

# Cytoskeletal proteins with N-terminal signal peptides: plateins in the ciliate *Euplotes* define a new family of articulins

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## Summary

Protistan cells employ a wide variety of strategies to reinforce and give pattern to their outermost cortical layers. Whereas some use common cytoskeletal elements such as microtubules, others are based on novel cytoskeletal proteins that are as-yet-unknown in higher eukaryotes. The hypotrich ciliate *Euplotes* possesses a continuous monolayer of scales or plates, located within flattened membranous sacs ('alveoli') just below the plasma membrane, and this provides rigidity and form to the cell. Using immunological techniques, the major proteins comprising these 'alveolar plates' have been identified and termed  $\alpha$ -,  $\beta$ -, and  $\gamma$ -plateins. The present report describes work leading to the molecular characterization of three plateins,  $\alpha$ 1 and  $\alpha$ 2 (predicted  $M_r$ s of 61 and 56 kDa) and a  $\beta/\gamma$  form ( $M_r=73$  kDa). All three proteins have features that are hallmarks of articulins, a class of cytoskeletal proteins that has been identified in the cortex of a wide variety of protistan cells, including certain flagellates, ciliates, dinoflagellates and *Plasmodium*. Chief among these common features are a prominent primary domain of tandem 12-amino acid repeats, rich in valine and proline, and a secondary domain of fewer, shorter repeating units.

However, variations in amino acid use within both primary and secondary repetitive domains, and a much more acidic character (predicted pIs of 4.7-4.9), indicate that the plateins represent the first proteins in a new subclass or family of articulins. This conclusion is supported by another novel feature of the plateins, the presence of a canonical hydrophobic signal peptide at the N-terminus of each derived platein sequence. This correlates well with the final cellular location of the plateins, which are assembled into plates within the membrane-limited alveolar sacs. To our knowledge, this is the first report in any eukaryote of cytoskeletal proteins with such start-transfer sequences. Confocal immunofluorescence microscopy, using antibodies to the plateins as probes, reveals that new alveolar plates (enlarging in cortical zones undergoing morphogenesis) label more faintly than mature parental plates. During plate assembly (or polymerization), the plateins thus appear to exist in a more soluble form.

Key words: Signal sequence, Membrane skeleton, Alveolata, Protist, Epiplasm, Pellicle, Cortical cytoskeleton, Repetitive protein domains, Ciliate expression library

## Introduction

Eukaryotic cells typically stabilize their plasma membranes with a more-or-less tightly associated layer of cytoskeletal proteins, forming a complex often referred to as the 'membrane skeleton'. A classic example has been the spectrin network linked (via ankyrins and several actin-binding proteins) to integral membrane glycoproteins at the cytoplasmic face of the erythrocyte plasmalemma (Bennett and Gilligan, 1993). Spectrin isoforms and ankyrin homologs have been identified in numerous other vertebrate cells (Dubreuil et al., 1997; Kordeli, 2000), indicating that the membrane skeleton is a functionally critical, and perhaps ubiquitous, element of cell surfaces.

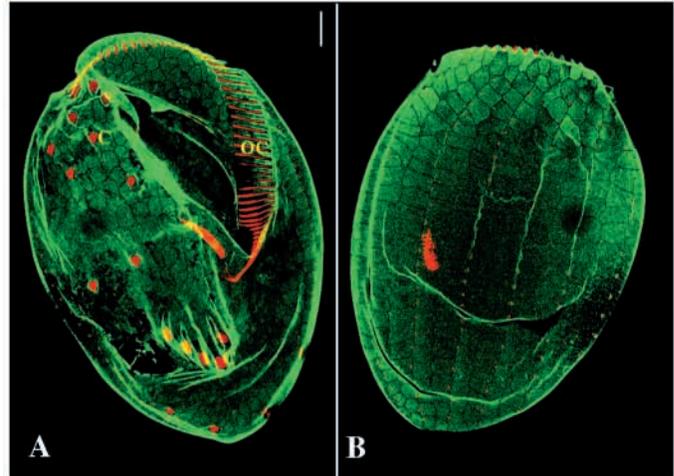
Ciliated protozoans, as large cells living in a wide range of potentially disruptive environments, have evolved a variety of strategies for strengthening and reinforcing their outermost surface. A monolayer of flattened membranous sacs (termed

'cortical alveoli') is characteristic of ciliates, subtending the plasma membrane and effectively isolating the cytoplasm from the environment by three membrane layers. Additionally, various cytoskeletal elements are found in this outer cortical layer (often termed the 'pellicle'). Some are similar to cytoskeletal structures well represented in other eukaryotes, such as microtubules, which are widely used supporting elements in ciliate cortexes (Grim, 1982; Fleury and Laurent, 1995; Adoutte and Fleury, 1996). However, the other well-known eukaryotic supportive elements, namely actin-myosin complexes and intermediate filaments, are not commonly used to support the cortex. In their stead are often found layers of microfilamentous material (Adoutte and Fleury, 1996). The protein composition of most such layers is unknown; in a few, cytoskeletal proteins have been identified that are, to date, well characterized only in protists (cf. Bouck and Ngô, 1996).

Examples of such novel cytoskeletal proteins are the tetrins, first described in *Tetrahymena* (Honts and Williams, 1990; Brimmer and Weber, 2000), and the epiplasmins of *Paramecium* (Nahon et al., 1993; Coffe et al., 1996) and other protists (Huttenlauch et al., 1998b; Bouchard et al., 2001). Perhaps the most well characterized and widespread of protist cytoskeletal proteins are the articulins. These proteins were first described in the cortex of the euglenoid *Euglena gracilis* (Marrs and Bouck, 1992), where they assemble into articulating strips below the plasma membrane. Articulon-like proteins have also been identified beneath the plasma membrane of the parasitic protists *Plasmodium* (Stahl et al., 1987; Bowman et al., 1999; Tchavtchitch et al., 2001) and *Toxoplasma gondii* (Mann and Beckers, 2001) at certain life-cycle stages. Immunological evidence indicates that similar proteins are found in dinoflagellates as well (Bricheux et al., 1992; Huttenlauch et al., 1998b). Among ciliates, the clearest evidence for the presence of articulins is in *Pseudomicrothorax dubius*. These cells possess a thick, continuous filamentous layer termed the 'epiplasm', situated in the cytoplasm immediately below the cortical alveoli (Peck et al., 1991). The two major epiplasmic proteins in *P. dubius* have been characterized (Huttenlauch et al., 1995; Huttenlauch et al., 1998a) and shown to have properties quite similar to the articulins of *Euglena*. The hallmark of the articulins is a core of numerous tandemly repeating 12-amino acid (a.a.) units, rich in valine and proline (VP-rich).

The cortical cytoskeleton of euplotid ciliates is disposed in a different fashion than in most other ciliates. In these cells, the surface is supported by a monolayer of tightly abutted 'alveolar plates' (APs; Fig. 1), so called because the individual polygonal scales of the assemblage occupy the spaces within the membranous cortical alveoli (Ruffolo, 1976; Hausmann and Kaiser, 1979; Geyer and Kloetzel, 1987; Williams et al., 1989; Hausmann and Hülsmann, 1996). The major proteins making up these APs in various species of *Euplotes* have been identified and partially characterized (Williams et al., 1989; Williams, 1991; Kloetzel, 1991). Electrophoretic evidence suggests that at least three subunit forms of these proteins exist in the plates. On the basis of peptide mapping and genetic data, Kloetzel has proposed that each subunit is encoded by a separate gene locus in *Euplotes aediculatus* (Kloetzel, 1991; Kloetzel et al., 1992), and has termed the 125, 99 and 97/95 kDa electrophoretic variants the  $\alpha$ -,  $\beta$ - and  $\gamma$ -platein forms, respectively (Kloetzel, 1993). Confocal immunofluorescence results reported in the present study show that these platein forms, while co-localized within mature APs, display significant differences in solubility.

In the work presented here, we have used anti-platein antibodies to screen an expression library of *Euplotes* genes, and have isolated and cloned a gene encoding one of the closely related  $\beta$ - or  $\gamma$ -platein subunits. Taking advantage of new peptide sequence information and a PCR-based strategy, two additional platein genes have been cloned; these encode very similar  $\alpha$ -platein isoforms. The derived protein sequences of these three genes indicate that the plateins display long tandem runs of VP-rich dodecamer repeats, and clearly are members of the articulon class of cytoskeletal proteins. However, distinct differences in amino acid composition and arrangement indicate that the plateins make up a separate family within the articulins. Moreover, all three plateins predict



**Fig. 1.** General features of *Euplotes*. Projection of optical sections passing through the ventral (A) or dorsal (B) surfaces of a cell decorated (in green) with anti-E antiserum directed against proteins of the alveolar plates (Williams et al., 1989), and decorated (in red) with the 20H5 antibody directed against centrins at the bases of the cilia (Sanders and Salisbury, 1994). On the ventral surface, clusters of cilia form the oral ciliature (OC) and the locomotory cirri (C); alveolar plates on this surface are irregular in shape. On the dorsal surface, the cilia are aligned into antero-posterior rows (in red), between which alveolar plates are regularly patterned.

canonical start-transfer sequences at their N-termini, which correlates well with the final intra-alveolar location of the assembled skeletal plates. N-terminal sequencing of a  $\gamma$ -platein directly demonstrates that the predicted signal peptide is removed from the mature protein. To our knowledge, the plateins are the first cytoskeletal proteins from any eukaryotic cells described to date that feature such N-terminal signal sequences.

