Ciliary Microtubule Capping Structures Contain A Mammalian Kinetochore Antigen

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Abstract. Structures that cap the plus ends of microtubules may be involved in the regulation of their assembly and disassembly. Growing and disassembling microtubules in the mitotic apparatus are capped by kinetochores and ciliary and flagellar microtubules are capped by the central microtubule cap and distal filaments. To compare the ciliary caps with kinetochores, isolated *Tetrahymena* cilia were stained with CREST (Calcinosis/phenomenom esophageal dysmotility, sclerodactyly, telangiectasia) antisera known to stain kinetochores. Immunofluorescence microscopy revealed that a CREST antiserum stained the distal tips of cilia that contained capping structures but did not stain axonemes that lacked capping structures. Both Coomassie blue–stained gels and Western blots probed with CREST antiserum revealed that a 97-kD antigen copurifies with the capping structures. Affinity-purified antibodies to the 97-kD ciliary protein stained the tips of cap-containing *Tetrahymena* cilia and the kinetochores in HeLa, Chinese hamster ovary, and Indian muntjak cells. These results suggest that at least one polypeptide found in the kinetochore is present in ciliary microtubule capping structures and that there may be a structural and/or functional homology between these structures that cap the plus ends of microtubules.

T HE distal tips of cilia and flagella are bound to microtubule capping structures, or caps, that link the ends of the central pair and doublet microtubules to the ciliary membrane (Dentler, 1980, 1987; Dentler and LeCluyse, 1982a, 1982b). In *Tetrahymena* and *Chlamydomonas*, there are two morphologically distinguishable caps, the distal filament plugs that link the A-tubules to the membrane, and the central microtubule cap that links the central microtubules to the membrane (Dentler and Rosenbaum, 1977; Dentler, 1980). Each of these capping structures are linked to the microtubules by a small plug inserted into the lumen of each A- and central microtubule (Dentler, 1980). The capping structures are fully assembled during the initial stages of ciliogenesis and are tightly attached to the distal "+" ends of the microtubules throughout ciliary and flagellar growth (Dentler, 1980; Portman et al., 1987). The capping structures also block the assembly of tubulin onto ciliary microtubules in vitro (Dentler and Rosenbaum, 1977; Dentler and LeCluyse, 1982a). Since the caps are firmly bound to the sites of microtubule assembly (and, in some flagella, the sites of microtubule disassembly), they may have a role in the regulation of ciliary and flagellar microtubule assembly (Dentler, 1987; 1989).

One approach to studying the capping structures and characterizing the proteins that link them to the microtubules is to purify them from the axonemes. As a first step, methods were developed to release the capping structures from ciliary axonemes with a minimum of axonemal disruption. Purified

Tetrahymena thermophila axonemes were extracted with 75 mM $MgCl_2$, which releases the distal filament plugs and the central microtubule caps (Suprenant and Dentler, 1988). The intact axonemes were separated from the released capping structures by centrifugation, leaving cap-free axonemes that were pelleted and capping structures, with some membranes and microtubule fragments, in the supernatant. However, further purification of the capping structures was not accomplished because of the lability of the structures and the lack of a known polypeptide to purify.

An alternative approach to study the caps is to obtain antibodies against capping proteins and use these antibodies as probes for subsequent characterization. We have shown that the capping structures are similar to the kinetochores bound to mammalian and avian chromosomes in that they both bind to the plus ends of assembling and disassembling microtubules in vivo. Some of the most commonly used probes for kinetochore proteins are autoantibodies obtained from human patients with the CREST (Calcinosis/Raynaud's phenomenon/Esophageal dysmotility/Sclerodactyly/Telangiectasis)[1] variant of scleroderma; these patients contain antibodies that bind antigens found in kinetochores of a variety of cell types (Brenner et al., 1981; Moroi et al., 1980; Cox et al., 1983; Earnshaw et al., 1984; Valdiva and Brinkley, 1985; Kingwell and Rattner, 1987). Because of the similarities between ki-

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1. *Abbreviation used in this paper:* CREST, calcinosis/Raynaud's phenomenon/esophageal dysmobility/sclerodactyly/telangiectasis.

netochores and the ciliary microtubule capping structures, the ability of CREST antisera to recognize antigens in the ciliary caps was examined. The results shown here reveal that CREST antisera recognize a 97-kD antigen in fractions containing ciliary capping structures but not in axonemes from which the caps are removed, and the affinity-purified 97-kD antibodies stain the distal tips of isolated *Tetrahymena* cilia that contain capping structures. Moreover, antibodies affinity-purified from CREST sera against the 97-kD *Tetrahymena* ciliary capping protein stain kinetochores in cultured mammalian cells. These results provide the first identification of a polypeptide associated with the capping structures and suggest that proteins associated with the ciliary microtubule capping structures are related to those found in kinetochores.

Materials and Methods

Isolation and Fractionation of Cilia

Tetrahymena thermophila, Strain SB-711, were grown to mid-late log phase in 2% proteose peptone. Cells were harvested, washed in proteose peptone, and deciliated with 1 mg/ml dibucaine. Cilia were purified as described by Suprenant and Dentler (1988) but were washed in TMESDP (25 mM Tris-HCl, pH 7.4, 3 mM $MgSO_4$, 0.1 mM EGTA, 250 mM sucrose, 1 mM DTT, 0.3 mM PMSF). Cilia were demembranated by suspending them in TMESDP, adding Triton X-100 to 0.5% and incubating the suspension for 20 min at 4°C. Axonemes were sedimented at 17,400 g at 4°C and then resuspended in TMESDP to a final concentration of 1 mg/ml. The capping structures were released from the axonemes by adding 1 M $MgCl_2$ to a final concentration of 75 mM, incubating for 20 min, 4°C, and then sedimenting the axonemes at 17,400 g, 20 min, 4°C. The pellets were suspended in TMESDP and pellets and supernatants were examined by negative stain EM to insure that the supernatant contained capping structures while the axoneme-containing pellets lacked them. For most experiments, the 17,400 g supernatant was centrifuged at 48,400 g, 30 min, 4°C, to sediment membrane vesicles and fragments of central pair microtubules. The capping structures remained in the supernatant.

Light Microscopy

For immunofluorescence microscopy, a drop of purified cilia was placed on a glass coverslip and fixed in 3.7% formaldehyde in PBSA (10.6 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 137 mM NaCl, 2.68 mM KCl, 3% BSA [Fraction V; Sigma Chemical Company, St. Louis], pH 7.4) for 20 min at room temperature. Cilia were permeabilized by washing the coverslips containing cilia in PBSA and treating with 0.1% Triton X-100 in PBSA for 90 s. Coverslips were then washed to remove the detergent, incubated in CREST antisera (generous gifts from Dr. Bill Brinkley and Dr. William Earnshaw) at 1:5,000 dilutions in PBSA for 2 h at room temperature, washed in PBS, incubated in FITC goat anti-human IgG (Zymed Laboratories, South San Francisco, CA) for 1 h room temperature, washed in PBS, and mounted on glass slides in antibleaching solution (1 mg/ml p-phenylenediamine in PBS and glycerol, pH 8.0 [Johnson et al., 1981]). Cilia were viewed with a microscope equipped with an epifluorescence unit containing a 450–490-nm excitation filter and 520–560-nm barrier filter. Most samples were photographed using a 63 × 1.4 NA objective lens, 10× projection eyepiece, and an SIT camera (DAGE-mti, Michigan City, IN). The image was frame-averaged with an Image Sigma digital image processor (Hitschfel Optical Instruments, Inc., St. Louis, MO) and photographed from a monitor (WV 5370; Panasonic) with a 35-mm camera and 400 ASA film (T-MAX; Eastman Kodak Co., Rochester, NY), which was developed in T-Max developer (Eastman Kodak Co.).

