminal deletions (positions 183-200); P4-P7, single amino acid replacements of peptides spanning positions 183-200. Replacements are indicated in bold. The additional cysteines (in italics) present at the N-terminal ends were used to couple the peptides to ovalbumin with Sulfo-MBS. Reactivity of the peptides coupled to ovalbumin was measured in a dot blot assay using a chemiluminescence detection system. Ovalbumin reacted with Sulfo-MBS in the absence of peptide was used as control (Control). +++, strong reaction; +, weak reaction; –, no reaction. Note that in P4, conservative replacement of R190 by lysine completely abolished reactivity with mAb 4B5F3.

coupled to ovalbumin, peptide P1 reacted strongly with the 4B5F3 antibody (Fig. 3C), confirming the results of the fusion protein analysis. Peptides P2 and P3 were completely negative (Fig. 3C). The combined results show that the region between amino acid residues 182 and 200 harbors the 4B5F3 epitope. To confirm this result and to identify some single amino acid residues that constitute the 4B5F3 epitope, a second set of experiments used synthetic peptides with a single amino acid replacement in four of the 19 residues of this region (residues typed in bold in Fig. 3D). Replacement of R190 by lysine completely abolished the reactivity of the peptide P4 with 4B5F3 in dot blots (Fig. 3D). Replacement of D191 by alanine reduced the reactivity, but replacement of either E193 or R196 by alanine had no effect on the reactivity with the antibody (Fig. 3D).

In conclusion, the 4B5F3 epitope lies within residues 183-200 of the central core domain of articulin p60. Amino acid replacement studies of 19-mer peptides show that R190 does not allow a conservative replacement without loss of antibody reactivity, while replacement of D191 has a less severe effect and two other replacements further towards the C terminus have no effect.

Interspecific cross-reaction of antibodies recognizing articulin and epiplasmin-specific epitopes

The antibodies recognizing either articulin core domain epitopes (anti-p60 core domain 018 and mAb 4B5F3) or epiplasmin epitopes (anti-epiplasmin 018 and mAb CTS-32, the latter raised against polypeptides of the Triton X-100-soluble fraction of a cortical preparation of the ciliate *Paramecium tetraurelia* and demonstrated to specifically label the epiplasmins by Nahon et al., 1993) were used to determine whether articulins and epiplasmins are present in other protists.

