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The cruciated microtubule-associated fibers of the green alga *Dunaliella bioculata* consist of a 31 kDa SF-assemblin

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SUMMARY

Cytoskeletons of *Dunaliella bioculata*, the biflagellate wall-less green alga, were isolated and analyzed using a monoclonal and a polyclonal antibody raised against SF-assemblin, the major protein of the two striated microtubule-associated fibers of the alga *Spermatozopsis similis*. Indirect immunofluorescence showed antigenic structures associated with the four microtubular flagellar roots. SDS-PAGE followed by immunoblot analysis revealed a cross-reacting polypeptide of 31 kDa. This protein of *D. bioculata* was isolated using gel filtration chromatography in 8 M urea and in vitro reassembly of striated fibers. Microsequencing of the purified protein yielded various peptides, which could be aligned along the sequence of SF-assemblin from *S. similis*.

A complete sequence of the *Dunaliella* protein was obtained by cDNA cloning. It documents the non helical head domain followed by a helical rod domain with a 29 residue repeat pattern based on four heptads followed by a skip residue. Compared to SF-assemblin of *S. similis* the SF-assemblin of *Dunaliella* has a shorter head and a slightly longer rod domain. The two algal SF-assemblins share only 57% sequence identity. We conclude that SF-assemblin and related proteins in various protists are representatives of a new class of α-helical proteins characterized by the ability to form a special segmented coiled coil and to assemble into striated fibers of 2 nm protofilaments in vivo and in vitro.

Key words: SF-assemblin, *Dunaliella bioculata*, Segmented coiled coil, Microtubule associated fiber

INTRODUCTION

The basal bodies of flagellate/ciliate eukaryotic cells are commonly associated with microtubular and fibrous flagellar roots (Pitelka, 1969). The latter can be classified into at least two types, which differ in their ultrastructure and biochemical composition (Lechtreck and Melkonian, 1991a). One is contractile (system-II-fibers) and consists predominantly of Centrin/Caltractin, a calcium-binding EF-hand type protein (reviewed by Melkonian et al., 1992). In general centrin-related proteins are associated with the centrosome of eukaryotes (Lee and Huang, 1993; Ogawa and Shimizu, 1993; Errabolu et al., 1994).

The second type of fibrous flagellar roots (system I fibers) is composed of fine filaments (2-4 nm diameter), which exhibit a narrowly cross-striated banding. They are apparently non-contractile (Dingle and Larson, 1981). Here we refer to the kinetodesmal fibers of ciliates (Rubin and Cunningham, 1973; Williams et al., 1979; Hyams and King, 1985), the flagellar rootlets of the amoeboflagellate *Naegleria gruberi* (Larson and Dingle, 1981), the striated rootlets of the ciliate gill epithelium of molluscs (Stephens, 1975), and the striated microtubule-associated fibers of flagellate green algae (Lechtreck and Melkonian, 1991b). Recently, the striated microtubule-associated fibers of the flagellate green alga *Spermatozopsis similis* were isolated, reassembled in vitro, and a 34 kDa protein (SF-assemblin) was identified as the major constituent (Lechtreck and Melkonian, 1991b). Interestingly, the only protein found in the data banks with a significant homology to SF-assemblin is β-giardin, a 30 kDa protein from the parasitic protozoan flagellate *Giardia lamblia* (Holberton et al., 1988; Weber et al., 1993). β-giardin is located in striated fibers associated with the complex microtubular structure of the sucking disk (Peattie et al., 1989). SF-assemblin and β-giardin consist of a small non-helical N-terminal head domain and a rod domain of 253 amino acids. They show a striking structural similarity over the entire rod domain i.e. a 29 residue repeat pattern based on four heptads followed by a skip residue (Weber et al., 1993). A similar structure for proteins forming microtubule-associated bundles of 2 nm filaments in an archezoan protist and a green alga indicates that related molecules may be widespread among eukaryotes.

Despite their structural homology, SF-assemblin and β-giardin show only low sequence identity (<20%). Therefore we were interested in the evolutionary conservation of the structure and sequence of SF-assemblin. For this purpose the cytoskele-

tons of the marine green flagellate *Dunaliella spp.*, which contain striated fibers associated with all four microtubular roots in contrast to the two striated fibers described in *Spermatozopsis similis*, were analyzed with antibodies against SF-assemblin. The immunoreactive 31 kDa protein was purified from *D. bioculata*, reassembled into striated fibers and the sequence was determined from cDNA clones. SF-assemblin from *Spermatozopsis* and the homologous protein of *Dunaliella* share only 57% sequence identity, indicating that although the protein structure is retained, sequence divergence has occurred.

MATERIAL AND METHODS

Strains and culture conditions

Dunaliella bioculata (strain number SAG 19-4, Sammlung von Algenkulturen, University of Göttingen), *D. primolecta* (SAG 183.80), *D. minuta* (PLY 430, Plymouth Culture Collection, Plymouth, UK), and *D. tertiolecta* (CCMP MCIIIA, Provasoli-Guillard Center for Culture of Marine Phytoplankton, West Boothbay Habor, Maine, USA) were cultured in the artificial sea water medium ASP-H (McFadden and Melkonian, 1986). The culture conditions were 15°C, 20 mE $\times$ m^{-2} $\times$ second^{-1}, and a L/D-cycle of 14/10 hours. The procedures for culturing *Spermatozopsis similis* (strain number SAG B 1.85) have been described (Lechtreck and Melkonian, 1991b).

Production of monoclonal antibodies against SF-assemblin

Isolated basal apparatuses of *S. similis* were purified by sucrose gradient centrifugation, resuspended in PBS (1.5 mg/ml), sonified, and mixed with Complete Freund's Adjuvant (Sigma). Three month old female mice ((C57 BL/6 $\times$ BALB/c)F_1) were injected subcutaneously with 190 μg antigen and after four weeks again with 120 μg antigen without Freund's Adjuvant. Five days after the final boost the spleens were removed and the cells were fused to the myeloma cell line X63-Ag8.653 (Kearney et al., 1979). Hybridoma cells were produced following the method of Köhler and Milstein (1976) and plated into microtiter plates with mouse peritoneal macrophage feeder cells. Once hybridoma colonies appeared the supernatants were analyzed against purified SF-assemblin by ELISA (Bremerich et al., 1995). Six positive clones were identified and clone BAS 6.5 was subcloned by limited dilution.

Immunolocalization techniques

Cells of *D. bioculata* and *D. tertiolecta* were harvested by centrifugation (100 ***g***, 5 minutes, 15°C), washed once with culture medium (60-100 ***g***, 2-5 minutes, RT), resuspended in MT/Mg^{2+} (30 mM Hepes, 15 mM KCl, 5 mM Na-EGTA, 5 mM $MgSO_4$, pH 7) and lysed by the addition of an equal volume MT/Mg^{2+} including 0.2 or 2% Nonidet P-40. After 5 minutes the cytoskeletons were fixed with 3% paraformaldehyde (final concentration in MT/Mg^{2+}) for 15-30 minutes on ice, and either washed (200 ***g***, 2 minutes, RT) or allowed directly to adhere to polylysine-coated coverslips. Cells of *D. minuta* and *D. primolecta* were concentrated as described above, fixed with 2-4% paraformaldehyde (final concentration in ASP-H omitting Ca^{2+}) for 30 minutes on ice, and subsequently lysed by the addition of an equal volume 0.2 or 2% Nonidet P-40 in MT/Mg^{2+} for 5-10 minutes. Cytoskeletons were washed and allowed to adhere to polylysine-coated coverslips. All following steps were as described (Lechtreck and Melkonian, 1991b). Anti-SF-assemblin-IgG (1:60-100 in PBS including 2% BSA; for origin and specificity of the antibody see Lechtreck and Melkonian, 1991b) or undiluted hybridoma supernatant containing the monoclonal antibody BAS 6.5 were used as primary antibodies; anti-rabbit- or anti-mouse-IgG conjugated to tetramethylrhodamine isothiocyanate (Sigma) were diluted 1:40-100.