Cultured HeLa cells were grown on coverslips in DME containing 10% FCS, fixed by immersion in −20°C methanol for 6–8 min and rinsed in PBS containing 0.1% Triton X-100. Coverslips containing the cells were incubated in primary antibody for 60 min at 37°C in a humid chamber. Coverslips were rinsed for 10 min in PBS and incubated in FITC labeled anti-human antibody (Boehringer-Mannheim, diluted 1:20 in PBS) for 40 min. Coverslips were then rinsed and mounted in PBS:glycerol containing 25 μg/ml Hoechst 33258. Photographs were taken on T-Max 400 ASA film and developed with an effective ASA of 1,600 using T-Max developer.

Electrophoresis and Affinity Purification of Antibodies

Separation of polypeptides on one and two dimensional gels was carried out essentially as described in Suprenant et al. (1985). To concentrate the relatively dilute capping structures, polypeptides in the $MgCl_2$ supernatant were precipitated with 10% perchloric acid. For comparison, cap-free axonemes were also acid-precipitated. Acid precipitates were rinsed with water before suspension in electrophoresis sample buffer. To insure that acid precipitation did not select for any polypeptides, acid-precipitated samples were compared with samples concentrated by filtration (PM 10, Amicon Corp., Danvers, MA). Samples concentrated with each method had identical polypeptide composition but the acid-precipitated samples were more consistent from preparation to preparation, presumably because of less degradation by proteolysis.

SDS-gels were composed of 4–12% gradients of acrylamide buffered at pH 8.3. Isoelectric focusing gels were prepared as described by Suprenant et al. (1985). For comparison of different antibodies, single well gels were run and blots were cut into narrow strips for staining or testing with antibodies. After running, gels were briefly soaked in 49.6 mM Tris, 384 mM glycine, 20% (vol/vol) methanol, 0.1% (wt/vol) SDS (Pierce Chemical Co., Rockland, IL) (King et al., 1986) and blotted onto nitrocellulose sheets (Schleicher & Schuell, Inc., Keene, NH) in the same buffer for 60 min, 0.9 A in a blotting apparatus (Idea Scientific, Corvallis, OR).

For detection of antigens, nitrocellulose was blocked with blocking buffer (5% Carnation dry milk, 0.05% Tween 20, 0.15 M NaCl, 10 mM Tris-HCl, pH 7.25) for 20 min with one change. Blots were incubated in CREST antisera diluted 1:5,000 in blocking buffer for 1–2 h at room temperature. Blots were washed four times with blocking buffer and incubated in peroxidase-labeled goat anti-human IgG (Cappel Organon Teknika, West Chester, PA) for 1 h. Blots were then washed with 50 mM Tris-HCl, pH 6.9, and developed as described by King et al. (1986). Blots were photographed on Technical Pan Film (Eastman Kodak Co.) with a yellow filter and developed in HC-110 developer (Eastman Kodak Co.). Affinity purification of the SH-CREST antibodies from blots was carried out using the procedure of Smith and Fisher (1984).

Results

Isolation and Analysis of Capping Structure Proteins

Tetrahymena axonemes contain two types of capping structures, the distal filaments, attached to the distal tips of each A-tubule, and the central microtubule cap, attached to the distal tips of the central pair microtubules (Fig. 1 *A*). These capping structures can be released if the axonemes are extracted for 10–20 min with 50–75 mM $MgCl_2$, and the capping structures can be separated from the axonemes by centrifugation (Suprenant and Dentler, 1988). The pelleted axonemes are free of capping structures (Fig. 1 *B*) and distal filaments and central microtubule caps are found in the supernatant (Fig. 1, *C* and *D*). In addition to the capping structures, the supernatants contain pieces of axonemal microtubules as well as fragments of the ciliary membrane. Further purification of the capping structures has not been accomplished because of the lability of the structures once separated from the microtubules.

When the polypeptides in the $MgCl_2$ extracted axonemes and cap-containing supernatant are separated by SDS-PAGE, the cap fraction can be seen to contain many axonemal proteins (Fig. 2), as predicted by electron microscopy (see Suprenant and Dentler, 1988). The amount of protein composing the relatively small capping structures is easily lost among a few fragments of axonemes and detergent-resistant membrane vesicles. Although many microtubule proteins, including tubulin and dynein, are found in the cap fraction, there are several bands that consistently appear enriched when compared to the axonemal fraction. Since the axoneme fraction lacks the capping structures and the $MgCl_2$ extract (supernatant) contains them, an initial search for capping
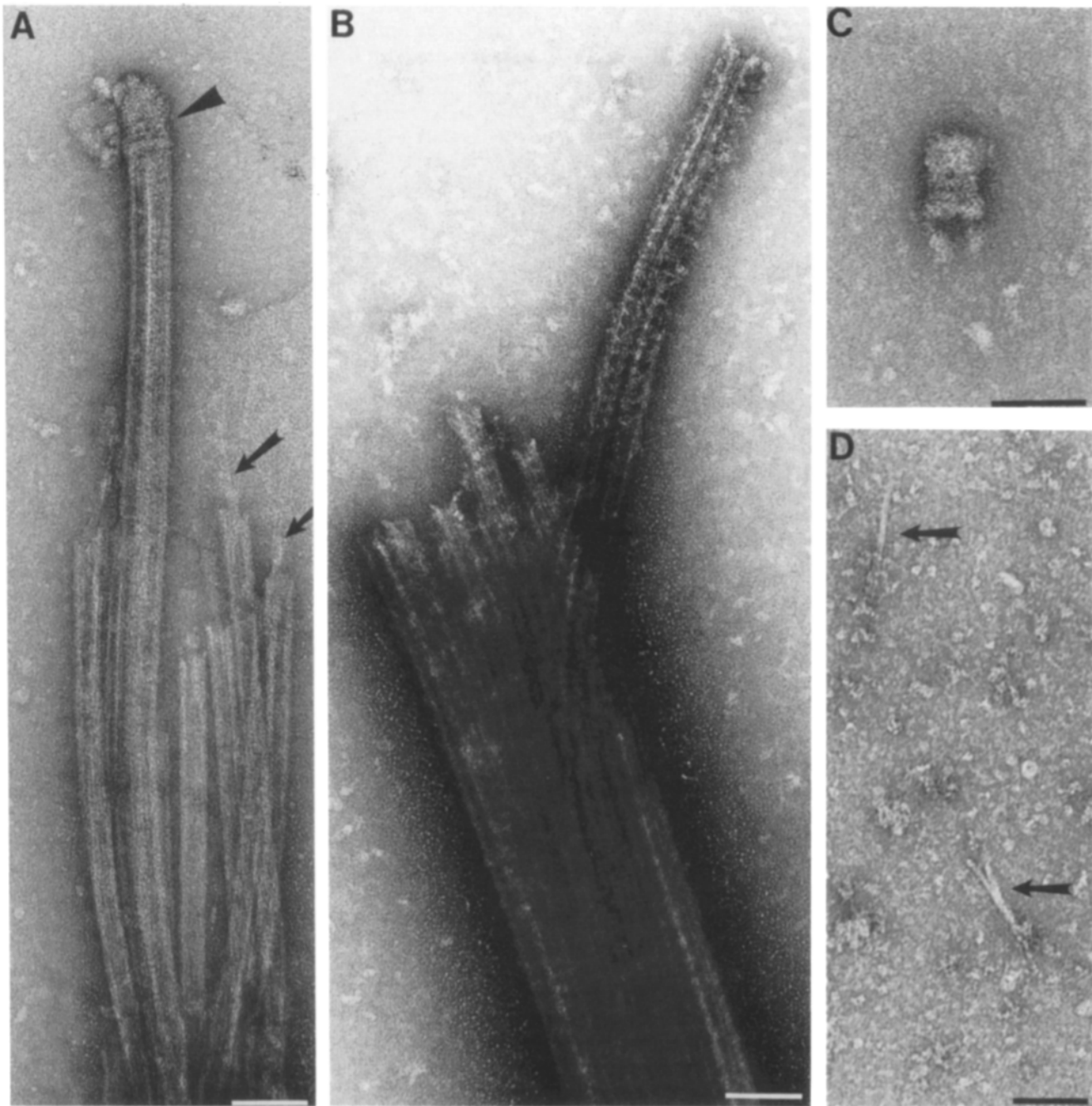
Figure 1. Release of capping structures from axonemes by MgCl₂ extraction. When whole cilia (*A*) are incubated in 75 mM MgCl₂, both the central pair caps (*arrowhead*) and the distal filaments (*arrows*) are released from the microtubules. Upon centrifugation, intact cap-free axonemes are found in the pellet as shown in *B* and the caps and distal filaments are found in the supernatant (*C* and *D*). Bar, 0.1 μm.

