Purification and Characterization of a Basal Body-associated Ca\textsuperscript{2+}-binding Protein

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Abstract. Isolated basal body complexes from the unicellular alga, *Chlamydomonas reinhardtii* were found to contain a low molecular mass acidic polypeptide, distinct from calmodulin, but with biochemical features in common with members of the calmodulin family of calcium-binding proteins. These common characteristics included a relative low molecular mass of 20 kD, an experimentally determined acidic pI of 5.3, an altered electrophoretic mobility in SDS-polyacrylamide gels in the presence of added calcium, and a calcium-dependent binding to the hydrophobic ligand phenyl-Sepharose which allowed its purification by affinity chromatography. The relatedness of the basal body–associated 20-kD calcium-binding protein (CaBP) to calmodulin was confirmed by amino acid compositional analysis and partial peptide sequencing of the isolated protein. A rabbit antibody specific for the 20-kD CaBP was raised and used to determine by indirect immunofluorescence the cellular localization of the protein in *Chlamydomonas* cells. In interphase cells the antibody stained intensely the region between the paired basal bodies, two fibers extending between the basal bodies and the underlying nucleus, and an array of longitudinal filaments surrounding the nucleus. The two basal body-nuclear connecting fibers were identified in thin-section electron micrographs to be narrow striated fiber roots. In mitotic cells the 20-kD CaBP was specifically associated with the poles of the mitotic spindle at the sites of the duplicated basal body complexes.

The temporal and spatial disposition of microtubules in eukaryotic cells is determined in large part by centers which regulate their assembly (for a review see reference 6). In animal cells the major microtubule-organizing center of interphase cells is the centrosome consisting of one or more pairs of centrioles surrounded by amorphous electron-dense material (pericentriolar material). At the end of interphase the centrosome is duplicated to generate the poles of the mitotic spindle. At the completion of mitosis the duplicated centrosomes are partitioned between daughter cells. The correct positioning of the centrosome before the assembly of the mitotic spindle, and the equal partitioning of these complexes into daughter cells is crucial to the fidelity of cellular reproduction and determination of cytoplasmic organization (for reviews see references 32, 34).

In the unicellular, flagellated alga *Chlamydomonas* the basal body complex with its pair of basal bodies is the functional homologue of the centrosome in animal cells. In interphase cells the basal body complex functions not only as the well-recognized site for the assembly of the microtubules of the flagella, but also as the apparent organizing center for an interphase cortical array of microtubules (11, 38, 51). Like the centrosome in animal cells, the basal body complex undergoes a transformation when the cells enter mitosis. The flagella and interphase cytoplasmic microtubules are disassembled and the basal body complex is duplicated. In mitotic cells the daughter complexes define the polarity of the intranuclear mitotic spindle (10). At the completion of chromosomal segregation the duplicated basal body complexes are the apparent organizing centers for several distinct sets of microtubules that participate in nuclear and cytoplasmic cleavage (11, 23).

Methods have been described previously for isolating enriched preparations of basal body complexes from interphase *Chlamydomonas* cells (15, 45). Basal body complexes isolated from *Chlamydomonas* have been shown to serve as the initiating centers for the in vitro assembly of microtubules (45) and the in vivo assembly of asters in *Xenopus* oocytes (16).

We have initiated a biochemical analysis of the proteins that are present in purified preparations of basal body complexes from *Chlamydomonas*. The long-term objectives of these studies are to define by biochemical methods and mutant analyses the structure–function relationships of specific basal body–associated proteins. In this study, we describe the purification and characterization of one of these components, a novel basal body–associated 20-kD calcium-binding protein (CaBP).\textsuperscript{1}

\textsuperscript{1} Abbreviations used in this paper: CaBP, calcium-binding protein; SFR, striated fiber root; TBS, Tris-buffered saline.
Materials and Methods

**Materials**

NP-40 was purchased from Accurate Chemical & Scientific Corp. (Westbury, NY), trysiol from Mobay Chemical Corp. (Pittsburgh, PA), phenyl-Sepharose CL-4B from Pharmacia Fine Chemicals (Piscataway, NJ), cyano- gen bromide from Fisher Scientific Co. (Pittsburgh, PA), trypsin (1-p-tosyl-L-lysino-2-phenylthyl) chloromethyl ketone [TPCK] treated) from Worthington Biochemical Corp. (Freehold, NJ), Alu-Gel-S from Serva (Heidelberg, Germany), and tritocellulose from Schleicher & Schuell, Inc. (Keene, NH). 10-well microscope slides were purchased from Carlson Scientific (Peotone, IL), formamide was from Electron Microscopy Sciences (Fort Washington, PA) or Ladd Research Industries, Inc. (Burlington, VT), glutaraldehyde from Polysciences, Inc. (Warrington, PA), and phenylendiamine from Sigma Chemical Co. (St. Louis, MO).