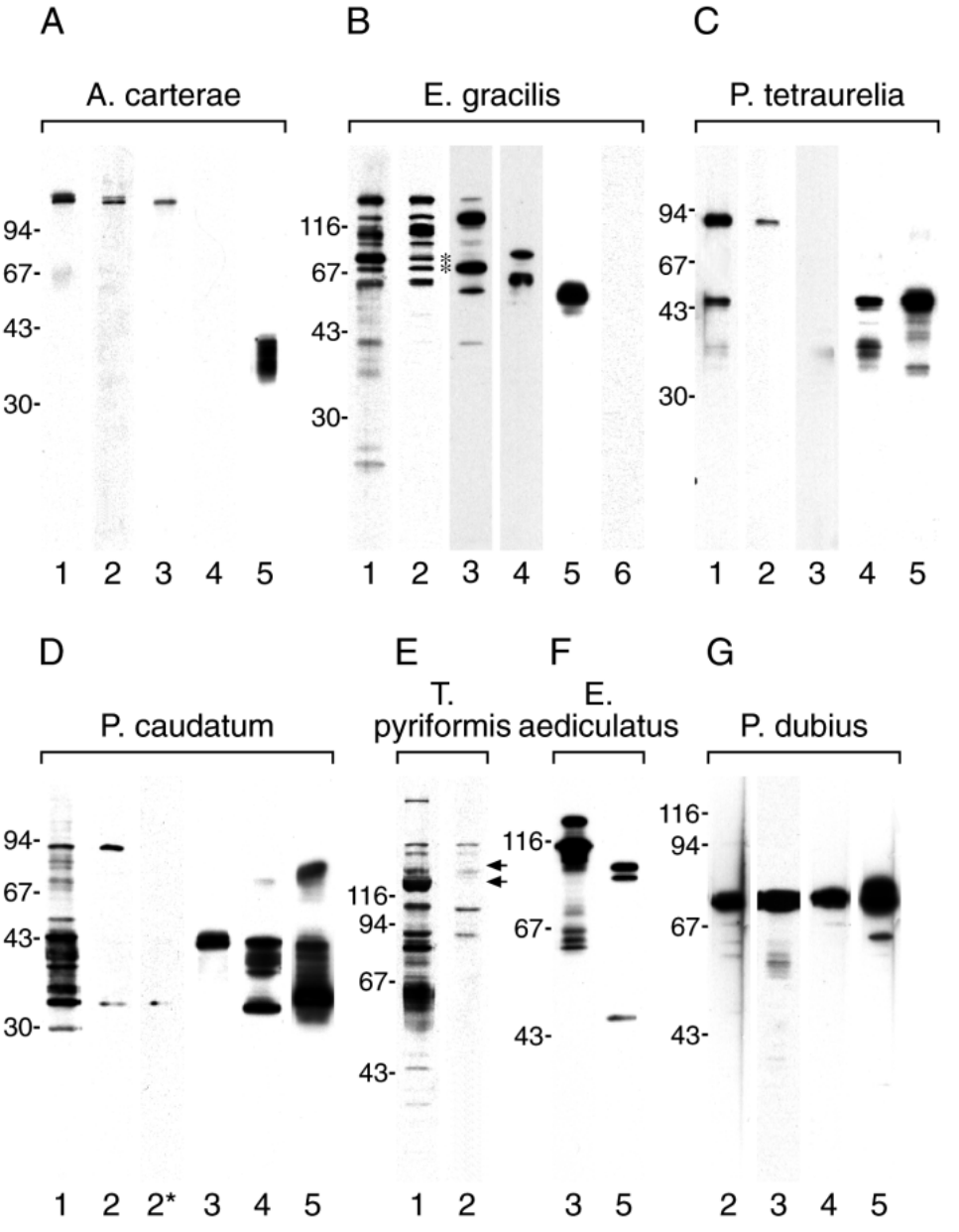

Fig. 4. Interspecific cross-reaction of articulin-specific antibodies and anti-epiplasmin antibodies. Lysates of entire cells of *Amphidinium carterae* (A) and *Euglena gracilis* (B), cortical preparations of *Paramecium tetraurelia* (C), *Paramecium caudatum* (D), *Tetrahymena pyriformis* (E) and *Euplotes aediculatus* (F), and epiplasm isolates of *Pseudomicrothorax dubius* (G) were separated by SDS-PAGE (12% in A-D, 8% in E and 10% in F and G). Polypeptides were transferred to nitrocellulose membranes and probed with unfractionated serum 018 raised against total epiplasm proteins of *P. dubius* (lanes 1), polyclonal antibodies of serum 018 affinity selected against a fusion protein containing the central VPV-motif core domain of *P. dubius* articulin p60 (lanes 2), articulin-specific mAb 4B5F3 (lanes 3), polyclonal antibodies of serum 018 affinity selected against *P. tetraurelia* epiplasmins (lanes 4), mAb CTS-32 raised against epiplasmins (lanes 5) or, as a control, polyclonal antibodies of serum 018 affinity selected against bacterial proteins of non-transformed bacteria (lane 6 in B). As a further control, a gel blot of a *P. caudatum* cortical preparation was incubated with peroxidase-conjugated goat anti-rabbit IgG alone (lane 2* in D). The positions of *E. gracilis* articulins p80 and p86 are indicated by asterisks in B. The positions of *T. pyriformis* polypeptides B and C at 135 and 125 kDa, as revealed by reaction with antibodies specific for either band B or C (Williams et al., 1995), are indicated by arrows in E. The relative mobilities of marker proteins β-galactosidase (116 kDa), phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa) and catalase (30 kDa) are indicated.

Lysates of entire cells of the dinoflagellate *Amphidinium carterae* were subjected to SDS-PAGE and blots were probed with the different antibodies. Unfractionated serum 018 reacts with two closely migrating bands of approx. 130 kDa (Fig. 4A, lane 1). The same bands are recognized by the anti-p60 core domain antibodies (Fig. 4A, lane 2). The faster migrating band is also recognized by antibody 4B5F3 (Fig. 4A, lane 3). These observations strongly suggest that articulins are present in this dinoflagellate. Affinity-selected anti-epiplasmin antibodies (anti-epiplasmin 018) do not react with *Amphidinium* polypeptides (Fig. 4A, lane 4), but antibody CTS-32 recognizes a series of polypeptides in the range of approx. 35-40 kDa (Fig. 4A, lane 5), but not the bands of approx. 130 kDa, identified as articulins, indicating that epiplasmins are also present.

Blots of entire cell lysates of the euglenoid flagellate *Euglena gracilis* show a complex pattern of reactive polypeptides with unfractionated serum 018 (Fig. 4B, lane 1). A similar pattern of polypeptides of 60 to >130 kDa is recognized by the anti-p60 core domain 018 antibodies (Fig. 4B, lane 2), while antibody 4B5F3 reacts with only a subset of these polypeptides (Fig. 4B, lane 3). The specificity of the affinity selection and the immunostaining is demonstrated by the absence of labeling on a blot of *E. gracilis* cell lysate incubated with antibodies affinity-purified from serum 018 on a nitrocellulose strip cut from a blot of non-transformed *E. coli* lysate (Fig. 4B, lane 6). The relative staining intensity of individual bands differs depending on which of the antibodies was used (Fig. 4B, lanes 1-3). Based on Coomassie Blue staining after SDS-PAGE, articulins p80 and p86 are quantitatively the major polypeptides of cortical preparations of *E. gracilis*. Their positions are indicated by asterisks in Fig. 4B. Following immunolabeling, numerous other bands are labeled as intensely or more intensely than p80 and p86; however, since labeling intensity depends upon the reactivity of the antibodies with individual polypeptides, it does not directly reflect polypeptide abundance. Interestingly, several polypeptides that are recognized by the articulin-specific antibodies have much higher apparent molecular masses than articulins p80 and p86, which indicates that additional articulins exist in *Euglena*.