## Materials and Methods

### Cell culture

Both isolates of *E. aediculatus* utilized in these studies originated in France. Clones used in the previous identification of the plateins (Kloetzel, 1991) were originally collected by Dieter Ammermann near Marseilles. They were cultured at room temperature in modified Pringsheim's medium and fed *Tetrahymena*, as described (Kloetzel, 1991). These strains were used for platein peptide sequencing, for anti-platein antibody production, and in the PCR reactions leading to the cloning and sequencing of the  $\alpha$ -platein genes. Another strain of *E. aediculatus* was isolated on the campus of the Université Paris-Sud in Orsay. Cultures of these cells were used to create the expression and genomic libraries from which the gene encoding  $\beta/\gamma$ -platein was isolated and cloned, and for confocal microscopy. These cells were cultured in commercial Volvic mineral water with *Tetrahymena* as food source.

Preparation of cortical residues of *Euplotes*, as a means for enrichment of plateins, basically followed the Triton-high salt protocol (Williams et al., 1989) with modifications reported elsewhere (Williams and Honts, 1995).

### Antibody production and purification

The production of monoclonal antibodies (mAbs) against *E. aediculatus* plateins has been described (Kloetzel, 1991). The present

studies used mAb PL-5 (which recognizes all platein forms in this species) and mAb PL-3 (recognizing only the  $\beta$  and  $\gamma$  isoforms of platein, but not the  $\alpha$  form).

To obtain an antibody specific for  $\alpha$ -platein, polyclonal antisera were raised in rabbits against *Euplotes* cortical proteins separated by SDS-PAGE and transferred onto nitrocellulose membranes, following methods that have been described previously (Kloetzel, 1991). Nitrocellulose strips containing the 125 kDa  $\alpha$ -platein band were excised, sonicated to a fine slurry in PBS, and used for immunizations. Whole sera that reacted positively with the 125 kDa  $\alpha$ -platein band in immunoblots were affinity purified using  $\alpha$ -platein bands blotted onto PVDF membrane after electrophoretic separation, following described protocols (Harlow and Lane, 1988). The final eluate (containing affinity-purified antibody that we designated AP-2) yielded much lower backgrounds in immunoblotting and immunofluorescence staining protocols than did whole serum.

Polyclonal antibodies (pAbs) against the two main electrophoretic plate protein bands of *Euplotes eurytomus* ('anti-E' serum) (Williams et al., 1989) were kindly provided by Norman Williams.

### Peptide sequencing

*Euplotes* cortical extracts were separated by SDS-PAGE, blotted onto PVDF membranes, and stained with Coomassie Blue R-250. Strips of membrane bearing individual platein bands were excised and incubated with TPCK-trypsin for 24 hours at 37°C. The resultant peptides were separated by HPLC and sequenced with an ABI 477A Protein Sequencer (for details, see Matsudaira, 1993; Fernandez et al., 1994).

### cDNA expression library construction

Messenger RNAs were isolated (Quick-Prep micro-mRNA purification kit; Pharmacia) from a *Euplotes* culture allowed to grow slowly overnight at 16°C in a dilute solution of dried milk (0.05% in mineral water) to avoid interference from *Tetrahymena* food organisms. Double-strand cDNAs were synthesized by random priming using a cDNA synthesis kit (Amersham). The cDNA rapid adaptor ligation kit, cDNA cloning module  $\lambda$ gt11 and  $\lambda$ -DNA in vitro packaging module (Amersham) were used to construct the library in the  $\lambda$ gt11 vector. A total of  $2.5 \times 10^6$  pfu recombinant phages were obtained.

### Expression library screening and recombinant sequence analysis

The cDNA library was screened with an antibody raised against plateins (E-band) from *E. eurytomus*, following standard procedures (Sambrook et al., 1989).  $0.8\text{--}1.0 \times 10^5$  plaques were screened; positive plaques were excised and subjected to three further rounds of expression screening prior to characterization of inserts. Amplification of positive  $\lambda$ gt11 clones was performed using 2.5  $\mu$ l of phage suspension in 50  $\mu$ l PCR reactions at pH 9.0. The amplification products were cloned into the *Sma*I site of the vector pUC 18 for sequencing.

Some clones from the two  $\lambda$ gt11 libraries were sequenced by the Genome Express company (Grenoble, France). Others were sequenced manually or with the Vistra automatic sequencer (Amersham) using the DNA cycle sequencing kit from Amersham. Sequences of  $\alpha$ -platein genes were obtained by automated DNA sequencing (ABI Prism Model 373A; PE Applied Biosystems) using Big Dye methodology supplied by the manufacturer. The sequences obtained were compared with the non-redundant sequence databases using the ExPASy interface to the SIB BLAST network service (Altschul et al., 1997). The nucleotide sequences of the three platein genes reported here have been submitted to GenBank™, under accession numbers AY124989 ( $\alpha$ 1), AY124990 ( $\alpha$ 2), and AY124991 ( $\beta/\gamma$ ).

### Southern and northern blot analyses

Blotting protocols with agarose-separated nucleic acids followed those described (Sambrook et al., 1989).  $\beta/\gamma$ -platein probes cut from recombinant plasmids were labeled using the Megaprime DNA labeling kit (Amersham);  $\alpha$ -platein probes (from PCR reactions) were labeled using random hexanucleotide primers. Hybridizations (using Hybond-N membranes; Amersham) were carried out overnight at 65°C in 5 $\times$  Denhardt's solution, plus 0.5% SDS, and 10 mM EDTA (except Southern blots with  $\beta/\gamma$ -platein probes, which used 0.5 M sodium phosphate pH 7.2, 7% SDS and 1 mM EDTA). 100 mg/ml denatured tRNA was included in northern hybridizations.

### Expression analysis

Total RNA was extracted from an actively growing vegetative culture of *Euplotes*, since nucleic acids from the food organism (*Tetrahymena*) do not cross-hybridize with platein probes. PolyA<sup>+</sup> RNA was prepared from other *Euplotes* cultures using the QuickPrep mRNA Kit (Pharmacia). Reverse-transcript (RT-)PCR analysis was performed in two separate reactions. cDNAs from total RNA reactions, using M-MLV reverse transcriptase (Eurobio, France), were amplified with  $\beta/\gamma$ -platein-specific primers; amplification of polyA<sup>+</sup> RNA reactions, using reverse transcriptase from Pharmacia Biotech, utilized  $\alpha$ 1- and  $\alpha$ 2-specific primers.

### Indirect immunofluorescence

Immunolabeling was performed on permeabilized and fixed whole cells as described previously (Fleury, 1991; Jeanmaire-Wolf et al., 1993). Ghosts were produced by treatment for 1 minute with 0.25–1% Triton X-100 in PHEM buffer (Schliwa and van Blerkom, 1981), then fixed for 1 hour in 2% paraformaldehyde (Sigma) in PHEM buffer at room temperature and washed three times in PBS, pH 7.4, containing 10 mM EGTA, 2 mM MgCl<sub>2</sub>, 3% BSA, 0.1% Tween 20 (this buffer was used for all subsequent steps). The cells were incubated for 1 hour in the presence of the different primary antibodies (working dilutions: 1/5 for PL-3 and PL-5 mAb supernatants; 1/100 for AP-2 affinity-purified pAb; 1/200 for Anti-E pAb). After two washes in the same buffer, the cells were incubated for 1 hour with secondary antibody (Alexa Fluor 488-conjugated goat anti-mouse or Alexa Fluor 568-conjugated goat anti-rabbit; Molecular Probes) at 1/100 to 1/200 dilution. Following three washes, cells were mounted in Citifluor medium (City University, London, UK) and observed with conventional epifluorescence (Leitz) or with a Biorad MRC 1024 confocal microscope equipped with a Nikon Diaphot 300 inverted microscope and a krypton/argon laser (Service d'Imagerie Cellulaire, Orsay, France). Z-series acquisition was obtained with a Nikon Plan Apo 60 $\times$  oil immersion objective, using 522/DF35 and 598/40 filters for green and red light, respectively. Individual focal plane projections were saved as separate files, then merged and colorized using Adobe Photoshop (Adobe Systems, San Jose, CA).