The mouse monoclonal anti-α tubulin antibody used in this study was raised in this laboratory against the protein isolated from *Chlamydomonas* and reacts with all species of α-tubulin present in the cell. The preimmune and immune sera from a rabbit immunized with vertebrate calmodulin (47) was generously provided by L. J. Van Eldik. The rabbit anti-Tetraselmis 20-kD striated fiber root protein antisera (40) was kindly provided by J. L. Salisbury. FITC-conjugated and rhodamine-conjugated goat anti-rabbit IgG antibodies (Fab fragments) were obtained from Cappel Laboratories (Malvern, PA) and FITC-conjugated goat anti-mouse IgG antibodies from Fisher Biotech (Orangeburg, NY). Alkaline phosphatase-conjugated goat anti-rabbit IgG antibodies, 5-bromo-4-chloroindoxyl phosphate, and nitroblue tetrazolium were obtained from Promega Biotech (Madison, WI).

**Strains and Cell Culture**

*Chlamydomonas reinhardtii* wild-type strain 137c was grown in Tris-acetate-phosphate media (14). Equilibrium labeling of cellular proteins with [35S]sulfate was achieved by growing the cells as lawns on agar plates containing low sulfate Tris-acetate-phosphate media and [35S]sulfate as previously described (4). For the immunoblotting experiments on whole cell lysates, the immunocytochemical localization studies, and the electron microscopic analyses the cells were grown in liquid cultures at 25°C under constant light conditions (10,000 lux) with agitation.

A culture of the marine alga *Tetraselmis striata* (CW 490) was obtained from the Bigelow Laboratory for Ocean Sciences, West Boothbay Harbor, ME. The cells were grown as lawns on 1% agar-containing growth media at 20°C under constant light conditions (10,000 lux). The growth medium used here was developed by Dr. Ralph Lewin (Scripps Institute of Oceanography) and contained per liter: 750 ml filtered seawater, 250 ml distilled water, vitamins were added: cobalamin B-12 (1.0 μg/liter), thiamine B-1 (1.0 μg/liter) and niacin at multiple subcutaneous sites, and the animals were bled from the ear by venipuncture.

**Protein Isolation and Purification**

Basal body complexes were isolated from mating mixtures of wild-type strain 137c mt+ and mt− gametes as previously described (45) with minor modifications that increased the purity of the preparations (to be described in detail elsewhere). In brief, the basal body complexes were isolated from wall-less, deflagellated mated cells lysed in the presence of 1% NP-40 in a solution containing 10 mM Tris–HCl, 0.1 mM Na2EDTA, 50 U/ml trysiol, pH 7.5, at 4°C (TE buffer). The basal body complexes were fractionated from other cellular components by a series of low speed differential centrifugations and purified, after a second extraction with the nonionic detergent, NP-40, by sedimentation on a sucrose step gradient.

For purification of the 20-kD CaBP, pellets of basal body complexes containing ~200 μg of protein (sp act 100,000 cpmp) were resuspended in 450 μl of TE buffer, to which 150 μl of 2 M NaCl in TE buffer was added. After a 15-min incubation on ice, the suspension was centrifuged at 30,000 rpm in an SW 60 rotor (Beckman Instruments, Inc., Palo Alto, CA) for 40 min at 4°C. The supernatant was collected and an appropriate volume of 0.1 M NaCl in TE buffer was added to yield a final concentration of 1 M NaCl. The samples were applied by gravity to a 1-ml (0.5 cm inside diameter × 5.0 cm) column of phenyl-Sepharose CL-4B preelutted in column buffer (10 mM Tris–HCl, 1 mM CaCl2, 0.5 M NaCl, pH 8.0, at 23°C). After the sample was loaded, the column was washed with column buffer and then with column buffer minus 0.5 M NaCl until the 35S counts in the eluates dropped to baseline levels. The bound 20-kD CaBP was eluted by application of a solution containing 10 mM Tris–HCl and 10 mM EGTA, pH 8.0, at 23°C. 0.5-ml fractions were collected at a flow rate of 20 ml/h. These fractions containing 35S counts after application of the EGTA-containing buffer were pooled and analyzed by one- and two-dimensional gel electrophoresis.