Anti-epiplasmin 018 antibodies react with two bands in the 65-70 kDa range of entire *Euglena* cell lysates (Fig. 4B, lane 4), while antibody CTS-32 very strongly recognizes one band at approx. 60 kDa (Fig. 4B, lane 5). Taken together, the results indicate that epiplasmins might also be present in *Euglena*; however, the complex pattern of anti-articulin reactive polypeptides and the limited resolution of the one-dimensional separation make it difficult to exclude that some of the epitopes recognized by the anti-epiplasmin antibodies (Fig. 4B, lane 4) might reside on the same polypeptides that are recognized by the articulin-specific antibodies. Therefore, lysates of entire *Euglena* cells were separated by two-dimensional gel electrophoresis and blots were probed with either mAb 4B5F3 (Fig. 5A) or mAb CTS-32 (Fig. 5B). Comparison of the staining patterns of the two-dimensional separations clearly demonstrates that the two antibodies recognize different polypeptides.

In the ciliate *Paramecium tetraurelia*, a single polypeptide at approx. 90 kDa as well as a series of polypeptides at 35-45 kDa are recognized on blots of cortical preparations with the

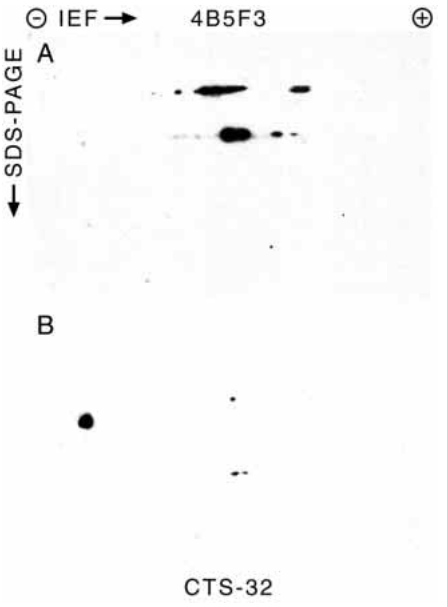

Fig. 5. Two-dimensional gel electrophoresis of lysates of entire cells of *Euglena gracilis*. Polypeptides were separated by IEF in the first dimension and by 12% SDS-PAGE in the second dimension. They were transferred to nitrocellulose membranes and probed with mAb 4B5F3 (A) or mAb CTS-32 (B). Only relevant portions of the blots are shown.

unfractionated serum 018 (Fig. 4C, lane 1). Anti-p60 core domain 018 antibodies react exclusively with the 90 kDa polypeptide (Fig. 4C, lane 2), while anti-epiplasmin 018 antibodies recognize only the 35-45 kDa polypeptides (Fig. 4C, lane 4). The group of 35-45 kDa polypeptides is identified as epiplasmins on the basis of their reaction with the antibody CTS-32 (Fig. 4C, lane 5). Antibody 4B5F3 does not react with *P. tetraurelia* (Fig. 4C, lane 3; Curtenaz et al., 1994). These results indicate that both articulins and epiplasmins are distinct constituents of the membrane skeleton of this organism.

A similar conclusion can be drawn from the results obtained with *Paramecium caudatum*. While the number of polypeptides of cortical preparations that react with unfractionated serum 018 is higher in *P. caudatum* than in *P. tetraurelia* (Fig. 4D, lane 1), only one band at approx. 90 kDa is recognized by the antibodies affinity selected against the p60 core domain (Fig. 4D, lane 2). Staining of a second band in the lower molecular mass range is due to a non-specific reaction with the secondary antibody, since this band is also labeled when incubation with the primary antibody is omitted (Fig. 4D, lane 2*). As for *P. tetraurelia*, anti-epiplasmin 018 antibodies (Fig. 4D, lane 4) and antibody CTS-32 (Fig. 4D, lane 5) recognize several epiplasmins at 35-45 kDa. Labeling of the lower molecular mass bands in lanes 4 and 5 is at least in part non-specific, since a band in this range is labeled when the primary antibody is omitted (Fig. 4D, lane 2*). In addition, CTS-32 reacts strongly with a band at approx. 70 kDa (Fig. 4D, lane 5) that is also recognized by the anti-epiplasmin 018 antibodies, although much more weakly (Fig. 4D, lane 4). Antibody 4B5F3 recognizes two closely migrating bands at approx. 43 kDa (Fig. 4D, lane 3). To decide whether the epitope recognized by antibody 4B5F3 resides on the same