structure proteins was made by examining gels for polypeptides present in the supernatant but not in the cap-free axonemal pellet.

A prominent 97-kD polypeptide appears in the cap fraction in virtually all preparations and has no corresponding band in the axonemal fraction (Fig. 2). Although other bands are enriched in the cap fraction, the 97-kD band appears consistently in all fractions examined by one and two dimensional electrophoresis. The 97-kD polypeptide migrates with a pI of ∼7.4 and has no counterpart in the cap-free axoneme fraction (Fig. 3, *A* and *C*). Since the capping structures are

released by MgCl₂ extraction, as shown by EM, these polypeptides enriched in the cap fraction are likely candidates to comprise part of the capping structures.

CREST Antigens Are Associated with the Capping Structures

To identify CREST antigens in ciliary fractions, axonemal polypeptides were separated by one-dimensional SDS-PAGE and Western blots stained with the SH-CREST antiserum. This antiserum stained a 97-kD polypeptide and, occasion-
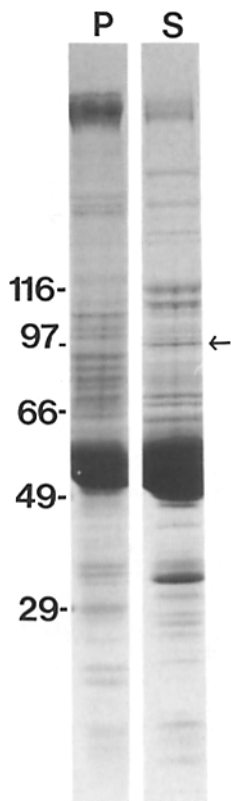
P   S

116-
97.   ←
66-
49-
29-

Figure 2. Analysis of the protein composition of the MgCl₂ cap fraction (*S*) and the cap-free axonemal fraction (*P*) by SDS-PAGE. Although many microtubule proteins, including tubulin and dynein, are found in the cap fraction, there are several bands that are consistently enriched when compared to the axonemal fraction. The prominent 97-kD band (*arrow*) is one band that reliably appears in the cap fraction but has no corresponding band in the axonemal fraction.

ally, a few lower molecular mass polypeptides. To identify polypeptides that copurify with the capping structures, axonemes were extracted with MgCl₂ and centrifuged to separate cap-free axonemes from the capping structures found in the supernatant. Western blots stained with the antiserum revealed that the 97-kD antigen fractionates with the capping structures and is not present in the cap-free axonemes (Fig. 4). Similar results were found when the polypeptides were separated by two-dimensional electrophoresis (Fig. 3): the 97-kD polypeptide was the only polypeptide stained in the cap-containing supernatant and was absent in the cap-free axonemal pellet (Fig. 3, *B* and *D*). A 34-kD polypeptide in the axoneme (Fig. 3 *D*) or in the capping structure fraction (Fig. 4, MgS) was occasionally stained with SH-CREST fraction but, unlike the 97-kD polypeptide, the 34-kD polypeptide did not appear in all preparations of axonemes or capping structures. Additionally, a few low molecular mass polypeptides were stained in axoneme and capping structure fractions but these bands were also stained in controls run with the secondary antibody but not with SH-CREST.

These results suggested that the SH-CREST antiserum may stain polypeptides associated with the capping structures. To test this possibility, whole cilia and MgCl₂-extracted (cap-free) cilia were incubated in the CREST antiserum and examined by immunofluorescence microscopy to determine if the CREST stained the ciliary tips. Initial studies revealed that the distal tips of isolated cilia were brightly stained with the SH-CREST (not shown), which supported the suggestion that CREST antiserum recognized ciliary tip proteins.

Affinity-purified Antibodies against the Ciliary 97-kD Polypeptide Stain the Distal Tip of Tetrahymena Cilia and Mammalian Kinetochores

To determine if the 97-kD polypeptides stained on immunoblots are present at the ciliary tip, the antibodies to the 97-kD polypeptides were affinity-purified from nitrocellulose blots and were used for immunofluorescence microscopy. The affinity-purified antibodies were tested by immunoblotting as shown in Fig. 4 (lanes *3* and *3'*) and found to bind exclusively to the 97-kD protein in the cap fraction and to no proteins in the axonemal fraction. The 34-kD polypeptide that occasionally stained in the capping or axonemal fraction was not stained with the affinity-purified antibody. To insure that the antibodies to the 97-kD polypeptide were not directed against carbohydrates, which could be collected near the membrane-binding regions of the capping structures, Western blots of glycosylated membrane proteins of both *Tetrahymena* cilia and *Chlamydomonas* flagella were stained with the affinity-purified 97-kD antibodies. None of the membrane proteins were stained with the antisera (data not shown).

Immunofluorescence staining of *Tetrahymena* cilia was carried out using the affinity-purified 97-kD antibodies. The affinity-purified antibodies stained the distal tips of whole cilia (Fig. 5, *A–F*) but did not stain the cap-free MgCl₂-extracted axonemes (Fig. 5, *G* and *H*). The distal tips of the cilia were distinguished from the proximal end because they tend to be slightly splayed or often tapered, as the central pair extends outward from the doublets. In many cilia, the central pair microtubules are visible and the fluorescently labeled central pair cap is separated from the stained caps at the ends of the A-microtubules (Fig. 5 *B*). The majority of the axoneme (*small arrow*) is stained at a background level as are the cap-free axonemes (*open arrows*), and cilia stained only with the secondary antibody (Fig. 5 *I*). To insure that the lack of staining was not because of the inaccessibility of the antibody to the microtubules, cilia were splayed by the addition of ATP, which separated doublet microtubules, and were stained with the CREST antiserum. Under these conditions, no fluorescence was observed along the microtubules (data not shown). These results confirmed that the 97-kD polypeptides stained on immunoblots are localized at the distal tips of *Tetrahymena* cilia and that they are released from the axonemes under the same conditions used to release the microtubule capping structures.