Immunogold electron microscopy was carried out as described previously (Lechtreck and Melkonian, 1991b), but using 0.5% Nonidet P-40 for cell lysis. The monoclonal antibody BAS 6.5 (diluted 1:1) was detected with anti-mouse-IgG conjugated to 5 nm gold particles (Sigma).

Immunoblot analysis

Basal apparatuses of *Dunaliella* were enriched as described below. Basal apparatuses of *S. similis* were isolated as described (Lechtreck and Melkonian, 1991b). Cytoskeletal proteins were separated by SDS-PAGE (Laemmli, 1970) and transferred to a PVDF-membrane (Millipore Corp.). Membrane strips were quenched extensively in PBS containing 2% BSA or 3% gelatine from cold water fish (Sigma), incubated in diluted anti-SF-assemblin-IgG (1:500 in PBS containing BSA or gelatine) or BAS 6.5 (1:5) for 90 minutes, washed 8-10 times for 2 minutes with PBS containing 0.05% Tween-20 or 0.5% BSA, and incubated in anti-rabbit- or anti-mouse-IgG conjugated to peroxidase (Sigma, 1:500-1000). After extensive washing antibody conjugates were detected by the use of 4-chloro-1-naphthol.

Reassembly of paracrystals

A 30-40 l sample of a culture of *D. bioculata* (about 5×10^6 cells/ml) was concentrated by a tangential flow system (Millipore Corp., Bedford, MA, USA), washed once with ASP-H (890 ***g***) and once with MT. Cells were resuspended in ~80 ml MT and mixed with an equal volume of 2% Triton X-100 in MT. Cytoskeletons were centrifuged (1,700 ***g***, 10 minutes, 15°C), washed 3 times with decreasing concentrations of Triton X-100 (0.5, 0.25, and 0.1%) and twice with MT. Disintegration of cytoskeletons during resuspension results in the loss of most of the axonemal and cytoskeletal microtubules. Finally basal apparatuses were pelleted at 18,000 ***g*** for 20 minutes at 4°C and stored at −20°C.

Frozen basal apparatuses were resuspended in 30 ml MT (2 M in NaCl) by homogenization (50 strokes in a 30 ml tissue homogenizer; Kontes Glass Co., Vineland, NJ) and extracted overnight at 4°C. The suspension was centrifuged at 48,500 ***g*** (4°C, 30 minutes). The pellet was resuspended in 2.5 ml 20 mM Tris-HCl, 50 mM KCl, 1 mM DTT, pH 7.8, containing 8 M urea and extracted for 2 hours at RT. To remove insoluble material the suspension was centrifuged at 18,000 ***g*** for 1 hour and filtered (0.22 μm, Millex-GV, Millipore). Soluble proteins were separated by gel filtration on a HiLoad 26/60 Superdex 200 column calibrated by molecular mass standards (Pharmacia, Freiburg, Germany). All fractions were analyzed by immunodot blots with the polyclonal anti-SF-assemblin-IgG. Fractions containing the immunoreactive 31 kDa protein were dialyzed overnight against either reassembly buffer A (150 mM KCl, 10 mM MES, 2 mM EDTA, 0.1 mM DTT, pH 6.25) or reassembly buffer B (as buffer A but containing 180 mM KCl at pH 7).

Amino acid sequence analysis of the striated fiber protein

The 31 kDa protein of *D. bioculata* used for microsequencing was purified by a modified enrichment procedure. The NaCl-extracted basal apparatuses (see above) were resuspended in 5 M urea in MT and extracted for 5 hours at 4°C. Insoluble material was removed by centrifugation for 1 hour at 200,000 ***g***, 4°C. The supernatant was dialyzed for 14 hours at 4°C against reassembly buffer B. Paracrystals were harvested at 48,500 ***g*** (30 minutes, 4°C). Finally a 31 kDa protein from *D. bioculata* was purified by SDS-PAGE, transferred to a PVDF membrane and digested with trypsin or endoproteinase Asp-N (Bauw et al., 1989). The peptides released by each digest were subjected to HPLC and the profiles screened by automated sequencing using an Applied Biosystems sequenator (model A470, Weiterstadt, Germany) or a Knauer model 810 (Berlin, Germany). Both instruments operated with an on-line PTH amino acid analyzer.

Cloning and sequencing of cDNAs coding for *Dunaliella* SF-assemblin

Total RNA from *D. bioculata* was isolated by the phenol/SDS method

basically following the method of Palmiter (1974). To purify mRNA we used an oligo-dT-cellulose column (Boehringer, Mannheim, Germany). A λZapII cDNA library was constructed using a cDNA synthesis and cloning kit (Stratagene, Heidelberg, Germany) according to the instructions of the manufacturer with the following modification: first-strand synthesis was done for 15 minutes, 45°C; 20 minutes, 50°C; and 20 minutes, 55°C, with reverse transcriptase from Gibco BRL (Eggenstein, Germany). This library was screened with a monoclonal antibody directed against SF-assemblin of *S. similis*. The only positive clone (clone 1) obtained was incomplete as shown by partially sequencing (Sequenase or Δtaq DNA polymerase non-cycle sequencing, USB, Cleveland, Ohio) after in vivo excision of the pBluescript phagemid. Therefore the cDNA library was screened again using the randomly labeled insert of clone 1 as a probe. Three positive clones with different insert sizes were obtained and sequenced completely (clone 2) or in parts (clone 3 and 4). Polymerase chain reaction was performed in a PTC-100 programmable thermal controller (MJ Research, Inc., Watertown, Massachusetts) under the following conditions: after 5 minutes at 98°C the DNA polymerase (AmpliTaq, Perkin Elmer, 1 U/25 μl) was added followed by 30 cycles of 1 minute at 95°C, 1 minute at 58°C, and 90 seconds at 72°C extended by 2 seconds/cycle.

Whole mount electron microscopy

For whole mount electron microscopy 4 μl of suspended particles were applied to copper grids coated with Pioloform (Plano GmbH, 3550 Marburg, Germany) and allowed to adhere for 2-15 minutes. The grids were stained with 1% uranyl acetate for 1-5 minutes and observed with an electron microscope (CM 10, Philips, Kassel, Germany).

RESULTS

Antibodies to SF-assemblin identify a cross reacting 31 kDa protein in the cruciated striated fibers of *Dunaliella bioculata*

The cytoskeleton of *Dunaliella bioculata* resembles that of *Chlamydomonas reinhardtii* comprising a cruciate microtubular flagellar root system of the 4-2-4-2 type typical for chlorophycean algae (Moestrup, 1978). Two nucleus basal body connectors, characterized by the presence of centrin, link the basal bodies to the nucleus (Schulze et al., 1987; Merten et al., 1995, Fig. 4B). This study deals with the striated microtubule-associated fibers (SMAFs; system-I-fibers) which underly all four microtubular roots (Hyams and Chasey, 1974; Melkonian and Preisig, 1984; Marano et al., 1985).