## Results

### Sequences of platein peptides

Trypsin digestion and peptide sequencing was carried out on individual  $\alpha$ -,  $\beta$ - and  $\gamma$ -platein bands from *E. aediculatus* cortical extracts separated by SDS-PAGE and transferred to PVDF membranes. Table 1 shows the a.a. sequences of several peptides successfully identified. Note the similarity of many of the peptides from the  $\beta$ - and  $\gamma$ -platein variants (e.g., their respective f52 fractions;  $\beta$ -f72 and  $\gamma$ -f71). The one-dimensional tryptic peptide maps published earlier (Kloetzel, 1991) also showed several apparently common peptide bands generated from the  $\beta$ - and  $\gamma$ -plateins. Many of these new

**Table 1. Sequences of tryptic peptides derived from  $\alpha$ -,  $\beta$ - and  $\gamma$ -plateins**

Tryptic peptides	Sequences <sup>†</sup>
Alpha peptides	
f30	TYETSYVAPAGGYR
f42	YNE[W]VEEV[HA]PR
f45	VVEEPVY[W]APR
f55	LPDAVFTYTDEHHTPYTYEYVVDVHIIPTLE[YYDR]
Beta peptides	
f50	TW[Y]****VPEYVQR
f52	*PVTSYQTV***EYVP[H]VDV[V]PE
f55	*VVYEAVQDNPLSIVNE[E]K
f69	<u>IAEPV</u> *TEPI
f72	<u>EPV[W]TQPVVV</u> [SSP/A]
Gamma peptides	
f43	T[H]PVDEPEFITR
f52	<u>PVTSYQTVDEVEYVPHVDV</u> VPEVQHR
f71	<u>EPVWTQPVVVEPA</u> *[T]N *[AF]
f72	<u>IAEPVWTQPV</u> **

<sup>†</sup>Square brackets indicate tentative assignment; asterisks indicate unknown residue; underlining indicates regions common to  $\beta$ - and  $\gamma$ -plateins.

peptide sequences were useful in confirming the identity of the isolated platein genes and, in the case of the  $\alpha$ -platein peptides, in designing oligonucleotide primers for the amplification and ultimate isolation of two  $\alpha$ -platein genes.

#### Identification of a gene encoding $\beta/\gamma$ -platein in the expression library

We took advantage of the availability of antibodies against plateins to screen a *E. aedicularis* expression library. Five positive phages were obtained in the first round of anti-platein screening with anti-E serum, three of which ( $\lambda$ W1,  $\lambda$ W2 and  $\lambda$ W3) remained positive after further rounds of immunoscreening. The nucleotide sequence of each cDNA insert shows an open reading frame (ORF) that is in-frame with the  $\beta$ -galactosidase sequence; the three ORFs largely overlap, yielding an assembled total sequence encoding 553 a.a. that is rich in valine and glutamic acid and displays numerous internal repeats. Probing a Southern blot of total cellular DNA with the insert of phage  $\lambda$ W1 revealed a single band at around 2 kb (data not shown), indicating the existence of a macronuclear gene ('minichromosome') with a coding capacity of approximately 666 a.a. (74 kDa).

To obtain the entire sequence of this protein, we screened a macronuclear genomic library of *E. aedicularis* with the  $\lambda$ W1 insert as probe. One of the six positive clones found in the first round of screening was further analyzed and corresponds to the complete macronuclear molecule. The insert is 2069 bp long and has the typical features of *E. aedicularis* 'minichromosomes': a single ORF of 1935 nucleotides (n.t.), with short adenine and thymine (AT)-rich 5' leader (46 n.t.) and 3' trailer (65 n.t.) sequences. Most of the 5' duplex C<sub>4</sub>A<sub>4</sub> telomeric repeats are missing. The deduced 644 a.a. sequence included the previously determined partial sequence from the expression library. The correspondence of the sequence obtained with several  $\beta$ - and  $\gamma$ -peptides (cf. Fig. 3) indicates that this gene encodes either  $\beta$ - or  $\gamma$ -platein; the macronuclear gene sequence contains  $\beta$ -fragments f50, f52, f55 (with a single

mismatch), f69 and f72 (with two mismatches: E instead of SS) as well as  $\gamma$ -fragments f52, f72 and parts of fragments f43 and f71. Very interestingly, one  $\gamma$ -peptide (f71) resembles the COOH-terminal part of the  $\lambda$ W2 clone (EPVWTQPVVVEPAWTNPA), whereas the corresponding sequence of the genomic clone is EPVWTQPVVVEPAWTQPV. This suggests that  $\beta$ - and  $\gamma$ -platein proteins differ near their C-terminal extremities, and that the insert of phage  $\lambda$ W2 derives specifically from a  $\gamma$ -platein gene. The genomic clone sequence determined (GenBank AY124991) thus probably represents a  $\beta$ -platein gene.

#### Identification of two $\alpha$ -platein genes using PCR

A different approach, independent of the expression library, was used to identify the genes encoding  $\alpha$ -platein. We took advantage of the sequences determined for four  $\alpha$ -platein-specific peptides (Table 1), one of which (f55) is quite long (31 residues), to devise a PCR strategy. On the basis of the sequences of  $\alpha$ -f55 and of  $\alpha$ -f30 (14 residues), six oligonucleotide primers (termed AP1-AP6) were designed in both forward and reverse combinations (Table 2) and used for PCR, with *E. aedicularis* DNA as the template. A single amplified fragment of 1025 bp was obtained, using the primer combination AP2 + AP3. This fragment was cloned and sequenced; within the derived a.a. sequence, peptides corresponding to f30 and f55 were confirmed (although minor substitutions occurred towards the end of the long f55 peptide). Additionally, the exact sequence of peptide  $\alpha$ -f42 was found, indicating that this amplified PCR product corresponded to a portion of an  $\alpha$ -platein coding region.

In order to obtain the sequence of the entire macronuclear gene encoding  $\alpha$ -platein, we utilized a strategy termed RATE-PCR (rapid amplification of telomere extremities) (Di Giuseppe et al., 2002), based on the organization of *Euplotes* macronuclear genes as linear DNA molecules terminating in telomeres of known sequence. The strategy used as primers two internal oligonucleotides, termed AP7 and AP8, designed on the basis of the sequence determined for the amplified fragment, in combination with an oligonucleotide corresponding to the telomere sequence. Using this strategy, we cloned and sequenced two fragments that both overlapped the original 1025 bp fragment; this allowed the reconstruction of an entire macronuclear  $\alpha$ -platein-encoding gene. This gene, named the  $\alpha$ 1-platein gene (GenBank AY124989), consisted of a coding region of 1611 nucleotides, with 5' and 3' flanking regulatory regions of 87 and 317 nucleotides, respectively (including the presumed euplotid telomeres). Within the deduced a.a. sequence, a precise match for the fourth  $\alpha$ -platein peptide (f45) was found.

During the analysis of clones obtained from RATE-PCR, we found a second clone that only partially overlapped the previously determined  $\alpha$ 1 coding sequence. It differed in the 5' flanking noncoding sequence, thus suggesting the existence of a second  $\alpha$ -platein gene. Confirmation of the C-terminal sequence of a second  $\alpha$ -platein gene and details of its 3'-untranslated region were obtained by a RACE-PCR strategy, utilizing *E. aedicularis* polyA<sup>+</sup> RNA. The complete sequence of the coding region of this gene, named the  $\alpha$ 2-platein gene, was determined (GenBank AY124990). The existence of two

**Table 2. Nucleotide sequences of the primers used for PCR amplifications of the *Euplotes aediculatus*  $\alpha$ -platein genes**

Primer name	Nucleotide sequence (5'-3')	Origin/specificity (peptide)
AP-1	TAT GTI GCI CCI GCI GGI CCI CCI TAT GG	f30 (a.a. 6-14)
AP-2*	CTA TAI CCI CCI GCI GGI GCI ACA TA	f30 (a.a. 6-14)
AP-3	GTI TTT ACI TAT ACI GAT GAI CAT CAT AC	f55 (a.a. 5-14)
AP-4*	GTA TGA TGI TCA TCI GTA TAI GTA AAI AC	f55 (a.a. 5-14)
AP-5	CCI TAT ACI TAT GAI TAT GTI GAT GT	f55 (a.a. 15-24)
AP-6*	ACA TCI ACI ACA TAI TCA TAI GTA TAI GG	f55 (a.a. 15-24)
AP-7	ATG GTA CCA CAT CAA TGA CAA ATT	$\alpha$ 1 (n.t. 1236-1259)
AP-8*	AGG AAC GTG GAT TTC TTC GAC	$\alpha$ 1 (n.t. 888-908)
AP-9	GAC CGC TCA GCC AGT AAC AAC CG	$\alpha$ 2 (n.t. 152-174)
AP-10*	TTA CCA TCT TCT GTA TCC ACC	$\alpha$ 1 (n.t. 1680-1700); and $\alpha$ 2 (n.t. 1572-1592)
AP-11*	TTG GAA CGC TAA CTT CTT CAA CA	$\alpha$ 1 (n.t. 1079-1101); and $\alpha$ 2 (n.t. 971-993)
AP-12	AAG GCC GCA CTA CCA CCA GGT C	$\alpha$ 1 (n.t. 611-632)
AP-13	CAC CAA AGC AAA AGT ACC ACT ATT CA	$\alpha$ 2 (n.t. 619-644)
AP-14	TGT TGA AGA AGT TAG CGT TCC AA	$\alpha$ 1 (n.t. 1079-1101); and $\alpha$ 2 (n.t. 971-993)
5' $\alpha$ 1TEL	GTT ATA TTG ATA ATG GGT TTT AAG	$\alpha$ 1 (n.t. 40-63)
3' $\alpha$ 1TEL*	CTC GAT AAA ATG TTT GCG CAT TTA	$\alpha$ 1 (n.t. 1941-1964)
C4A4TEL	CCC CAA AAC CCC AAA ACC CC	(C4A4) 5'-telomeres

\*Indicates reverse primers.