To determine if the ciliary 97-kD polypeptide is antigenically related to kinetochore-associated polypeptides, the affinity-purified 97-kD antibodies were applied to HeLa cells fixed at various times during the cell cycle. The 97-kD antibodies stained the kinetochore regions of interphase and mitotic cells (Fig. 6). Similar results were obtained with Chinese hamster ovary (CHO) and Indian muntjak cells (data not shown). Serum from a diffuse scleroderma patient that does not exhibit the CREST scleroderma syndrome was used as a control for immunofluorescence staining, since this patient's serum does not stain kinetochores. These results clearly show that the ciliary 97-kD polypeptides are antigenically related to the kinetochore proteins. However, the kinetochore-associated antigen recognized by the 97-kD antibodies is not known because blots containing kinetochore proteins were not stained by the affinity-purified 97-kD antibodies.
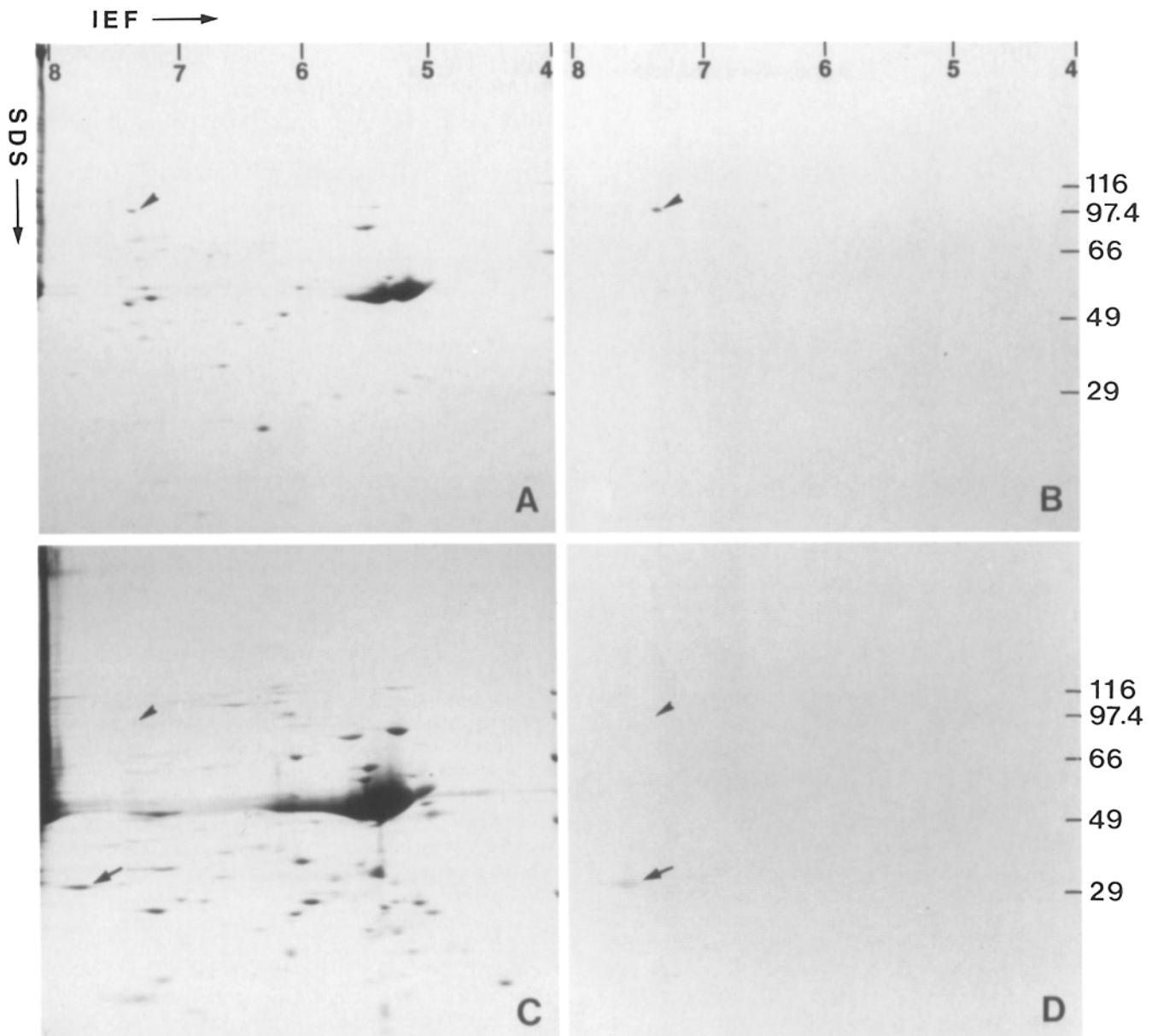
Figure 3. Comparison of the protein composition of the MgCl₂ cap fraction with the cap-free axonemal pellet on 2-D gels. *A*, Coomassie blue–stained gel of the MgCl₂ cap fraction; *B*, immunoblot of the cap fraction stained with the SH-CREST antiserum; *C*, Coomassie blue–stained gel of the cap-free axonemal fraction; and *D*, immunoblot of the cap-free axonemes stained with the SH-CREST antiserum. The arrowhead in *A* shows the stained 97-kD polypeptide present in cap fractions and not in cap-free axonemal fractions (compare with *C*). The 97-kD polypeptide in the cap fractions is the only polypeptide stained on 2-D immunoblots with SH-CREST antiserum. A 34-kD polypeptide stained by CREST is occasionally observed in the axonemes.

Do All CREST Antisera Recognize the 97-kD Polypeptide?

Most of this study was carried out using the SH-CREST antiserum previously characterized by Balczon and Brinkley (1987). The antiserum was chosen for its high titre and cross-reactivity in a variety of animal and plant kinetochores. To compare the SH-CREST antiserum with that obtained from other patients, GS-CREST serum was obtained from another CREST patient, UB serum (that does not stain kinetochores) was obtained from a patient with diffuse scleroderma, and "normal" human sera were obtained from three volunteers at the University of Kansas. None of the "normal" human sera