Cytoskeletons of four different species of *Dunaliella* were analyzed with a previous described polyclonal antibody and a new monoclonal antibody raised against SF-assemblin which is the principal protein of the SMAFs of *Spermatozopsis similis* (Lechtreck and Melkonian, 1991b). The specificity of this monoclonal antibody for SF-assemblin is shown by immunoblotting (Fig. 1, lanes 7, 8) and by preembedding immunogold electron microscopy, which revealed an exclusive labelling of the SMAFs of *S. similis* (Fig. 2). Thus, the monoclonal anti-SF-assemblin recognizes the same protein and structures in *S. similis* as the polyclonal anti-SF-assemblin (Lechtreck and Melkonian, 1991b).

When isolated cytoskeletons (nucleo-flagellar apparatuses) of *Dunaliella bioculata* are stained with either monoclonal (Fig. 3A,B) or polyclonal anti-SF-assemblin (Fig. 3D-E) cruciate structures emerging from the basal bodies are seen by immunofluorescence. Two oppositely oriented fluorescent fibers branch distally into two subfibers of unequal length (Fig. 3E). The longer subfibers are termed s-α SMAFs, the shorter ones s-β SMAFs (according to Marano et al., 1985, and Moestrup and Hori, 1989). The two other oppositely oriented fibers are unbranched and are termed d-SMAFs. Indirect immunofluorescence of isolated cytoskeletons from additional *Dunaliella spp.* (*D. tertiolecta*, *D. primolecta*, *and D. minuta*) always resulted in a cruciate and branched pattern of antigenic structures as in *D. bioculata* (Fig. 3F-H). We found similar length distributions of the antigenic structures in all species analyzed: s-α > d > s-β. Double immunofluorescence microscopy using anti-β-tubulin revealed that the microtubular roots are accompanied by the anti-SF-assemblin binding fibers over about 75% of their length (not shown).

Proteins of isolated cytoskeletons of *D. bioculata* were separated by SDS-PAGE and transferred to a PVDF-

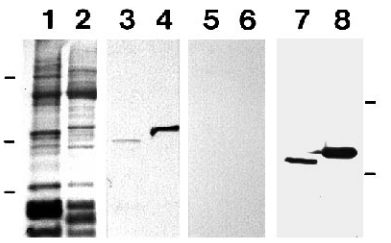


Fig. 1. Immunoblot analysis comparing proteins from the basal apparatuses of *D. bioculata* (lanes 1,3,5, and 7) and *S. similis* (lanes 2,4,6, and 8). Lanes 1 and 2, Amido Black stained blot. Lanes 3 and 4, immunoblots of basal apparatuses stained with polyclonal anti-SF-assemblin-IgG showing a slight difference in apparent molecular masses of the immunoreactive proteins in *D. bioculata* (lane 3) and *S. similis* (lane 4). Lanes 5 and 6, immunoblots of basal apparatuses probed with preimmune serum. Lanes 7 and 8, immunoblots probed with the monoclonal anti-SF-assemblin. Note the more equal staining of the immunoreactive proteins compared to lanes 3 and 4. Molecular mass markers (Dalton Mark VII-L, Sigma) indicated left (lanes 1-6, 12% SDS-PAGE) are, from top to bottom: 66, 29, and 20.1 kDa, and for lanes 7 and 8 (5-16% gradient SDS-PAGE), indicated at the right: 66 and 29 kDa.

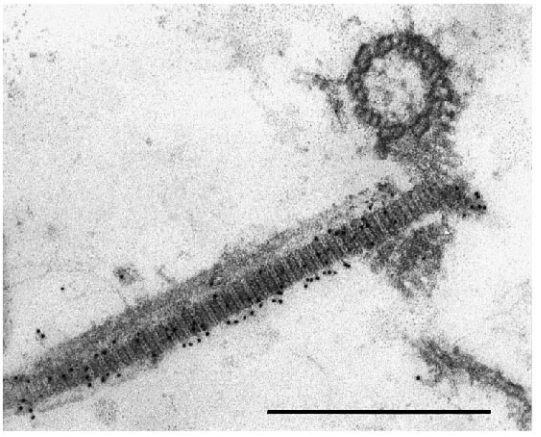
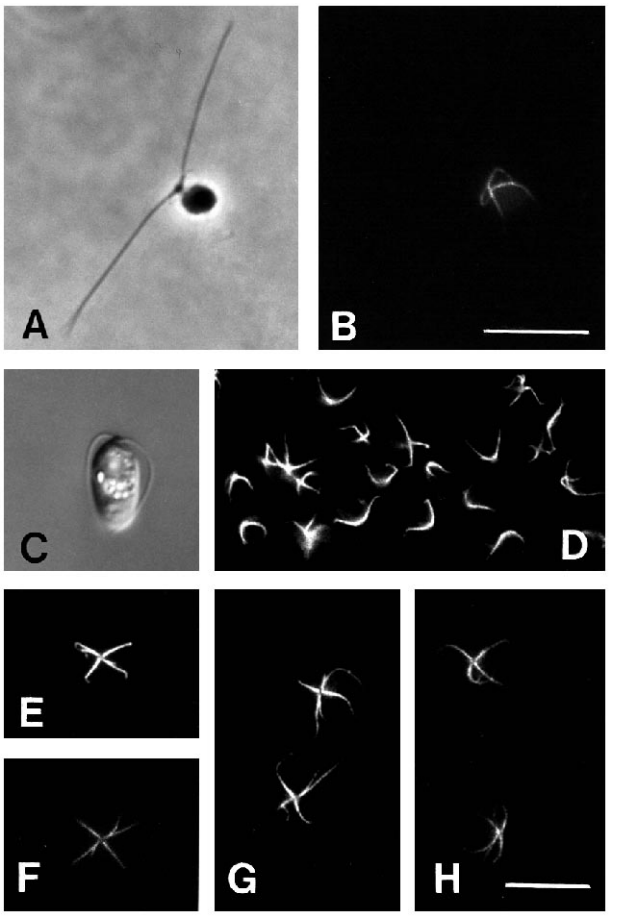


Fig. 2. Thin section through an isolated cytoskeleton of *S. similis* probed with monoclonal antibody BAS 6.5 directed against *Spermatozopsis* SF-assemblin and anti-mouse-IgG conjugated to 5 nm gold. Note the extensive and specific labeling of the striated fiber by the 5 nm gold particles and the two associated microtubules. Bar, 0.4 μm.



membrane (Fig. 1, lane 1). When the membrane strips were probed either with polyclonal anti-SF-assemblin IgGs or with monoclonal anti-SF-assemblin (Fig. 1, lanes 3, 7) a single 31 kDa protein was detected in *D. bioculata* whereas in *Spermatozopsis similis* a 34 kDa band was recognized (compare Fig. 1, lanes 3, 7 with 4, 8). A 31 kDa protein was also detected in *D. tertiolecta, D. primolecta*, and *D. minuta* (not shown).

