Striated Microtubule-associated Fibers: Identification of Assemblin, A Novel 34-kD Protein That Forms Paracrystals of 2-nm Filaments In Vitro

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Abstract. Microtubule-associated fibers from the basal apparatus of the green flagellate alga *Spermatozopsis similis* exhibit a complex cross-striation pattern with 28-nm periodicity and consist of 2-nm filaments arranged in several layers. Fibers enriched by mechanical disintegration and high salt extraction (2 M NaCl) of isolated basal apparatuses are soluble in 2 M urea. Dialysis of solubilized fibers against 150 mM KCl yields paracrystals which closely resemble the native fibers in filament arrangement and striation pattern. Paracrystals purified through several cycles of disassembly and reassembly are greatly enriched (>90%) in a single protein of 34 kD (assemblin) as shown by SDS-PAGE. A rabbit polyclonal antibody raised against assemblin labels the striated fibers as shown by indirect immunofluorescence of isolated cytoskeletons or methanol permeabilized cells and immunogold EM.

Two-dimensional electrophoresis (isoelectric focusing and SDS-PAGE) resolves assemblin into at least four isoforms (*a–d*) with pI's of 5.45, 5.55, 5.75, and 5.85. The two more acidic isoforms are phosphoproteins as shown by in vivo $^{32}PO_4$-labeling and autoradiography. Amino acid analysis of assemblin shows a high content of helix-forming residues (leucine) and a relatively low content of glycine.

We conclude that assemblin may be representative of a class of proteins that form fine filaments alongside microtubules.

FINE filaments with a diameter of 2–4 nm have been detected in various eukaryotic cell types (Roberts, 1987). In contrast to the major cytoskeletal filament classes (microfilaments, intermediate filaments, microtubules) fine filaments are apparently very heterogenous in their protein composition. The molecular weights of the respective proteins range from 14 kD (major sperm protein [MSP])[1]; Sepsenwol et al., 1989) to >1,000 kD (titin; Wang et al., 1984). Nearly always they are mechanically and biochemically highly stable (Holberton and Ward, 1981; Linck and Langevin, 1982; Honts and Williams, 1990) and thus could perhaps serve a major stabilizing function in the eukaryotic cytoskeleton. Fine filaments are arranged as extensive networks (Ris, 1985) or tight bundles (microribbons, tektins, kinetodesmal fibers, system I fibers) and are often associated with other protein filaments of the cytoskeleton. Microtubule-associated fine filaments occur in organelles that are exposed to considerable mechanical stress, e.g., flagella (Linck and Langevin, 1982), the sucking disk of *Giardia lamblia* (Crossley and Holberton, 1983*a*), or feeding organelles (Honts and Williams, 1990). Despite these common functional properties of fine filaments the relationship between the different filament-forming proteins remains at present unknown and requires further analysis.

The basal apparatus of flagellate green algae (a functional homologue to the centrosomal complex of animal cells) contains two main types of filamentous structures (Melkonian, 1980). One type consists of 4–8-nm filaments, undergoes Ca^{2+}-modulated contraction and contains the 20-kD EF-hand phosphoprotein centrin (Salisbury and Floyd, 1978; Salisbury et al., 1984; Salisbury et al., 1988; Huang et al., 1988*a*, *b*). Such centrin-containing filaments connect basal bodies with each other (connecting fibers; McFadden et al., 1987), with the nucleus (nucleus–basal body connectors, system II fibers, rhizoplasts; Wright et al., 1985) or occur within the flagellar transition region (Schulze et al., 1987; Sanders and Salisbury, 1989). They have been implicated to play an important role in various motile functions of the basal apparatus (Salisbury, 1989*a*; Melkonian et al., 1991). A second type of filamentous structure in the basal apparatus consists of fine (2 nm) filaments arranged in cross-striated bundles that run parallel to flagellar root microtubules (Hoffman, 1970). These bundles have been termed system I fibers or striated microtubule-associated components (Micalef and Gayral, 1972; Melkonian, 1980; Floyd et al., 1980). Immunological studies have indicated that system I fibers do not contain centrin (Melkonian et al., 1988). In this study we describe the isolation and characterization of system I fibers from *Spermatozopsis similis* (Preisig and Melkonian, 1984), a unicellular, naked green flagellate related to *Chlamydomonas* (Melkonian and Preisig, 1984). We identify a novel 34-kD protein consisting of several isoforms that

1. *Abbreviation used in this paper*: MSP, major sperm protein.

can form paracrystals in vitro which closely resemble native system I fibers. The characteristics of system I fibers indicate a relationship to other fine filament structures.

Materials and Methods

Strains and Culture Conditions

S. similis (Preisig and Melkonian, 1984) (strain no. B 1.85 of the Sammlung von Algenkulturen Göttingen; Schlösser, 1986) was cultured in aerated (2 l/min) and stirred 10-liter flasks in a modified WARIS-solution (McFadden and Melkonian, 1986). The culture conditions were 15°C, 20 $\mu E \times m^{-2} \times s^{-1}$, and a L/D-cycle of 14/10 h.

Preparation of Basal Apparatuses

Basal apparatuses of *S. similis* were isolated essentially as described by Lechtreck et al. (1989) with the exception that $MgSO_4$ was omitted from the MT buffer. 30–60 liters of cells were concentrated with a tangential flow filtration system (Millipore Corporation, Bedford, MA), washed twice (600 *g*, Sorvall Dupont RC 28S; rotor GS3). The cells were resuspended in MT-buffer (50 ml/10-liter culture) and lysed by adding an equal volume of 2–3% Triton X-100 in MT buffer (lysis buffer). The cytoskeletons were then pelleted at 1,500 *g*, for 15 min in a Labofuge I (Heraeus Christ GmbH, D-3360 Osterode, Germany) and washed three times with decreasing concentrations of Triton X-100 (1, 0.5, and 0.25%) and finally several times with MT buffer. The cytoskeletons were resuspended by homogenization (40 strokes in a 30-ml tissue homogenizer; Kontes Glass Co., Vineland, NJ) in MT-buffer including 0.05% NaN_3 and pelleted at 15,000 *g* (centrifuge 2K15, Sigma Chemical Co., Osterode, Germany). The supernatants were discarded and the pellets stored up to four weeks at −20°C.

Reassembly of Paracrystals

Frozen basal apparatuses were resuspended in 30 ml MT-buffer with 2 M NaCl by homogenization (20–30 strokes, see above) and extracted for 3–5 h at 4°C (with agitation). The suspension was centrifuged at 48,500 *g* at 4°C for 30 min (RC 28S; rotor SS34). The pellet was resuspended in 4–8 ml MT buffer including 2 or 2.5 M urea (disassembly buffer) and extracted for 3–5 h at 4°C. After extraction the suspension was centrifuged for 1 h at 200,000 *g*, 4°C (OTD-Combi rotor, TST 60.4, 39,000 rpm; Sorvall DuPont). The supernatant was dialyzed for 8–16 h at 4°C against a 50-fold volume of reassembly buffer (150 mM KCl, 10 mM MES, 2 mM EDTA, 1 mM DTT, pH 6.25) with three or more changes. The paracrystals were pelleted at 48,500 *g*, 30 min, 4°C (as above) and dissolved again in disassembly buffer. The disassembly/reassembly process was repeated up to five times. In some cases the reassembled structures were washed in 1 M NaCl (in reassembly buffer) for ~30 min.

Electrophoretic Analyses

(*a*) SDS PAGE was carried out according to the method of Laemmli (1970) in slab gels containing 12% polyacrylamide and 0.1% SDS. (*b*) Two-dimensional electrophoresis (first dimension, isoelectric focusing; second dimension, SDS-PAGE) was performed according to O'Farrell (1975) with the following modifications: after focusing, the IEF-gels were incubated for 5 min in SDS sample buffer (Laemmli, 1970) followed by 2 min in 270 mM iodoacetamide and placed directly on top of the stacking gel. 7.5% total ampholines obtained from Bio-Rad Laboratories (Cambridge, MA) (pH 3–10) and Pharmacia Fine Chemicals (Piscataway, NJ) (pH 4–6, pH 5–8, and pH 3.5–5) were used for isoelectric focusing in the first dimension. For IEF and SDS-PAGE a Minigel-system (Renner GmbH, Dannstadt, Germany) was used. Gels were stained with Coomasie Brilliant blue. (*c*) For protein blot analysis, proteins separated on gels were electrophoretically transferred to a PVDF membrane (Millipore Corp.) using a semi-dry blot apparatus (Graphoblot, Organogen GmbH, Göttingen, Germany) according to the instructions of the manufacturer. (*d*) Proteins were electroeluted from gel slices with an electroelution apparatus (Orpegen GmbH, Heidelberg, Germany) using SDS-PAGE running buffer or Tris/borate-buffer (160 mM Tris, 40 mM borate, 0.1% SDS, pH 8.6).