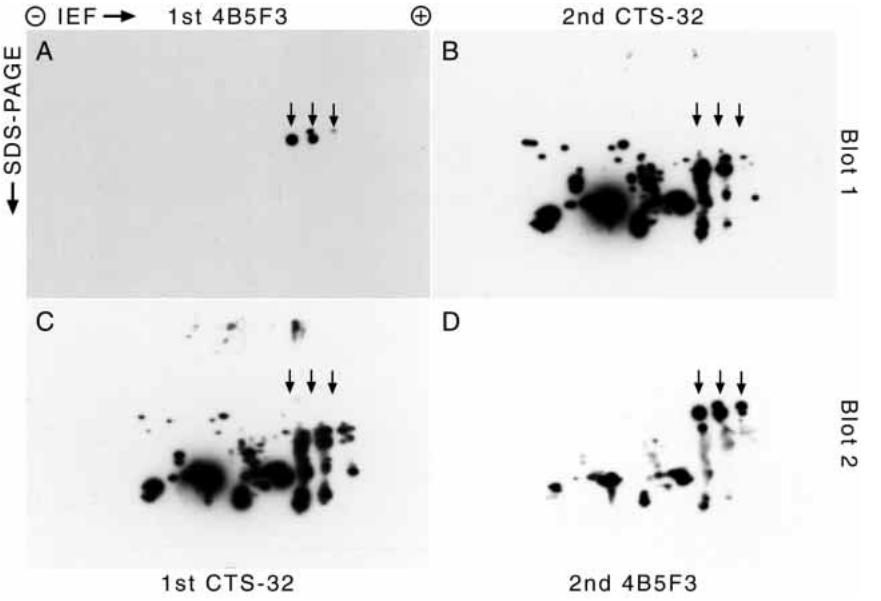

Fig. 6. Two-dimensional gel electrophoresis of cortical preparations of *Paramecium caudatum*. Polypeptides were separated by IEF in the first dimension and by 12% SDS-PAGE in the second dimension. They were transferred to nitrocellulose membranes and probed first with mAb 4B5F3 (A) followed by mAb CTS-32 (B) (Blot 1), or probed first with mAb CTS-32 (C) followed by mAb 4B5F3 (D) (Blot 2). The positions of spots that react with mAB 4B5F3 are indicated by arrows. Only relevant portions of the blots are shown.

polypeptides that are recognized by antibody CTS-32, *P. caudatum* cortical preparations were separated by two-dimensional gel electrophoresis and blots were probed with the two mAbs. Antibody 4B5F3 reacts with 4-5 spots (Fig. 6A,D), while antibody CTS-32 reacts with a large number of spots (Fig. 6B,C), as previously described for two-dimensional separations of *P. tetraurelia* epiplasmins (Nahon et al., 1993). Successive staining of each blot with the other antibody (Fig. 6B,D) allowed a precise alignment of the staining patterns. It clearly demonstrates that the two antibodies recognize different polypeptides. In conclusion, in *P. caudatum*, as in *P. tetraurelia*, epiplasmins as well as articulins are separate polypeptide constituents of the membrane skeleton. Interestingly, in *P. caudatum*, some of the articulins are detected at approx. 43 kDa, in the molecular mass range of the epiplasmins. The latter observation explains the failure of our initial attempts to select for anti-epiplasmin antibodies using 1D-blots of *P. caudatum* cortical preparations. Antibodies eluted from these blots react with epiplasmins as well as articulins (not shown) since both types of proteins are found in the same molecular mass range and consequently both are present on the blots used as the affinity matrix.

In the ciliate *Tetrahymena pyriformis*, three major epiplasm polypeptides named bands A, B and C, at 235, 135 and 125 kDa, respectively, have been described. Using several mAbs, each recognizing one of the three polypeptides, it was found that these three epiplasm proteins have overlapping, but independent, distributions within the epiplasm (Williams et al., 1995). Neither of the mAbs employed in the present study reacts with any of these three polypeptides, nor with any other polypeptides of *T. pyriformis* cortical preparations (not shown; Nahon et al., 1993; Curtenaz et al., 1994). In contrast, unfractionated serum 018 cross-reacts with many polypeptides of the cortical preparations (Fig. 4E, lane 1), and anti-p60 core domain antibodies recognize 4-5 bands at 90-170 kDa (Fig. 4E, lane 2). None of the latter bands, however, co-migrates with any of the major epiplasm polypeptides A, B or C of *T. pyriformis*, as revealed by direct comparison of blots stained with mAbs specific for either bands A, B or C. The positions of bands B and C are indicated by arrows in Fig. 4E, lane 2; no labeling was obtained with the mAb specific for band A, but the high molecular mass of the latter (235 kDa) places it well above the molecular mass of the bands we label with the articulin-specific anti-p60 core domain antibodies.

Polypeptides of cortical preparations of the hypotrich ciliate *Euplotes aediculatus* have been previously shown to be labeled with both antibody 4B5F3 (Curtenaz et al., 1994) and antibody CTS-32 (Nahon et al., 1993). These data, however, did not resolve whether the same, or different, polypeptides were recognized by the two antibodies. Blots from the same gel were therefore used to analyse the labeling pattern. Comparison of these immunoblots reveals that different polypeptides are recognized by the two mAbs (Fig. 4F, lanes 3, 5).

