Major Epiplastic Proteins of Ciliates Are Articulins: Cloning, Recombinant Expression, and Structural Characterization

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Abstract. The cytoskeleton of certain protists comprises an extensive membrane skeleton, the epiplastic, which contributes to the cell shape and patterning of the species-specific cortical architecture. The isolated epiplastic of the ciliated protist Pseudomicrothorax dubius consists of two major groups of proteins with molecular masses of 78–80 kD and 11–13 kD, respectively. To characterize the structure of these proteins, peptide sequences of two major polypeptides (78–80 kD) as well as a cDNA representing the entire coding sequence of a minor and hitherto unidentified component (60 kD; p60) of the epiplastic have been determined. All three polypeptides share sequence similarities. They contain repeated valine- and proline-rich motifs of 12 residues with the consensus VPVP- V-V-V-. In p60 the central core domain consists of 24 tandemly repeated VPV motifs. Within the repeat motifs positively and negatively charged residues, when present, show an alternating pattern in register with the V and P positions. Recombinant p60 was purified in 8 M urea and dialyzed against buffer. Infrared spectroscopic measurements indicate 30% β-sheet. Electron microscopy reveals short filamentous polymers with a rather homogenous diameter (~15–20 nm), but variable lengths. The small polymers form thicker filaments, ribbons, and larger sheets or tubes. A core domain similar to that of P. dubius p60 is also found in the recently described epiplastic proteins of the flagellate Euglena, the so-called articulins. Our results show that the members of this protein family are not restricted to flagellates, but are also present in the distantly related ciliates where they are major constituents of the epiplastic. Comparison of flagellate and ciliate articulins highlights common features of this novel family of cytoskeletal proteins.

The membrane skeleton of eukaryotic cells is part of the cytoskeleton. It is involved in the organization and maintenance of cell shape and functions in the generation and stabilization of specialized membrane domains. This submembraneous organization is usually dictated by microfilamentous structures containing actin and a variety of actin-binding proteins. Particularly well-analyzed examples are, for instance, the membrane skeletons of vertebrate red blood cells (for review see Bennett, 1990) and the intestinal brush border cells of various vertebrates (for review see Louvard, 1989). An actin-based subplasma membrane organization occurs not only in metazoan cells, but is also typical for some protists such as Dictyostelium and Physarum (for reviews see Schleicher and Noegel, 1992; Stockem and Brix, 1994).

In contrast, other protists possess a unique cortical cytoplasm: the epiplastic. The epiplastic is always in close contact with a membrane (Peck, 1977; Grain, 1986). In flagellates like Euglena it is the plasma membrane, in ciliates it is the inner membrane of the alveolus, a membrane system that closely apposes the plasma membrane (Peck, 1977; Bricheux and Brugerolle, 1986, 1987) and is thought to be involved in regulation of the intracellular calcium level (Stelly et al., 1991). It is generally believed that the epiplastic functions in maintaining cell shape. Moreover, there is experimental evidence that it is involved in pattern-forming processes like cortical organelle positioning (Aufderheide, 1983; Peck, 1986). In ciliates, cilia or ciliary complexes with their basal bodies that are homologous to centrioles in metazoans, are among these cortical organelles. They are polar structures, and their precise and asymmetrical arrangement defines the anterioposterior polarity (corresponding to the normal swimming direction) and the left−right or circumferential asymmetry of these unicellular organisms. The cortical patterns are species specific. Moreover, they have to be precisely reproduced during cell division: during the equatorial mode of cell division the anterior division product has to reconsti-
The epiphasm of the ciliate *Pseudomicrothorax dubius* is a thick, continuous, proteinaceous layer (Peck, 1977). It includes distinct regions such as the terminal plates and the docking sites, where basal bodies and trichocysts are attached. These regions, as well as the ribs extending between adjacent rows of terminal plates, are precisely positioned relative to one another (for a detailed ultrastructural description of the epiphasm see Peck, 1977; Peck et al., 1991). The epiphasm of *P. dubius* can be isolated in milligram quantity in a highly purified form, free of cortical filaments (microtubules, microfilaments). This has allowed a detailed biochemical analysis (Huttenlauch and Peck, 1991; Peck et al., 1991). The epiphasm of *P. dubius* consists of two major groups of polypeptides, 78–80 and 11–13 kD, as well as a series of minor components of 18–62 kD. The group of 78–80-kD proteins consists of at least three different polypeptides and numerous isoelectric variants. Immunoelectron microscopy revealed that at least one of these proteins is located throughout the entire epiphasm, while many of the minor components, which are glycosylated, are located at the outer surface of the epiphasm, or might even be membrane proteins that are tightly bound to the epiphasm (Curtenaz and Peck, 1992). Ultrastructural analysis of some protists such as dinoflagellates and entodiniomorphid ciliates indicates a filamentous substructure of the epiphasm with filament diameters of 2–3 nm (Viguès et al., 1984; Cachon et al., 1987; Viguès and David, 1989). In other protists, including *P. dubius*, the epiphasm appears homogenous with no obvious indication for the existence of filaments. Moreover, in no case have filamentous proteins been observed by electron microscopy in material obtained by in vitro reconstitution experiments of *P. dubius* epiphasm (Peck et al., 1991).

To unravel the nature of the epiphasmic proteins of ciliates we have carried out primary sequence analysis of several epiphasmic polypeptides of *P. dubius*. We present here partial peptide sequences of two of the major constituents as well as the full-length cDNA sequence of one minor species. All three polypeptides show sequence similarities and contain repeated valine- and proline-rich, 12-residue-long motifs with the consensus sequence VPV-...V-V-V-. Similar amino acid repeats have recently been described for two major epiphasmic proteins of the flagellate *Euglena gracilis* (Marris and Bouck, 1992), which have been named articulins.

Articulins have no significant sequence similarity to other known proteins. The presently known flagellate articulin and the ciliate epiphasmic proteins described here show overall sequence similarity, both are characterized by repeated VPV motifs with characteristic charge distribution in their central domains, and thus belong to the same class of cytoskeletal proteins. Members of this novel protein family comprise the major protein constituents of the epiphasm of ciliates as well as flagellates.