$\alpha$ -platein genes was confirmed by a Southern blot analysis of *E. aediculatus* macronuclear DNA. Using the  $\alpha$ 1-platein coding region as probe, a tight doublet of bands appeared (data not shown).

#### Evidence for platein gene expression

Macronuclear gene-sized molecules of hypotrich ciliates are usually transcribed, and pseudogenes are rarely found. However, to determine whether both  $\alpha$ -platein genes are truly expressed, we carried out northern blot and RT-PCR analyses. Although the northern blot showed only a single band (data not shown), it is likely that at this resolution two messengers of similar size would overlap. Indeed, the RT-PCR experiment revealed the expression of both messengers (Fig. 2A). The

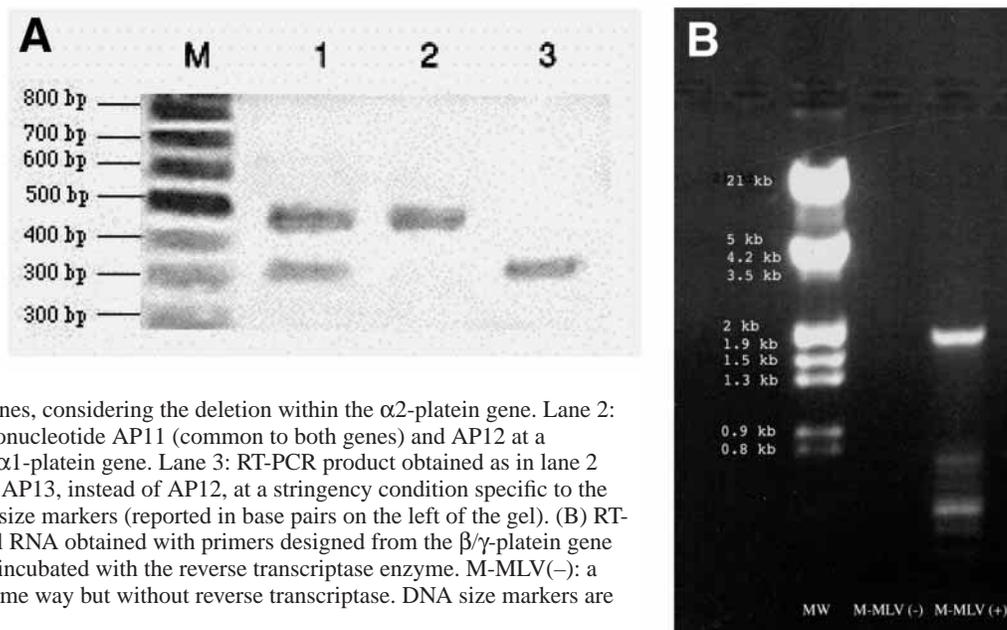
difference in their lengths confirms the presence of an insertion of 40 a.a. in the  $\alpha$ 1-platein gene with respect to the  $\alpha$ 2-platein gene (cf. Fig. 3). From the clones produced using the amplified cDNA products, 150 were screened; those corresponding to  $\alpha$ 1 and  $\alpha$ 2 cDNAs were equally represented, suggesting that the transcribed products of the two genes are also likely to be equally represented.

Since the original screen that uncovered the  $\beta/\gamma$ -platein gene sequence was performed on a cDNA expression library using anti-platein antibodies, it is reasonable to assume that this gene is also expressed in the cell. However, to demonstrate this directly, northern and RT-PCR analyses were performed. Hybridization on a northern blot gave a single band at approximately 1.9-2.0 kb (data not shown). Expression of the gene was also confirmed by RT-PCR; amplification of reverse-

**Fig. 2.** Analysis of the expression of the platein genes. (A) Products of the  $\alpha$ -platein RT-PCR experiments (see text), stained with ethidium bromide. Lane 1:

RT-PCR products obtained using oligonucleotides AP11 and AP12 at low annealing stringency so that they bind to both genes; the two bands correspond in size to

the expected fragments of the two genes, considering the deletion within the  $\alpha$ 2-platein gene. Lane 2: RT-PCR product obtained using oligonucleotide AP11 (common to both genes) and AP12 at a stringency condition specific for the  $\alpha$ 1-platein gene. Lane 3: RT-PCR product obtained as in lane 2 except for the use of oligonucleotide AP13, instead of AP12, at a stringency condition specific to the  $\alpha$ 2-platein gene. Lane M: molecular size markers (reported in base pairs on the left of the gel). (B) RT-PCR amplification product from total RNA obtained with primers designed from the  $\beta/\gamma$ -platein gene sequence. M-MLV(+): RNA sample incubated with the reverse transcriptase enzyme. M-MLV(-): a control RNA sample treated in the same way but without reverse transcriptase. DNA size markers are shown to the left.



$\alpha$ 1-Platein	$\alpha$ 2-Platein	$\beta/\gamma$ -Platein
1 <b>MRFIGYFLILSLIIGSVYVQA</b>	1 <b>MKIIGYFLILSLIIGSVYVQA</b>	1 <b>MRLVLCIYLLLSVGFVGMAYGYKRNL</b>
22 QIVKPAATTTTKPAAPAKPAAQK	22 QTAQPVTTAATKPAAKTTATKPAATTATKT	25 <b>GEAAIPKAAATGSTKAPVTAATA</b>
45 TVAKPATTTVAKPAVARAPVTVA	52 VAPAKPAAAKPLATTTVAKPAVARAPVTVA	48 <b>PKTTTTAAKAIPTTTRAAATPAWG</b>
69 PAWTA PVATQ RFVE	83 PAWTA PVTQ RFVE	72 TAAARPVGTYYPVGGVVEPGVARAE
83 PAWTA PVATQ RFVE	97 PAWTA PVAIQ RVVE	98 PVAYGARPASVYAGYAPAFAYEAFPT
97 PAWTA PVATQ RFVE	111 PAWTA PVATQ RFVE	125 <b>VPEVQRRVGYET</b>
111 PTWTA PVAIQ RVVE	125 PAWTA PTWVE	137 <b>VPEVERRTNYAT</b>
125 PAW	135 PAWRE	149 <b>VVDYATVQRAEV</b>
RE	140 PAWTQ PVWTE QVVREPVRVVEE	161 <b>VPEVERRTWYETVD</b>
130 PAWRD	162 PVWVA	175 <b>VPEVQRTQV</b>
135 PAWTQ PVWTE QVVREPVRVVEE	167 PREYA	185 <b>VPERVARPVTSYQT</b>
157 PVWVA	172 PVTQV	199 <b>VDEVEVPVHVDV</b>
162 PREYA	177 EAPKQKYHYSTREYENKLPDAVFYTDDEHH	211 <b>VPEVQHRTRSEV</b>
167 PVAVV	207 <b>TPHTYEVDDVH</b>	223 <b>VNEPEWRTRYQ</b>
172 EEP RPHYHQVTREYENKLPDAVFYTDDEHH	219 <b>IPHTLEYNDERVLVD</b>	235 <b>VVEPEYRTRYET</b>
202 <b>TPHTYEVDDVH</b>	233 HPVEVPETIM	247 <b>VDEYEEVRVYIT</b>
214 <b>IPHTLEYTDERVLVD</b>	243 VPHTYQYEEAIT	259 <b>VDEPEFIRHTI</b>
228 HPVEVPETIM	255 VPKTVEYQEIQM	271 <b>VMEPEYRTRTEV</b>
238 VPHTYQYEEAIT	267 VPHPWVEEIH	283 <b>VNDVQYVTRYQ</b>
250 VPKTVEYQEQLI	277 VPHTYQYEQIM	295 <b>VDEPEFRTRTEV</b>
262 VPHPWVEEIH		307 <b>VDPVQYVTRYQ</b>
272 VPHTYQYEQIM		319 <b>VDEPEFRTRTQL</b>
284 <b>VPHTYEQEAIT</b>		331 <b>VPEVRQ</b>
296 VPRTVEVKM		339 <b>VDEPEYIT</b>
304 <b>VPHTFEYEE</b>	289 <b>VPHTYEVVEEVS</b>	347 <b>VNDIEYVTRQQV</b>
314 <b>VPHEYQEQIM</b>	301 VPRTVEYQEIQM	359 <b>VDEPEYRAREE</b>
324 <b>VPHTYEVVEEVS</b>	313 <b>IPHTYEQEAVT</b>	371 <b>VDEVEYVERVEQ</b>
336 VPRTVEYQEIQM	325 <b>VPRTVEYQEEIM</b>	383 <b>VPEYRTRTEV</b>
348 VPHTYEQEAVT	337 <b>VPRDYEQEQIM</b>	393 <b>VPEIQYVTRQA</b>
360 VPRTVEYQEQLI	349 <b>VPHEYQVVEQVA</b>	404 <b>VREDPEIRYHTEH</b>
372 VPHDYEQEEIM	361 <b>VPRTYEVETID</b>	417 <b>VPEVQRKRLYE</b>
384 VPHQCQIVEQVA	373 <b>VPHTYEQEEIM</b>	429 <b>VDEVREKTYET</b>
396 VPRTYEVVETID	385 <b>IPREYQWDEEYI</b>	441 <b>KDDVTYTEHAEPAPQYEEKCVVVEAVODNPL</b>
408 VPHTYEQEEIM	397 <b>VPRYNEWVEEVH</b>	471 <b>SIVNEKGWTEVPRHQWIDGKQLIERPIY</b>
420 <b>IPREYQWDEEYI</b>	409 <b>APRTYEVVTEQYDV</b>	501 <b>VVPEQRPIHNRWQTAIVPEPTVLEPRTIAE</b>
432 <b>VPRYNEWVEEVH</b>	423 <b>VPREYVVEEVH</b>	532 <b>PVWTE</b>
444 <b>APRTYEVVTEQYDV</b>	435 <b>NPQIERYQDVE</b>	537 <b>PIWTE</b>
458 <b>VPREYVVEEVH</b>	447 <b>VPRTVYRNEQVA</b>	547 <b>PVWTE PIRTQ</b>
470 <b>NPQIERYQDVE</b>	459 <b>VPRTVAEEDRV</b>	557 <b>PVWTE PIRTQ</b>
482 <b>VPRTVYRNEQVA</b>	471 <b>VPRTVAVEQDVE</b>	567 <b>PVWTE PIRTQ</b>
494 <b>VPRTVAEEDRV</b>	483 <b>VPRTYESTVAVP</b>	572 <b>PVVVE</b>
506 <b>VPRTVAVEQDVE</b>	495 <b>TGGYRRW</b>	577 <b>PAWRE PAWRE PAWRE</b>
518 <b>VPRTYESTVAVP</b>		592 <b>PVWTQ</b>
530 <b>AGGYRRW</b>		597 <b>PVVVE</b>
		602 <b>PAWTQ</b>
		607 <b>PVVVD</b>
		612 <b>PVWFE</b>
		617 <b>EVPVKTEAAKTEPAKTEAAATEKERR</b>