**PAGE**

The two-dimensional resolution of polypeptides was achieved by separating the proteins in the first dimension by nonequilibrium pH gradient electrophoresis and in the second dimension by SDS-polyacrylamide gradient electrophoresis. The methods used were those previously reported for the resolution of *Chlamydomonas* flagellar proteins (36), with the following modifications: the samples were solubilized directly in the first dimension sample buffer and loaded within 4 h or frozen at −20°C for subsequent analysis. The electrophoresis in the first dimension was extended from 14 to 16 h at a constant current of 2 mA; the second-dimension SDS gel was formed with a 5–13% gradient of acrylamide. In the studies on the effects of added CaCl2 vs. EGTA on the electrophoretic mobilities of proteins in the SDS dimension, 1 mM CaCl2 or 1 mM EGTA was included in the second dimension sample loading buffer as well as in the buffers used to form the stacking and running gel.

The analytical and preparative resolution of polypeptides on one-dimensional SDS gels was performed with the 5–13% gradient gels used in the second dimension of the two-dimensional gel system. For immunoblotting experiments the proteins were separated on 15% SDS–polyacrylamide slab gels as described by Laemmli (27).

**Amino Acid Compositional Analysis and Peptide Sequencing**

The 20-kD CaBP in isolated basal body complexes was resolved on preparative one-dimensional SDS gels. The protein was electroeluted and electroalyzed using the procedure of Hunkapiller et al. (20). Stain was removed and the SDS concentration reduced by precipitating the protein with 80% (vol/vol) ethanol. The eluted protein was characterized by amino acid compositional analysis and then subjected to digestion with cyanogen bromide or trypsin (TPCK-treated) by using previously described protocols (31). Approximately 200 pmol of protein was used in each digest. The digests were fractionated essentially as described previously (42) on an RP-300 microbore column (1.0 × 250 mm; Brownlee Labs, Santa Clara, CA) at a flow rate of 70 μl/min using HPLC (model No. 130A; Applied Biosystems, Inc., Foster City, CA). The eluting solvents were 0.1% (vol/vol) aqueous trifluoroacetic acid and 60% (vol/vol) aqueous acetonitrile containing 0.08% (vol/vol) trifluoroacetic acid. Aliquots of selected fractions were subjected to acid hydrolysis (6 M HCl with 1% [wt/vol] phenol) and amino acid analysis using a Picotag system (Waters Associates, Milford, MA) (5). Automated Edman degradations were performed with a protein sequencer (model No. 470A; Applied Biosystems, Inc) and PTH amino acid derivatives separated with an on-line PTH analyzer (model No. 120A, Applied Biosystems, Inc.) coupled to a laboratory automation system (model No. 3357; Hewlett-Packard Co., Palo Alto, CA).

Amino acid sequence comparisons and database searches were carried out with the FASTN and FASTP programs (29) supplied by the National Biomedical Research Foundation. The National Biomedical Research Foundation, Genbank, EMBL databases were used.

**Antigen Preparation and Immunization**

Two young female New Zealand white rabbits were immunized on days 1, 27, 125, 213, 240, and 276 with gel-purified basal body–associated 20-kD CaBP. The initial immunization and the first two booster injections were made with homogenized gel pieces containing ~50 μg of protein emulsified with an equal volume of a 1:1 mixture of Freund's complete adjuvant and Alu-Gel-S. Subsequent immunizations were with 25 μg of electroeluted gel-purified 20-kD CaBP in Freund's incomplete adjuvant. Injections were given at multiple subcutaneous sites, and the animals were bled from the ear by venipuncture.

**Preparation of Chlamydomonas and Tetraselmis Whole Cell Lysates**

Pellets containing 1 × 10^6 cells were resuspended in 1 ml of SDS lysis buffer (10 mM Tris–HCl, pH 7.5, 0.1 mM Na2EDTA, 1 mM phenylmethylsulfonfonyl fluoride [PMSF], 1% [vol/vol] β-mercaptoethanol, and 1% [vol/vol]
Sds). The SDS cell lysates were sonicated and then heated at 95°C for 5 min. Insoluble material (predominantly cell walls and starch) was removed by centrifuging the lysates in a microfuge for 15 min. The chlorophyll and SDS concentrations were diminished by precipitating the proteins with 10 vol of cold 95% ethanol. After an overnight incubation at -20°C, the ethanol precipitates were pelleted by centrifugation in a rotor (model No. JA-20; Beckman Instruments, Inc.) at 8,000 rpm for 15 min at 4°C, freeze-dried, and resuspended in gel electrophoresis sample buffer.