specifically stained ciliary tips when assayed by immunofluorescence microscopy. Each of the normal human sera contained antibodies that bound to several axonemal polypeptides but only one had a weak reaction with a polypeptide migrating near the 97-kD CREST antigen (Fig. 7). The GS-CREST serum showed a strong reaction with a band that comigrated with the 97-kD SH-CREST antigen (Fig. 8) but the UB antiserum stained none of the ciliary polypeptides (Figure 8). These results show that two CREST antisera, previously demonstrated to stain mammalian kinetochores, stain polypeptides in the *Tetrahymena* capping structure fraction and that a scleroderma serum that does not stain kineto-
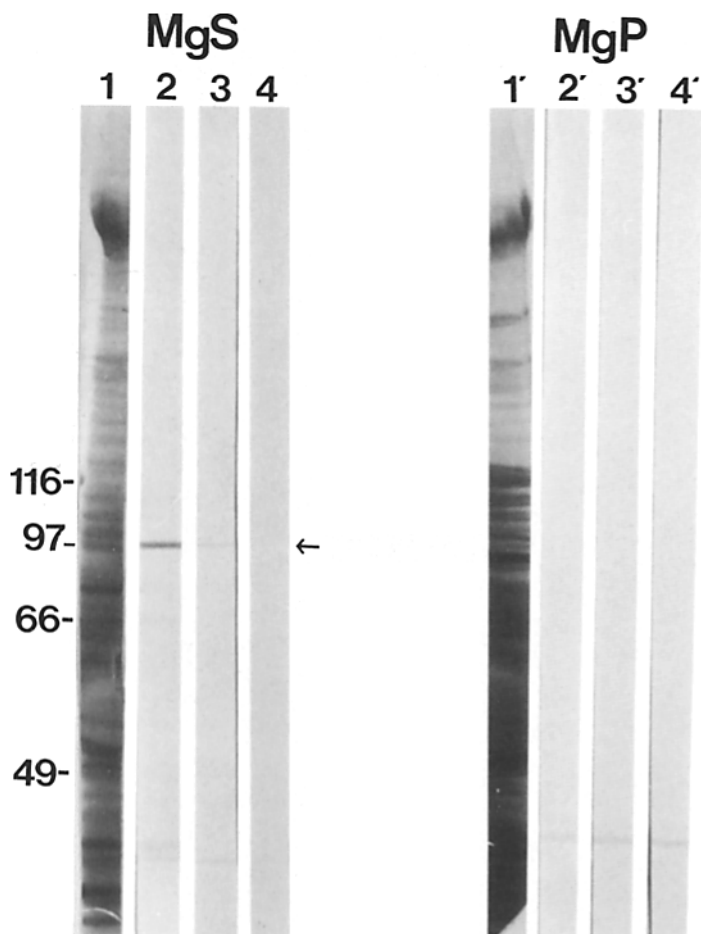
Figure 4. Analysis of the MgCl$_2$ cap fraction (*MgS*) and the cap-free axonemal fraction (*MgP*). The Amido black stained blots (lanes *1* and *1'*) show the total proteins. Both fractions were incubated with the SH-CREST antiserum (1:5,000 in PBSA) followed by an HRP-labeled secondary antibody (lanes *2* and *2'*). Lanes *4* and *4'* were not exposed to CREST but were incubated with the secondary antibody. These results show that the CREST antiserum recognizes a 97-kD protein found in the cap fraction (*arrow*) but not found in the axonemal fraction. Lanes *3* and *3'* show the binding of the antibodies that were affinity-purified from blots containing the 97-kD antigen.

chore proteins does not stain *Tetrahymena* capping structure polypeptides.

Discussion

The mechanisms that regulate microtubule assembly in cells are not well understood but several lines of evidence point to the possibility that important regulatory events occur at the ends of microtubules. Most of the microtubule assembly observed in vivo and in vitro occurs by the addition of tubulin to the plus ends of the microtubules, with the opposite, minus, ends generally being associated with nucleation centers, including the centrosome, centrioles, or the basal bodies of cilia and eukaryotic flagella. Therefore, most studies of microtubule assembly are focused on events that occur at the plus ends of microtubules. Many cytoplasmic microtubules are dynamic and rapidly assemble and disassemble in vitro (Mitchison and Kirschner, 1984) and in vivo (Schultze and Kirschner, 1988; Cassimeris et al., 1988), but there is also a population of microtubules that appear to be selectively stabilized in vivo (Khawaja et al., 1988; Cassimeris et al., 1988). Although numerous explanations of the selective stabilization of microtubules by MAPs or by posttranslational modifications of tubulin have been suggested, there is an increasing body of evidence that indicates that the ends of individual microtubules may be capped and stabilized by

yet unidentified factors (see Khawaja et al., 1988). These factors may attach to the ends of microtubules or they may be distributed along them and stabilize segments such that rapid microtubule disassembly may occur up to the point at which a stabilizing factor may be found (see Schultze and Kirschner, 1988; Margolis et al., 1986).

At least one of these stabilizing mechanisms may involve the binding of the plus end of a microtubule to a capping structure. One example of a capping structure is the kinetochore attached to chromosomes in dividing eukaryotic cells. The kinetochore is attached to the plus ends of microtubules (Huitorel and Kirschner, 1988; Euteneur and McIntosh, 1981; Telzer and Haimo, 1981) and their attachment to the kinetochore stabilizes the microtubules to disassembly (Salmon and Begg, 1980; Rieder, 1982; Kirschner and Mitchison, 1986). In addition to stabilizing the microtubules, the kinetochore is the site of microtubule assembly during metaphase and a site of microtubule disassembly as the chromosomes are pulled to the poles during anaphase (Gorbsky et al., 1987; Koshland et al., 1988; Mitchison et al., 1986; Mitchison, 1989a). Thus, the kinetochores are sites at which the plus ends of microtubules are captured, are elongated or shortened, and may be the sites of motors that translocate chromosomes (Brinkley et al., 1988; Koshland et al., 1988). Structural studies of kinetochores have not clearly revealed the nature of the attachment between the
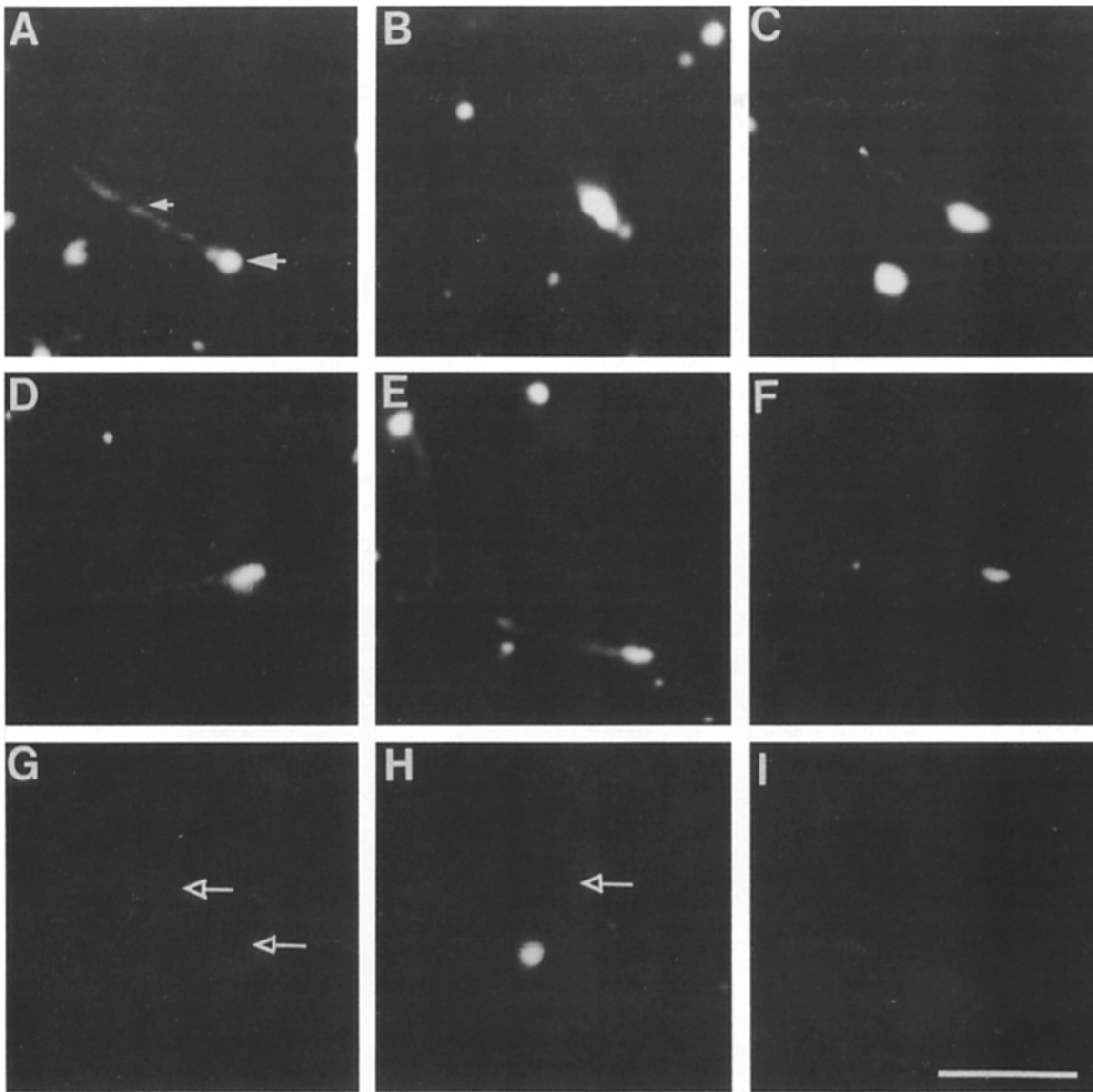
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Figure 5. Immunofluorescence microscopy of *Tetrahymena* cilia with affinity-purified SH-CREST antibody. Whole cilia (*A–F*) and MgCl₂ extracted cap-free axonemes (*G* and *H*) were incubated in affinity-purified 97-kD antibody and observed by fluorescent microscopy as described above. Whole cilia are brightly labeled at the distal tip (*large arrow*). The distal tip can be distinguished from the proximal tip because it tends to be more splayed. Also, in many of the cilia the central pair is visible and the fluorescently labeled central pair cap appears to be separated from the rest of the fluorescent staining (*B*). The rest of the axoneme (*small arrow*) is stained at a background level as are the cap-free axonemes (*open arrows*) and the negative controls, which contain no primary antibody (*I*). The cap-free axonemes and the negative controls were photographed at the same exposure as the cilia with caps; however, they were developed for less time so they would still be visible. Bar, 5 μm.