**Materials and Methods**

**Cultures and Epiphasm Preparation**

*P. dubius* strain N5b was cultivated, harvested, and the epiphasm was prepared as described (Peck et al., 1991).

**Electrophoretic Procedures**

SDS-PAGE using 7.5 or 10% (w/v) polyacrylamide slabs was performed as described (Huttenlauch and Peck, 1991). The *M*, values of the polypeptides were determined from mobilities on SDS-PAGE compared with the following protein standards of *M*, × 10⁻³: phosphorylase b (94), BSA (67), and ovalbumin (43). Two-dimensional gel electrophoresis and electrophoretic transfer of proteins to nitrocellulose sheets was as described (Huttenlauch and Peck, 1991) except that transfer was carried out at 240 mA for 8 h.

**Immunolabeling of Gel Blots**

Immunolabeling of gel blots was carried out as described (Stick, 1988). Antisera 018 and 015 (Peck et al., 1991) were diluted 1:220. The secondary antibody was alkaline phosphatase-conjugated goat anti–rabbit IgG (Dianova, Hamburg, FRG) diluted 1:2,000.

**Isolation of Epiphasmic Proteins, Preparation of Fragments and Peptide Sequences**

Epiphasmic polypeptides separated by NEPHGE followed by SDS-PAGE were visualized by staining with 0.1% Coomassie brilliant blue R in 45% (vol/vol) methanol, 10% (vol/vol) acetic acid. Individual spots were excised from 15 gels and stored at −70°C until use. Electrophoretic protein concentration, transfer to poly(vinylene difluoride) membrane, digestion with endopeptidase Asp-N or trypsin, HPLC of the resulting peptides, and microsequencing were essentially as described by Ungewickell et al. (1994).

**RNA Techniques and Northern Analysis**

RNA from *P. dubius* was isolated according to Chomczynski and Sacchi (1987). Poly(A)+ RNA was isolated by selection on oligo(dT)-cellulose (Boehringer Mannheim Biochemicals, Mannheim, FRG) using standard procedures (Maniatis et al., 1982). Northern blots of RNA separated on denaturing formaldehyde agarose gels were hybridized in 5× SSC at 65°C overnight with a 500-bp PCR fragment (generated with primers P4 and P5) radiolabeled with [³²P]dCTP (3,000 Ci/mmol, Amersham Buchler GmbH, Braunschweig, FRG) to high specific activity using a random prime kit (Pharmacia Diagnostics AB, Upsala, Sweden). Filters were washed two times 30 min each in 1× SSC, 1% SDS at 65°C, and once for 30 min in 0.5 M Na-phosphate, pH 7.0, at 65°C. Filters were exposed to x-ray films (XAR-5; Eastman Kodak Co., Rochester, NY) at −70°C using intensifying screens for 2–3 d.

**Oligonucleotides**

Peptide amino acid sequences were used to synthesize degenerated oligonucleotides (L, inosine) of the coding as well as the noncoding strand: spot 4 peptide e (DROIEKP) sense primer P1 5′GAT/CAGAT/CAIATIGAI-AAICCS′ and (PVYIEVE) antisense primer P2 5′TCTACT/TCTIAI-ITIAIACIGG3′; peptide g: (EPVPFYA) antisense primer P3 5′GCIACI- GGIACIGGCTTTCTC′. Oligonucleotides deduced from the nucleotide sequence of the PCR products obtained in the first round of RT-PCR: spot 4 peptide e (VFVPYVR) sense primer P4 5′GTCCAGGATCTCCCAGTGGAGG3′, antisense primer P5 5′CAGTGGCACCCTAACGTGG-A3′. Oligonucleotides deduced from the partial cDNA 3′- to the amplification of the 5′ end of the cDNA: antisense primer P6 5′CAACT- GCGCCGCTTGGCCTGCTG3′; antisense nested primer P7 5′GCCGCCT- TGAACTGCTGGTCCTGAGG3′ Oligonucleotide designed to amplify the open reading frame of 5′ flanked by restriction sites BamHI and HindIII: sense primer with BamHI site P8 5′CGGGATCCATGATGTCGCCACCTGCACGATC′.

**Polymerase Chain Reaction**

First-strand cDNA was synthesized from total RNA using the Gene Amp reverse transcriptase-PCR kit from Perkin-Elmer Cetus (Norwalk, CT). 1 μg total RNA was primed per reaction with a mixture of random hexanucleotides following the manufacturer’s protocol. PCR was carried out with combinations of degenerated primers (0.4 μM each) deduced from the cDNA sequences. Primer combinations that gave specific products were P1/P2 and P1/P3. The cycling parameters were: 4 cycles (1 min, 94°C, 1 min, 42°C; 2.5 min, 72°C), and 30 cycles (1 min, 94°C, 1 min, 50°C; 2.5 min, 72°C), and a final polymerization step (12 min, 72°C). To appropriate aliquots of the reaction mixture 0.1 vol of all four dNTPs (10 mM) and 1...
μl Klenow polymerase (5 U/μl) (Boehringer Mannheim Biochemicals) was added. After incubation at 37°C for 15 min individual fragments were separated on agarose gels (1-2.2%) and electroeluted. The fragments were treated with polynucleotide kinase (Boehringer Mannheim Biochemicals) under standard conditions for 1 h, extracted with phenol/chloroform and precipitated with ethanol. The fragments were then cloned into the EcoRV restriction site of the Bluescript KS vector (Stratagene Corp., La Jolla, CA) and subjected to double-stranded sequencing by the dideoxy chain termination method with either T7 and T3, or KS and SK primers using the Sequenase kit (United States Biochemical Corp., Cleveland, OH).