**Immunoblotting**

Samples resolved on one- and two-dimensional polyacrylamide gels were electrophoretically transferred to 0.2-μm nitrocellulose paper as described (46). The blots were rinsed briefly in Tri-buffered saline, 50 mM Tris-HCl, pH 7.4, 0.2 M NaCl. (TBS) and the proteins fixed to the nitrocellulose by incubating the blots for 15 min in 0.2% glutaraldehyde buffered in TBS. After repeated washing in TBS over a period of 30 min, the blots were blocked for 1 h in TBS containing normal dry milk, BLOTTO (22). The blots were incubated for 1 h at room temperature in primary antisera diluted in BLOTTO, rinsed in TBS, and then incubated for 1 h at room temperature with an alkaline phosphatase-conjugated goat anti-rabbit antibody made up in BLOTTO. The blots were washed in several changes of TBS and containing 0.05% NP-40 before they were developed with 5-bromo-4-chloroindoxyl phosphate and nitroblue tetrazolium by the methods recommended by the distributor (Promega Biotec., Madison, WI).

**Indirect Immunofluorescence Staining of Fixed Chlamydomonas Cells**

Wild-type Chlamydomonas cells from logarithmic phase liquid cultures were harvested by centrifugation and incubated for 30-60 min at 25°C in a medium containing the Chlamydomonas cell wall-degrading enzyme, autolysin. This medium represents the supernatant fraction obtained from mating experiments and was prepared essentially as described previously (44). The cells without walls were fixed in 3% formaldehyde in 0.1 M KH2PO4 buffer (pH 7.4) for 20-30 min at room temperature. The fixed cells were washed 3× in PBS by pelleting the cells at 1,400 rpm for 4 min in a Beckman Microfuge 11, and the pellets were finally resuspended in 0.5 ml distilled water. 5-μl aliquots were spotted onto the wells of 10-well microscope slides and the cells were allowed to air dry onto the slides. The fixed, adsorbed cells were permeabilized by immersing the slides in a solution containing 0.5% NP-40 in PBS for 2 min. The slides were then rinsed in distilled water and the chlorophyll in the cells extracted by immersing the slides in three changes of ice cold acetone for 5 min each. After a brief rinse in distilled water, excess liquid was removed from around the wells with cotton swabs. 10-μl aliquots of primary antibodies diluted in PBS were applied to each well and the slides placed in a humidified chamber at 37°C for 30-45 min. Slides were then rinsed in PBS and incubated for 15 min in a blocking solution containing 0.1% BSA, 0.01% NaN3 in PBS (with three changes of the solution every 5 min). Secondary antibodies diluted in PBS were applied in 10-μl aliquots to each well and the slides were incubated at 37°C for 30-45 min. The slides were rinsed in PBS, dipped in distilled water, swabbed, and mounted in a medium containing p-phenylenediamine, prepared as previously described (1).

For double-label immunofluorescence staining, the cells were reacted with the primary antibodies and subsequently with the secondary antibodies mixed to appropriate dilutions. For DNA staining with propidium iodide, 10-μl aliquots of RNase A (100 μg/ml; heat-treated) were applied to the fixed cells adsorbed to the 10-well microscope slides before incubation in the primary antibody. The slides were placed in a humidified chamber at 37°C for 30-45 min. Slides were then rinsed in PBS and incubated for 15 min in a blocking solution containing 0.1% BSA, 0.01% NaN3 in PBS (with three changes of the solution every 5 min). Secondary antibodies diluted in PBS were applied in 10-μl aliquots to each well and the slides were incubated at 37°C for 30-45 min. The slides were rinsed in PBS, dipped in distilled water, swabbed, and mounted in a medium containing p-phenylenediamine, prepared as previously described (1).

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**Electron Microscopy**

Chlamydomonas cells were fixed and processed for thin-section electron microscopy as previously described (17, 43). The sections were examined and photographed using a JEOL 100 CX electron microscope.

**Results**

**Identification of a Basal Body-Associated Calcium-binding Protein Distinct from Calmodulin**

Basal body complexes were isolated from Chlamydomonas cells grown in the presence of [35S]sulfate under conditions in which the cellular proteins were equilibrium-labeled to high specific activity (~100,000 cpn/mg protein). The complexes were isolated from wall-less, mated gametes by methods based on a previously described procedure (45). By thin-section electron microscopic criteria, the preparations were highly enriched for basal body complexes with little or no contamination from other cellular components. A detailed account of the ultrastructure of the isolated complexes and a comparative analysis of basal body-associated proteins with those found in isolated flagellar axonemes from Chlamydomonas will be described elsewhere.