Antigen Preparation and Immunization

Paracrystals were subjected to SDS-PAGE and gel-purified 34-kD protein was used to immunize a female rabbit ("German Grey Giant"). The 34-kd protein was electroeluted from the preparative gels and after extensive dialysis against 10 mM ammoniumbicarbonate followed by 10 mM Hepes including 2 mM EDTA (pH 8), the eluate containing 10–20 μg of protein was mixed with complete (first injection) or incomplete (booster injections on days 13, 46, 101, and 306) Freud's adjuvant and injected (10–20 μg) subcutaneously at different sites into the rabbit. An IgG fraction was prepared from the immune serum by two ammonium sulfate precipitations (1.75 M) and affinity chromatography using a 5 ml protein A Sepharose column (Pharmacia-LKB S-75182 Uppsala, Sweden).

Immunoblotting

Isolated cytoskeletons or basal apparatuses were electrophoresed and transferred to a PVDF-membrane. After extensive blocking with PBS/BSA (3%) the membrane strips were incubated with the anti-34-kD IgG fraction (1:1,000, 2.4 μg IgG/ml in 3% PBS/BSA) for 60–90 min, washed three times for 20 min with PBS/BSA (0.5%), incubated with anti-rabbit IgG peroxidase conjugate (Sigma Chemical Co.) diluted to 1:1,000 in PBS/BSA (3%) for 60–90 min and washed again five times for 10–20 min in PBS/BSA (1%). 4-chloro-1-naphtol was used as substrate.

Indirect Immunofluorescence

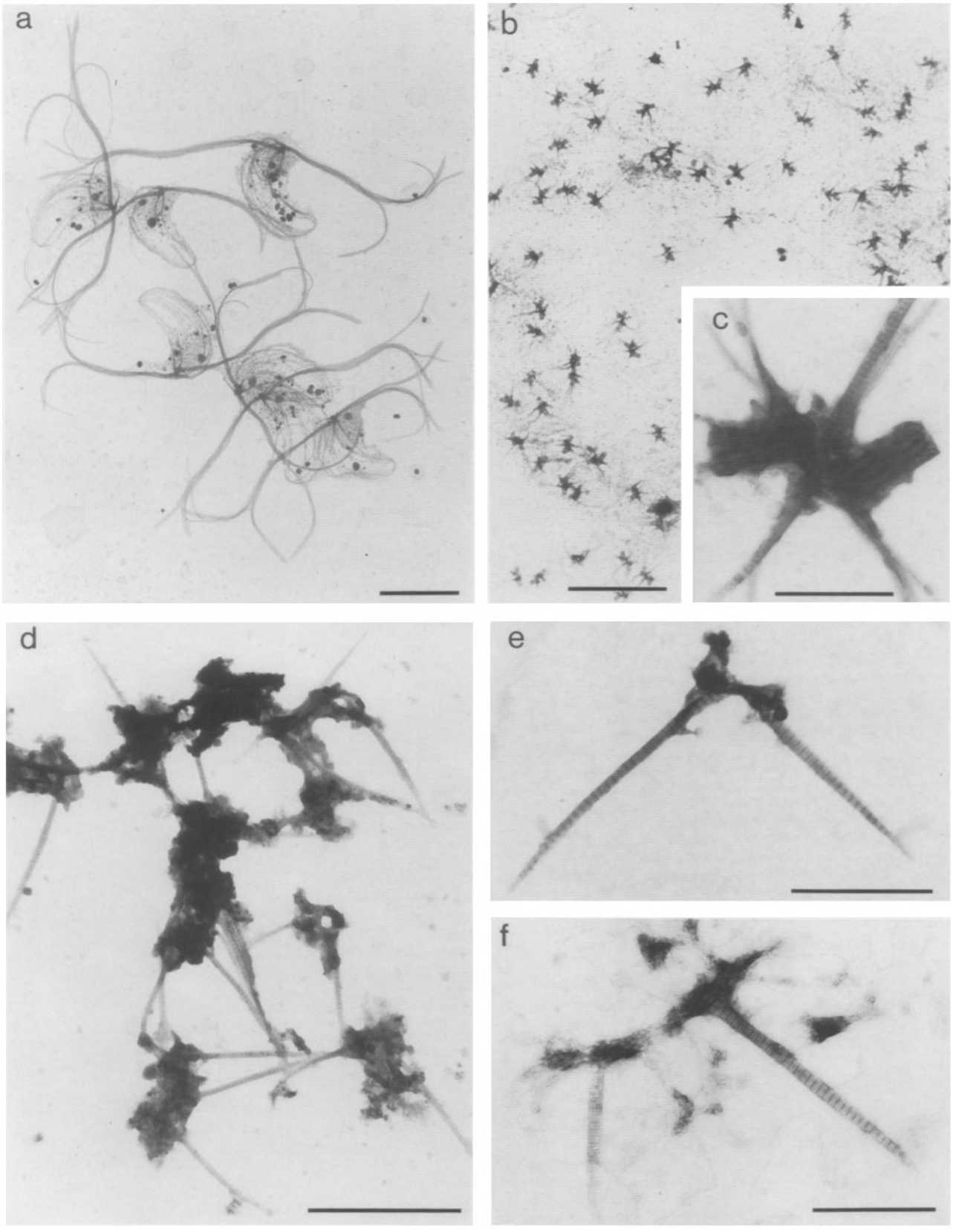
Isolated cytoskeletons. Cells were harvested and washed with a modified MT-buffer (30 mM Hepes, 15 mM KCl, 5 mM $MgSO_4$, 5 mM EGTA, pH 7) by low-speed centrifugation. Cells were lysed by addition of an equal amount of 1% Triton X-100 in modified MT buffer, immediately followed by fixation with 3% freshly prepared paraformaldehyde and 0.25% glutaraldehyde (final concentration in modified MT buffer) for 30 min. During the last 10–15 min of the fixation the cytoskeletons were allowed to adhere to precleaned polylysine-coated coverslips. The coverslips were washed with PBS, quenched for 30 min with PBS/BSA (2%) and then incubated with anti-34-kD IgG (1:40 in 2% PBS/BSA) for 90 min at 37°C. Subsequently, coverslips were rinsed several times with PBS, quenched again with 2% BSA in PBS for 10 min and incubated with anti-rabbit IgG conjugated to TRITC (1:40 in 2% PBS/BSA) for 90 min at 37°C.

Methanol-extracted Cells. Cells were fixed to polylysine-coated coverslips and rinsed with methanol at −20°C for 90 s. Coverslips were treated as described for isolated cytoskeletons (see above).

Double Immunofluorescence. Cytoskeletons were isolated and processed as described above. Monoclonal anti-α-tubulin (mAb 1-2.3; courtesy of Dr. D. J. Asai, Santa Barbara, CA) and anti-34-kD IgG were applied together (at 1:250 and 1:80 dilution in 2% PBS/BSA) for 90 min at 37°C. Anti-mouse IgG conjugated with FITC (Boehringer Mannheim GmbH, Mannheim, Germany) and anti-rabbit IgG conjugated with TRITC (Sigma Chemical Co.) (1:50 and 1:80 in 2% PBS/BSA) were applied together.

After extensive washing the coverslips were mounted in glycerol/PBS (2:1) including 0.1% *p*-phenylenediamine (Sigma Chemical Co.). Cells and cytoskeletons were observed with an inverted microscope (IM 35; Zeiss, Oberkochen, Germany) using 100× oil immersion objective. Photographs were taken with phase contrast (Tech-Pan; Eastman Kodak, Rochester, NY) or fluorescence film (HP5; Ilford, Knutsford, Cheshire, England).