Reactivity of anti-epiplasmin antibodies with epiplasm polypeptides of *Pseudomicrothorax dubius*

The reactions of serum 018, the anti-p60 core domain antibodies and the mAb 4B5F3 with epiplasm proteins of *Pseudomicrothorax* and with the bacterially expressed *Pseudomicrothorax* articulin p60 were described above (Figs 1, 2). In contrast to what was observed in the other species, in one-dimensional separations the mAb CTS-32, raised against *Paramecium* epiplasmins, shows reactivity with the same group of epiplasm bands that is recognized by the articulin-specific antibodies (Fig. 4G, compare lanes 2-4 with lane 5). Since the 78-80 kDa band consists of at least three different polypeptides and numerous isoelectric variants (Peck et al., 1991), we separated the polypeptides of the *Pseudomicrothorax* epiplasm by two-dimensional gel electrophoresis and probed the blots with either mAb 4B5F3 or mAb CTS-32. The results show that both antibodies recognize spots 1-3 (Fig. 7A,C). These spots represent isoelectric variants of articulin 1 (Peck et al., 1991; Huttenlauch et al., 1998). Neither antibody recognizes spot 4 (Fig. 7A,C), recently identified as articulin 4 (Huttenlauch et

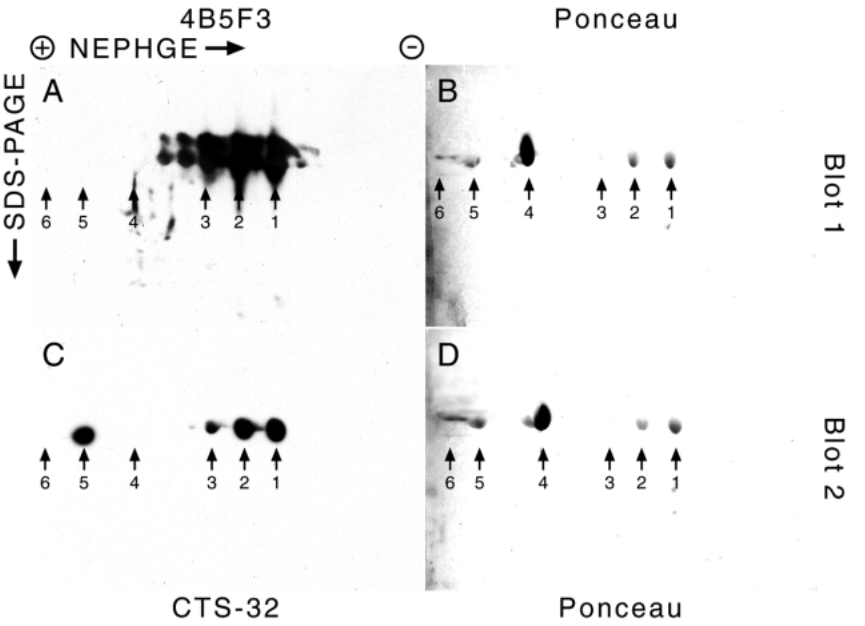

Fig. 7. Two-dimensional gel electrophoresis of the epiplasm of *Pseudomicrothorax dubius*. Polypeptides were separated by NEPHGE in the first dimension and by 10% SDS-PAGE in the second dimension. They were transferred to nitrocellulose membranes and first stained with Ponceau Red (B,D) and then probed with mAb 4B5F3 (A) or with mAb CTS-32 (C). Major spots of the 78-80 kDa group of polypeptides are indicated by arrows and numbered 1-6. Spots 1-3 represent isoelectric variants of articulin 1 and spot 4 represents articulin 4. The amount of protein charged on gels was chosen to allow Ponceau Red staining after transfer. This results in overstaining in A due to the strong reactivity of mAb 4B5F3 with *P. dubius* articulin 1. Background staining in B and D is due to staining of ampholytes with Ponceau Red. Note that mAb CTS-32 reacts with spot 5 in addition to the reaction with articulin 1 (spots 1-3). Only relevant portions of the blots are shown.

al., 1998). It should be noted that the reaction of antibody 4B5F3 is much stronger than that of CTS-32 for articulins 1-3. The two mAbs appear to recognize different epitopes on the 78-80 kDa polypeptides, since CTS-32, but not 4B5F3, recognizes spot 5 (Fig. 7C), a polypeptide that can be distinguished from articulins 1 and 4 by peptide mapping (Peck et al., 1991), but has not yet been sequenced.