**cDNA Library Construction and Screening**

An oligo (dT)-primed cDNA library was synthesized from *P. dubius* poly(A)⁺ RNA and ligated unidirectionally into XZAP vector arms using the XZAP-cDNA synthesis kit (Stratagene Corp.). The library was packaged in Gigapack II Gold packaging extract (Stratagene Corp.) and plated on *E. coli* SURE cells (Stratagene Corp.). The library had a complexity of >2 × 10⁷ plaque-forming units with average insert sizes between 0.7 and 2 kbp. The library was amplified once on *E. coli* SURE. About 1 × 10⁶ pfu of the amplified library were screened with a ~500-bp PCR fragment (obtained in an RT-PCR with primers P4/P5) radiolabeled with [³²P]dCTP (3,000 Ci/mmol) (Amersham Corp.) to high specific activity using a random primer kit (Pharmacia Diagnostics AB). Library screening, plaque purification, conversion of XZAP into Bluescript plasmid, DNA isolation, subcloning, and sequencing were by standard methods (Maniatis et al., 1982) and according to manufacturer’s instructions. Four independent phage clones were isolated that represented partial cDNA clones encoding p60. The cDNA insert of the largest clone (termed pBst SK 3-1) was sequenced on both strands.

Amplification of the 5' end of the cDNA encoding p60 was done with the gene-specific primers P6 and P7 using the 5'-Amplifinder RACE kit (Clontech, Palo Alto, CA) following the protocol of the manufacturer. Cycling parameters of the PCR step were: 40 cycles (45 s, 94°C; 45 s, 60°C; 2 min, 72°C), and a final polymerization step (7 min, 72°C). The resulting PCR fragment was cloned into the EcoRV restriction site of the Bluescript KS vector (Stratagene Corp.) (pBst KS 5' p60).

**Plasmid Constructions and Protein Expression**

To obtain a clone containing the entire coding sequence of p60 the StyI/Styl restriction fragment of plasmid pBst SK 3-1 containing the bases +71 to +1714 of the cDNA (numbering with respect to the cDNA sequence given in Fig. 4) was cloned into pBst KS S' p60 that had been linearized with StyI. Clones containing the StyI/Styl fragment in sense orientation were identified by PCR and restriction fragment analysis. p60 was cloned into Toyosite. Cycling parameters of the PCR were: 40 cycles (45 s, 94°C; 45 s, 60°C; 2 min, 72°C), and a final polymerization step (7 min, 72°C). The resulting PCR fragment was cloned into the EcoRV restriction site of the Bluescript KS vector (Stratagene Corp.) (pBst KS S' p60).

**Deglycosylation of Epiplasmic Proteins**

Deglycosylation of *P. dubius* epiplasmic proteins with N-glycosidase F (Boehringer Mannheim Biochemicals) was essentially as described by Huttenlauch and Peck (1991).

**Spectroscopy**

For Fourier-transform-infra-red (FTIR) spectroscopy, the urea solution was concentrated to 8 mg/ml protein and dialyzed for 48 h at 4°C against D2O containing 10 mM Tris-HCl at pH 8.0 and 1 mM 2-mercaptoethanol. The spectrum was recorded on a spectrometer (IFS25; Bruker Instruments, Karlsruhe, FRG) using a CaF2 cell with a 50-μm Teflon spacer. 20 interferograms were taken in the range 400-4,000 cm⁻¹ with a resolution of 2 cm⁻¹. Fourier self-deconvolution was in part performed with software provided by the laboratory of H. H. Mantsch (Institute for Biodiagnostics, Winnipeg, Canada) (Kauppinen et al., 1981). Line narrowing of the slightly smoothed spectrum was performed using a triangular apodization function, assuming an initial line-width of half-width at half-height = 17 cm⁻¹ with a line-narrowing factor of κ = 2.0. The components in the amide I region of the spectrum were fitted with Gaussian bands by using a deconvoluted spectrum over the range from 1,598 to 1,695 cm⁻¹ (Byler and Susi, 1986). A constraint on the fitting was that all lines but the less well-defined random coil band at 1,641 cm⁻¹ should have comparable widths that are consistent with the deconvolution parameters.

**Electron Microscopy**

Purified, recombinant p60 in 8 M urea buffer was dialyzed against 10 mM Tris-HCl, pH 8.0, 1 mM MgCl₂, or 1 mM CaCl₂, and 1 mM 2-mercaptoethanol. Dialysis was performed at room temperature using 0.02 μm filters (Millipore Corp., Milford, MA). Protein concentration was 0.5 mg/ml. Specimens were negatively stained using either 1% uranyl acetate or 2% phosphotungstic acid adjusted to pH 7.5 with NaOH.

**Results**

The protein pattern of the isolated epiplasm of *P. dubius* consists of two major groups of polypeptides with molecular masses of 78,000-80,000 and 11,000-13,000, respectively, as well as several minor components of 18,000-62,000. The 78,000-80,000-mol wt group of polypeptides is resolved into more than 10 spots by two-dimensional-gel electrophoresis (Peck et al., 1991; see Fig. 1). Peptide map analysis of six major spots (1-6; Fig. 1) indicated that some spots 1-5 have been subjected to amino acid sequence analysis in this study.

1. Abbreviation used in this paper: FTIR, Fourier-transform-infra-red.

**Figure 1.** Two-dimensional gel electrophoretic analysis of epiplasm from the ciliate *P. dubius*. Epiplasmic polypeptides were separated by NEPHGE in the first dimension, and by SDS-PAGE in the second dimension. Upper portion of a Coomassie brilliant blue-stained gel is shown. Major spots of the 78,000-80,000-mol wt group of polypeptides are numbered 1-6. Spots 1 and 4 have been subjected to amino acid sequence analysis in this study.
of these spots represent isoelectric variants of the same polypeptides (1, 2, 3/5, 6, respectively) (Peck et al., 1991). In this report we have focused our sequence analysis on spots 1 and 4. They differ in their peptide maps and represent the two major components of the 78,000–80,000-mol wt group of polypeptides of P. dubius epiplasm (Peck et al., 1991).