microtubule end and the chromosome, although the ends of the microtubules are surrounded by filamentous material, at least part of which is chromatin (Rieder, 1982; Ris and Wit, 1981; Roos, 1977). Since the kinetochores appear to be the site of a motor that must maintain a connection to assembling and disassembling microtubules, models have been proposed showing the kinetochores forming a collar that surrounds individual microtubules with "motors" reaching from the collar to the outer surface of the microtubule (Huitorel and Kirschner, 1988; Koshland et al., 1988). Ultrastructural studies have not, to date, revealed the structure of the collar.

We chose to study capping structures in cilia and flagella because they share certain features with the kinetochore. The capping structures are bound to the plus ends of the doublet and central microtubules at the site of microtubule growth and disassembly in vivo (Allen and Borisy, 1974; Binder et al., 1975; Rosenbaum et al., 1969; Dentler and Rosenbaum, 1977; Sale and Satir, 1977; Mesland et al., 1980), they are present throughout the assembly and disassembly of ciliary and flagellar microtubules in vivo (Dentler and Rosenbaum, 1977; Dentler, 1980; Portman et al., 1987), and they maintain a tight physical connection to the microtubules, as shown by their attachment to the ciliary tips during ciliary beating (Dentler and LeCluyse, 1982*a*) and their inhibition of micro-
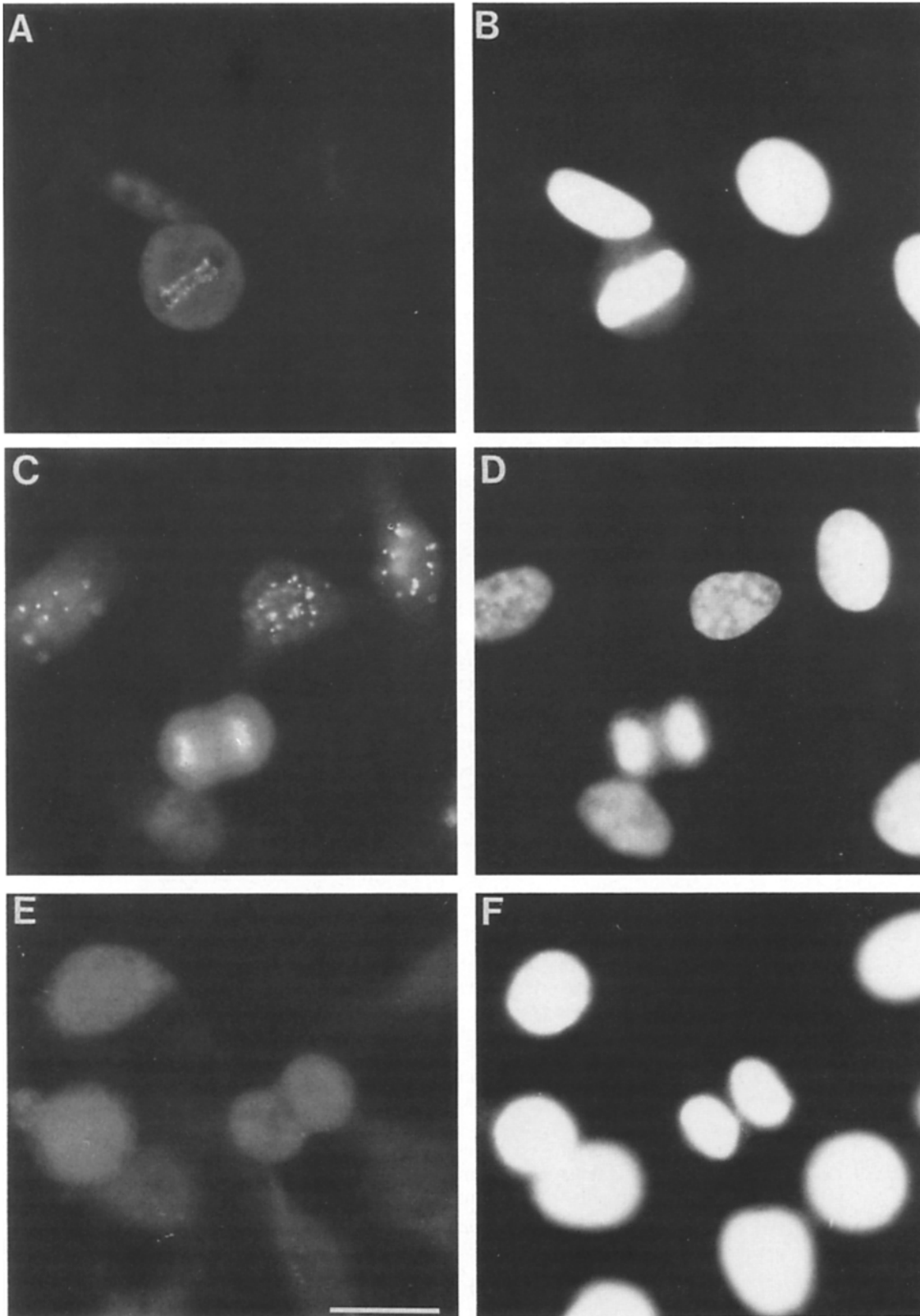
Figure 6. Immunofluorescent staining of HeLa cells double-labeled with CREST antiserum and Hoechst 33258. Affinity-purified 97-kD antibody stains the kinetochores of HeLa cells (*A*). A similar staining pattern is found when whole SH-CREST antiserum is used (*C*), but the kinetochores are not stained when antiserum from a patient with diffuse scleroderma is used (*E*). Hoechst 33258 stains the DNA (*B*, *D*, and *F*). Bar, 20 μm.
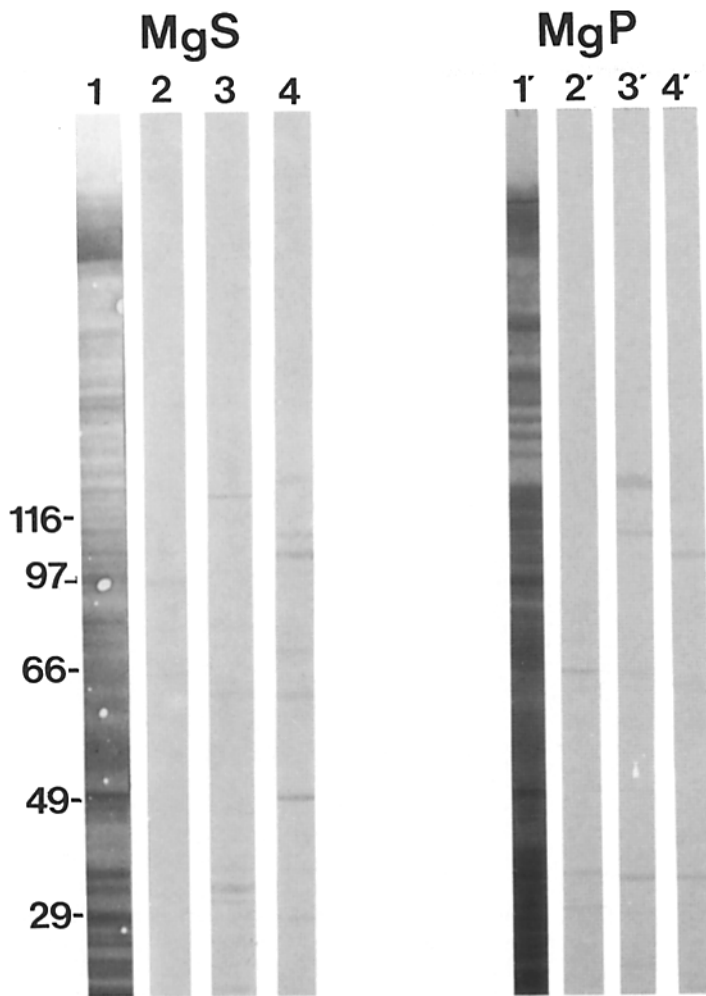
Figure 7. Immunoblots of $MgCl_2$ cap fractions (MgS) and the cap-free axonemal fractions (MgP) with three "normal" human sera. All three sera contain antibodies to cap and axonemal proteins. The serum tested in lane *2* shows a weak reaction with a band in the 97-kD region of the cap fraction but did not react with polypeptides in the axonemal fraction. None of these sera selectively stained the ciliary caps when examined by immunofluorescence microscopy.