Figure 1. (*a*) Isolated cytoskeletons of *S. similis*. Negatively stained whole mount preparation. Cells were washed in MT buffer and lysed with Triton X-100 (1–1.5%). (*b*) Negatively stained whole mount preparation of isolated basal apparatuses. Basal apparatuses were obtained from isolated cytoskeletons by homogenization and differential centrifugation. (*c*) Negatively stained, isolated basal apparatus at higher magnification from a preparation such as that shown in *b*. The isolated basal apparatus of *S. similis* consists of the two interconnected basal bodies, the proximal portions of the four microtubular flagellar roots and two striated microtubule-associated fibers (system I fibers) accompanying two of the microtubular roots. (*d–f*) Negatively stained pairs of striated microtubule-associated fibers obtained from isolated



basal apparatuses by extraction with 2 M NaCl and homogenization. Each pair consists of two system I fibers of unequal length (*e* and *f*) and amorphous material at their proximal ends interconnecting the two fibers. In partially disintegrated pairs of system I fibers the connecting material appears to be fibrillar and may represent remnants of the distal connecting fiber (*f*). Bars: (*a* and *b*) 5 μm; (*c*, *e*, and *f*) 0.5 μm; and (*d*) 2 μm.

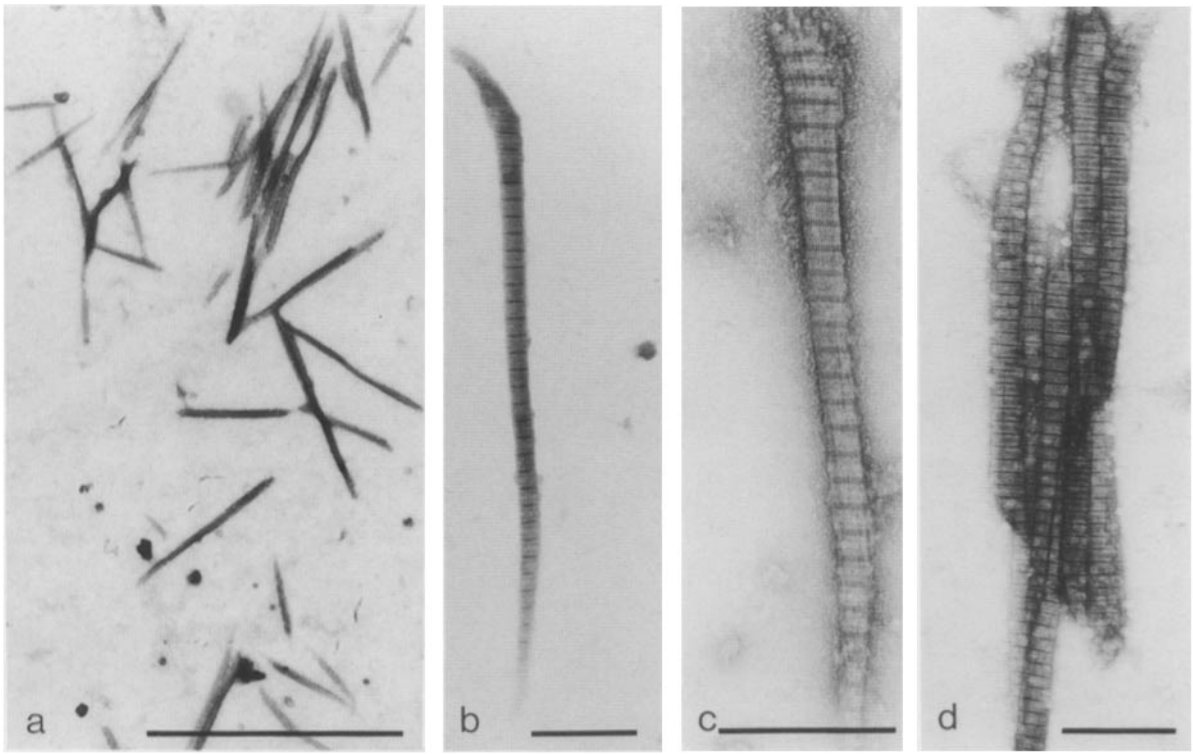


Figure 2. Negatively stained, isolated striated microtubule-associated fibers (system I fibers). (*a*) Overview of a preparation of isolated individual system I fibers. Pairs of system I fibers were treated for 1 h with DNAse and RNAse (0.25 mg/ml each in MT buffer with 2 mM $MgSO_4$, pH 6, 35°C) and then homogenized (80 strokes). (*b*) Negatively stained, single system I fiber after sonication of pairs of system I fibers. The complex striation pattern is visible. (*c*) Negatively stained, single system I fiber isolated as in *b*, but stained with 0.5% phosphotungstic acid for 10 min before staining with uranyl acetate. Following this staining protocol, protofilaments running the length of the fiber are more clearly revealed. (*d*) Laterally aligned, negatively stained, system I fibers isolated as in *a*. The cross-striation pattern of laterally aligned fibers is often in phase. Bars: (*a*) 2 μm; (*b–d*) 0.2 μm.

Immunogold Electron Microscopy

Cells were washed by centrifugation in modified MT buffer, lysed by the addition of an equal volume of 2% Triton X-100 in modified MT buffer, immediately followed by fixation with freshly prepared paraformaldehyde (1% final concentration in modified MT buffer) for 5 min. They were then washed twice by low-speed centrifugation in the presence of the fixative. All subsequent steps were performed as described previously (McFadden et al., 1987). The anti-34-kD IgG was used at a 1:300 final dilution. Samples were finally transferred to agar, dehydrated, embedded, sectioned, and described by McFadden and Melkonian (1986).

In Vivo Phosphate ($^{32}PO_4$) Labeling

Cells were washed three times with phosphate-free culture medium (WEES-P). Carrier-free $^{32}PO_4$ (Amersham-Buchler GmbH, Braunschweig, Germany; 8.9 Ci/mmol) was added (0.1 mCi/ml) and the cells were incubated for 45 min at 22°C in the dark. After incubation cells were washed twice with WEES-P and twice with MT buffer, resuspended in MT buffer and lysed by addition of an equal volume of MT buffer including 2% Triton X-100. The cytoskeletons were pelleted by centrifugation (top speed, microfuge B; Beckman Instruments GmbH, Düsseldorf, Germany) and resuspended in lysis buffer (O'Farrell, 1975), homogenized, and centrifuged to remove undissolved residues. Two-dimensional electrophoresis and electroblotting was preformed as described above. Autoradiographs were recorded on x-ray film (Hyperfilm-βmax; Amersham Buchler GmBH) for 12–48 h at −20°C. Subsequently the blots were immunostained (see above) or stained with AuroDye (Janssen Pharmaceutical Co., Beerse, Belgium).

Amino Acid Analysis

Paracrystals were subjected to SDS-PAGE. After brief staining with Coomassie blue the 34-kD protein was electroeluted with Tris-borate buffer (see above). The protein was dialyzed against distilled water, freeze dried, and destained with 90% acetone, 10% HCl (0.1 M). The pelleted and freeze-dried protein was hydrolyzed in 6 N HCl, 0.05% thioglycolic acid for 20 h at 100°C, and vacuum dried. The hydrolysate was analyzed with a Biotronic Amino acid Analyzer 6001. *o*-phthaldialdehyde (opa) was used as fluorescence marker.

Negative Staining of Whole Mounts

4 μl of suspended particles were applied to pioloform-coated copper grids and allowed to adhere for 2–15 min. The grids were stained with 1% uranyl acetate in distilled water for 90 s–5 min and then observed with an electron microscope (CM 10; Philips Electronic Instruments, Inc., Piscataway, NJ) or an Elmiskop (Siemens Corp., Iselin, NJ).

Results

Isolation of Striated Microtubule-associated Fibers

S. similis is a naked, biflagellate green alga that resembles *C. reinhardtii* in its major ultrastructural features (Preisig and Melkonian, 1984; Melkonian and Preisig, 1984). We have previously shown that detergent treatment of cells yields cytoskeletons, which retain the shape of live cells and may be assumed to have undergone little alteration during the isolation procedure (McFadden et al., 1987; Lechtreck et al., 1989). Thus, reactivated cytoskeletons were shown to perform some motility phenomena of the cell including centrin-mediated basal body reorientation (McFadden et al., 1987).