To isolate polypeptides for protein sequencing, purified epiplasm was subjected to NEPHGE/SDS-PAGE. Individual spots were cut from the gels. Polypeptides from several gels were electrophoretically concentrated into a single gel slot, transferred electrophoretically to a polyvinylene difluoride membrane, and treated with endoproteinase Asp-N or with trypsin. Peptides released from the membrane were subjected to HPLC, and the resulting elution profile was screened by microsequencing. Sequence information of some 300 residues of spot 1 and 200 residues of spot 4 were obtained in total. Assuming a molecular mass of 80,000 for each of the polypeptides this would account for about 40 and 30%, respectively, of the complete sequences. About two-thirds of the peptides analyzed from both spots are rich in valine and proline, and more strikingly, these fragments show an alternating pattern of these two amino acids (VPV repeats). Alignments of the sequences are shown in Fig. 2 together with the consensus sequence of the VPV repeats of the epiplasmic protein p60 for which the complete amino acid sequence has been deduced by cDNA sequencing (see below). Although peptides from both spots display the same pattern of VPV-repetitive sequences, no identical peptide was obtained from both components. This holds also for the nonrepetitive peptide sequences obtained (results not shown). Thus it seems that spots 1 and 4 are indeed the products of two distinct genes as previously deduced from comparative peptide map analysis (Peck et al., 1991).

To generate hybridization probes for cDNA library screening we used reverse transcriptase-PCR. Degenerated oligonucleotide primers were synthesized based on selected peptide sequences (see Materials and Methods). The peptide sequence e of spot 4 (Fig. 2) was of sufficient length to allow the design of a primer pair from the same fragment. Two alternative primers with opposite orientation were synthesized for the other peptide sequences selected. Random primed cDNA was used as template in PCR amplifications with primer pairs in all possible combinations. With the two primers deduced from sequence e of spot 4, a single product of the expected size was obtained. This product, 79 bp in length, encoded the amino acid sequence situated between the two primers (underlined in Fig. 2). Since no size prediction could be made for the products of the other PCR reactions, all products were subcloned into plasmid vectors and sequenced from both ends. One product, 300-bp in length, encoded VPV-repetitive sequences. All other fragments were products of false priming; they either encoded completely unrelated sequences or had no open reading frame. The 300 bp product had been generated in a PCR with the downstream primer of peptide e (P1), and the upstream primer of peptide g (P3) of spot 4 (Fig. 2; see Materials and Methods). Unexpectedly, this fragment did not encode the amino acids downstream of primer P1, but a rather similar sequence. We obtained a similar result in a PCR that was carried out to amplify the cDNA fragment spanning the gap between the two previously isolated PCR products. In this PCR, perfect matching primers (P4, P5) deduced from the nucleotide sequence of these fragments were used. A 500-bp cDNA fragment was amplified that represented a 5' extension of the 300-bp PCR product. While the 3' end of this fragment perfectly matched the 5' end of the 300-bp product, the sequence at its 5' end was not identical to the sequence of the fragment from which the 5' primer had been deduced. The whole region encoded VPV repeats, but none of these were identical with peptide sequences of either spot 4 or 1. We therefore assumed that, while the 79-bp PCR product was derived from the cDNA encoding spot 4 polypeptide, the other two PCR products represented cDNA fragments coding for an additional epiplasmic protein. From the analysis of the complete cDNA sequence of this polypeptide we could infer that the cross-hybridization of the PCR primers was due to the high degree of amino acid similarity between these two polypeptides.
When Northern blots of poly(A+)-enriched RNA were probed with the 32P-labeled 500-bp PCR fragment a single RNA of about 2,100 bp was detected. No hybridization was found with up to eight times the amount of poly(A-) RNA, indicating that the corresponding mRNA is in the polyadenylated fraction (Fig. 3). The 500-bp PCR fragment was used in subsequent cDNA library screening. 1 x 10^6 plaques of a XZAP cDNA library of oligo dT primed P. dubius mRNA were screened under high stringency. Four cross-hybridizing clones were isolated. The largest clone (3-1), contained a cDNA insert of 1832 bp. It has an open reading frame of 1699 bp (566 amino acids) that codes for a VPV-repetitive protein. Its sequence perfectly matched that of the 300- and 500-bp cDNA fragments obtained by PCR. However, the insert of clone 3-1 was shorter than expected from the Northern blot analysis, and more significantly, it did not contain a methionine start codon. The first methionine of clone 3-1 is situated COOH-terminal of four VPV repeats, ~200 codons downstream of the 5' end. To obtain a full-length clone, we carried out a primer extension reaction with poly(A+) RNA, ligation of an anchor oligonucleotide to the 5' end of the single stranded cDNA, followed by PCR with a cDNA-specific nested primer and a primer complementary to the anchor sequence (see Materials and Methods). We isolated a 397-bp cDNA fragment that extended the existing cDNA by 261 bp. The complete cDNA is 2093 bp long (Fig. 4). This size is in good agreement with the results of Northern blot analysis, indicating that a full-length cDNA clone had been generated. The cDNA contains an open reading frame encoding a polypeptide of 567 amino acid residues. The methionine initiation codon is embedded in a sequence favorable for translational initiation (Kozak, 1989). It is preceded by four in-frame stop codons.

Codon usage in some ciliates like Paramecium, Tetrahymena, Stylonychia, and Oxytricha, differs from the universal genetic code by translating either UAA or UAG as glutamine and by the use of UGA as the sole termination codon (Caron and Meyer, 1985; Helftenbein, 1985; Kuchino et al., 1985; Preer et al., 1985; Hanyu et al., 1986). In other ciliates, like Euplotes, UGA is translated as cysteine, and UAA functions as termination codon (Cohen et al., 1990; Kaufmann et al., 1992). Our analysis of the cDNA encoding P. dubius p60 shows no direct evidence that an unusual genetic code is used. If the UAG codon at the end of the open reading frame would code for glutamine, as in other ciliates, the polypeptide would be extended by 16 residues. UAA would then function as termination codon, since it is the only in-frame stop codon present in the 3' untranslated region of the p60 mRNA.