Structure and in vitro reassembly of striated microtubule-associated fibers

Cytoskeletons of *D. bioculata* were isolated by treatment of the cells with a non-ionic detergent. In the presence of magnesium, the nuclear skeleton remains linked to the basal bodies by two nucleus-basal body connectors (Fig. 4A,B). Isolated cytoskeletons of *D. bioculata* were disintegrated by homogenization and most of the axonemal microtubules and the microtubular roots removed by differential centrifugation. The remaining fraction is enriched in basal apparatuses, which were subsequently

Fig. 3. Indirect immunofluorescence of four species of *Dunaliella*. (A-E) *Dunaliella bioculata.* (A) Phase contrast of an isolated cytoskeleton. (B) Corresponding immunofluorescence of the cytoskeleton depicted in A with monoclonal antibody BAS 6.5 and anti-rb-IgG-TRITC. Bar, 10 μm. (C) Living cell embedded in agarose photographed with Nomarski optics.
(D,E) Immunofluorescence image obtained with polyclonal anti-SF-assemblin-IgG of isolated cytoskeletons in detail (E) and overview (D). (F-H) Anti-SF-assemblin immunofluorescence (polyclonal antibody) of isolated cytoskeletons of *D. tertiolecta* (F), *D. minuta*. (G), and *D. primolecta* (H). Note that two of the four roots of each cytoskeleton are brached (E-H). Bar (C-H) 10 μm.

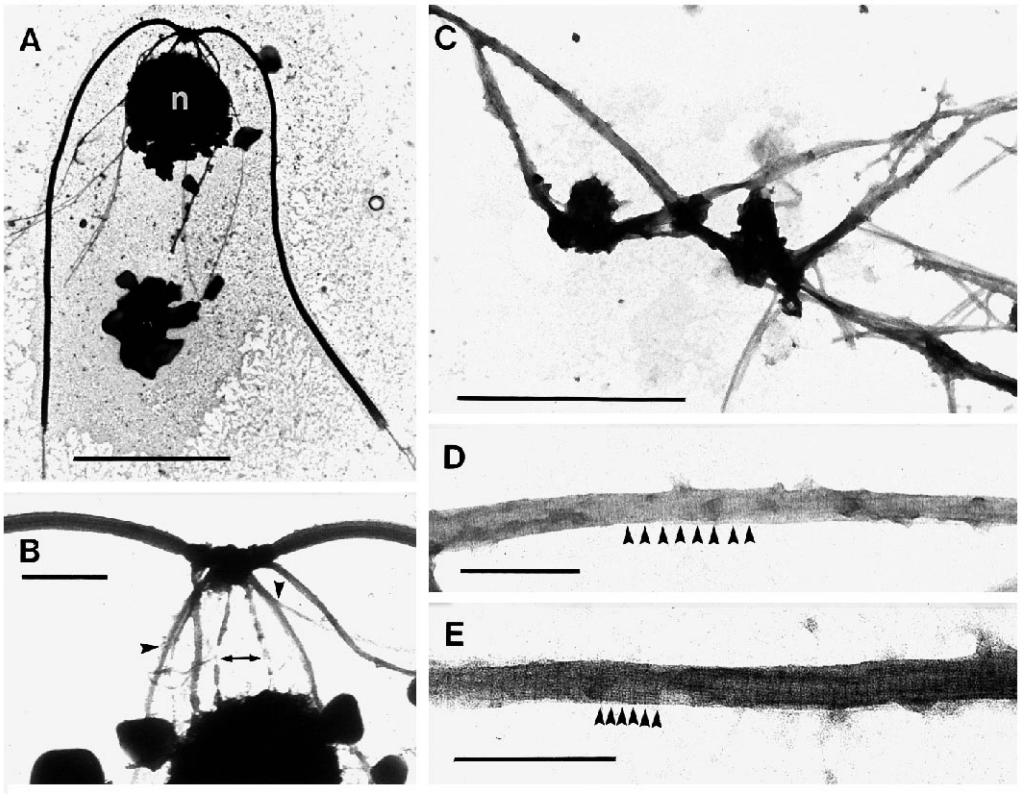


Fig. 4. (A) Cytoskeleton of *D. bioculata* isolated in the presence of 5 mM $MgSO_4$ in MT buffer. n, nuclear remnant. (B) Detail of an isolated cytoskeleton showing the two nuclear-basal body-connectors (double-headed arrow) and the four microtubular roots. The two branched s-roots are marked with arrowheads. (C) Basal body-flagellar root-complexes obtained from isolated basal apparatuses by homogenization and extraction with 2 M sodium chloride.
(D) Striated flagellar root in detail. The 28 nm cross striations are indicated with arrowheads.
(E) Striated flagellar root displaying a 15 nm periodicity (indicated with arrowheads), a phenomenon already described for paracrystals of *Spermatozopsis* SF-assemblin (Patel et al., 1992). Note the 2 nm protofilaments.
Bars: (A) 5 μm; (B and C) 1 μm; (D and E) 0.2 μm.
(A-E) Negatively stained whole mount preparations.

extracted with 2 M NaCl. The high salt insoluble material is represented by pairs of striated fibers (one s and one d fiber) connected to one basal body (Fig. 4C). The s-SMAF split into two subfibers about 0.5 μm from their origin near the basal bodies (the 1.9 μm ± 0.5 μm long s-α SMAF (*n*=4) and the 1.3 μm ± 0.4 μm long s-β SMAF (*n*=4)). The d-SMAFs have an average length of 2.1 μm ± 0.5 μm (*n*=5). The SMAFs have a maximum width of 73 nm (± 18.5 nm, *n*=18 for the s-SMAF) or 64 nm (± 19 nm, *n*=7 for the d-SMAF). The striated fibers display a complex cross-striation pattern of 28.5 nm (± 1.9 nm, *n*=26) and are composed of longidudinally oriented filaments of 2-3 nm diameter (Fig. 4D,E).

The SMAFs of *D. bioculata* can be dissolved in 8 M urea. When insoluble particles are removed by centrifugation and filtration a 31 kDa protein is enriched in the supernatant (>50% of total protein; calculated by densitometric scans of stained SDS gels, Fig. 5, lane 3). This protein constitutes about 4% of the total protein in isolated basal apparatuses (Fig. 5, lane 1) and 20% in the NaCl insoluble fraction (Fig. 5, lane 2). Most of the 31 kDa protein remains insoluble when the basal body-striated fiber complexes are extracted with ≤3 M urea (not shown). Separation of the 8 M urea extract by gel filtration chromatography yields fractions almost exclusively consisting of the 31 kDa protein (>90%, Fig. 5, lane 4). Since the 31 kDa protein elutes between 29 and 48 kDa it seems to resemble a monomeric state in 8 M urea (not shown). After dialysis of the 31 kDa protein against reassembly buffer B (pH 7) fibers (paracrystals) exhibiting a distinct cross striation pattern of 15 nm and longitudinally arranged protofilaments are formed (Fig. 6B,E). The protein composition of the paracrystals is shown in Fig. 5, lane 5. Dialysis against reassembly buffer A (pH 6.25) yields only non-striated fibers (Fig. 6C,F). The reassembly of striated paracrystals is also possible by dialysis of 5 M or 8 M urea extracts of basal body-striated fiber complexes against reassembly buffer B without further purification of the 31 kDa protein (Fig. 6A,D). In this case paracrystals are associated with amorphous material and the 31 kDa protein constitutes only about 60% of the total protein (not shown).