When isolated cytoskeletons (Fig. 1 *a*) are homogenized,

Table I. Solubility Properties of Striated Microtubule-associated Fibers from S. similis

Compound	Concentration	Solubility
Triton X-100	1.5%	−
Nonidet P-40	1.5%	−
NaCl	2 M	−
KCL	1 M	−
Urea	2 M	+
KI	0.4 M	+
N-Lauroylsarcosine	0.1 M	+
Low ionic strength buffer (10 mM Hepes, 2 mM EDTA, pH 8)	(<10 mM)	+

Isolated basal apparatuses (for treatments with Triton X-100, Nonidet P-40, and NaCl) or pairs of system I fibers (all other treatments) were homogenized in the different compounds in MT buffer (except for treatment in low ionic strength buffer) for 1–3 h (24 h in case of low ionic strength buffer).

axonemes, and basal body–associated microtubules are largely removed and basal apparatuses can be enriched by differential centrifugation (Fig. 1, *b* and *c*). Isolated basal apparatuses consist of the two interconnected basal bodies plus the proximal parts of the four flagellar roots including two striated microtubule–associated fibers (system I fibers, cf., Melkonian, 1980; Fig. 1 *c*). The microtubular components of the basal apparatuses can be dissolved by extraction with sodium chloride (2 M; Fig. 1 *d–f*). The insoluble particles are pairs of system I fibers with remnants of distal connecting fibers at their proximal ends (Fig. 1, *e* and *f*). System I fibers in each isolated pair are of unequal length (about 1.1 and 0.8 μm; $n = 44$). Disintegration of pairs of system I fibers by sonication or extensive homogenization yields single fibers (Fig. 2).

Ultrastructure of Striated Microtubule-associated Fibers

When intact, isolated system I fibers exhibit an overall polarity with a pointed, distal, and a hooked, proximal end (Fig. 2 *b*). They are cross-striated with a constant periodicity of 28 nm ($n = 20$), consisting of a dark gap (~7 nm) and a light zone of 21 nm with a complex interbanding pattern (Fig. 2, *b* and *c*). In some cases a longitudinal perodicity consisting of alternating dark and light lines (center-to-center spacing 3 nm; $n = 12$) is seen to run along the length of the fibers indicating the presence of regularly spaced sheets of 2-nm protofilaments. Similarly spaced sheets of protofilaments are also visible in cross-sections through system I fibers of isolated cytoskeletons (see Fig. 10, *f* and *g*). Isolated system I fibers tend to aggregate, often exhibiting an in-phase cross-striation pattern (Fig. 2 *d*). A detailed structural analysis of the striated microtubule-associated fibers of *S. similis* and a comparison with reconstituted fibers (paracrystals, see below) is presented elsewhere (Patel et al., manuscript submitted for publication).

In Vitro Reassembly of Striated Microtubule-associated Fibers

The system I fibers are stable in high salt buffers (2 M NaCl in MT buffer) but can be solubilized in urea (2 M) or other dissociating agents (1 M KI), and in low salt buffer (<10 mM, pH 8). The solubility properties of system I fibers are summarized in Table I. If urea extracts (2–2.5 M) of system I fiber pairs are dialyzed against reassembly buffer (see Materials and Methods), the solution turns turbid because of the formation of paracrystals visible with EM (Fig. 3). The paracrystals exhibit a regular cross-striation pattern of 28 nm periodicity as in isolated system I fibers (Figs. 3 and 4). They consist of 2-nm protofilaments, which are laterally aligned and extend along the length of the paracrystal (Fig. 4, *b* and

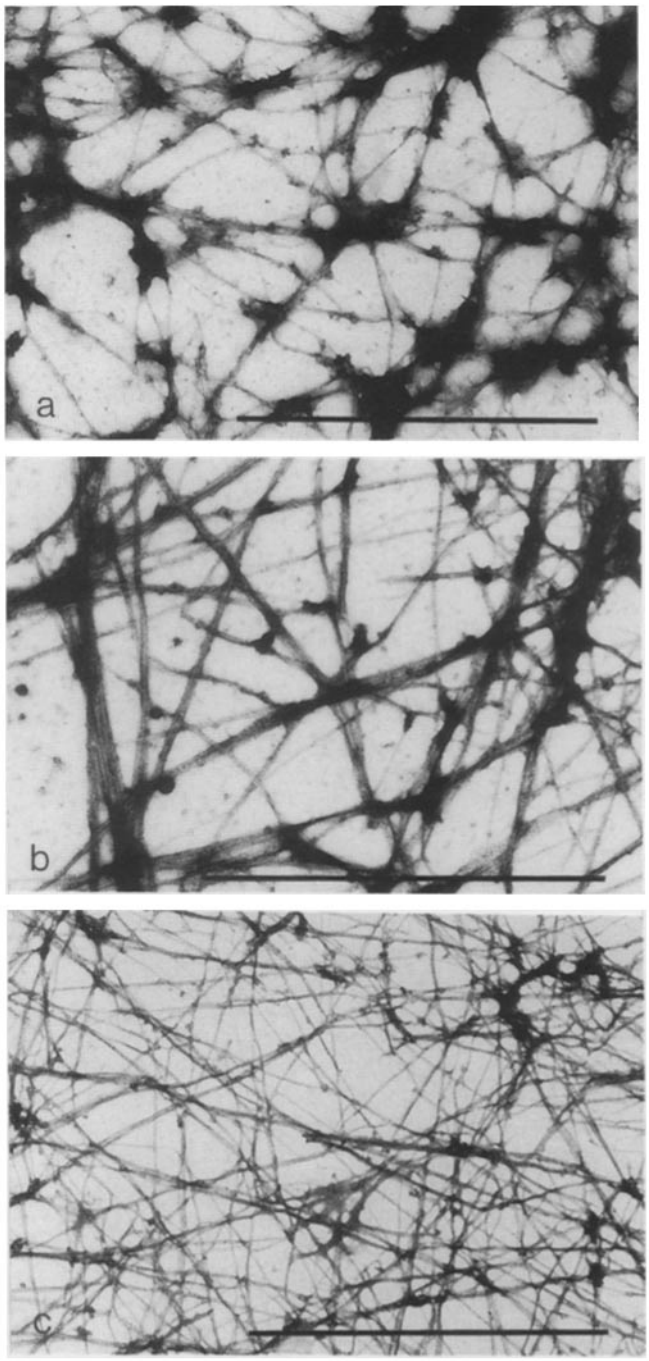


Figure 3. Negatively stained preparations of reassembled paracrystals following one (*a*), two (*b*), or three (*c*) cycles of disassembly/reassembly of isolated pairs of system I fibers from a single preparation. Amorphous material, partially coating the paracrystals, present after the first cycle of disassembly/reassembly (*a*), is progressively lost during subsequent disassembly/reassembly cycles (compare *a* with *c*). Bar, 2 μm.

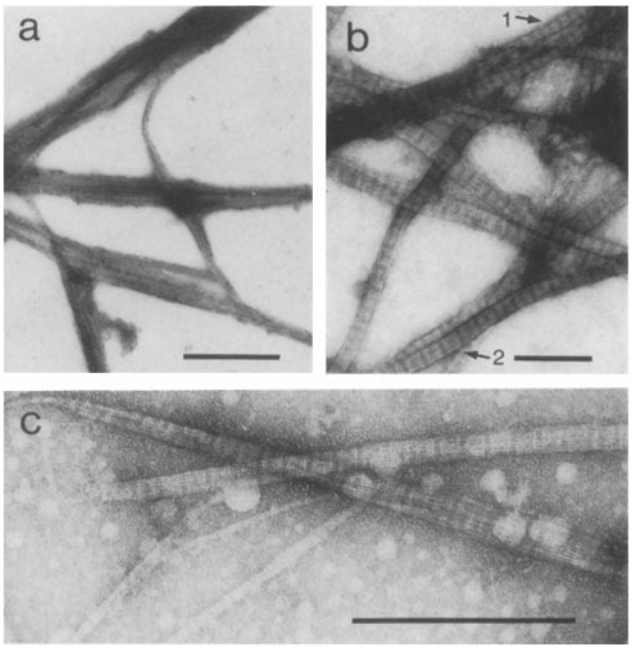


Figure 4. Negatively stained, reassembled paracrystals from system I fibers at higher magnification. (*a*) Paracrystals after one cycle of disassembly/reassembly showing laterally aligned paracrystals with partially in phase cross-striation pattern. (*b*) Paracrystals after two cycles of disassembly/reassembly. In this preparation paracrystals with two types of cross-striation periodicities have been found (15 nm [1] or 30 nm [2]). (*c*) Paracrystals after three cycles of disassembly/reassembly. Paracrystals of variable length and thickness occur and protofilaments can be clearly seen. Bars: (*a*) 0.5 μm; (*b*) 0.1 μm; (*c*) 0.25 μm.