tubule assembly onto capped microtubules in vitro (Dentler and Rosenbaum, 1977; Dentler and LeCluyse, 1982*b*). Moreover, the assembly and disassembly of capped microtubules are associated with motility: the assembly and disassembly at the plus ends of kinetochore microtubules moves chromosomes and assembly and disassembly at ciliary and flagellar tips moves the tips of the ciliary membranes and associated material (such as the ciliary crowns attached to respiratory cilia) (Dentler, 1989). Major advantages to studying the ciliary capping structures include their accessibility, since they are at the distal tips of most, if not all, cilia and flagella (Dentler, 1987; 1989), and the potential that their functions can be studied by the use of mutants defective in flagellar microtubule assembly. Finally, in contrast to kinetochores or other postulated structures associated with the plus ends of microtubules, we understand the major structural features of the caps. Both the central microtubule cap, attached to the central microtubules, and the distal filaments, attached to each of the A-microtubules, are attached to their microtubules by short plugs (Dentler, 1980; 1984; Suprenant and Dentler, 1988). Rather than forming a collar surrounding the microtubules, as proposed for the kinetochores, the ciliary and flagellar capping structures are attached to the ends of microtubules by short plugs inserted into the microtubule lumen and by plate or bead structures that cross the ends of

the microtubules. Since the capping structures are linked to the growing ends of microtubules throughout ciliary assembly (and disassembly), it is not unreasonable to suggest that the kinetochores, or a portion of the kinetochore, may be linked to microtubules in a similar manner (Figure 9).

We propose that the 97-kD polypeptide identified by the CREST antisera is specifically bound to the distal ends of ciliary microtubules and that it is associated with a microtubule capping structure. This is based on several lines of evidence. First, the 97-kD polypeptide is one of the few polypeptides that is selectively released from isolated *Tetrahymena* axonemes with $MgCl_2$. Extraction of axonemes with low concentrations of $MgCl_2$ or $CaCl_2$ selectively releases the capping structures from isolated *Tetrahymena* cilia (Suprenant and Dentler, 1988). Second, both crude antiserum and 97-kD affinity-purified antiserum stain the distal tips of isolated *Tetrahymena* cilia and axonemes but not axonemes extracted with $MgCl_2$, which releases the capping structures. The lack of tip staining of $MgCl_2$-extracted axonemes is not because of some change in the accessibility of the antibody to the interior of the axoneme because no staining was seen when extracted axonemes were induced to disintegrate into individual doublet and central microtubules by the addition of ATP. The 97-kD antigen is, therefore, part of the capping structure and is present at the tips of both the central and
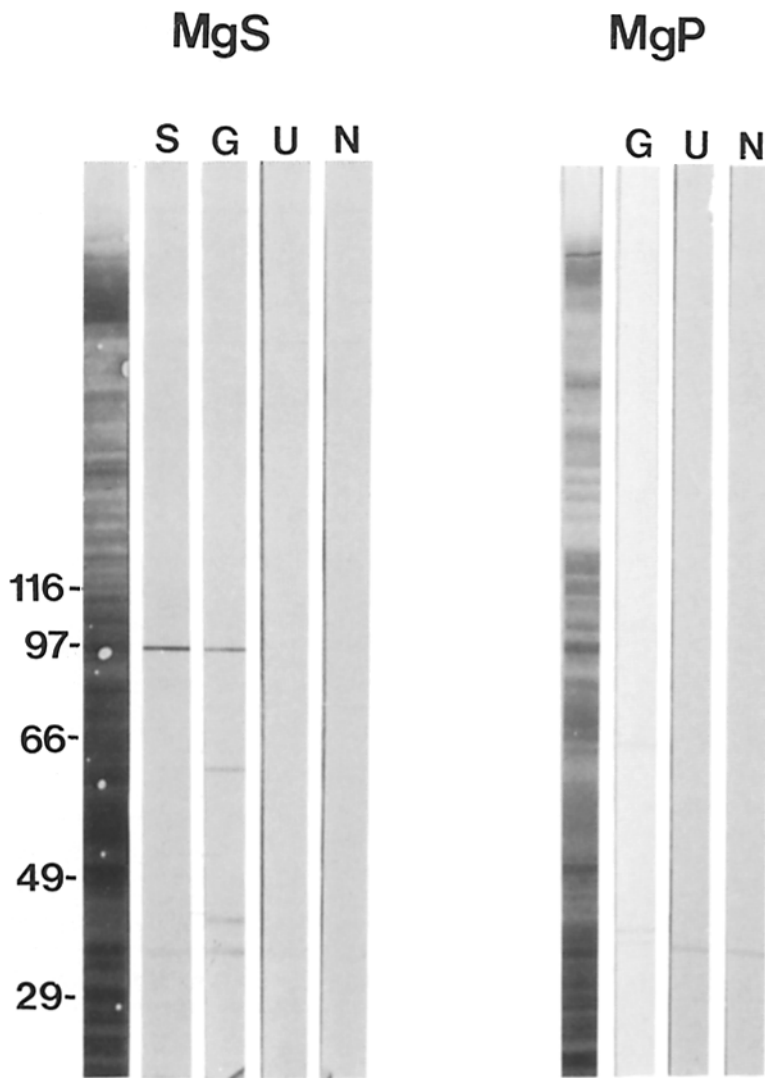
Figure 8. Immunoblots of MgCl₂ cap fractions (MgS) and the cap-free axonemal fractions (MgP) stained with two CREST antisera, SH (*S*) and GS (*G*). Both the GS-CREST and SH-CREST antisera contain antibodies against the 97-kD polypeptide found exclusively in the cap fractions. The GS-CREST also contains antibodies against other polypeptides in the cap and axonemal fraction. The UB-antiserum, from a patient who does not exhibit the CREST syndrome, does not contain antibodies to cap or axonemal proteins and does not stain axonemes assayed by immunofluorescence. The negative control (*N*) contains no primary antibody.