The epiplas is part of the membrane skeleton of protists. In ciliates like P. dubius, it is in close contact with the inner membrane of the alveolus, while in flagellates like Euglena, which lack alveoli, it underlies directly the plasma membrane. In Euglena, articulins interact with the membrane by noncovalent binding to a major integral plasma membrane protein (Rosier et al., 1990). For ciliates, it is not known how the corresponding interaction with membranes occurs. We therefore searched for potential membrane-spanning domains in the sequence of p60. The NH2-terminal domain of p60 contains a segment of predominantly hydrophobic residues (positions 37-74) with hydrophobic indices $\geq 1.5$. However, this region does not display the typical features of a transmembrane domain. It is nearly twice as long as a typical membrane-spanning domain, and it is not flanked by charged amino acids. One might therefore assume that p60 does not directly interact with membranes.

When the full-length synthetic RNA is translated in vitro in a rabbit reticulocyte lysate in the presence of [35S]methionine, a single major polypeptide with an apparent molecular mass of ~60,000 is detected by fluorography (Fig. 5, lane 1). This is in good agreement with the molecular mass of 60,998, calculated from the predicted protein sequence. The molecular mass of the polypeptide (p60) encoded by this cDNA is, however, much lower than the 78-80 kD determined for the major epiplastic polypeptides of spots 1 and 4 by SDS-PAGE (Peck et al., 1991). To decide whether this discrepancy was due to differences in the gel systems used in the two investigations, epiplastic polypeptides and in vitro synthesized p60 were separated on the same gel. The epiplastic 78-80-kD group is separated into two closely spaced bands on 7.5% SDS-PAGE with apparent masses of 70,500 and 80,000 (Fig. 5, lane 2) well separated from the in vitro synthesized p60 protein (Fig. 5, lane 1).

We have also expressed p60 in E. coli. For this purpose, the complete coding region was cloned into a bacterial expression vector (pINDU; Bujard et al., 1987). The resulting fusion protein contained the 567 amino acids of p60, as well as four vector derived amino acids (MRGS) at the NH2 terminus. The NH2-terminal sequence of the fusion protein was confirmed by protein sequencing (results not shown). A total-cell lysate of bacteria transformed with pINDU-p60 (Fig. 5, lane 3) and, as a control, a lysate of bacteria transformed with pINDU carrying an unrelated and smaller cDNA insert (Fig. 5, lane 4) were separated on the same gel as the epiplastic polypeptides and the in vitro synthesized p60. Lysates of the pINDU-p60-transformed bacteria show a prominent band at 60 kD that is absent in the control lysate. This band migrates at the same position as the protein synthesized in the reticulocyte lysate (Fig. 5, compare lanes 3 and 1).

A rabbit polyclonal antiserum against total epiplasm...
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Figure 4. cDNA sequence and deduced amino acid sequence of P. dubius p60. Amino acid residues are represented by single letters above the first base of each codon. Numbering of amino acid residues is in italics. Numbering of nucleotides starts with +1 at the ATG initiation codon. The stop codon TAG is designated by an asterisk. The 24 VPV-repeated motifs of the core domain are underlined. A putative polyadenylation signal (AAATAAT) is designated by a dotted line. These sequence data are available from GenBank under accession number L41557.

The antiserum (018; Peck et al., 1991) was used to characterize further the bacterially expressed p60. This antiserum had previously been shown to react with epitplasmic proteins, but not with other cytoskeletal elements. On Western blots it labels the major components at 78-80 kD, as well as some quantitatively minor bands (Fig. 5, lane 7; see also Peck et al., 1991). The antiserum recognizes p60 in total bacterial cell lysates (Fig. 5, lane 6). The specificity of the reaction is demonstrated by the complete absence of staining of the control lysate (Fig. 5, lane 5). Taken together,
Figure 5. Characterization of in vitro translated and bacterially expressed *P. dubius* articulin p60. p60 translated in a coupled in vitro transcription–translation system in the presence of [35S]methionine (lane 1), polypeptides of an epiplasmic isolate of *P. dubius* (lanes 2, 7, 9–12), total cell lysate of *E. coli* BL 21 expressing *P. dubius* p60 (lanes 3, 6, and 8), total cell lysate of *E. coli* BL 21 expressing a control plasmid (see Materials and Methods) (lanes 4 and 5) were separated on SDS-PAGE (7.5%). In vitro synthesized polypeptides were detected by fluorography (lane 1). Polypeptides in lanes 2–4 were stained with Coomassie brilliant blue, and polypeptides in lanes 5–7 were electrophoretically transferred to nitrocellulose membrane and probed and detected with antisera 018 as described in Materials and Methods. Samples shown in lanes 7–9 were separated on the same gel. Note that the recombinant p60 is detected by serum 018 (lane 6). p60 is a minor component in isolated epiplasm (lane 7). Lanes 8–12: gel electrophoretic comparison of bacterially expressed p60 (lane 8) with epiplasmic polypeptides of *P. dubius* deglycosylated with N-glycosidase F (lanes 9 and 11), and mock- and incubated epiplasmic polypeptides (lanes 10 and 12). Polypeptides were separated on SDS-PAGE (7.5%), electrophoretically transferred to nitrocellulose membrane, and probed and detected with either antiserum 018 as described in Materials and Methods. Serum 018 recognizes protein epitopes of epiplasmic polypeptides, while serum 015 recognizes protein, as well as carbohydrate epitopes. The absence of immunoreactivity with serum 015 of the lower molecular weight range it is not glycosylated. N-glycosidase F, which cleaves a broad range of asparagine-bound N-glycans, has been shown to effectively deglycosylate several of the minor epiplasmic polypeptides (Huttenlauch and Peck, 1991). The effectiveness of the enzyme has been monitored by the loss of reactivity with antisera 015, which recognizes the glycomoieties of these proteins (Fig. 5, compare lanes 11 and 12).

**P. dubius** p60 Is an Articulin

The protein encoded by the cDNA described here has a molecular mass of 60,998 and a calculated isoelectric point of ~4.8. It contains an unusually high number of valine and proline residues (valine ~20%, proline ~14%; Table I). In the central core domain, which comprises about two-thirds of the total length of the polypeptide, valine plus proline accounts for more than 45% of the residues (Table I). The core domain consists of 24 tandem repeats of 12 residues that are occasionally separated by two to eight residue long linkers. The repeats show an alternating pattern of valine and proline residues (VPV repeats) (Fig. 6). Strikingly, these repeats resemble those of *Euglena* articulins p80 and p86 (Marrs and Bouck, 1992) and thus *P. dubius* p60 represents a ciliate homologue of the flagellate articulins (see below).