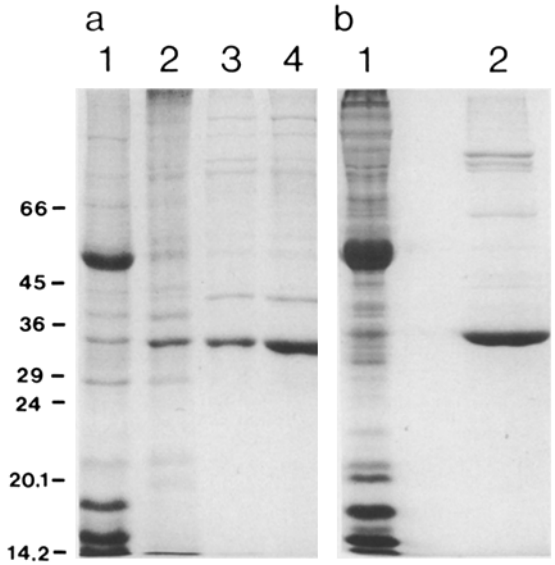


Figure 5. SDS-PAGE analysis of basal apparatuses, isolated system I fibers, and paracrystals from *S. similis*. A 34-kD protein (assemblin) is enriched by NaCl extraction (2 M) of basal apparatuses and several cycles of disassembly/reassembly of paracrystals. (*a*) Lane *1*, isolated basal apparatuses; lane *2*, pairs of isolated system I fibers; lane *3*, paracrystals after one cycle of disassembly/reassembly; lane *4*, paracrystals after two cycles of disassembly/reassembly. Lanes *1–4* are from a single preparation. (*b*) Lane *1*, isolated basal apparatuses; lane *2*, paracrystals after five cycles of disassembly/reassembly. Molecular weight markers indicated at the left are from top to bottom: Bovine albumin (66,000), ovalbumin (45,000), glyceraldehyde-3-phosphate dehydrogenase (36,000), carbonic anhydrase (29,000), trypsinogen (24,000), trypsin inhibitor (20,100), α-lactalbumin (14,200).

c). Reassembled paracrystals can be very long (>10 μm) and then exceed the length of the system I fibers severalfold. Individual paracrystals are pointed at both ends, the width of the paracrystals varies between 40–80 nm (maximum 140-nm width). In our preparations paracrystals often formed lateral aggregates with matching cross-striations (Fig. 4 *a*).

The paracrystals can be solubilized and reassembled several times (up to four times were tested) using the above procedure. Whereas reconstituted paracrystals after the first reassembly are interconnected by conspicuous amorphous material, they gradually loose the amorphous material during subsequent reassemblies (Fig. 3). Paracrystals reassembled more than three times sometimes exhibit only an indistinct cross-striation pattern (not shown).

Protein Composition of Striated Microtubule-associated Fibers

SDS-PAGE of preparations of isolated basal apparatuses (as in Fig. 1 *b*) resolves more than 50 polypeptides with tubulin being the major protein (~35%; based on densitometric analysis of Coomassie-stained gels; Fig. 5, *a* and *b*, lane *1*). Most of the tubulin and several other proteins were extracted from the basal apparatuses by 2 M NaCl. The remaining pairs of system I fibers when analyzed by SDS-PAGE reveal a 34-kD protein (~25% of total protein determined as above) and several minor proteins of mainly higher apparent molecular mass (Fig. 5 *a*, lane *2*). The 34-kD protein is further enriched (~50%; not shown) by solubilization of system I fiber pairs in urea (2–2.5 M) and removal of the insoluble residue (200,000 *g* centrifugation). These data (and corresponding results obtained with other extraction protocols, not shown) indicated that the 34-kD protein could be the principal protein component of system I fibers. We have therefore used the reassembly properties of system I fibers as a means for further enrichment of the protein. Paracrystals (first reassembly) when analyzed by SDS-PAGE consist predominantly of the 34-kD protein (at least 65%; Fig. 5 *a*, lane *3*). Additional extractions of the paracrystals with 1 M NaCl and repeated cycles of disassembly and reassembly (three to five times) yields almost pure (>90%) polymers of the 34-kD protein (Fig. 5 *b*, lane *2* and see Fig. 7). Using the above purification protocol we routinely obtain 20–30 μg of 34-kD protein from about 10^{11} cells (5 mg basal apparatus protein). The concentration of nonassembled 34-kD protein in a reassembly cycle is about 100 nM (3 μg/ml, $n = 4$). Because of its capabilities to form highly ordered paracrystals, we propose that the 34-kD major protein of striated microtubule-associated fibers from green algae (i.e., system I fibers) be called assemblin.

Characterization of a Rabbit Antibody against the 34-kD Protein from Striated Microtubule-associated Fibers

Paracrystals (third-fifth reassembly) were resolved on preparative one-dimensional SDS-PAGE. The 34-kD protein

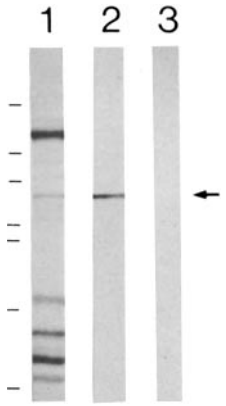


Figure 6. One-dimensional immunoblot analysis of basal apparatus proteins following SDS-PAGE and electrophoretic transfer to a PVDF-membrane. Lane *1*, amido black-stained protein blot of isolated basal apparatuses. Lane *2*, immunoblot of basal apparatuses probed with anti-assemblin IgG (1:1,000) and anti-rabbit IgG peroxidase-conjugate (1:1,000) showing a single immunoreactive band at 34 kD (*arrow*). Lane *3*, immunoblot of basal apparatuses labeled with preimmune serum (1:330) and anti-rabbit IgG peroxidase-conjugate (1:1,000) with no immunoreactive band visible. Molecular weight markers indicated on the left are the same as in Fig. 5.

was cut from the gels, electroeluted, dialyzed, and used to immunize a rabbit. Before immunization an aliquot of the eluate was subjected to SDS-PAGE to verify the purity of the antigen (not shown). Figure 6 shows a one-dimensional immunoblot analysis of basal apparatus proteins from *S. similis* (lane *1*) probed with anti-assemblin IgG (lane *2*) or preimmune serum (lane *3*). The anti-assemblin IgGs specifically stain a single molecular mass band of 34 kD. A two-dimensional immunoblot analysis of whole cytoskeletons again reveals specific labeling of the 34-kD protein which is here resolved into four immunoreactive isoforms (Fig. 8, *a* and *c*).

The 34-kD Protein of Striated Microtubule-associated Fibers Consists of Several Isoforms

When analyzed by two-dimensional electrophoresis (IEF, pH 4.5–6.5, SDS-PAGE) the 34-kD protein from reassembled paracrystals resolves into at least four isoforms (Fig. 7). The major isoform, named alpha-assemblin, has a pI of 5.85. The more acidic isoforms are accordingly termed beta (pI 5.75), gamma (pI 5.55), and delta (pI 5.45). Although the four isoforms were always found in the paracrystal preparations, in some preparations up to five additional isoforms

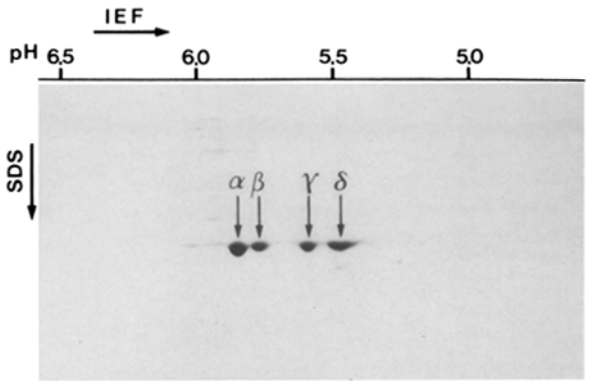


Figure 7. Analysis of paracrystals after three cycles of disassembly/reassembly by two-dimensional gel electrophoresis with isoelectric focusing (2,500 Vh) in the first dimension (*IEF*) and SDS-PAGE (12% polyacrylamide concentration) in the second dimension (*SDS*). After staining with Coomassie Brilliant blue four different acidic isoforms of the 34-kD protein (α, β, γ, δ) were found. 7.5% total ampholines were used in the following ratio: pH 4–6; pH 5–8; pH 3–10 = 1:1.5:1.5.