In order to analyse whether mAb CTS-32, like mAB 4B5F3, recognizes an epitope within the central VPV-motif domain of *Pseudomicrothorax* articulin 1, we expressed in vitro Flag epitope-tagged full-length articulin 1 (WT, Fig. 8A), as well as a C-terminally truncated version of articulin 1 that contains the head as well as the complete VPV-motif domain, but lacks the tail domain (ΔC, Fig. 8A). The two polypeptides were probed with mAb CTS-32, mAb 4B5F3, as well as with an anti-Flag epitope-specific mAb (for details, see Materials and methods). Since the Flag epitope resides at the N termini of the polypeptides, both versions can be detected with the Flag-specific mAb (Fig. 8B, lanes 1 and 2). Both polypeptides reacted with mAb 4B5F3, as expected (Fig. 8C, lanes 1 and 2; see Fig. 8A for the position of the 4B5F3 epitope). In contrast, only the full-length polypeptide, but not the C-terminally truncated polypeptide, showed reactivity with mAb CTS-32 (Fig. 8D, lanes 1 and 2). Staining of an additional band at approx. 50 kDa, seen in Fig. 8D, is due to a cross-reaction of

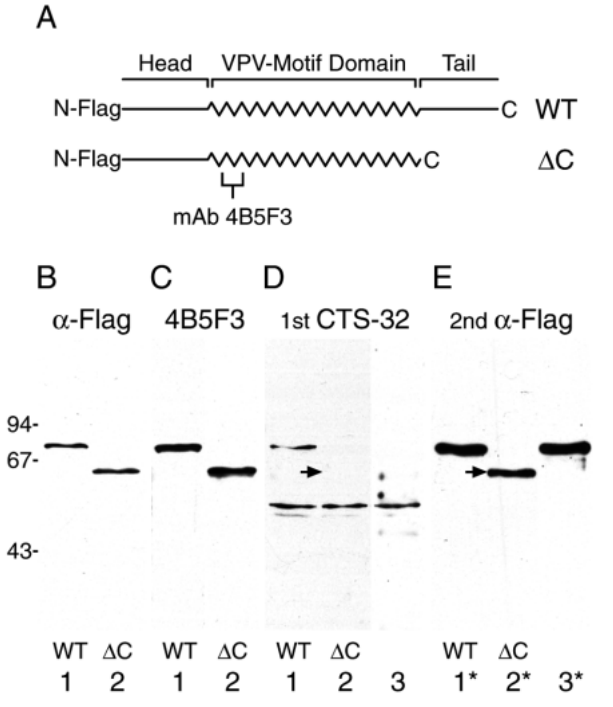

Fig. 8. Mapping of the mAb CTS-32 epitope of *Pseudomicrothorax dubius* articulin 1. (A) Schematic representation of articulin 1 constructs used in the epitope mapping. The central VPV-motif domain of articulin 1 is drawn as a zig-zag line and the region to which the epitope of mAb 4B5F3 was mapped based on sequence comparison with articulin p60 is indicated. All constructs carry a Flag epitope tag at their N terminus (N-Flag). The deletion construct of articulin 1 (ΔC) lacks the entire tail domain; WT, full-length articulin 1. Polypeptides were translated in an in vitro reticulocyte lysate system, separated by 10% SDS-PAGE, transferred to nitrocellulose membranes and probed with antibodies (B-E). Full-length articulin 1 (lanes 1), articulin 1 lacking the tail domain (lanes 2), full-length articulin 4 (lanes 3). Blot B was probed with anti-Flag mAb M2 (α-Flag), blot C with mAb 4B5F3, and blot D with mAb CTS-32. The presence of ΔC and articulin 4 in blot D was demonstrated by reprobing the blot with anti-flag mAb M2 (blot E, lanes 1*-3*). The position of ΔC in blot D, lane 2, is indicated by an arrow. Since the reaction of mAb CTS-32 with articulin 1 is relatively weak, long exposure times were necessary. Under these conditions reaction of mAb CTS-32 with a polypeptide of the reticulocyte lysate is detected in all three translation reactions (blot D, lanes 1-3). The relative mobilities of marker proteins phosphorylase b (94 kDa), bovine serum albumin (67 kDa) and ovalbumin (43 kDa) are indicated.

this mAb with a polypeptide of the reticulocyte lysate. This band is also labeled with lysates that were primed with RNA encoding polypeptides that do not react with mAb CTS-32, such as articulin 4 (Fig. 8D, lane 3). The presence of the ΔC polypeptide in lane 2 of Fig. 8D was verified by subsequent staining of the blots shown in Fig. 8D with the anti-Flag-specific mAb (Fig. 8E, lane 2*). The latter staining also confirmed the presence of Flag epitope-tagged articulin 4 in lane 3 of Fig. 8D (Fig. 8E, lane 3*). In conclusion, the mAb CTS-32 epitope is located in the tail domain of articulin 1 rather than in the VPV-motif domain characteristic of articulins.

DISCUSSION

The existence of common epitopes among proteins of the membrane skeleton of protists has been established by several studies using polyclonal antisera as well as mAbs (Bricheux and Brugerolle, 1986; Vigues et al., 1987; Nahon et al., 1993; Curtenaz et al., 1994; Sghir and David, 1995). These studies revealed (1) intra- as well as inter-specific cross-reaction of membrane skeletal proteins, (2) frequently, a marked heterogeneity with respect to the number of constituents and their molecular sizes, and (3) often unexpected patterns of cross-reaction with respect to the evolutionary relationships as deduced by other morphological and molecular criteria for the organisms under study. Articulins and epiplasmins are among the most well-defined of these membrane cytoskeletal proteins.