Fig. 1 contains an autoradiogram of the two-dimensional resolution of 35S-labeled polypeptides fixed in isolated basal body complexes from Chlamydomonas. The major polypeptide components are the α- and β-tubulin subunits that comprise over 50% of the 35S label (the heavily exposed components in the 55-kD region). In the acidic, low molecular mass region of the two-dimensional map (boxed area) a minor protein was identified (labeled C) that comigrated with the previously isolated and characterized Chlamydomonas flagellar calmodulin (12, 48). One of the characteristics of calmodulin is that its electrophoretic mobility in SDS-polycrylamide gels is increased in the presence of calcium, a characteristic that apparently reflects the ability of the protein to bind calcium even in the presence of SDS (7). To obtain further evidence that component C was calmodulin, two-dimensional maps of the proteins found in isolated basal body complexes were generated in which either 1 mM EGTA or 1 mM CaCl2 was present during electrophoresis in the SDS dimension. As previously reported for Chlamydomonas calmodulin (12), the migration of component C in the SDS dimension shifted from an apparent Mr of ~20,000 to ~18,000 in the presence of added calcium (bottom of Fig. 1). Strikingly, in the same region of the gel, a second less acidic component with an apparent pI of 5.3 (CaBP in Fig. 1) was also observed to migrate faster in the SDS dimension when subjected to electrophoresis in the presence of 1 mM CaCl2 vs. 1 mM EGTA. The migration of this component shifted from an apparent Mr of 22,000 to 20,000. Because of its prevalence in isolated basal body complexes and its apparent calcium-binding properties, we sought to purify and further characterize this protein at the biochemical level. Since the molecular masses of acidic, low molecular mass calcium-binding proteins such as calmodulin and troponin C correspond more closely to their observed relative mobilities in SDS-polycrylamide gels run in the presence of calcium (24), we assume that the true molecular mass of the basal body-associated calcium-binding protein identified in this report is likely to be ~20 kD. Therefore, the protein will be referred to as a 20-kD CaBP.

**Purification of the 20-kD CaBP**

The 20-kD CaBP was found to be partially solubilized from isolated basal body complexes by incubating the complexes in a high salt (0.5 M NaCl) solution. Fig. 2 A contains an
Figure 1. An autoradiogram of the two-dimensional resolution of 35S-labeled proteins found in purified basal body complexes from *Chlamydomonas*. The sample containing 10 μg of protein (sp act ~100,000 cpm/μg) was subjected to nonequilibrium pH gradient electrophoresis (NEPHGE) followed by SDS gel electrophoresis (SDS). In the acidic, low molecular mass region of the gel (boxed area) are two components (CaBP and C) that were found to migrate faster in the SDS dimension when subjected to electrophoresis in the presence of 1 mM CaCl₂ vs. 1 mM EGTA (bottom). The circles in the bottom panels designate the positions (as revealed by Coomassie Blue staining) of two Mr standards, soybean trypsin inhibitor (20.1 kD) and α-lactalbumin (14.4 kD) whose relative mobilities were not altered in the presence of calcium. The minor more acidic component labeled C was identified as *Chlamydomonas* calmodulin. The more prevalent less acidic polypeptide labeled CaBP was further purified and characterized in this study.
Gel electrophoretic analysis of the extraction and purification of the 20-kD CaBP from isolated basal body complexes. (A) An autoradiogram of the one-dimensional resolution of proteins present in isolated basal body complexes (BB), the pellet fraction of high salt-extracted basal body complexes (HSP), the corresponding high salt supernatant fraction (HSE), and the pooled EGTA-eluted peak fractions from a phenyl-Sepharose column that contained the purified 20-kD CaBP (PS). The samples loaded in the first three lanes contained equal numbers of counts (100,000 cpm). The sample loaded in the PS lane contained 10,000 cpm. (B) An autoradiogram of the two-dimensional resolution of the purified 20-kD CaBP. The sample loaded contained 5,000 cpm. B corresponds in the molecular mass dimension with A.

Amino Acid Compositional and Peptide Sequence Analysis

The proteins found in isolated basal body complexes were resolved by preparative one-dimensional SDS gel electrophoresis. The region of the gels containing the 20-kD CaBP was excised and the electroeluted protein was characterized by amino acid analysis. Table I compares the amino acid composition of the 20-kD CaBP with the composition of Chlamydomonas calmodulin as calculated from its amino acid sequence (30). While the 20-kD CaBP showed an amino acid composition similar to calmodulin (in its prevalence of acidic amino acids and its high content of charged amino acids), it was clearly distinct from calmodulin. The 20-kD CaBP differed from Chlamydomonas calmodulin specifically in its higher content of arginine, isoleucine, and serine residues.