**Fig. 3.** Amino acid sequences of three plateins, derived from their respective nucleotide sequences. The proteins are arrayed to display their respective repetitive domain structures. N-terminal signal sequences (predicted by SignalP V1.1 for  $\alpha$ 1 and  $\alpha$ 2; directly determined for  $\beta/\gamma$ ) are highlighted in yellow. The primary repetitive sequences (VP-rich 12-mers, with some degeneracy) are indicated in red; the secondary proline-rich pentameric repeats are shown in blue. Note the inversion of primary and secondary repeat domains between the  $\alpha$ - and  $\beta/\gamma$ -plateins. Sequences found within one of the  $\alpha$ -isoforms, but missing from the other, are highlighted in light gray; the corresponding gaps in the other isoform (representing deletions from the aligned sequences) are indicated with lines. Sequences corresponding to the tryptic peptides from  $\alpha$ -platein (Table 1) are underlined. For  $\beta/\gamma$ -platein, the  $\beta$ -tryptic peptides are in double underline, and the  $\gamma$ -peptides (and actual N-terminal  $\gamma$  sequence) in single underline; sequences common to both are in wavy underline. Residues that show very high prediction values as phosphorylation substrates ( $\geq 0.9$  output values using NetPhos 2.0) are highlighted in darker gray.

transcribed RNA from vegetative cells with a  $\beta/\gamma$ -specific pair of primers gave a major band at the expected size (approximately 2 kb; Fig. 2B).

#### Sequence features of the encoded platein proteins

The  $\beta/\gamma$ -platein gene sequence was the first to be obtained. The predicted protein, of 74.9 kDa, displayed several interesting properties, as first revealed by analysis of its a.a. composition. Particularly abundant residues included valine, glutamate, threonine, proline and arginine; these five a.a. accounted for more than 60% of its 644 residues. The four major charged residues were notably abundant, in sum almost 30% of the total

protein; negatively charged a.a. (D + E) greatly outnumbered positively charged (K + R), yielding a predicted pI of 4.88.

Conceptual translation of the  $\alpha$ 1- and  $\alpha$ 2-platein gene sequences revealed encoded proteins (of 536 and 501 a.a., respectively) that were also predicted to be highly acidic, and similarly rich in V, E, P and T. As shown in Fig. 3, they are very similar in overall sequence,  $\alpha$ 1 having one insert of 40 a.a. not found in  $\alpha$ 2, and lacking one 5 a.a. insert found only in  $\alpha$ 2. In contrast to their overall highly charged, acidic backbones, the N-termini of the plateins are by far the most hydrophobic portions of the molecules. When evaluated by the SignalP V1.1 program (Nielsen et al., 1997), the N-termini of all three sequenced plateins meet the criteria for canonical

**Table 3. Analysis of amino acid use within the primary 12-mer repetitive domains of plateins, with articulins 1 of *Pseudomicrothorax dubius* for comparison**

Articulins 1 ( <i>P. dubius</i> ) (30 central repeats)													
Consensus	V	P	V	P	V	P	V	P	V	P	V	P	360
+	4	0	2	0	7	1	6	0	3	1	4	3	+31
-	0	<b>10</b>	1	9	0	5	0	7	1	5	0	4	-42
Charge consensus	+	-	(+)	-	+	-	+	-	+	-	+	(-)	73 (-11)
Residue No.	1	2	3	4	5	6	7	8	9	10	11	12	
α-Platein-1 ( <i>E. aediculatus</i> ) (28 C-terminal repeats)													
Consensus	V	P	R/H	T	Y	E	Y	Q/V	E	Z	I/V	-	336
+	0	0	<b>13</b>	0	0	0	3	0	0	0	2	0	+18
-	0	0	0	5	1	<b>17</b>	3	5	<b>20</b>	<b>13</b>	0	2	-66
Charge consensus			+	-	(-)	-		-	-	-	(+)	(-)	84 (-48)
Residue No.	1	2	3	4	5	6	7	8	9	10	11	12	
α-Platein-2 ( <i>E. aediculatus</i> ) (24 C-terminal repeats)													
Consensus	V	P	R/H	T	Y/V	E	Y	Q/V	E	Z	V/I	-	288
+	0	0	<b>13</b>	0	0	0	2	0	0	0	2	0	+17
-	0	0	0	5	0	<b>15</b>	2	5	<b>18</b>	<b>12</b>	0	2	-59
Charge consensus			+	-		-		-	-	-	(+)	(-)	76 (-42)
Residue No.	1	2	3	4	5	6	7	8	9	10	11	12	
β/γ-Platein ( <i>E. aediculatus</i> ) (27 central repeats)													
Consensus	V	P/D	E	V/P	E	Y	R/V	T	R	Y	Z	T/V	324
+	0	1	0	1	3	4	<b>15</b>	2	<b>16</b>	0	0	0	+42
-	0	9	<b>27</b>	1	<b>14</b>	3	0	1	1	1	<b>12</b>	1	-70
Charge consensus		-	-		-	(+)	+	(+)	+	(-)	-	(-)	112 (-28)
Residue No.	1	2	3	4	5	6	7	8	9	10	11	12	

Consensus residues at each position within the 12-mer repeat (bold) are those whose frequency at that position is at least twice that of the next most common residue (minimum of 10 uses for the consensus residue). Charged residues (+=R or K; -=D or E) at each position are also noted, and summed for all repeats in the right-hand column. Notable are the deviation of the plateins from the VPVPV... consensus and the strict alternation of positively and negatively charged residues (substituting for V and P, respectively, at particular repeat positions) of articulins. Note also the very large excess of negatively charged residues (red numerals) in plateins, compared with articulins. (Z=E or Q).