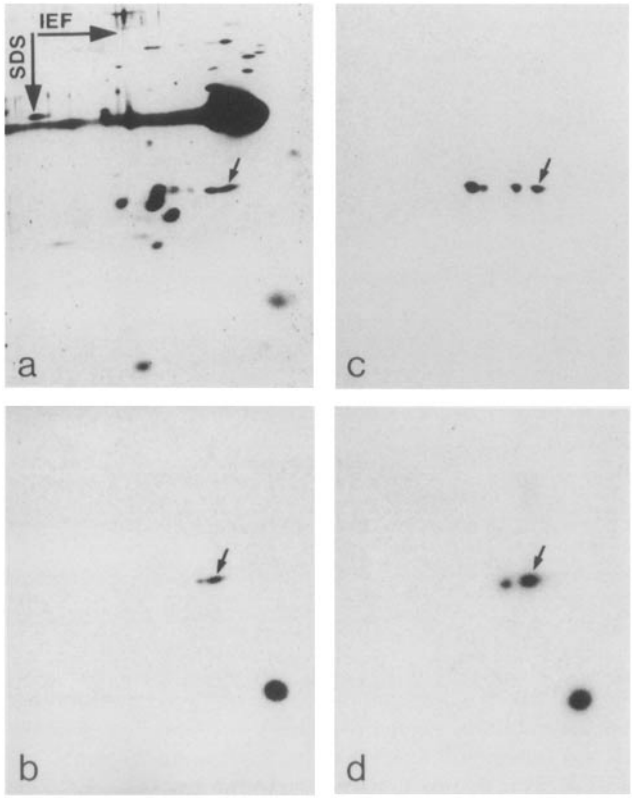


Figure 8. Analysis of in vivo phosphorylation of the 34-kD protein from striated microtubule-associated fibers. Cells of *S. similis* were incubated in $^{32}PO_4$ (0.1 mCi/ml) for 45 min before detergent lysis. Two parallel two-dimensional gels (*a*/*b* and *c*/*d*) of the cytoskeletons were run (2,450 Vh) and the proteins electrophoretically transferred to PVDF-membrane. 7.5% total ampholines were used in the following ratio: pH 4–6; pH 5–8; pH 3–10 = 1:1:1.35. (*a*) Protein blot stained with Aurodye; the gel was overloaded to also reveal minor protein spots. The small arrows in all blots indicate delta assemblin. (*b*) Corresponding autoradiogram to *a* (12-h exposure on x-ray film). (*c*) Immunoblot analysis of cytoskeletons with anti-assemblin IgG and anti-rabbit peroxidase-conjugate (both at 1:1,000 dilution). (*d*) Corresponding autoradiogram to *c* (12-h exposure on x-ray film). Anti-assemblin reacts with all four acidic isoforms of the protein, but with no other protein(s) of the cytoskeleton (compare *a* and *c*). The two autoradiograms (*b* and *d*) show that the two most acidic isoforms of assemblin (gamma and delta) are phosphoproteins, no ^{32}P-label is detected in the two other isoforms (alpha and beta) at this exposure. Because significantly more ^{32}P-label is incorporated into the more acidic delta isoform of assemblin and the gamma and delta isoforms are present in about equal quantities (see *a*), we suggest that delta-assemblin is phosphorylated at multiple sites. The most heavily labeled phosphoprotein in the cytoskeleton of *S. similis* is β-centrin (pI 5.25; *b* and *d*).

(one between beta and gamma assemblin, up to four in an alkaline pH range [>pH 6]) were detected (not shown).

The 34-kD Protein of Striated Microtubule-associated Fibers Is A Phosphoprotein

Cells of *S. similis* rapidly incorporate $^{32}PO_4$ label into at least three cytoskeletal proteins as resolved by two-dimensional electrophoresis and autoradiography (Fig. 8). Two of the phosphorylated proteins correspond to gamma and delta

Table II. Amino Acid Composition of the 34-kD Protein from Striated Microtubule-associated Fibers of S. similis

Amino acid	pmol/sample	Percent
Ala	2950	10.4
Arg	1599	5.6
Asx	3341	11.7
Cys	ND	–
Glx	4871	17.1
Gly	1591	5.6
His	339	1.2
Ile	1854	6.5
Leu	3019	10.6
Lys	1439	5.0
Met	333	1.2
Phe	1044	3.7
Pro	ND	–
Ser	2553	9.0
Thr	1646	5.8
Trp	ND	–
Tyr	172	0.6
Val	1721	6.0

ND, not determined.

assemblin (Fig. 8, compare anti-assemblin IgG immunoblot [*c*] with corresponding autoradiograph [d]). The more acidic delta assemblin is labeled to about a threefold higher intensity than gamma assemblin (Fig. 8 *d*), although both isoforms occur in approximately similar protein quantities (Fig. 7 and 8 *a*). The protein with the highest phosphorylation turnover has a pI of 5.25 and a molecular mass of 20,000, and can be identified as β-centrin. Prolonged exposure of the autoradiographs reveals several additional minor phosphoproteins including weak labeling in the position of alpha and beta assemblin (not shown).

Amino Acid Composition of the 34-kD Protein

The 34-kD protein from paracrystals was purified by SDS-PAGE, electroeluted, and characterized by amino acid analysis (Table II). The results emphasize a high content of leucine and isoleucine and a comparatively low value of glycine. Hydrophobic amino acids constitute 38.3% of the total amino acids. Preliminary amino acid analysis (11 amino acids) of small amounts (<1 μg) of the four isoforms of assemblin (alpha–delta; purified by 2-*D*-electrophoresis) indicate that all isoforms are very similar in amino acid composition (not shown).

Immunolocalization of the 34-kD Protein from Striated Microtubule-associated Fibers

The anti-assemblin IgG was used to stain isolated cytoskeletons or methanol extracted cells of *S. similis* by indirect immunofluorescence. In cytoskeletons of interphase cells the antibody specifically stains a wedge-shaped structure with two branches of unequal length at the anterior end of the cytoskeleton (Fig. 9, *a* and *b*). Double immunofluorescence with anti-α-tubulin and anti-assemblin reveals that the two branches of the wedge-shaped structure converge near the flagellar bases (Fig. 9, *e–g*). In methanol fixed cells a similar pattern of anti-assemblin immunofluorescence is present but the longer branch often extends into a thin filament (Fig. 9, *c*, *d*, and *h*). Preimmune controls exhibited no fluorescence (not shown).