The consensus sequences for the 12 residue repeats of the core domain are VPVP–V–V–Vp (P. dubius p60), VPVPVeV–V–Ve (E. gracilis p80), and VPVPpVeV–V–k–Ve (E. gracilis p86; Marrs and Bouck, 1992), respectively. Uppercase letters represent occupancy in >50% of the repeats, while lowercase letters indicate >33% (see Fig. 6 for details). The sequences are compatible with a series of alternating V and P residues, which is relatively strict for p60, and somewhat more relaxed for few positions of the *E. gracilis* proteins. V positions never show a proline, while a few P positions can display a valine.

| Amino Acid | NH₂-terminal head | Core | COOH-terminal tail |
|------------|-------------------|------|-------------------|
| Val        | 20.28             | 8.51 | 27.75             | 8.75 |
| Pro        | 13.93             | 9.22 | 17.63             | 6.25 |
| Gin        | 11.29             | 14.89| 9.54              | 12.50|
| Gly        | 6.88              | 12.06| 0.58              | 25.00|
| Ala        | 6.53              | 14.89| 2.02              | 11.25|
| Ile        | 6.17              | 4.26 | 7.80              | 2.50 |
| Glu        | 4.94              | 1.42 | 7.51              | 0.00 |
| Leu        | 4.41              | 10.64| 1.73              | 5.00 |
| Asp        | 3.70              | 0.71 | 5.78              | 0.00 |
| Arg        | 3.35              | 2.13 | 4.05              | 2.50 |
| Phe        | 3.35              | 4.96 | 1.16              | 10.00|
| Thr        | 3.00              | 2.13 | 3.18              | 3.75 |
| Asn        | 2.82              | 5.67 | 0.87              | 6.25 |
| Lys        | 2.65              | 0.71 | 4.05              | 0.00 |
| Ser        | 2.65              | 5.67 | 1.16              | 3.75 |
| Tyr        | 2.47              | 0.71 | 3.47              | 1.25 |
| His        | 0.88              | 0.71 | 1.16              | 0.00 |
| Met        | 0.71              | 0.71 | 0.58              | 1.25 |

* NH₂-terminal domain, residues 1–141; core domain, residues 142–487; COOH-terminal domain, residues 488–567. The frequency of amino acids is given in percent.

* p60 does not contain cysteine and tryptophan residues.
The number of valine (V) and proline (P), as well as positively charged residues, is given below the alignment. Uppercase letters, residue in >50% of the repeats; lowercase letters, residue in >33% of the repeats.

**Sequence alignment of VPV-repeat motifs of Euglena gracilis**

- **articulin p60**: 24 repeats
- **articulin p80**: 33 repeats
- **articulin p86**: 36 repeats

The NH2- and COOH-terminal domains flanking the central core show quite different amino acid sequences. Both domains are rich in glycine and alanine, as well as phenylalanine. These three amino acids together comprise >45% of all residues of the COOH-terminal domain (Table I). More generally, with the exception of glutamine, that is found with similar frequency in all three domains, as well as those amino acids that occur with low overall frequency, the abundance of most amino acids shows a characteristic segregation between the central core domain and the two terminal domains (Table I). The terminal domains contain few charged residues (NH2-terminal domain, 7 out of 141 residues; COOH-terminal domain, 2 out of 80 residues). Moreover, the first 100 residues of the NH2-terminal domain are completely devoid of charged residues (Fig. 4). Also, in the central repeat domain, a segregation of charged residues is found: the last six repeats lack charged amino acids, while several repeats in the first two-thirds of the central domain are particularly rich in acidic and basic amino acids (Fig. 6).

The COOH-terminal domain contains the motif Thr-Pro-Gly-Arg at positions 542 to 545. This sequence conforms to the proposed consensus site for phosphorylation by p34cdc2 protein kinase (Moreno and Nurse, 1990). Phosphorylation of *P. dubius* epiplasmic proteins has not yet been analyzed. However, other cytoskeletal proteins of the ciliate cortex, i.e., the polypeptides that constitute the ciliary rootlets (kinetodesmal fibres) show hyperphosphorylation during their disassembly in the course of cell division (Sperling et al., 1991). The role of phosphorylation in mitotic disassembly and reassembly of nuclear lamins has been analyzed in detail in vertebrate cells (Heald and McKeon, 1990; Peter et al., 1990, 1991). Here, p34cdc2 is involved directly in the mitotic disassembly of the nuclear lamina.

**Spectroscopic and Electron Microscopic Analysis of Recombinant p60**

Secondary structure analysis of the core domain using the method of Chou and Fasman (1974) predicts many short stretches of β-sheets for this region. The FTIR spectrum of p60 has a pronounced maximum at 1,641 cm⁻¹, indicative of random coil structure. A second maximum is situated at 1,616 cm⁻¹, a region that is typical for β-sheet or extended chain bands (Fig. 7 A). A detailed analysis yields ~50% random coil, and ~28% β/extended-chain contributions (Table II). The overall spectral shape can be compared with random-coil polypeptides such as polylysine at neutral pH. Low wavenumber β-strand bands of high intensity have predominantly been observed in denatured proteins which aggregate and have been attributed to intermolecular interactions.