Articulins of the flagellate *Euglena* (Marrs and Bouck, 1992) and the ciliate *Pseudomicrothorax* (Huttenlauch et al., 1995, 1998) are the only membrane skeletal proteins for which complete primary sequence data are available. These articulins are members of the same family of proteins, characterized by a common tripartite molecular structure, and by repetitive articulin-specific, 12-residue-long VPV-motifs in particular (Marrs and Bouck, 1992; Huttenlauch et al., 1995, 1998). To date, only short, partial sequences have been determined for the epiplasmins of *Paramecium* (Coffe et al., 1996). It has therefore not been possible to decide whether the epiplasmins of *Paramecium* belong to the same protein family as the articulins or whether they represent another type of epiplasm protein. We therefore deemed it pertinent to determine the distribution of articulins and epiplasmins in parallel and on the same blotted material from a variety of protists using specific immunological probes.

Newly available molecular tools allowed us to isolate articulin-specific and anti-epiplasmin polyclonal antibodies by affinity selection, and also permitted the identification of individual epitopes and protein domains recognized by an articulin-specific mAb and a mAb raised against epiplasmins. These antibodies show wide species cross-reactivity, allowing insight into the distribution of articulins and epiplasmins in diverse protists.

Specificity of the antibodies used in this study

The hallmark of articulins is the central core domain of repetitive motifs of alternating valine and proline residues, the VPV-motif. Antibodies recognizing motifs within the core domain should therefore be valuable reagents for identifying articulins. The central core domain of *Pseudomicrothorax* articulin p60, expressed as a bacterial fusion protein, was used to affinity purify articulin-specific polyclonal antibodies. The entire antiserum (018) was raised against polypeptides of an entire epiplasm preparation of *Pseudomicrothorax* (Peck et al., 1991), and it shows broad interspecific reactivity (Vigues et al., 1987; this study). The affinity-selected antibodies retain their reactivity with p60 and the p60 core domain, and they also recognize the major articulins of *Pseudomicrothorax*. The specificity of the affinity selection for articulins is demonstrated best by the results obtained with *Paramecium tetraurelia*. The unfractionated serum 018 recognizes at least two groups of polypeptides with widely different electrophoretic mobilities, whereas the affinity-selected antibodies no longer react with the faster migrating polypeptide(s). The latter were identified as epiplasmins by their reactivity with mAb CTS-32, in agreement with the results of Nahon et al. (1993). The affinity-selected antibodies recognize, however, the polypeptides with the higher electrophoretic mobility, that were therefore tentatively identified as articulins. In the complementary experiment, antibodies of the same serum affinity purified against epiplasmins from *P. tetraurelia* cortical preparations selectively recognize epiplasmins of this species, but not articulins.

The second articulin-specific antibody used in this study was mAb 4B5F3, which has been previously characterized (Curtenaz and Peck, 1992). Similar to the polyclonal serum, mAb 4B5F3 shows broad intra- and interspecific reactivity, including cross-reaction with two euglenoids. Curtenaz et al. (1994) therefore speculated that mAb 4B5F3 might be directed against a conserved epitope within the central VPV-repeat domain of articulins. The mapping of the 4B5F3 epitope to 19 residues within the central core domain of *Pseudomicrothorax* articulin p60, reported here, proves this assumption was correct. Furthermore, the presence of a nearly identical sequence in *Pseudomicrothorax* articulin 1 and a very similar sequence in *Euglena* articulin p80, as well as the absence of such a sequence in *Pseudomicrothorax* articulin 4 and *Euglena* articulin p86, corresponds well with the observed reactivity of this mAb. Moreover, our mutational analysis of the 4B5F3 epitope demonstrates that a single conservative replacement of a particular residue (arginine to lysine at position 190 of p60) results in complete loss of antibody reactivity. As demonstrated for articulin 4 of *Pseudomicrothorax*, this mAb does not recognize all articulins and, most probably, its interspecific cross-reactions will not necessarily follow the accepted ideas of evolutionary distances separating the organisms under study; this situation has been observed previously with other mAbs (Riemer et al., 1991; Nahon et al., 1993). Thus lack of reactivity with mAb 4B5F3, as seen for e.g. in *Paramecium tetraurelia* and *Tetrahymena pyriformis* cortical preparations (see also Curtenaz et al., 1994), does not prove that articulins are absent. This interpretation is corroborated by the results obtained with the polyclonal anti-articulin antibodies, which demonstrate that articulins are present in these species. Although not characterized in such detail, the same principles discussed above for mAb 4B5F3 are valid for mAb CTS-32.

In several cases the patterns of reactive polypeptides obtained with the two mAbs and the patterns obtained with their corresponding affinity-selected polyclonal antibodies overlap, but they are not identical. This observation is not

surprising in light of what has been discussed above with respect to the reactivity of mAb 4B5F3, and also taking into account that the affinity-selected antibodies, due to their polyclonal nature, will almost certainly show a broader reactivity. For some polypeptides the only difference is the relative labeling intensity observed between the polyclonal antibodies and the corresponding mAb.