A-microtubules. More definitive localization of the antigen must await studies of isolated capping structures with purified antibodies directly labeled with colloidal gold.

The 97-kD polypeptide recognized by the SH-CREST antiserum and reported here is the first polypeptide identified to be associated with the ciliary microtubule capping structures. Is it related to previously identified kinetochore antigens? The major kinetochore antigens recognized by the SH-CREST antiserum used in this study include a prominent 80-kD protein and a slightly less prominent 18-kD protein (Valdiva and Brinkley, 1985). The 80-kD protein may be identical to the kinetochore-associated 80-kD CENP-B protein described by Earnshaw et al. (1987), but the location and function of the 80-kD kinetochore-associated protein (or proteins) is not well understood. Earnshaw (1987; Earnshaw et al., 1987) suggested that the 80-kD CENP-B protein is an acidic DNA-binding protein. Other evidence suggests that the 80-kD protein is a tubulin-binding protein. A tubulin 80-kD protein complex binds to antitubulin affinity columns (Balczon and Brinkley, 1987), and the 80-kD antigen recognized by the SH-CREST serum binds to N₃-labeled tubulin in vitro. When probed on blots, the 18-kD protein recog-

nized by SH-CREST and not the 80-kD protein binds DNA. The location and function of the 80-kD kinetochore antigen is, therefore, not well understood.

Although antibodies affinity-purified from the ciliary 97-kD cap protein stain kinetochores it is likely that the 97-kD ciliary protein is not identical to the 80-kD CENP-B protein identified by Earnshaw et al. (1987). In addition to having different molecular masses, the polypeptides differ in charge, with the CENP-B protein being an acidic protein, pI 5.2-5.7 (Earnshaw et al., 1987) and the ciliary 97-kD protein having a slightly basic pI of 7.4 (this report). Based on the SH-CREST serum used in this study, the 80-kD kinetochore antigen and the 97-kD ciliary antigen do not share antigenic sites. Despite repeated attempts, affinity-purified antibodies against the ciliary 97-kD protein did not stain blots of kinetochore proteins and affinity-purified antibodies against the kinetochore 80-kD protein failed to stain blots of ciliary proteins. Since the affinity-purified 97-kD antibodies stain kinetochores (by immunofluorescence) these results are consistent with the possibility that we have identified a kinetochore-associated protein not previously identified on immunoblots. Additional studies using other antibodies against the
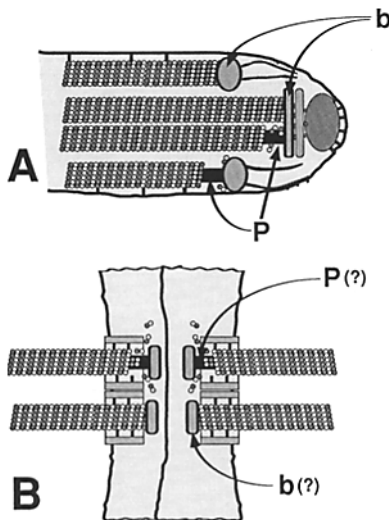
Figure 9. Diagram comparing the capping structures at the tips of a *Tetrahymena* cilium (*A*) with a possible structure for kinetochores (*B*). Plug structures (*P*) are known to insert into the lumens of the ciliary (*A*) and central tubules, but plugs have not been identified in association with the ends of kinetochore microtubules. Plate or bead-shaped structures (*b*) are attached to the ends of the ciliary microtubules and may be related to dense material of the outer kinetochore plate in which the kinetochore microtubules end (see Rieder, 1982; Brinkley et al., 1989). Identification of the site of the 97-kD ciliary antigen in the cilium and kinetochore should help clarify the structures common to these two capping structures.

97-kD ciliary proteins will be necessary to confirm this possibility.

Finally, since there is no evidence for the presence of DNA (let alone centromere-specific sequences) at the tips of cilia and flagella, it is unlikely that the DNA-binding properties of CENP-B are shared with the 97-kD ciliary cap protein. Thus, the 97-kD ciliary capping protein may share certain antigenic sites with 80-kD antigens in kinetochores (for example, the sites recognized by immunofluorescence assays) but it is unlikely that it is identical to the CENP-B protein.

The precise identification of the location of the antigens associated with kinetochores and ciliary capping structures remains to be determined. Based on ultrastructural evidence alone, it is likely that the ciliary capping structures are composed of many different proteins (Dentler, 1984; LeCluyse and Dentler, 1984; Suprenant and Dentler, 1988). The identification of the 97-kD antigen is an important start in the characterization of these structures and, hopefully, will provide a tool with which to isolate and identify additional microtubule capping structure proteins.

The assembly and disassembly of ciliary and flagellar microtubules is similar to that of kinetochore-associated microtubules in that each appears to be at least partly regulated by a switch at or near the site of tubulin addition or loss (see Mitchison, 1989*b*; McIntosh et al., 1989). While we do not know if a common switching mechanism is associated with each of these types of microtubules, the discovery of related antigenic sites suggests that at least some of the proteins in these plus-end caps are related to one another. If there are similar types of switches regulating microtubule assembly and disassembly in cilia and kinetochores, then future studies

of ciliary capping structures, using both biochemical approaches and the use of mutants of flagellar growth, should make a significant contribution to the understanding of mechanisms regulating cell division and of microtubule assembly in general.

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