Electron microscopic analysis of purified recombinant p60 reveals rod-shaped, short filamentous structures, with diameters of ~15–20 nm (Fig. 8 A). Their length distribution is heterogeneous, ranging from 20 nm for single round “subunits” to about 120 nm (Fig. 8 A, inset). The longer filaments seem to be formed by the smaller subunits in longitudinal array. In addition, a tendency for the formation of larger structures is noticed. These are about 25–30-nm-thick filaments with lengths in the μm range (Fig. 8 A).
Figure 7. FTIR spectrum of recombinant P. dubius articulin p60. A shows the deconvoluted spectrum in the amide I region. It also includes the fitted bands. Abscissa is given as wavenumber. The quality of the fitting is shown in the box below A, which gives the difference between the deconvoluted curve and the sum of the fitted bands in a 10-fold amplification. For assignments of the fitted bands see Table II. B: SDS-PAGE (12%) of recombinant P. dubius articulin p60 after purification. The gel was run from left to right; arrowheads mark top and bottom of the separation gel. The protein fraction shown here was used to record the FTIR spectrum (this figure), as well as the electron micrographs shown in Fig. 8.

and broader, large aggregates which often appear to be tubes. The latter often branch, forming interconnected, wavy networks (Fig. 8 B). The presence of divalent cations does not seem to change significantly their appearance, but the structures look smoother and straighter. To exclude the possibility that the larger structures formed on the grid during the staining procedure, samples were also centrifuged for 15 min at 20,000 g before staining. About 25% of the protein was pelleted. Amino-terminal sequencing of pellet and supernatant provided the same sequence. The supernatant showed images like those given in Fig. 8 A inset.

Table II. Secondary Structure Assignment of Fitted FTIR Bands of Purified, Recombinant P. dubius Articulin p60

| Band | Position (cm⁻¹) | Percent area | Secondary structure feature |
|------|----------------|--------------|-----------------------------|
| 1    | 1,641.2        | 49.4         | Random coil                 |
| 2    | 1,616.0        | 16.7         | β                           |
| 3    | 1,628.1        | 4.7          | β                           |
| 4    | 1,675.6        | 6.1          | β                           |
| 5    | 1,666.7        | 8.6          | β-turn*                     |
| 6    | 1,687.5        | 2.9          | β-turn                      |
| 7    | 1,675.6        | 10.9         | α                           |

The β-sheet bands (2–4) amount to ~30%.

*The band at 1666.7 cm⁻¹ could not unambiguously be assigned.

Discussion

Here, we report sequence results on epiplastic proteins of the ciliated protist Pseudomicrothorax dubius. The sequence of a complete cDNA encoding a polypeptide of 60 kD (p60), that represents a quantitatively minor component of the epiplasm, as well as partial peptide sequences of two distinct major components with molecular masses of 78,000–80,000 are presented. Previous studies (Peck et al., 1991) demonstrated that the 78-80-kD group of polypeptides can be separated into several components by two-dimensional-gel electrophoresis. Based on peptide map analysis, several of these components represent isolectric variants of the same polypeptides (spots 1, 2, and 3; see Fig. 1), while others seem to reflect products of separate genes (spots 1 and 4; see Fig. 1). Our sequence data directly confirm this view. From the apparent molecular masses of the two major polypeptides, we calculate that about one-third of their sequences are covered by our peptide sequence analysis. Although the proteins are clearly related, none of the peptide sequences obtained from the
two components are identical. This strongly suggests that the two components (spot 1 and 4) are indeed the products of two distinct genes.

**Epiplastic Proteins of P. dubius Are Characterized by VPV-repetitive Motifs**

The most striking finding of this study concerns the identification of a 12-amino acid-long repeat sequence in all three epiplastic proteins. This motif resembles that of *Euglena* articulins (Marrs and Bouck, 1992). It is tandemly repeated 24 times in p60. Comparison of the peptide sequences obtained for the two major components with the complete amino acid sequence of p60 indicates that all three *P. dubius* proteins have a similar molecular architecture.

Secondary structural prediction rules of the sequence in the central domain with its 24 VPV repeats indicates a high content of β-sheet, and FTIR spectroscopical measurements of the bacterially expressed protein show that p60 contains ~30% β-sheet. Comparison of the repeats of all three *P. dubius* polypeptides reveals a remarkable distribution of charged amino acids. Positively and negatively charged residues show an alternating distribution in register with the pattern of valine and proline residues. In a β-sheet structure, this distribution would result in the separation of positively and negatively charged residues into two layers above and below the polypeptide backbone, which could have an important function in the structural organization of the epiplastic proteins. Although the VPV-repeat consensus of spots 1 and 4 polypeptides is still based on incomplete sequence information, and therefore might be biased, the 24 repeats of p60 are fully characterized by the complete cDNA.

**Electron Microscopical Observations**

The epiplasm of *P. dubius* appears homogeneous in electron microscopical ultrathin sections. Reconstitution experiments with total epiplastic preparations have failed to detect filamentous structures (Peck, 1977). The electron microscopical appearance of recombinant p60 shows that the majority of the molecules are found in elongated or short filamentous forms. These have a uniform diameter (~15–20 nm), but variable length distribution. They seem to be constructed of smaller roundish units with a diameter similar to the thickness of the short filaments. The small polymers have a tendency to form thicker and longer filaments, ribbons or tubes, and large sheets. p60, however, is a minor component of the epiplasm. It remains to be seen whether the major articulins form similar structures in vitro, and how these structures are related to the epiplastic organization in vivo.