The intact Chlamydomonas 20-kD CaBP was subjected to automated Edman degradation, but the amino terminus of the protein was found to be blocked. The protein was digested with cyanogen bromide or trypsin, and three selected peptides resolved by reverse-phase HPLC chromatography were sequenced. Fig. 3 contains the amino acid sequences for one peptide generated from a trypsin digest (TP-21) and two peptides resulting from a cyanogen bromide cleavage (CB-17 and CB-8). TP-21 and CB-17 were found to overlap in amino acid sequence by 7 residues, providing data on a 31-residue linear sequence of the protein. As seen in Fig. 4, this 31-residue segment of the 20-kD CaBP has a linear homology with sequences found toward the carboxy termini of both Chlamydomonas calmodulin (30) and rabbit skeletal muscle troponin C (9). The homologous regions in these well-characterized calcium-binding proteins includes the 12 residues that comprise the fourth calcium-binding loop.

Characterization of a Rabbit Antibody Raised against the 20-kD CaBP

The 20-kD CaBP present in isolated basal body complexes was resolved on preparative one-dimensional SDS-polyacrylamide gels, and then used to immunize rabbits. Fig. 5
Table I. Amino Acid Compositions of Chlamydomonas Proteins

| Amino acid | 20-kD CaBP* | Calmodulin† |
|------------|-------------|-------------|
| Ala        | 14.4        | 13          |
| Arg        | 12.9        | 6           |
| Asp        | 19.9        | 24          |
| Cys        | ND          | 0           |
| Glu        | 25.8        | 28          |
| Gly        | 14.3        | 13          |
| His        | 1.0         | 3           |
| Ile        | 11.6        | 6           |
| Leu        | 14.5        | 11          |
| Met        | 5.8         | 9           |
| Phe        | 8.9         | 9           |
| Pro        | 4.2         | 2           |
| Ser        | 8.6         | 5           |
| Thr        | 10.8        | 12          |
| Tmk‡       | 0           | 0           |
| Trp        | ND          | 0           |
| Tyr        | 2.6         | 1           |
| Val        | 7.7         | 8           |

* Values shown are mole per 20,000 g as estimated from analysis of gel-eluted protein.
† Values shown are residues per molecule calculated from the amino acid sequence (30).
‡ Tmk = N\(^\text{\textsuperscript{-}}\)trimethyllysine.

Figure 3. Amino acid sequences of selected peptides from a trypsin digest (TP-21) and a cyanogen bromide cleavage (CB-17 and CB-8) of the purified Chlamydomonas 20-kD CaBP. Peptides TP-21 and CB-17 overlapped in amino acid sequence by 7 residues (underlined residues) providing data on a 31-residue linear sequence of the native 20-kD CaBP.

shows an immunoblot analysis of Chlamydomonas whole cell proteins separated on an SDS gel and probed with a rabbit anti-20-kD CaBP preimmune serum (lane 2) and immune serum (lane 3). The rabbit immune serum specifically stained a single molecular mass band in whole cell extract. Adjacent strips of the nitrocellulose transfer were also probed with the preimmune serum (lane 4) and immune serum (lane 5) from a rabbit immunized with vertebrate CaBP. Lanes 2 and 3 were incubated in 1:1,000 dilutions of the (lane 2) preimmune and (lane 3) immune sera from a rabbit immunized with the Chlamydomonas 20-kD CaBP. Lanes 4 and 5 were incubated in 1:100 dilutions of the (lane 4) preimmune and (lane 5) immune sera from a rabbit immunized with vertebrate calmodulin (47). The immunoreactive bands were detected with an alkaline phosphatase-conjugated secondary antibody. The two immune sera specifically recognized single bands electrophoretically distinct from each other.

Figure 4. Amino acid sequence comparison of the composite 31-residue linear sequence of the Chlamydomonas 20-kD CaBP with (A) Chlamydomonas calmodulin, residues 114–144 (30), and (B) rabbit skeletal muscle troponin C, residues 121–151 (9). .: identical residues; .: conservative substitutions (as determined by methods referenced in Materials and Methods).