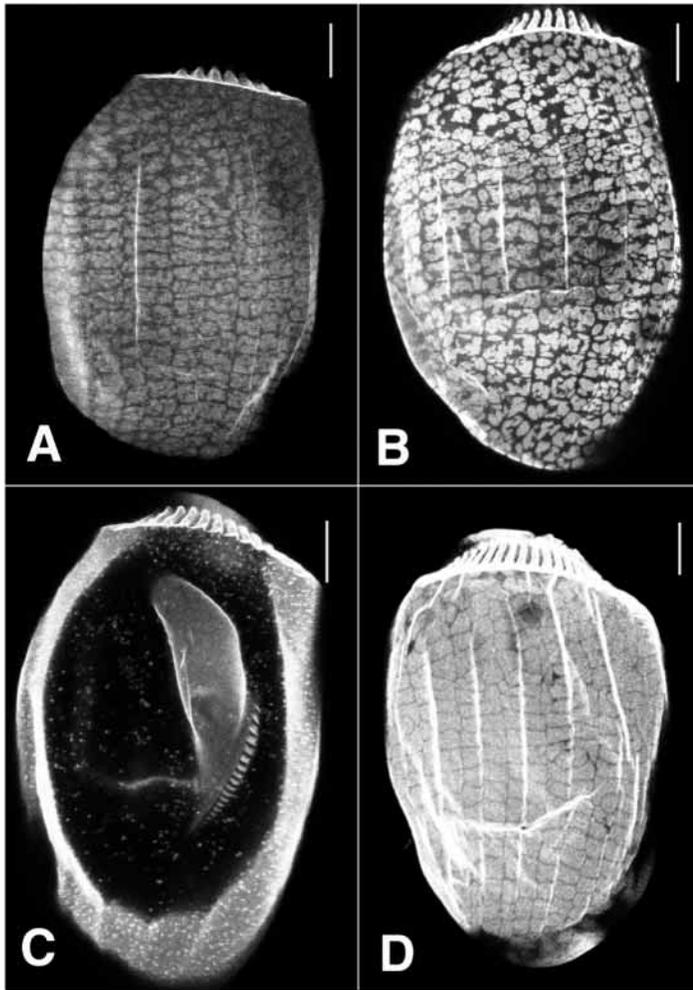
start-transfer signal peptides. This program also predicts the most likely signal cleavage sites, indicated for α1 and α2 in Fig. 3. The N-terminal peptide sequence from authentic γ-platein has been determined directly. Its sequence is GEAAATPKAAATGS[t][t]A[q]V, where [x] indicates an uncertain assignment. A corresponding sequence is found (underlined) in our derived β/γ-platein, beginning with residue 25: GEAAATPKAAATGSKAPV. This correspondence provides strong evidence that the predicted signal sequence is indeed cleaved from the mature platein in vivo. The non-matching residues later in the respective peptides lend slightly more weight to the suggestion (made above) that the 'β/γ' sequence determined actually represents a β-platein gene.

A search for potential phosphorylation sites in the platein sequences was performed, using the NetPhos 2.0 program (Blom et al., 1999). Numerous residues were identified, particularly in β/γ-platein, that show a strong likelihood of being phosphorylated (output values ≥0.9); these sites are highlighted in Fig. 3.

When the predicted α-protein sequences were searched against the BLAST database (Altschul et al., 1997), a potential relative was identified: a cytoskeletal protein from *E. gracilis*

named articulins (Marrs and Bouck, 1992). Similar articulins from the ciliate *P. dubius* have been identified and sequenced (Huttenlauch et al., 1998a). The primary articulins characteristic is a core of 12-a.a. repeats, with a VPV... consensus. Consequently, the platein sequences were scanned, and all three were constructed along a similar plan (Fig. 3). For example, α1-platein can be arranged with 28 VP-initiated tandem repeats – dodecamers, with some degeneracy (in the form of 8-, 10- or 14-residue units) – and α2 has 24 similar primary repeats. In β/γ-platein, 316 central residues (nearly half of the total molecule) can be arranged in 27 such repeats, most of them 12 a.a. in length. Rather than VPV, most α-platein core repeats in this arrangement initiate with VPH or VPR, and β/γ-repeats with VPE or VDE (cf. Table 3).

Another characteristic of the known articulins sequences is the presence of a set of shorter secondary repeat sequences. A search through the β/γ-platein sequence indeed showed additional repeats C-terminally. These secondary repeats are most easily arranged as 17 proline-initiated pentamers; included are three exact tandem decapeptides and three exact tandem pentapeptides (Fig. 3). In the α-plateins, secondary repeats are represented by 15 (α1) or 14 (α2) proline-initiated



**Fig. 4.** Immunofluorescence localization of  $\alpha$ - and  $\beta/\gamma$ -plateins in whole permeabilized cells. The dorsal surface of cells decorated with anti- $\alpha$ -platein (A and C) or anti- $\beta/\gamma$ -platein (B and D) are shown. Before staining, cells were permeabilized/extracted with either mild (0.25%; A and B) or stronger (1%; C and D) concentrations of Triton X-100. After mild permeabilization, plates are clearly visible after decoration with the  $\alpha$ -platein antibody (A), while appearing irregularly decorated with the anti- $\beta/\gamma$ -platein (B). However, after strong extraction, the plates are no longer detected with the anti- $\alpha$ -platein (C); many small vesicles appear just beneath the cell cortex, as seen in the center of this cell. With the same treatment, the plates are fully and strongly decorated with the anti- $\beta/\gamma$ -platein (D). Bars, 10  $\mu$ m.

pentamers, similar in sequence to those of  $\beta/\gamma$ -platein. Notably, these secondary repeats in the  $\alpha$ -platein isoforms are located on the N-terminal side of the primary core 12-mer repeats, instead of C-terminally as in  $\beta/\gamma$ -platein. In fact, the primary 'core' VP-rich dodecamer repeats in both  $\alpha$ -plateins are not at all central, but reside within seven residues of the respective C-termini.

#### $\alpha$ - and $\beta/\gamma$ -plateins co-localize but show differences in solubility

In the course of studies on the immunofluorescence localization of the plateins in cells, it became apparent that the pattern of antibody staining was dependent upon the concentration of the membrane permeant (Triton X-100) used during cell processing. Therefore, we analyzed the staining pattern of interphase cells under two conditions of permeabilization, 0.25% and 1% Triton X-100. After permeabilization with the lower concentration of Triton, the plates were fully decorated with the affinity-purified serum AP-2 (specific for  $\alpha$ -platein), but only partially decorated with the two mAbs PL-3, specific for the  $\beta/\gamma$ -plateins, and PL-5, which recognizes all three plateins on immunoblots (Fig. 4A,B). When the Triton concentration was increased to 1%, the plates were no longer clearly demarcated with the AP-2 antibody; the cell surface stained uniformly but less intensely,

and many small vesicles were detected in the cytoplasm (Fig. 4C). The plates were fully stained with the PL-3 antibody under these more-stringent extraction conditions (Fig. 4D). These results suggest that the AP-2 target is located on proteins ( $\alpha$ -plateins) that are at least partly solubilized by the same treatment that retains the PL-3 epitope (presumably on  $\beta/\gamma$ -plateins), now fully accessible within the plates. Under these conditions, the PL-5 antibody gave a pattern on interphase cells (full plate staining) similar to that observed with the PL-3 antibody.

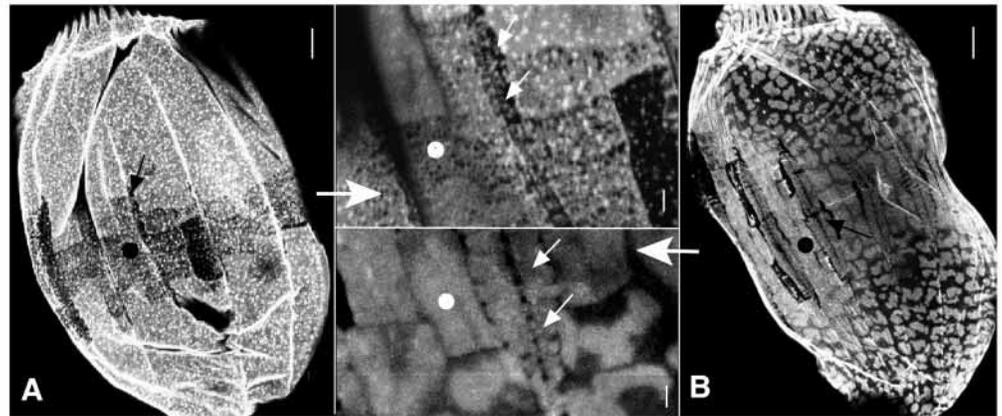
The accumulation of plateins in new APs formed during cellular reproduction was also followed. The pattern of appearance of new plates during pre-division morphogenesis has been described in detail from silver-stained preparations (Chatton and Seguela, 1940; Wise, 1965; Ruffolo, 1976). Briefly, plate assembly follows a two-step process. New miniature plates first appear in close association with proliferating basal bodies, both on the ventral and the dorsal sides; these new APs then gradually enlarge and spread across the cell surface, while parental plates are resorbed. This process leads to a complete renewal of the ventral surface, except in the oral area where old plates are retained and passed to the anterior daughter cell. On the dorsal side, where basal body duplication begins in the equatorial region of the ciliary rows, only two-thirds of the APs are initially replaced. Immunofluorescence suggests that the two platein forms ( $\alpha$  vs.  $\beta/\gamma$ ) in these new plates do not exhibit the same behavior. As in interphase cells, some  $\alpha$ -plateins are partially solubilized: with the AP-2 antibody, the staining of the new plates even after mild (0.25% Triton) extraction appeared reticulated against a fluorescent background (Fig. 5A). These plates were not stained at all after 1% Triton pre-treatment. By contrast, the new plates were fully stained with the PL-3 antibody after both 0.25% (Fig. 5B) and 1% Triton extraction. This indicates that the  $\beta/\gamma$ -plateins are less soluble than  $\alpha$ -plateins in assembling plates, as well as in fully formed ones. It appears, however, that the  $\beta/\gamma$ -epitopes are more accessible in newly forming plates than they are in mature APs.