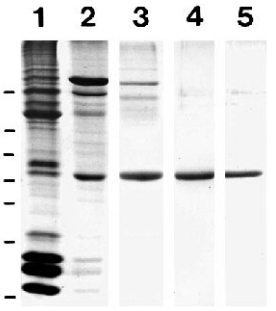


Fig. 5. SDS-PAGE analysis documenting the enrichment of the 31 kDa striated fiber protein from *D. bioculata*. (A) lane 1, isolated basal apparatuses; lane 2, NaCl (2 M) insoluble fraction enriched in striated fibers; lane 3, proteins solubilized in 8 M urea from the NaCl insoluble fraction; lane 4, 31 kDa protein purified by gel filtration chromotography; lane 5, protein composition of reassembled paracrystals. Molecular mass markers are given at the left: 66, 45, 36, 29, 24, 20.1, and 14.2 kDa.

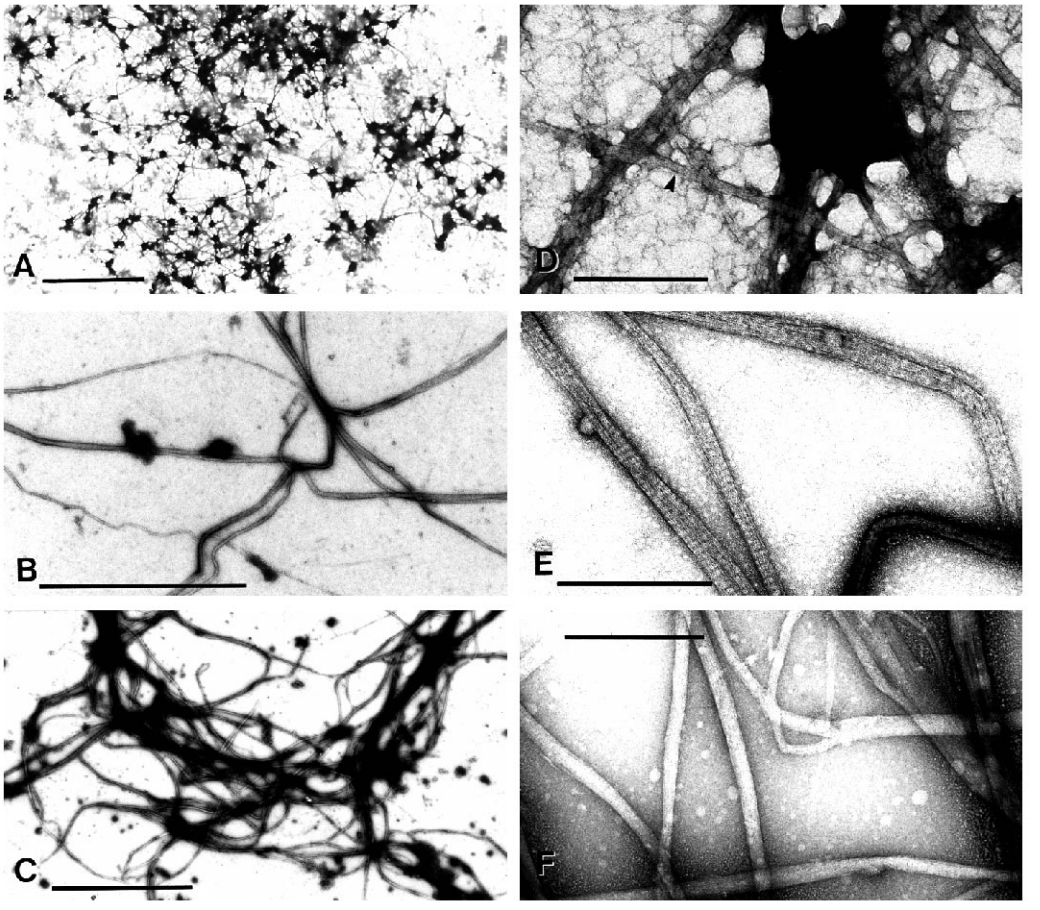


Fig. 6. Negatively stained paracrystals in overview (A,B,C) and detail (D,E,F) reassembled from extracted striated fibers of *D. bioculata*. (A and D) Paracrystals reassembled at pH 7 from 8 M urea extracts of basal body-striated fiber-complexes. Arrowhead: cross-striation of paracrystals. (B and E) Paracrystals reassembled at pH 7 from 8 M urea extracts of the 31 kDa protein purified by gel filtration chromatography. Paracrystals reveal cross striations. Note the protofilaments running longitudinal to the axis of the paracrystals. (C and F) As in B and E, but reassembled at pH 6.25 resulting in the formation of non striated paracrystals. Bars: (A,C) 4 μm; (B) 2 μm; (D,E,F) 0.25 μm.

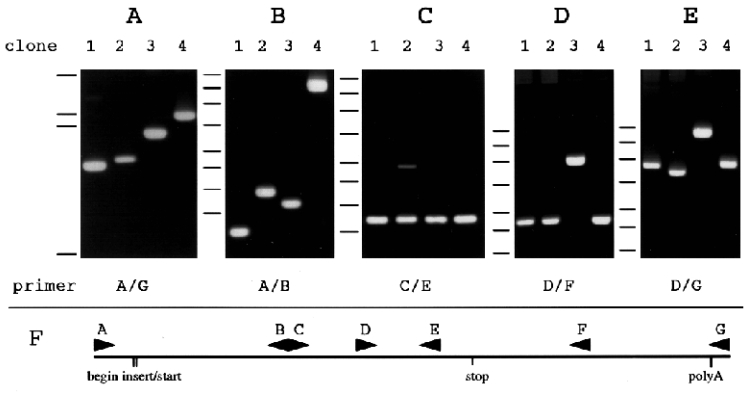


Fig. 7. Analysis of cDNA clones coding for the 31 kDa protein (SF-assemblin) of *D. bioculata* by PCR. The relative positions of the primers used are indicated in F. (A) Amplification of the complete inserts with the vector primers results in fragments of ~1,250 bp, ~1,350 bp, ~1,750 bp, and ~2,250 bp (clone 1-4, respectively). (B) Amplification of the 5′ part of the inserts using primers B and A results in fragments of ~350 bp ±70 bp for clones 1-3, while in clone 4 a 1,200 bp fragment was amplified. Clones 2 and 4 contain the complete coding region for the N-terminal part of the protein, while clones 1 and 3 have deletions at their 5′ end. Furthermore, clone 2 shows an internal deletion of 93 bp (s. text) and clone 4 contains a second insert of ~800 bp upstream of the cDNA of the 31 kDa protein. Using the primers C and E, fragments of equal size are ampified in all four clones (C), and PCR with the primers D and F reveals an insertion of ~450 bp in clone 3 (D). (E) The 3′ part of the coding region (as in D) and the complete 3′ non coding region were ampified with primer D and the reverse vector primer G showing a deletion of 60 bp in clone 2 as well as the insertion in clone 3. A 100 bp DNA ladder (Gibco BRL; B-E) or λ *Hin*dIII (A) were used as molecular mass markers, left side of each panel from the bottom in bp: A, 0.58, 1.98, 2.26, and 4.26; B and C, 300, 400, 500, 600, 800, 1,000 and 1,400; D and E, 400, 500, 600, 800, 1,000 and 1,400.

Microsequencing of peptides of the 31 kDa protein

The *Dunaliella* 31 kDa protein was enriched by in vitro reassembly of striated fibers, purified by SDS-PAGE and blotted onto PVDF membrane. In contrast to SF-assemblin of *S. similis* the 31 kDa protein has a blocked N-terminal end. Therefore blots were treated with endoproteinase Asp-N or trypsin. A total of 26 peptides with several overlaps were obtained. They were aligned along the rod domain of the sequence of *Spermatozopsis* SF-assemblin (bold letters in Fig. 8). Since the peptide sequences covered 199 residues of the rod domain the *Dunaliella* protein is identified as an SF-assemblin. Comparison with the sequence later deduced from the cDNA (see below) revealed only two mismatches. Residue 75 is a methionine instead of the glutamine in the peptide sequence and residue 106 is a cysteine instead of a glutamic acid.