Figure 5. Immunocytologic Localization of the 20-kD CaBP in Interphase and Mitotic Chlamydomonas Cells

In interphase cells the antibody specifically stained the anterior region of the cells in a pattern resembling the form of a round bottom flask (Fig. 7). Through-focus analysis revealed that this staining pattern reflected the specific localization of the 20-kD CaBP to (a) connections between the basal body pair (the rim of the flask), (b) two fibers extending from the basal bodies to the nucleus (the neck of the flask), and (c) a diffuse array of longitudinally oriented filaments surrounding the nucleus (the round bottom of the flask). In Fig. 7, c and d, the position of the nucleus relative to the distribution of the 20-kD CaBP is documented with propidium iodide staining of the nuclear DNA (after RNase treatment of the cells). The punctate
Figure 6. The rabbit anti-20-kD CaBP serum specifically recognizes the 20-kD CaBP present in isolated basal body complexes and resolved by two-dimensional gel electrophoresis. (A) The relevant portion of an autoradiogram of the nitrocellulose transfer of a two-dimensional resolution of polypeptides found in isolated basal body complexes. (B) The immunaoalkaline phosphatase staining of the nitrocellulose transfer after incubation in a 1:1,000 dilution of the rabbit anti-20-kD CaBP immune serum.

staining with propidium iodide seen in the cytoplasm reflects the localization of chloroplast DNA in the cells (26). The double-labeled images shown in Fig. 7, e and f, demonstrate that the 20-kD CaBP is associated with fibrous elements distinct from the extensive interphase cortical array of microtubules found in Chlamydomonas. Although not evident in the micrographs shown in Fig. 7, we have observed that the interphase flagella consistently showed a weak uniform fluorescence along their length when stained with the rabbit anti-20-kD CaBP immune serum.

At the ultrastructural level, the basal bodies of a pair in interphase Chlamydomonas cells have been shown to be connected by a single distal striated fiber and two smaller proximal striated fibers (38). The 20-kD CaBP is likely to be specifically associated with one or more of these fibers. The existence of connecting fibers between the basal bodies and the nucleus in Chlamydomonas has been previously documented (41, 52) but these fibers have not been identified at the ultrastructural level in Chlamydomonas reinhardtii. In an extensive analysis of cells fixed by routine methods for thin-section electron microscopy, rare images were obtained of two densely staining and extremely thin fibers extending from the basal bodies toward the nucleus. Fig. 8 contains an electron micrograph of a thin section which passes through the anterior region of an interphase Chlamydomonas cell. Two densely stained, narrow fibers are resolved that extend from the lateral sides of the basal bodies toward the nucleus. In the inset of Fig. 8 is an oblique section through a basal body with an en face view of a proximally tapered fiber that appears to be composed of thin filaments interrupted by cross-striations with a regular periodicity of ~160 nm. Based on their disposition and ultrastructure it is likely that these fibers represent the counterparts to the basal body-nuclear connectors visualized at the light microscopic level by immunofluorescence in this and previous studies (41, 52). The ultrastructural counterpart to the array of longitudinal filaments surrounding the nucleus detected at the immunofluorescence level was not visualized in our thin-section electron microscopic analysis of intact Chlamydomonas cells.

The distribution of the 20-kD CaBP, as revealed by indirect immunofluorescence staining, was dramatically different in Chlamydomonas cells fixed in mitosis. Fig. 9 contains double-labeled immunofluorescence images of a Chlamydomonas mitotic spindle stained with a mouse anti-α-tubulin mAb raised in this laboratory and the rabbit anti-20-kD CaBP immune serum. In Chlamydomonas the mitotic spindle is intranuclear (23) and the duplicated basal body complexes are found at the poles of the spindle, lying outside of the nuclear envelope (10). The anti-20-kD CaBP serum specifically and intensely stained the polar regions of the mitotic spindle. Serial thin-section analysis of three mitotic spindle poles did not reveal any specific structural features which might account for the staining pattern observed at the light microscopic level. In this region, parallel-oriented pairs of basal bodies, devoid of distal transition zone structures and interphase striated connecting fibers, were found (data not shown).

The Chlamydomonas 20-kD CaBP is Antigenically Related to the Tetraselmis 20-kD Striated Fiber Root Protein

The pattern of immunofluorescence staining of interphase Chlamydomonas cells observed with the immune 20-kD CaBP serum paralleled that which was previously observed with a rabbit antibody raised against the major 20-kD polypeptide component of the large contractile striated fiber roots of the marine alga, Tetraselmis (52). In this previous study the antibody was found to specifically recognize a Chlamydomonas protein of a similar molecular mass. The 20-kD CaBP purified and characterized in this study has been found.
Figure 7. Indirect immunofluorescence staining of *Chlamydomonas* cells fixed in interphase. (a and b) Images of cells that were incubated in 1:50 dilutions of the rabbit anti-20-kD CaBP (a) preimmune and (b) immune sera and stained with an FITC-conjugated secondary antibody. (c and d) Images of a cell stained with (c) the rabbit anti-20-kD CaBP immune serum and (d) propidium iodide. (e and f) Images of a cell double labeled with (e) the rabbit anti-20-kD CaBP immune serum detected with a rhodamine-conjugated secondary antibody and (f) a mouse anti-α-tubulin mAb detected with an FITC-conjugated secondary antibody. Bars: (a and b) 5 μm; (c and d) 2 μm; (e and f) 4 μm.

to be antigenically related to the *Tetraselmis* striated fiber root protein. The purified protein was found by immunoblot analysis to cross react with a rabbit antibody raised by Salisbury et al. (40) against the *Tetraselmis* striated fiber root protein (data not shown). In addition, as seen in Fig. 10, the antibody raised against the *Chlamydomonas* 20-kD CaBP specifically recognized a protein in *Tetraselmis* whole cell lysates with an electrophoretic mobility corresponding in size to the *Chlamydomonas* antigen.