## Discussion

### Plateins are articulins, yet display distinct differences

All three of the sequenced platein genes encode proteins with a set of common properties, clearly establishing them as members of the articulin class of cytoskeletal proteins. Interest in the articulins has increased as an ever-wider variety of

**Fig. 5.** Immunofluorescence localization of  $\alpha$ - and  $\beta/\gamma$ -plateins in dividing cells. Shown are the dorsal surface of cells decorated with anti- $\alpha$ - (A) and anti- $\beta/\gamma$ -plateins (B), with enlargements (large white arrows). The general appearance after decoration with the anti- $\alpha$ -platein (A) is as described in Fig. 4A, except in the area of ciliary duplication at the equator of the cell (black arrow), where the staining appears more punctuate (black circle). At higher magnification (top), the small new plates that appear concomitantly with the new cilia are virtually unlabeled (small white arrows); plates surrounding this area appear irregularly decorated (black, white circles). After treatment with anti- $\beta/\gamma$ -platein (B), only a partial staining of mature plates is seen, as in Fig. 4B. However, the new plates that appear along with the new cilia (black arrow in B, and small white arrows in the enlargement, bottom), as well as those surrounding this area (black circle in B, white circle in the enlargement), are fully decorated. Bars, 10  $\mu\text{m}$  (A, B); bars, 1  $\mu\text{m}$  (for the enlargements).



**Table 4. Comparison of features distinguishing plateins from other artikulins**

	Articulins	Plateins
Cellular location	Cytoplasmic (sub-plasmalemmal)	Within membranous cisternae (intra-alveolar)
N-terminal signal sequence	Absent	Present
Major repetitive domain ('core')	VP-rich 12-mers (VPVPV...)	VP-rich 12-mers (VP-charged)
Second repetitive domain (number of repeats)	<i>Pseudomicrothorax dubius</i> : 6-mers (G-rich), C-terminal (9-13) <i>Euglena gracilis</i> : Artic 80: 7-mers (APVT...), C-terminal (4) Artic 86: 7-mers (APVT...), N-terminal (4)	5-mers (P-rich, no G) $\alpha$ 1,2: N-terminal (15; 14) $\beta/\gamma$ : C-terminal (17)
Charged amino acids: total (%) (net charge)	<i>P. dubius</i> : Artic 1: 13.9% (+1) Artic 4: 16.1% (-12) <i>E. gracilis</i> : Artic 80: 19.2% (+1) Artic 86: 21.2% (+2)	$\alpha$ 1: 24.3% (-47) $\alpha$ 2: 24.0% (-39) $\beta/\gamma$ : 29.4% (-34)
Predicted pI: entire protein ('core' domain only)	<i>P. dubius</i> : Artic 1: 7.91 (5.02) Artic 4: 5.30 (4.85) <i>E. gracilis</i> : Artic 80: 7.86 (7.07) Artic 86: 8.19 (8.71)	$\alpha$ 1: 4.75 (4.43) $\alpha$ 2: 4.77 (4.39) $\beta/\gamma$ : 4.88 (4.66)
Anomalous gel retardation (gene-derived versus SDS-PAGE $M_r$ s; 'n'=apparent gel $M_r$ )	<i>P. dubius</i> : 69.7 $\rightarrow$ '78-80' kDa 59.9 $\rightarrow$ '78-80' <i>E. gracilis</i> : 72.1 $\rightarrow$ '80' 71.9 $\rightarrow$ '86'	$\alpha$ : 56.3-60.9 $\rightarrow$ '125' kDa $\beta/\gamma$ : 72.8 $\rightarrow$ '95-99'

Articulins described from the ciliate *P. dubius* (Huttenlauch et al., 1998a) and the euglenoid flagellate *E. gracilis* (Marrs and Bouck, 1992) are used to represent the standard artikulins. Amino acid composition and pI predictions for entire proteins (minus signal sequences for the plateins) and the major repeat core domains were derived using the ProtParam tool (ExPASy proteomics server of the Swiss Institute of Bioinformatics).

organisms has been identified that utilizes these proteins as important elements of their cortical cytoskeletons. Articulin homologs appear to exist in all taxa (see below). However, to date, these proteins have been well characterized only in unicellular eukaryotes, albeit an extremely wide evolutionary diversity of protists, including flagellates, dinoflagellates, apicomplexans and the ciliate *Pseudomicrothorax*. To these

groups now must be added another ciliate, the hypotrich *E. aediculatus*, which is only distantly related to *Pseudomicrothorax* (Lynn and Small, 2000).

Notable unifying features of the artikulins are summarized in Table 4, along with features of plateins that distinguish them from previously characterized artikulins. The following sections highlight points that deserve special attention.

### The primary ('core') articulon domain

An a.a. consensus in the tandem 12-mer repeats determined for the ciliate *Pseudomicrothorax* (and which also seems to typify the *Euglena* articulons) shows alternating V and P residues, with those residues not conforming strictly to the V or P positions often representing charged a.a. in an alternating + and - consensus arrangement, respectively (Huttenlauch et al., 1998a) (Table 3). A general conservation within this repetitive core domain almost certainly accounts for the observed crossreactivity of mAbs with different articulon forms, between similar (Williams, 1991; Kloetzel et al., 1992) and even distantly related species (Viguès et al., 1987; Bricheux et al., 1992; Curtenaz et al., 1994; Huttenlauch et al., 1998b).

While each *Euplotes* platein shows the hallmark articulon repeat motif of VP-rich 12-mers (ranging from 24-28 repeats in the three sequenced molecules), the consensus of these repeats clearly differs from the VPVPV... motif. The platein consensi are shown in Table 3, with articulon 1 from *P. dubius* for comparison. Each platein type shows a consensus 'fingerprint' that differs for the  $\beta/\gamma$ -platein versus the two  $\alpha$ -plateins; each in turn differs in significant ways from the *P. dubius* consensus. Notable are the strong preferences for acidic residues at distinct positions (e.g., glutamate in position 3 of  $\beta/\gamma$ -platein, positions 6, 9 and 10 of  $\alpha$ -plateins). Thus, the tendency for positively and negatively charged residues to alternate in the *P. dubius* consensus is not followed in the plateins; acidic residues can show strong preferences for occupying adjoining positions in the platein consensi. This reflects the overall much higher proportion of acidic residues in the plateins compared with other articulons (for example, almost a quarter of the residues in the  $\beta/\gamma$ -platein core repeat domain are aspartate or glutamate). The  $\alpha$ -plateins in particular are highly acidic, with net charges within the core domains alone of -50 and -44 for  $\alpha 1$  and  $\alpha 2$ , yielding predicted pIs for those regions of 4.43 and 4.39, respectively (cf. Table 4). By comparison, the most acidic of the described articulons, *P. dubius* articulon 4, has only a -19 net charge within its even longer primary domain.

The cortical alveoli, where APs are assembled, have been shown to be  $\text{Ca}^{2+}$  ion reservoirs in some ciliates (Stelly et al., 1991; Plattner et al., 1997; Plattner and Klauke, 2001). It thus seems reasonable to suggest that the abundant acidic residues in the platein core domain may function in  $\text{Ca}^{2+}$  binding, or even that  $\text{Ca}^{2+}$  ions might be included within *Euplotes* APs as part of their polymerization process. In another ciliate, *Coleps*, calcareous scales have been shown to assemble within the cortical alveoli (Huttenlauch, 1985).

### The second repetitive articulon domain

In described articulons, a secondary repeat domain is found that is shorter in the number of repeats and in the length of each repeating unit. In *E. gracilis* (Marrs and Bouck, 1992), these repeats number four in each protein, are heptads with a general consensus of APVT..., and can be located within either the N-terminal portion of the molecule (articulon 86) or the C-terminal portion (articulon 80). The *P. dubius* short repeats are glycine-rich hexamers, are more numerous (13 and 9 repeats for articulon 1 and 4, respectively), and are located near the C-termini of both forms (Huttenlauch et al., 1998a).

A second repetitive motif region is also found in *Euplotes*

plateins; however, the nature of the repeating units is novel. The most readily discerned repeats can be read as proline-initiated pentamers: 15 repeats in  $\alpha 1$ -, 14 in  $\alpha 2$ -, and 17 in  $\beta/\gamma$ -platein (Fig. 3). There is no single consensus, although PAW and PVW are common repeat triplets. Notable is the absence of glycines, prevalent in the secondary repeat domain of the other ciliate articulons (those of *P. dubius*), and the general proline/tryptophan richness of this region. One striking difference between the  $\alpha$ - and  $\beta/\gamma$ -plateins is in the overall design of the molecule; in both  $\alpha 1$  and  $\alpha 2$ , the P-rich pentamer domain is N-terminal to the primary core of 12-mer repeats, whereas in  $\beta/\gamma$ -platein, the pentamer domain is on the opposite side of the primary core, near the C-terminus.