Molecular cloning of cDNAs coding for the 31 kDa protein

A cDNA library with inserts sizes between 500-2,200 bp (average 1,290 bp, *n*=14) of *D. bioculata* was constructed and screened with the monoclonal antibody BAS 6.5 directed against SF-assemblin of *S. similis*. Among ~200,000 pfu one positive clone (clone 1) was identified and partialy sequenced revealing a 5′-deletion of the insert of 228 bp. Renewed probing of ~400,000 pfu with the insert of clone 1 yielded three positive clones. All four clones differed in the sizes of the inserts as determined by PCR (clone 1 ~1,250 bp, clone 2 ~1,350 bp, clone 3 ~1,750 bp, and clone 4 ~2,250 bp; Fig. 7A). Amplification of the 5′-region of the inserts using primers A and B resulted in fragments of ~280 bp (clone 1), ~400 bp (clone 2), ~360 bp (clone 3), and ~1,200 bp (clone 4; Fig. 7B). Complete (clone 2) or partial (clones 1, 3, and 4) sequencing demonstrated a deletion of the first 142 bp in clone 3, whereas clones 2 and 4 show identical 5′ noncoding and coding regions for the N-terminal part of the 31 kDa protein. However, clone 2 shows a deletion of 93 bp corresponding to residue 68 (VL) or 70 to residue 99 (VL) or 101 of the amino acid sequence and clone 4 contains an insertion of ~800 bp 5′ of the cDNA of the 31 kDa protein. Amplification of an internal part of the coding region results in fragments of ~350 bp with all four clones (Fig. 7C). Futhermore clone 3 contains an insertion of ~450 bp after nucleotide 827 of the constructed complete cDNA for the 31 kDa protein. Correspondingly PCR with primers D and F results in the amplification of a ~1,000 bp fragment when clone 3 is used as a template, while fragments of ~550 bp were amplified either with clones 1, 2, and 4 (Fig. 7D), or genomic DNA of *D. bioculata* as a template (not shown). Finally clone 2 shows a second deletion of 60 bp at the very end of the 3′ non coding region as shown by PCR and sequencing (Fig. 7E).

We have sequenced clone 2 completely and, with the exception of 308 bp near the 3′ end, in both directions. The two deletions of clone 2 (see above) were supplemented by partial sequencing of clones 1, 3 and 4 including the 5′ and 3′ parts of the inserts and the coding regions for the C-terminal part of the protein. Additionally most of the 3′ non coding regions of clone 1 and 3, and of the insertions in clone 3 and 4 were sequenced. The complete cDNA sequence for the 31 kDa protein given in Fig. 8 is available under accession number X90905, EMBL Nucleotide Sequence Database.

Sequence analysis of the 31 kDa protein

The cDNA sequence and the deduced amino acid sequence of the 31 kDa protein from *D. bioculata* are presented in Fig. 8. The protein sequence of 277 residues corresponds to a molecular mass of 31,377, in good agreement with the apparent value of 31 kDa determind by SDS-PAGE. The calculated isoelectric point is 5.40. The sequence identifies two distinct structural domains. The N-terminal 27 residues form a non-helical domain, which contains all three prolines of the protein. Starting with residue 28 the algorithm of Garnier et al. (1978) predicts a more or less uninterrupted α-helix and raises the possibility of a short β-sheet only in the N-terminal domain (not shown). The procedures of Chou and Fassman (1974) detect short β-sheets around residues 40, 100, 130, 225, and 255 and a series of strong α-helices for most of the rod domain (not shown). Both algorithms predict a non α-helical structure for a small region (~9 residues) at the C-terminus of the protein.

Fig. 9 shows an alignment of the 31 kDa protein of *D. bioculata* with SF-assemblin of *Spermatozopsis similis*. The sequence identity between the algal SF-assemblins is 57% and the similarity is 78%. The N-terminal head domain and the small presumptively non-helical C-terminal domain of both proteins differ in size. Both head domains contain conserved sequence motifs like (S/G)RPF and several SP-motifs (two or four for *D.*

```
gaaagaaaaa ggATGGCCAC TTCTGGGATG GTTTCCCCCA CCTCAGGCAG ACCGTTCAGC   60
             M  A  T   S  G  M   V  S  P  T   S  G  R   P  F  S
CCCATGCGCT CCAGTGTGCT GACCACTACC GGCTCAGCTA TCAAGCTTGA GCACGTATCC  120
P  M  R  S   S  V  L   T  T  T   G  S  A  I   K  L  E   H  V  S
GAAAAGTTTG CCGGGCTGTG GACTGACCTG GAGCAGGAGA AGCAGGCCCG ACGCATTGCT  180
E  K  F  A   G  L  W   T  D  L   E  Q  E  K   Q  A  R   R  I  A
GAGGCCACCC GAATGCAGCT TTTTCAGGAA TCCGTGCTGC GCCTGGAGAA GAGCATGGAG  240
E  A  T  R   M  Q  L   F  Q  E   S  V  L  R   L  E  K   S  M  E
GCTGAGGTGA AGAGGAGGGC CGAGTCAGAC AAGCAGCTGC AGGCCCACTT TGAGGGTGAA  300
A  E  V  K   R  R  A   E  S  D   K  Q  L  Q   A  H  F   E  G  E
CTGCGGGTGC TGCATGAGCG ATCAGCTTGC CAAGTGGCGG AAATGCACAA CTCCCTGAAG  360
L  R  V  L   H  E  R   S  A  C   Q  V  A  E   M  H  N   S  L  K
TCAGCCATTG ATTCCTTCTC TTCCAGAGTG CAAGACTTGC ATGCTGTCAT CAGGGAGGAG  420
S  A  I  D   S  F  S   S  R  V   Q  D  L  H   A  V  I   R  E  E
CGCGACCAAC GTCGCAATGA TATCGAACAC CTGGCCACAA GCCTGGTTGG CAAGGTCAAT  480
R  D  Q  R   R  N  D   I  E  H   L  A  T  S   L  V  G   K  V  N
GAGTGTGTGG CTGCCCTCGA TGAAGAGCGC ACAATCCGCA TGCAGGAGCA GGCCATGGCC  540
E  C  V  A   A  L  D   E  E  R   T  I  R  M   Q  E  Q   A  M  A
CTTAAGCGCT TTGGTGAGGA TATCAGCGGC GTTAACCACC GGGTGGACAC AGAGAAGGCA  600
L  K  R  F   G  E  D   I  S  G   V  N  H  R   V  D  T   E  K  A
CAAAGGGAGG CTGACCTGTC AGGCCTGCGT GTGGAGATTC ACGATGTGCT GGCCAACCGC  660
Q  R  E  A   D  L  S   G  L  R   V  E  I  H   D  V  L   A  N  R
AACATCCAGG ATGAGCAGTT CAAGACTGTC ACCCTGGATG AGCTGAACGC CTTCAAGGCT  720
N  I  Q  D   E  Q  F   K  T  V   T  L  D  E   L  N  A   F  K  A
GCCTTGGCCC TCGAGCGTGA GGAGCGTGTG GCTGAGGACG ATGAGATTGT GCAAGCTATC  780
A  L  A  L   E  R  E   E  R  V   A  E  D  D   E  I  V   Q  A  I
AATGACTACA CCCGGGCACT GCAGGAGGGC CTCAAGATGG TCAACATGCA GCACAACTCA  840
N  D  Y  T   R  A  L   Q  E  G   L  K  M  V   N  M  Q   H  N  S
GCCtaatggc gaggccacat gaactgtaag tgcgccaggg agcacaagct tttgggagac  900
A
aattccggcc aatccatccc cctgggctgg cctttgtcct cattttaatt tgtgttaggt  960
ccccctaat taacctggcc cttgccatgg agcgtgctct cttcatgctt ctgccatttc 1020
tcgagtctgt tgttctttga tcgtgcgaac gtttctgagg atcatctccc tctcgcgtta 1080
ggtaatggtg atcggacaca tttgccggct ttaaacattt accctgacga caatgctggt 1140
ggctgaccct tgacactctc cccaaaattt tttgcctctg tttcgatccg cagtgcctcc 1200
tgcatgcaga tctgctgtgc tgcactccaa tgttagcatg tcttcctgac agacaaaaca 1260
ttggtgacaa tgtctacatg tgaaggcaaa tctcggatgt tcagtgtacc aataaatgtc 1320
tgcttgtttg attttcaatg tggctttgtt tcactcattt gttgggcgat taagaaattc 1380
cttctggagg gcatgtgtaa cattgagagg tgg(a)21                          1434
```