Articulins and epiplasmins constitute two different classes of proteins

In all species probed, with the notable exception of *Pseudomicrothorax*, polypeptides reactive with antibodies directed against articulins are distinct from those reactive with antibodies directed against epiplasmins. A straightforward interpretation of these observations is that articulins and epiplasmins constitute two different classes of proteins. Moreover, in all species analysed, both types of proteins were detected. Therefore, both articulins and epiplasmins appear to contribute to the organization of the membrane skeleton in the protists we studied. The relative proportion that proteins of each of the two classes contribute to the organization of the membrane skeleton may, however, differ depending on the species. Staining intensity in blotting experiments is influenced by the reactivity of the particular antibodies with individual polypeptides. It is, therefore, not a direct measure of the abundance of a protein. Biochemical analysis has shown, for example, that articulins p80 and p86 are by far the most abundant polypeptides in pellicle preparations of euglenoids (Bricheux and Brugerolle, 1986, 1987; Vigues et al., 1987), and yet many other bands are as strongly labeled with the anti-articulin antibodies. In contrast, epiplasmins are the quantitatively major components of epiplasm preparations of *Paramecium* (Nahon et al., 1993), and they appear to be the major bands labeled with the anti-epiplasmin antibodies.

Immunological cross-reaction of mAb 4B5F3 and mAb CTS-32 with several of the species analysed here has been reported previously (Nahon et al., 1993; Curtenaz et al., 1994). Our results confirm these findings. Moreover, staining of lanes taken from the same blots, presented here, allowed a direct comparison of the patterns obtained with the two mAbs as well as with the affinity-selected polyclonal antibodies. In most instances, articulins show higher molecular masses than epiplasmins in our analysis, and in both classes of proteins a marked heterogeneity with respect to molecular masses is observed. The latter observation has been made previously in several other analyses (Vigues et al., 1987; Peck et al., 1991; Nahon et al., 1993; Curtenaz et al., 1994). Although proteolysis during the isolation procedure and sample preparation cannot be completely ruled out as the source of this heterogeneity, we feel it unlikely. In *E. gracilis*, for example, we detect, in addition to the two molecularly well-characterized articulins p80 and p86, other articulins with higher molecular masses. Evidence for the presence of additional articulins in euglenoids can be inferred also from an earlier report on immunological cross-reactions of epiplasm proteins (Vigues et al., 1987). Additionally, since mAb 4B5F3 recognizes a linear epitope, it should react also with some proteolytic peptide fragments, if present. This, however, is not observed (see for example lanes 3 in Fig. 4).

Taken together, our observations support the idea that two classes of proteins, articulins and epiplasmins, contribute to the membrane skeleton of many protists. We are aware that the final demonstration of this will require more sequence data, especially for the epiplasmins.

Reactivity of anti-epiplasmin antibodies with articulins of *Pseudomicrothorax*

Cross-reaction of the mAb CTS-32 directed against epiplasmins with polypeptide(s) of purified *Pseudomicrothorax* epiplasm has been reported by Nahon et al. (1993), and we have confirmed this observation. mAb CTS-32 reacts with *Pseudomicrothorax* articulin 1 and with polypeptide 5 (spot 5, see Peck et al., 1991; Huttenlauch et al., 1998), an epiplasm polypeptide that has not yet been characterized molecularly, and that is not recognized by the articulin-specific mAb 4B5F3. In addition, mAb CTS-32 recognizes articulin 1 translated in vitro, and experiments with in vitro translated, truncated polypeptides confine the epitope to the C-terminal domain of articulin 1. This immunological reaction may point to a biologically meaningful similarity between epiplasmins and articulin 1, rather than a fortuitous cross-reaction of this particular mAb, because the affinity-selected polyclonal anti-epiplasmin antibodies also recognize articulin(s) of the isolated epiplasm as well as bacterially expressed articulin p60 of *Pseudomicrothorax*. Furthermore, these antibodies do not recognize the central core domain of p60, indicating that, similar to the situation described above, they recognize epitope(s) within the terminal domain(s) of p60. Thus, both the mAb and the affinity-selected antibodies directed against epiplasmins recognize epitopes outside the central VPV-repeat domain, characteristic of the articulins, thus supporting the distinction between articulins and epiplasmins.

The reaction of mAb CTS-32 with articulin 1 is much weaker than that with epiplasmins of *Paramecium*, indicating that the epitope(s) recognized on articulin 1 are less abundant or slightly different than the epitope(s) recognized on *Paramecium* epiplasmins. Comparison of the sequence of the C-terminal domain of articulin 1 with the available short partial sequences of epiplasmins (Coffe et al., 1996) does not reveal obvious similarities.

The reaction of articulin 1 of *Pseudomicrothorax* with both an articulin-specific mAb and a mAb raised against epiplasmins may not a priori indicate a close relationship between articulins and epiplasmins, but the exact relationship between these two classes of proteins will probably become clearer only after complete sequence information for the epiplasmins becomes available.

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