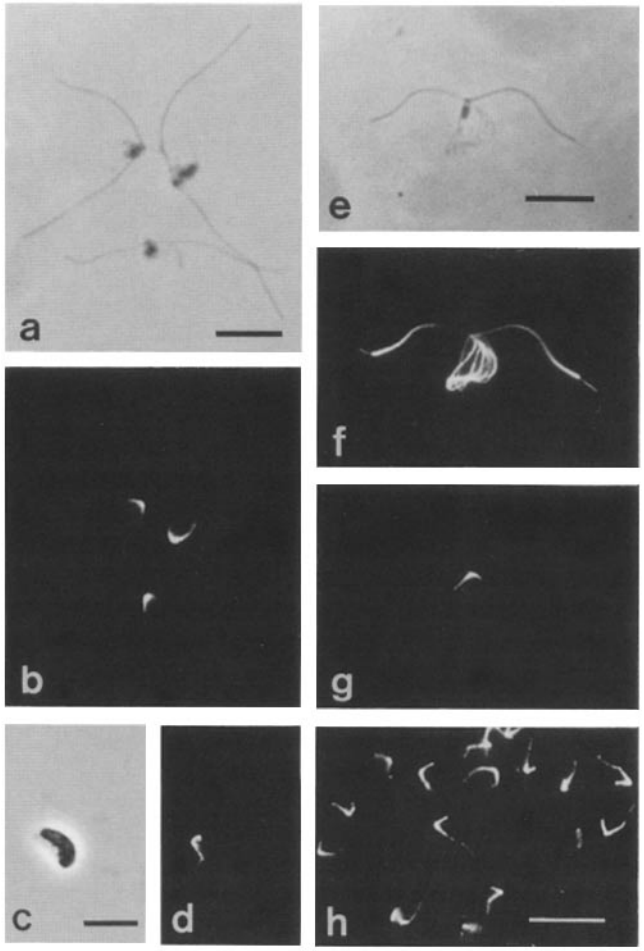


Figure 9. Indirect immunofluorescence of *S. similis*. (*a*) Phase contrast micrograph of three cytoskeletons. Bar, 5 μm. (*b*) Immunofluorescence of cytoskeletons depicted in *a* with anti-assemblin IgG (1:40 dilution) and anti-rabbit IgG-TRITC (1:40 dilution). The antibody specifically stains a wedge-shaped structure near the anterior end of the cytoskeletons. (*c*) Phase contrast micrograph of methanol-permeabilized cell of *S. similis*. Bar, 5 μm. (*d*) Anti-assemblin immunofluorescence of the cell shown in *c* illustrating the wedge-shaped structure with two branches of unequal length. (*e–g*) Indirect double immunofluorescence of a cytoskeleton using anti-assemblin and anti-α-tubulin. (*e*) Phase contrast micrograph of cytoskeleton. Bar, 5 μm. (*f*) Corresponding anti-α-tubulin immunofluorescence (mAb 1.23; 1:250 dilution) of the cytoskeleton shown in *e* using anti-mouse IgG-FITC (1:50 dilution) as secondary antibody. Notice labeling of the axonemes and cytoskeletal microtubules. The proximal parts of the axoneme is always less strongly labeled than the distal part. (*g*) Corresponding anti-assemblin immunofluorescence (1:80 dilution) of the cytoskeleton shown in *e* using anti-rabbit IgG-TRITC (1:80 dilution). The immunofluorescent wedge-shaped structure converges near the flagellar bases. (*h*) Anti-assemblin immunofluorescence (1:40 dilution) of methanol-permeabilized cells of *S. similis*. The two unequal branches of the wedge-shaped fluorescence signal often extend into a thin hairlike appendage. Bars: (*a–h*) 5 μm.

Immunogold EM localization of antigenicity in the cytoskeletons of *S. similis* showed extensive labeling over the entire exposed surface of the system I fibers (Fig. 10, *a*, *c*, *d*, and *f*). Other structures of the cytoskeleton (axoneme, basal body, nuclear skeleton, nucleus-basal body connector, distal

connecting fiber) are not labeled with gold particles, including the microtubules that accompany system I fibers (Fig. 10, *c*, *d*, and *f*). Although, as most clearly seen in cross-sections through system I fibers (Fig. 10, *f*), anti-assemblin labeling occurs exclusively on the surface of the fiber, postembedding immunogold EM using Lowicryl KM4 has demonstrated gold labeling throughout the entire fiber (not shown). In the preimmune controls no specific labeling of the system I fibers or any other parts of the cytoskeleton is seen (Fig. 10, *b*, *e*, and *g*).

Discussion

Biochemical Evidence for Two Types of Basal Body-associated Fibers

We have identified a 34-kD protein as the major component of the noncontractile, striated microtubule-associated fibers (system I fibers) in the green flagellate alga *S. similis*. System I fibers can be reassembled in vitro and repeated cycles of disassembly and reassembly yield paracrystals which are almost pure polymers of the 34-kD protein (assemblin). An antibody raised against the 34-kD protein labels the system I fibers of *S. similis* in immunofluorescence and immunogold EM. In immunofluorescence analyses of different green flagellates (e.g., *Chlamydomonas reinhardtii*, *Dunaliella spp.*, *Polytomella parva*) the anti-assemblin antibody labels structures in the pattern of system I fibers; by immunoblotting a 34-kD protein was detected in *C. reinhardtii* and *P. parva* whereas in the *Dunaliella spp.* a 32-kD protein was immunoreactive (data not shown). The latter result corroborates an earlier protein analysis of basal body-root complexes of *Dunaliella bioculata* which had indicated a 76-, 50-, and a 31-kD protein as predominant components (Marano et al., 1985). We conclude that assemblin is the major protein of the striated microtubule-associated fibers (system I fibers) of green algae.

Based on ultrastructural evidence, two types of basal body–associated fibers have previously been distinguished in flagellate green algae: microtubule-associated striated fibers with narrow cross-striations (<35 nm) also known as system I fibers, and contractile fibers, consisting of a bundle of 4–8-nm filaments, which often are cross-striated with a variable pattern (>80 nm) and known as system II fibers (for review see Melkonian, 1980; Lechtreck and Melkonian, 1991). System II fibers consist mainly of a 20 kD, Ca^{2+}-modulated phosphoprotein, termed centrin (synonym: caltractin; Salisbury et al., 1984; Salisbury et al., 1988; Huang et al., 1988*a*). Centrin or centrin-homologues have been localized in several basal body/centrosome-associated structures and are suggested to be (*a*) involved in many rapid motility mechanisms in protists, and (*b*) a universal component of the basal apparatus/centrosomal complex in eukaryotic cells (for reviews see Salisbury, 1989*a*,*b*; Melkonian, 1989; Melkonian et al., 1991). An antiserum raised against centrin from the system II fibers of the green flagellate *Tetraselmis striata* (Salisbury et al., 1984) labels the system II fibers and other basal body–associated structures (connecting fibers, flagellar transition region) in several green algae (Wright et al., 1985; McFadden et al., 1987; Schulze et al., 1987; Salisbury et al., 1987; Sanders and Salisbury, 1989) but not the system I fibers (Melkonian et al., 1988). With the identification of assemblin, a protein distinct from centrin in various biochemical properties, the existence of two principal types of basal body–associated fibers first proposed on structural grounds is now verified by biochemical evidence.

Noncontractile Striated Fibers: Relationship to System I Fibers

Eukaryotic flagella/cilia are nearly always anchored in the cell by striated fibers (Pitelka, 1969). They occur attached to the sensory cilia and in ciliated epithelia of multicellular organisms as well as in flagellate/ciliate protists. Some of these structures are of the contractile centrin type (see above). Others are apparently noncontractile and ultrastructurally resemble the system I fibers of green algae (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), and the striated rootlets of the molluscan-ciliated gill epithelium (Stephens, 1975). Unfortunately neither pure rootlet preparations nor in vitro reassembly of rootlet fibers was previously achieved. In consequence the proposed protein composition of the rootlet fibers (even when the same genus was used, see Rubin and Cunningham, 1973; Dingle and Larson, 1981; Hyams and King, 1985) varied considerably. The monospecific antibody raised against the major protein of system I fibers of *S. similis* (this study) may now be used to investigate the immunological relationships between noncontractile flagellar roots in diverse eukaryotic systems.

Microtubule-associated 2-nm Filaments and Intermediate Filaments Are Structurally Related

In the cytoskeleton of *Giardia lamblia* (Diplomonadida), an enteric parasite of the alimentary tracts of vertebrates, microtubules are associated with striated fibers forming the microribbons (Holberton and Ward, 1981). The striated fibers of *G. lamblia* consist of bundles of 2-nm filaments (Holberton, 1981) which can be reassembled in vitro and share similar solubility properties with the system I fibers (Crossley and Holberton, 1983*a*, 1985). Interestingly, a group of 29–38-kD proteins (the giardins) were found to be the major constituents of the striated fibers of *G. lamblia* (Crossley and Holberton, 1983*b*; Peattie et al. 1989). Two of the giardins (α,β) have been sequenced and shown to have a high α-helix content (Baker et al., 1988; Peattie et al., 1989; Aggarwal and Nash, 1989). The β-giardin sequence is suggestive of a continuous α-helical coiled-coil structure with a short nonhelical NH_2-terminal domain relating this protein to the k-m-e-f class of fibrous proteins (Holberton et al., 1988). The ultrastructural characteristics of system I fibers and paracrystals (this paper) indicate that the molecule is rod shaped. NH_2-terminal microsequencing of assemblin and assemblin fragments obtained by proteolytic digestion revealed that (*a*) six of seven detected prolines occur in the NH_2-terminal portion of the molecule and (*b*) the internal fragments obtained so far contain a high ratio of charged/apolar residues and several heptad motifs. The longest fragment when aligned to the β-giardin sequence exhibited considerable sequence similarity (unpublished data). We propose that assemblin and β-giardin are related proteins and