Fig. 8. cDNA sequence (upper rows) and deduced amino acid sequence (lower rows) of *D. bioculata* SF-assemblin. The underlined regions of the cDNA sequence were only sequenced in one direction. Amino acids given in bold letters were independently identified by microsequencing of peptides of the purified protein (see text). Non coding regions are given in small letters. The sequence is available from the EMBL/GenBank/DDBJ databases under accession number X90905.

```
head
    D.bioculata 1 MATSGMVSPTSGRPF..SPM....RS.SV...LTTT.G.
                  :|.:  ....:|||  ||:    || |.   :|.| :
      S.similis 1  PTPSPEARVASRPFLDSPLPGSPRSGSPTGYITATKAI
                                              |  :| |
      G.lamblia 1                          MSMFTSTRTLTQTMDKPDD

rod
    defgabcdefgabcdefgabcdefgabc*defgabcdefgabcdefgabcdefgabc*
 28        SAIKLEHVSEKFAGLWTDLEQEKQARRIAEATRMQLFQESVLRLEKSMEAE
           || |||||.|||..::.::| |||.||:|:|.|:|:: :|: :||||:|||
 39        SAGKLEHVAEKFSNFYNEIELEKQQRRMADAARFQMLTDSIAKLEKSLEAE
            | || :: ::   :   :| | : ||: :  |: :: : :  |:: :: |
 20 LTRSATETAVKLSNMNQRVSRFHDKMENEIEVRRVDDDTRVKMIKDAIAHLDRLIQTE


    defgabcdefgabcdefgabcdefgabc*defgabcdefgabcdefgabcdefgabc*
 79 VKRRAESDKQLQAHFEGELRVLHERSACQVAEMHNSLKSAIDSFSSRVQDLHAVIREE
    :|||||||||:|.|||:|::.|:||.| |:|:::..:|..:|::|. :|||||.:|:||
 90 IKRRAESDKQIQVHFESEVKGLQERTALQLADLQAAFKTSVDGLSRTMQDLHTIIKEE
     ::|  |  ::          :      ::  :   ::  :  ::  : ::   : ::
 78 SRKRQASFEDIREEVKKSADNMYLTIKEEIDTMAANFRKSLAEMGDTLNNVETNLQNQ


    defgabcdefgabcdefgabcdefgabc*defgabcdefgabcdefgabcdefgabc*
137 RDQRRNDIEHLATSLVGKVNECVAALDEERTIRMQEQAMALKRFGEDISGVNHRVDTE
    |:|||.||||||.|||.|||||||||||||. ||:.:.. ||.::.|:  |..::|||
148 REQRRSDIEHLAGSLVNKVNECVAALDEERISRMDAESKILKQIATDVFRVQEKIDTE
       :   |  |    :  :|:   ::  |   |            : :   :   :: |
136 IAIHNDAIAALRKEALKSLNDLETGIATENAERKKMYDQLNEKVAEGFARISAAIEKE


    abcdefgabcdefgabcdefgabcdefg*abcdefgabcdefgabcdefgabcdefg*
195 KAQREADLSGLRVEIHDVLANRNIQDEQFKTVTLDELNAFKAALALEREERVAEDDEI
    |: |||:|..|| |||:||:|||: ||.|.|:.|||:|.:|.|:.:|||||:.|||||
206 KGTREAELATLRSEIHEVLGNRNLSDEKFQTLVLDEINNLKSAVQMEREERISEDDEI
       ||  :     |       :   :       |:  :   :  |: ||   :| |
194 TIARERAVSAATTEALTNTKLVEKCVNEQLENVASEIRAIQEEIDREKAERKEAEDKI


    abcdefgabcdefgabcd
253 VQAINDYTRALQEGLKMVNMQHNSA 277
    |||:||||||||:||::||   ||
264 VQAVNDYTRALQDGLRIVN...NS  284
    |  ::|    :|:||  |
252 VNTLEDVVSKIQGGLSMVTKH     272
```

Fig. 9. Sequence alignment of the SF-assemblins from *D. bioculata* and *S. similis* with β-giardin. The sequence of the *Dunaliella* protein (upper rows) is aligned versus the *S. similis* protein (middle rows). Identical residues are connected by a vertical line. One or two points indicate homologous residues according to the programme of Needleman and Wunsch (1970). The sequence identity is 57%, similarity accounts to 78%. Residues which are identical in both green algal SF-assemblin and β-giardin (lower rows) are connected by vertical lines, two points indicate conserved substitutions. The predicted heptad arrangement in 29 residue repeats (Weber et al., 1993) is indicated (a to g). The first 29 residue repeat seems incomplete in SF-assemblin. Note the conserved skip residues, marked by a star, after 4 consecutive heptads. The N-terminal head domains of both SF-assemblins contain SP-motifs and in the case of *Dunaliella* a SPMR-motif (bold letters). These are potential sites for phosphorylation and the latter may be a site for the $p34^{cdc2}$ kinase (see text). Further conserved motifs in the head domains ((S/G)RPF and (L/I)TXT) are underlined.

bioculata and *S. similis*, respectively). A LTTT-motif in *Dunaliella* seems to be conserved in *Spermatozopsis* as ITAT. In the only other protein known to be homologous to SF-assemblin, β-giardin (see below) the corresponding sequence is LTQT (Fig. 9). One of the SP motifs, found only in *Dunaliella*, lies in the sequence context SPMR (residues 16-19) which could resemble a putative $p34^{cdc2}$ protein kinase consensus site indicative of mitotic phosphorylation (Moreno and Nurse, 1990).