### Anomalous retardation in electrophoretic mobility

The *E. gracilis* articulons, with apparent molecular masses of 80 and 86 kDa on SDS-PAGE gels, represent proteins whose predicted molecular masses (from the cloned genes) are about 72 kDa each. Articulons 1 and 4 from *P. dubius* migrate more aberrantly; their SDS-PAGE mobilities indicate proteins of 78-80 kDa, while their derived  $M_r$ s are 69.7 and 59.9 kDa, respectively. The mobilities of the plateins in SDS-PAGE are even more significantly retarded:  $\alpha$ -plateins migrate with an apparent  $M_r$  of 125 kDa, yet the proteins derived from their cloned genes predict proteins with  $M_r$ s of about 61 kDa ( $\alpha 1$ ) and 56.3 kDa ( $\alpha 2$ ). While  $\beta/\gamma$ -plateins migrate at 95-99 kDa by SDS-PAGE, the derived protein predicts a mass of about 73 kDa. This anomalous electrophoretic behavior may be related to the high proportion of charged residues in these proteins. Gumpel and Smith (Gumpel and Smith, 1992) found that an acidic repeat protein from *E. gracilis* (with an estimated pI of 3.56) showed similarly retarded gel migration, interpreted to be due to the high content of acidic residues in the protein. By contrast, the *E. gracilis* articulons, with relatively balanced charged residues and predicted pIs near 8.0, also show SDS-PAGE retardation. Thus, it is possible that the abundance and regular spacing of proline residues is the significant feature affecting gel retardation of the articulons. Both charged residues and prolines might contribute to a persistent secondary structure that is retained during electrophoresis. Neural-net-based secondary structure prediction programs (Rost, 1996) suggest that the platein molecules exist primarily in an extended form.

Post-translational modifications of the plateins could be an alternative (or additional) explanation for their anomalous gel migration. One report (Böhm and Hausmann, 1981) suggests that APs in *E. vannus* are coated with a material that reacts cytochemically with polysaccharide stains. While the protein sequences derived here from the platein genes reveal no consensi for N-terminal glycosylation, O-glycosylation prediction programs (Hansen et al., 1998) highlight a large number of potential sites, mostly located N-terminally (see below). It remains for future biochemical work to determine whether such glycosylation (or any other post-translational modifications) in fact occur.

### Signal peptides

All three plateins in *E. aediculatus* are distinguishable quite clearly from the previously described articulons by their putative N-terminal signal sequences. Unlike these other

articulins (and indeed all other known cytoskeletal proteins), which typically are assembled free in the cytoplasm, plateins are polymerized into structural elements (APs) within membrane-bound cisternae, the cortical alveoli. The N-terminal sequence of *E. aediculatus*  $\gamma$ -platein, determined directly here, matches the sequence of the derived  $\beta/\gamma$ -platein gene product, minus its first 24 residues (i.e., starting with residue 25; cf. Fig. 3). Similarly, the N-terminal sequences of two platein proteins extracted from *E. eurytomus* have been determined (N. Williams, personal communication); residues 4-11 of the upper platein band of this species are identical to those same sites in the *E. aediculatus*  $\alpha$ 1-platein sequence reported here, if the signal peptide predicted by the SignalP computer program is first removed. These results provide strong experimental support for the proposal that the postulated N-terminal signal sequences of both  $\alpha$ - and  $\beta/\gamma$ -platein forms are indeed cleaved to yield the mature proteins.

The presence of signal peptides on the plateins correlates well with their final intra-alveolar location, and raises questions for future work concerning the modes of plateins' synthesis, intracellular trafficking and polymerization into cytoskeletal plates. At this point, it will only be mentioned that many proteins similarly rich in proline residues have been shown to form strong 'interlocking networks' (Williamson, 1994), which has evident implications for the assembly and functioning of plateins (and other articulins) as cortical cytoskeletal elements. The assembly state of such proline-rich protein networks is known in many cases to be affected by reversible phosphorylation of the proteins (Williamson, 1994). Protein kinases can function within membrane-bound organelles of the secretory pathway (Drzymala et al., 2000); since the plateins predict significant numbers of phosphorylatable residues (cf. Fig. 3), it needs to be determined whether plateins are in fact phosphoproteins, and if so whether their phosphorylation state varies as fields of new cortical plates are assembled during pre-division morphogenesis. Regulation of the assembly state of another important cytoskeletal element (ciliary rootlets) by reversible phosphorylation has been shown to occur in *Paramecium* (Sperling et al., 1991).

#### Platein/articulin domains appear evolutionarily conserved

When used as queries in BLAST homology searches (Altschul et al., 1997), the  $\alpha$ -platein sequences identify the articulins from *Euglena*, then *Pseudomicrothorax*, among the highest scoring matches. However, BLAST searches using the full  $\beta/\gamma$  sequence reveal no described articulins among the first 100 responses; only if the  $\beta/\gamma$  core alone is submitted does a known (*Euglena*) articulin appear, well down the list. These results indicate that the  $\alpha$ -plateins are more-closely related to 'ancestral' articulins, and suggest that the  $\beta/\gamma$ -plateins are products of  $\alpha$ -platein gene duplications that have diverged significantly.

With the domain architecture of the plateins somewhat clear (Fig. 3), and with the increasing availability of fully sequenced genomes, it has proven instructive to narrow homology searches by using only selected domains as BLAST queries. Submitting the major articulin feature of  $\alpha$ -platein, the VP-rich core repeats, identifies potential homologs in virtually all taxa,

ranging from bacteria to humans. For example, a predicted VP-rich *Drosophila* protein (Adams et al., 2000) with a pronounced domain of 12-mer repeats and a likely N-terminal signal (AAF57876) yields a higher BLAST score than do even the other ciliate (*P. dubius*) articulins. Some vertebrate proteins may similarly employ platein-like domains. One projected human protein (XP\_092855) possesses 18 dodecamer repeats (most VP-initiated); another high-scoring protein predicted from the human genome database (Hs6\_7569) contains a core of 25 IP-initiated 12-mer repeats. Both of these human proteins show suggestions of membrane association (predicted trans-membrane helices). Most of the many putative articulin homologs uncovered are of unknown function or localization; however, it seems likely that they might assemble to perform cytoskeletal roles, as demonstrated for the articulins. If true, this would provide another instance [as in the case of the centrins (Salisbury et al., 1984; Chapman et al., 2000)] in which the identification and molecular characterization of new proteins from protists can prove useful in functional genomic studies of other organisms. As one example, a prokaryotic homolog of articulins has been found encoded within the recently sequenced genome of *Caulobacter crescentus* (Nierman et al., 2001). This protein (AAK22660; denoted a 'putative articulin') predicts an N-terminal signal sequence, suggesting that it might function (structurally) at the plasma membrane or within the periplasmic space.

Performing platein domain homology searches with the secondary P-pentamer repeat motif results in an entirely different set of responses. Particularly notable homologies are found among many insect proteins, typically secreted structural proteins such as those of the chorion or cuticle, or other secretory products (e.g., *Drosophila* salivary proteins). One example is peritrophin-95, an acidic secreted protein with 18 P-initiated pentamers at its C-terminus, which forms an extracellular mesh (peritrophic matrix) lining the gut of a larval dipteran (Casu et al., 1997). Other proteins with prominent domains of proline-initiated pentameric repeats (often referred to as extensins or proline-rich proteins, PRPs) are commonly found in plant cell walls (cf. Hong et al., 1987; Muñoz et al., 1998). The proline repeats in these wall proteins are presumed to form extended domains playing roles both structurally (stiffening the extension) and in binding rapidly and tightly to other proteins (cf. Williamson, 1994).

Even the N-termini of all three plateins (internal to the signal sequences) have an unusual common property; four a.a. (A, T, P, K) make up 80% of these short (50-60 residue) domains, which are correspondingly very basic (unlike the primary cores). BLAST searches reveal that sequences with this simple composition are characteristic of various mucins (heavily O-glycosylated secretory proteins) (Hanisch, 2001). The NetOGlyc 2.0 program (Hansen et al., 1998) identifies numerous T residues clustered within the N-terminal platein domains that predict a high likelihood of being O-glycosylated; such clustered sugar adducts could aid in stiffening this end of the plateins. Since O-glycosylation occurs in the Golgi apparatus (Hanisch, 2001), it would not be expected for cytoplasmically localized articulins.

Plateins thus can be viewed as composites of a modified (more anionic) articulin core domain, together with other domains differing significantly from those of the previously noted cytoplasmic articulins. The altered molecular

architecture of the platelets (including their signal sequences) is most probably related both to their different synthetic/trafficking paths (more like those of secretory proteins) and to the unique intra-alveolar environment within which the final assembled cytoskeletal product is formed.

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