**Epiplastic Proteins of Flagellates and Ciliates are Members of the Same Multigene Family, the Articulins**

The sequences of two epiplastic proteins of the flagellate *Euglena gracilis* have recently been described. These proteins were named articulins (Marrs and Bouck, 1992). Articulins and the ciliate polypeptide p60 described in this study show the same domain organization. All three ciliate proteins contain VPV repeats that match the consensus sequence of the articulin repeats. The complete sequence of an articulin from the ciliate *P. dubius* extends the results obtained from the two articulin sequences of the flagellate *E. gracilis*. Articulins have a tripartite sequence organization. A central core domain of repetitive 12 residue motifs starting with the consensus sequence VPVP... is the hallmark of this novel protein family (Marrs and Bouck, 1992). So far the number of repeats is either 33 (*E. gracilis* p80 and p86) or 24 (*P. dubius* p60). When present, the linkers between the repeats are maximally six to eight residues long. The terminal domains flanking the core differ in sequence and length. The NH2-terminal head domain harbors 76 (*E. gracilis* p80), 112 (*E. gracilis* p86), or 141 residues (*P. dubius* p60). The COOH-terminal tail domains are 67 (*P. dubius* p60), 110 (*E. gracilis* p86), or 149 residues (*E. gracilis* p80) long. Interestingly, the two terminal domains of each articulin show some similarity. They all contain a long segment free of charged residues. In the head domain, these segments cover 39 (*E. gracilis* p80), 62 (*E. gracilis* p86), or even 95 residues (*P. dubius* p60), while the tail domains display such segments with 33 (*E. gracilis* p80) or 55 continuous residues (*P. dubius* p60). An additional argument for a structural homology between heads and tails is, so far, restricted to the *Euglena* articulins. A heptapeptide around the sequence APVTYGA is found four times either in the head (*E. gracilis* p86) or in the tail domain (*E. gracilis* p80). The length variability of both terminal domains and the core domain opens the possibility that articulins can strongly vary in polypeptide molecular weight (see also below). Since the articulins preserve sequence principles rather than actual sequences the overall sequence identities are relatively low. The two *E. gracilis* articulins share only 37% identity (Marrs and Bouck, 1992), and the *P. dubius* articulin is even more remote with <20% identity. Despite these low identity values, the conservation of the sequence principles, as well as the overall structure of the polypeptides from *P. dubius* and *E. gracilis* is in favor of common ancestry, rather than convergent evolution. In addition, protein data bank search did not reveal any significant similarity to other proteins.

In conclusion, the epiplastic proteins of two distantly related groups of protists, the flagellates and the ciliates, which are viewed as representatives of different phyla, are members of the same protein family (for discussion of protist phylogeny see Baroin et al., 1988; Baroin-Tourancheau et al., 1992; Lee and Kugrens, 1992; Cavalier-Smith, 1993).

**Articulins Might Be the Major Constituents of the Epiplastic of Various Protists**

An epiplastic layer has been documented for many ciliate genera and several other protists including dinoflagellates and flagellates (for review see Grain, 1986). In all these organisms, the epiplasium shows a strikingly similar appearance and is always in close contact with cortical membranes, either the plasma membrane or the inner alveolar membrane. This apparent similarity has raised the question of whether the epiplastic proteins in different protists are homologous. Biochemical analysis of a variety of species revealed that the epiplasium is composed of a multitude of polypeptides with a great diversity in molecular masses (Dubreuil and Bouck, 1985, 1988; Williams, 1986;
Viguès et al., 1987; Peck et al., 1991; Nahon et al., 1993). Interestingly, heterogeneity in apparent molecular mass is even seen among closely related species (Williams, 1986). However, immunological studies with polyclonal as well as monoclonal antibodies indicate the presence of common epitopes in epiplasmic polypeptides of different molecular masses within a particular species, as well as between different protists (Viguès et al., 1987; Nahon et al., 1993; Curtenaz et al., 1994). Polyclonal antisera raised against epiplasmic proteins of distantly related protists, the ciliate *P. dubius*, the flagellate *Euglena acus*, and the dinoflagellate *Notothalia scintillans* (Viguès et al., 1987), showed cross-reaction of the major epiplasmic polypeptides of all three species. While the epiplasmic proteins of *P. dubius* and *Euglena* have similar ranges of apparent molecular masses (11–86 kD), polypeptides of much higher apparent molecular masses are found in *Notothalia* (45–220 kD; Viguès et al., 1987). With respect to the cDNA characterization of *P. dubius* articulin p60, it is interesting to note that sera raised against the euglenoid and the dinoflagellate proteins both recognize a quantitatively minor component of ~60 kD in epiplasmic preparations of *Pseudomicrothorax*. Interspecies cross-reactivity has also been observed with some mAbs (Nahon et al., 1993; Curtenaz et al., 1994). However, as often observed, such immunological cross-reactions can display unpredictable patterns with respect to the phylogenetic distances separating the organisms. Thus an mAb against *Paramaecium* epiplasmic proteins cross-reacts with major epiplasmic polypeptides of *Pseudomicrothorax* (articulins p78 and p80), *Euplotes*, and *Euglena acus*, but fails to recognize the corresponding polypeptides in much more closely related species such as *Tetrahymena* and *Colpidium*.

The finding that ciliate and flagellate epiplasmic proteins are members of the same protein family suggests that the repetitive VPV motifs of articulins might represent the common epitopes that are recognized by several of the antibodies directed against epiplasmic proteins, a hypothesis that now can be tested by using peptide sequences or recombinant articulins to produce bona fide articulin antibodies. It further could imply that the broad interspecies cross-reactivity of these antibodies indeed reflects common ancestry of epiplasmic proteins. We therefore assume that VPV proteins (articulins) are major epiplasmic constituents of most, if not all, protists that contain this structure. Given the length variability of both terminal domains and the core domain of flagellate and ciliate articulins, it seems likely that the large epiplasmic proteins of the dinoflagellate *Notothalia scintillans*, which show immunological cross-reactivity with the epiplasmic proteins of *Euglena acus* and *P. dubius* (Viguès et al., 1987) are most likely also articulins. Thus, the modular structure of the articulins could explain the diversity of molecular masses of epiplasmic proteins observed in different protists. This type of diversification is not without precedence. Intermediate filament proteins, one of the major classes of cytoskeletal proteins of metazoans, are only one example of how a modular architecture has been used to generate protein diversity while maintaining structural similarities (for review see Fuchs and Weber, 1994). The epiplasm is a very elaborate structure with numerous ridges and depressions in some protists, but it may be much simpler in others. Its structural organization is thus characteristic for each species examined so far. On the other hand, it is always associated with a cortical membrane along its external face, and cortical fiber systems and other organelles, e.g., mitochondria and secretory granules, are anchored to its internal face. The articulins, with their conserved core domain and more variable terminal domains, appear to be well adapted to a role of ensuring functions common to all epiplasms, yet permitting the epiplasmic structural diversity particular to each species. The demonstration that epiplasmic proteins of distantly related protists share common molecular principles will help to clarify the roles of core and terminal domains, as well as to define the limits of diversity within this novel family of skeletal proteins.

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