A segmented coiled coil with a 29 residue repeat pattern based on four heptads followed by a skip residue has been discussed for the rod domains of *Giardia* β-giardin and *Spermatozopsis* SF-assemblin (Holberton et al., 1988; Weber et al., 1993). The (28+1) residue system is also conserved in the rod domain of *Dunaliella* SF-assemblin. Both green algal proteins share 8 identical skip residues (indicated by * in Fig. 9) and a similar distribution of charged residues. 91% of the charged residues are identical or represent conservative exchanges in the two SF-assemblins. Sequence identities between the repeats are particularly high in repeat 5 and rather low in repeats 4 and 6. Structural analysis of the sequences with the programs used indicates, that the first 29 residue repeat of *Dunaliella* SF-assemblin is incomplete (as shown in Fig. 9).

DISCUSSION

The presence of four striated microtubule-associated fibers in the flagellar apparatus of *Dunaliella spp.* was first reported by Hyams and Chasey (1974). A previous analysis of the biochemical composition of basal body-striated fiber complexes from *D. bioculata* revealed that a 76 kDa, a 50 kDa, and a 31 kDa protein were enriched in the preparation (Marano et al., 1985). The present study shows that a 31 kDa protein related to SF-assemblin of *Spermatozopsis similis*, is the filament-forming subunit of the striated microtubule-associated fibers in

species of *Dunaliella*. A monoclonal and a polyclonal antibody raised against SF-assemblin from *Spermatozopsis similis* cross-react with the SMAFs of four different species of *Dunaliella*. While the polyclonal anti-SF-assemblin strongly labeled the SMAF of *Dunaliella* in immunofluorescence, the cross-reaction with the 31 kDa protein was only weak in immunoblots. The monoclonal antibody BAS 6.5, however, reacts strongly with SF-assemblin and the 31 kDa protein in immunoblots and shows excellent results in screening cDNA libraries. The deduced amino acid sequence identifies the 31 kDa protein of *D. bioculata* as SF-assemblin. The two algal SF-assemblins now available show 57% sequence identity.

In *Chlamydomonas reinhardtii*, *Polytomella agilis* and other flagellate green algae including the prasinophyte *Nephroselmis olivacea* the polyclonal anti-SF-assemblin identifies cross-reacting flagellar roots by indirect immunofluorescence and 34 kDa proteins in immunoblots (our unpublished observations). We therefore conclude that SF-assemblins are the major components of the striated microtubule-associated fibers in green algae.

Although the SF-assemblins from *Spermatozopsis* and *Dunaliella* share 57% sequence identity the two proteins differ somewhat in solubility properties in urea, and ability to form striated paracrystals at pH 6.25. Urea extracts of striated fibers from *D. bioculata* were reassembled in vitro into paracrystals. Only small quantities of the protein could be extracted from preparations of striated fiber with 2 M or 3 M urea. Best results were obtained with concentrations of urea higher than 5 M wheras SF-assemblin of *Spermatozopsis* is largely soluble already in 2 M urea. The different solubility properties may be related to the presence of other components of the striated fibers or to intrinsic differences between the two proteins such as different patterns of posttranslational modifications (i.e. phosphorylation), and different isoelectric points (5.4 and 5.0 for *Dunaliella* and *Spermatozopsis* respectively). *Spermatozopsis* SF-assemblin consists of at least four isoforms including two phosphorylated ones (Lechtreck and Melkonian, 1991b). Likely phosphorylation sites are four SP-motifs in the head domain. The head domain of *Dunaliella* SF-assemblin contains one SP-motif and a SPMR-motif. It remains to be seen whether the latter motif provides a site for the $p34^{cdc2}$ protein kinase during mitosis. In *Chlamydomonas reinhardtii* the striated microtubule-associated fibers are reorganized during mitosis and in metaphase SF-assemblin is restricted to two colon shaped structures located near the basal bodies (our unpublished observation).

The only protein presently known to be homologous (≈20% identity) to the algal SF-assemblins is β-giardin from the flagellate protozoan *Giardia lamblia* (Crossley and Holberton, 1983). The amino acid sequence of β-giardin shows two distinct domains: a short non-helical head domain and a α-helical rod domain (Holberton et al., 1988). This protein is located in the striated, microtubule-associated microribbons, which consist of several proteins of 28-36 kDa molecular mass (Peattie et al., 1989).

Spermatozopsis SF-assemblin and β-giardin show the same length of the α-helical rod domain, whereas the rod domain of the 31 kDa protein is four residues longer. Instead of the conventional coiled coil with a continuous apolar seam, as realized for example in tropomyosin, the rod domain of these three proteins appears to form a segmented coiled-coil with a 29 residue repeat pattern based on four heptads followed by a skip residue. Five of the 8 skip residues are identical in all three proteins. The preferred skip residues seem to be glutamine and glutamic acid: seven in β-giardin and six in each algal SF-assemblin. The distribution of charged residues is similar in SF-assemblins and less pronounced in β-giardin. Futhermore, in all three rods, the third and fourth heptads show the dominance of hydrophobic residues in the interior *a* and *d* positions, while the first two heptads of the repeat have a much less perfect hydrophobic pattern. The heptad faults created by the skip residues are regulary displacing the apolar seam in the coiled coil configuration (Marshall and Holberton, 1995). Interestingly other segmented coiled coils with periodicities of 24 and 25 residues have been predicted by two other cytoskeletal proteins of *Giardia* (Marshall and Holberton, 1993, 1995).

The head domain of β-giardin, initially reported to be only 6 residues long, is actually 13 residues longer (D. Holberton, unpublished; accession number X85958, EMBL/GenBank/DDBJ databases). Thus the size of the head domains varies between 31 residues (*Spermatozosis* SF-assemblin), 20 residues (*Dunaliella* SF-assemblin) or 19 residues (β-giardin). In all three proteins the head domain is rich in hydroxyamino acids and contains a (L/I)TXT-motif. While the green algal proteins are characterized by the presence of SP-motifs in their head domains, these are conspicuously absent from β-giardin.

The first comparison of an algal SF-assemblin with β-giardin revealing an 20% sequence identity over the rod domain indicated a strong drift in sequences versus a conservation in sequence principles (Weber et al., 1993). This seemed not surprising in view of the fact that the zooflagellate *Giardia* 'may be the most primitive of known eukaryotic organisms' (Marshall and Holberton, 1995; see also Sogin et al., 1989). Our results from *Dunaliella bioculata* show that even in closely related taxa of green algae there is a strong drift in the protein sequences of the two SF-assemblins, since they share only 57% sequence identity. In marked contrast, centrin, the major component of the second type of striated fibers in green flagellates shows 81% sequence identity for several algae (Bhattacharya et al., 1993).

The identification of homologous proteins in a parasitic archezoan flagellate and two green algae indicates that β-giardin, SF-assemblin and related proteins may be widespread among eukaryotes. Recently, the kinetodesmal fibers of *Paramecium caudatum* were isolated (Sperling et al., 1991) and shown to consist of a family of proteins of molecular mass 30-36 kDa with several, partially phosphorylated isoforms. We suggest that the kinetodesmal fibers of ciliates and the striated microtublule-associated fibers of green algae contain homologous proteins. Thus it appears that there exists a new family of cytoskeletal proteins which form segmented coiled coils and can pack into extensive cross-striated fibers, which may serve as stabilizing cytoskeletal element in flagellate/ciliate eukaryotic cells. While the kinetodesmal fibers and the microribbons of *Giardia* are heterogenous in their protein composition (Peattie et al., 1989; Sperling et al., 1991), the striated fibers of green flagellates can be assembled from a single polypeptide species (Lechtreck and Melkonian, 1991b; this study). The availability of cDNA clones coding for SF-assemblins now offers the possibility for a detailed study of domains involved in the formation of striated microtubule-associated fibers.

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