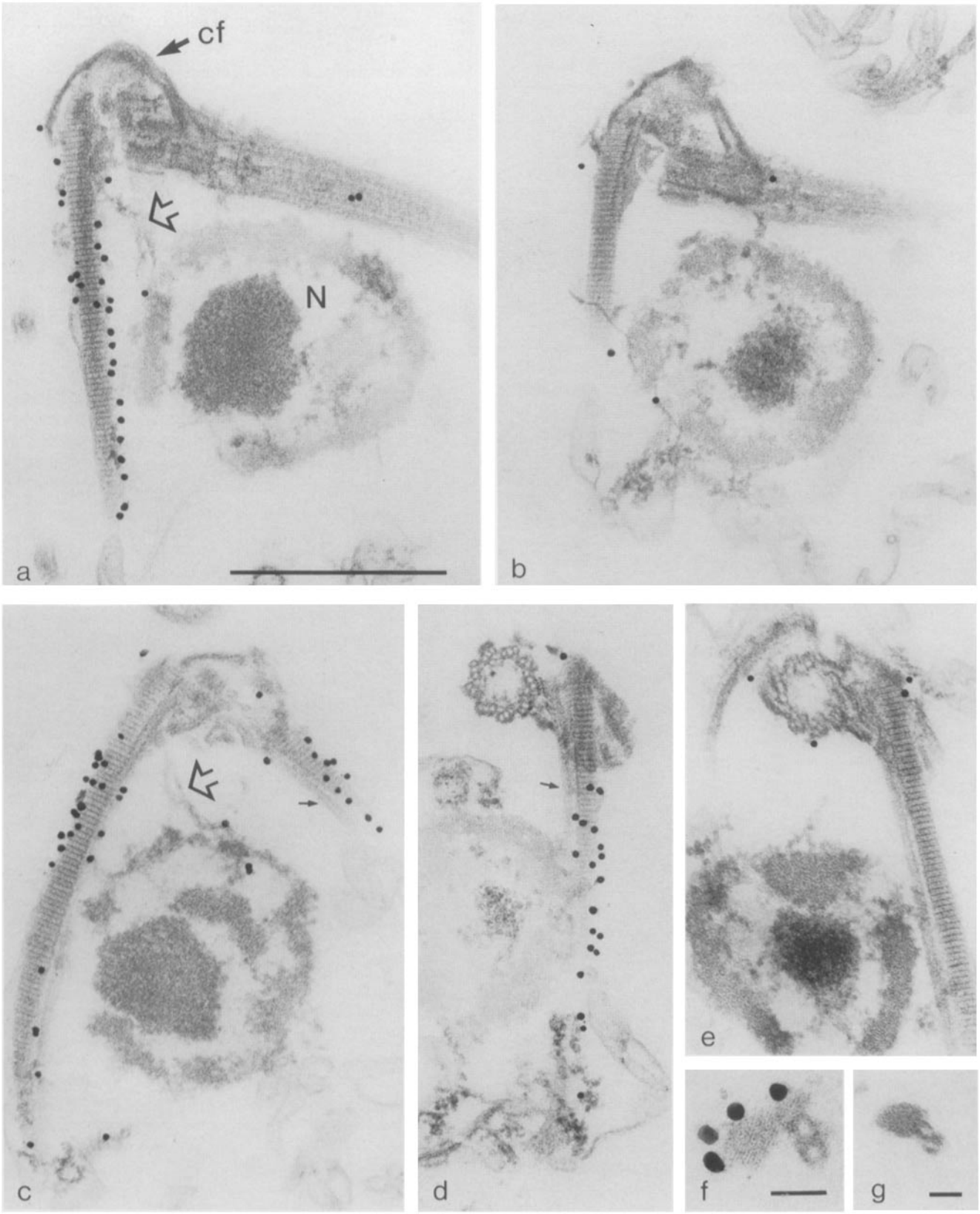


Figure 10. Ultrastructural localization of assemblin in *S. similis* by immunogold labeling of isolated cytoskeletons using the preembedding technique. (*a*, *c*, and *d*) Cytoskeletons treated with anti-assemblin IgG (1:300 dilution); (*b*, *e*, and *g*), cytoskeletons treated with preimmune serum (1:100 dilution). (*a* and *b*), Longitudinal sections through a system I fiber, the exposed surface of the system I fiber in *a* is immunoreactive, no immunogold labeling occurs on the surface of the system I fiber in the preimmune control (*b*). No other components of the cytoskeleton are immunoreactive with the anti-assemblin antibody (*N* = nuclear skeleton; *cf* = centrin-containing distal connecting fiber; *open arrow*, centrin containing nucleus-basal body connector, basal body). (*c*) A longitudinal section through parts of both system I fibers of

may belong to a protein family which forms cross-striated microtubule-associated bundles of 2-nm filaments. Tektins, proteins isolated from fine filaments associated with the axonemes of sea urchin sperm flagella (Mg 47–55 kD; Linck and Langevin, 1982), share several properties with the giardins and assemblin. The tektin filaments have a diameter of 2–3 nm and contain a high percentage of α-helical structure. The high α-helical content of giardin and tektin filaments is similar to that found in intermediate filaments. mAbs specific for tektins cross react with structures and subunits of intermediate filaments and the nuclear lamina suggesting that the two types of filaments are homologous in several parts of their structure (Chang and Piperno, 1987; Steffen and Linck, 1989). Although our anti-assemblin antibody did not cross react with selected intermediate filament proteins (vimentin, desmin; unpublished results), assemblin filaments and intermediate filaments can be enriched and reconstituted using similar protocols, indicating some structural similarity between the constituent proteins. This conclusion is supported by experiments demonstrating that the rod portion of glial fibrillary acidic protein, when expressed in vitro, forms cross striated paracrystals with a gap-overlap structure (Stewart et al., 1989). Proteolytic digestion of COOH- and NH_2-terminal domains or treatment with phosphate dissociates intermediate filaments into 2-nm subfilaments (Geisler et al., 1982; Aebi et al., 1983). We conclude that the relationship between the assemblin/giardin/tektin-filaments and intermediate filaments is based on structural similarities in the rod portions of the molecules.

Phosphorylation of Assemblin: Functional Significance

The principal proteins of microtubule-associated 2-nm filaments (giardins, tektins, assemblin) consist of several isoelectric variants. Our results show that the two most acidic isoforms of assemblin are phosphorylated. The observed different labeling intensity of the two isoforms could be related to differences in phosphate turnover or more likely to multiple phosphorylation. Phosphorylation of assemblin occurs constitutive in interphase cells. It may regulate the length and/or thickness of system I fibers by phosphorylation-dependent assembly/disassembly of subunits. The stability of intermediate filaments in vivo and in vitro is known to be regulated by phosphorylation/dephosphorylation events (Inagaki et al., 1987; Geisler and Weber, 1988; Peter et al., 1990). A more speculative function of phosphorylation/dephosphorylation could be an effect on the stiffness and tensile strength of the system I fibers. In this context we note that the elasticity of paracrystals reconstituted from the microtubule-associated protein tau changes in relation to the state of phosphorylation of the molecule (Hagestedt et al., 1989).

Functional Aspects of Striated Microtubule-associated Fibers in Green Algae

The function of striated microtubule–associated fibers of green algae is generally unknown (Goodenough and Weiss, 1978). The fibers have been implicated to serve an anchoring function for the flagella and to help absorb the mechanical stress generated by flagellar beating (Hyams and Borisy, 1975; Sleigh and Silvester, 1983). In accordance with this view we find that system I fibers are well developed and relatively long in the naked cell wall-less green flagellates *S. similis* (this study), *Dunaliella spp.*, and *Polytomella parva* in comparison to the much less developed and shorter system I fibers of *C. reinhardtii* (unpublished observations).

The complex consisting of 2-nm filaments and microtubules may universally serve as a cytoskeletal element of high stability and tensile strength in eukaryotic cells.

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a cytoskeleton treated with anti-assemblin IgG. Whereas both fibers are immunoreactive again no other components of the cytoskeleton react with the antibody. (*Open arrow*) Thin nucleus-basal body connector; (*small arrow*) root microtubule accompanying system I fiber. (*d* and *e*), Cross-sections through one basal body of a cytoskeleton with associated longitudinally sectioned system I fiber. Note that no structures directly attached to the basal body triplets are immunoreactive. (*Small arrow*) Root microtubule running parallel to system I fiber. (*f* and *g*), cross-sections through system I fibers and accompanying root microtubules. In *f* the surface labeling of the system I fiber by gold particles is especially evident. The cross sections show that system I fibers are constructed of several parallel sheets of electron-dense material (*f*). Bars: (*a–e*) 0.5 μm; (*f* and *g*) 50 nm.

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