**Discussion**

The purification and characterization of a 20-kD polypeptide component of isolated basal body complexes of the unicellular green alga *Chlamydomonas reinhardtii* has been described in this study. This basal body-associated protein displayed several biochemical features in common with members of the calcium-modulated protein family which includes calmodulin, troponin C, and parvalbumin (for reviews see references 25, 49). These common characteristics included a relatively low molecular mass of 20 kD, an experimentally determined acidic pl of 5.3, an altered migration in SDS–polyacrylamide gels in the presence of added calcium, and a calcium-dependent interaction with phenyl-Sepharose which allowed its purification by affinity chromatography. The relatedness of the basal body-associated 20-kD CaBP to calcium-modulated proteins was confirmed when its amino acid composition was compared to that of *Chlamydomonas*...
Figure 9. Double-labeled indirect immunofluorescence images of a Chlamydomonas intranuclear mitotic spindle stained with (a) a mouse anti-α tubulin mAb detected with an FITC-conjugated secondary antibody and (b) the rabbit anti-20-kD immune serum detected with a rhodamine-conjugated secondary antibody. Bar, 2 μm.

Figure 8. A longitudinal thin-section electron micrograph through the apical region of an interphase Chlamydomonas cell. Two densely stained, narrow fibers (arrows) extend from the distal region of the lateral sides of the basal body pair (BB) toward the nucleus. (Inset) An oblique section through a basal body with an en face view of a proximally tapered fiber that appears cross-striated with alternate dark and light segments (lines). Bars, 0.2 μm.

Calcium-modulated proteins are characterized by homologous calcium-binding domains that conform to a helix-loop-helix (EF hand) structure (25). The region of identified sequence relatedness included those residues in calmodulin and troponin C that constitute the E-α helix and calcium-binding loop of the fourth calcium-binding domain in the proteins. These data suggest that the 20-kD CaBP has calcium-binding domains structurally related to those found in calcium-modulated proteins. This has been confirmed through the isolation and characterization of a cDNA containing the entire coding region for the Chlamydomonas 20-kD CaBP (19).

Although a detailed biochemical analysis of the calcium-binding properties of the 20-kD CaBP has not yet been undertaken, we have recently determined that the purified protein effectively binds 45Ca2+ (unpublished results). Studies are in progress to determine the affinity and number of active calcium-binding sites in the 20-kD CaBP.

The basal body complex in interphase Chlamydomonas cells has been shown previously to be physically linked to the underlying nucleus by two connecting fibers (41, 52). These fibers were visualized at the light microscopic level by indirect immunofluorescence staining with antibodies raised against the major 20-kD protein component of large striated fiber roots (SFRs) isolated from the marine alga, Tetraselmis (40). The Chlamydomonas protein recognized by the antibody was identified as a protein with a molecular mass of ~20 kD and, like the Tetraselmis antigen, was found to consist of two acidic isoforms (52). Based on immunological
analyses, the 20-kD CaBP purified and characterized in this study has been found to be homologous to the Tetraselmis 20-kD SFR protein. Additional studies are needed to determine the exact relationship of the 20-kD CaBP single isoform purified in this study with the two previously identified isoforms (52).

In this study the two basal body-nuclear connectors were visualized at the ultrastructural level in thin sections to be narrow fibers with a cross-striated morphology. Based on their disposition and morphology, these fibers correspond to the rhizoplasts or system II striated fiber roots which have been previously demonstrated to be a characteristic of flagellated unicellular green algae (35). The large SFRs in Tetraselmis and their smaller counterparts in Chlamydomonas have been shown to be calcium-sensitive contractile fibers (40, 41, 52). In both cells calcium induces an in vivo and in vitro shortening of the SFRs that brings the basal body complex and the nucleus closer to each other. Although a biological function for SFR contraction in Chlamydomonas and related algae has not been established, it has been recently observed that experimental deflagellation of Chlamydomonas cells is correlated with contraction of the SFRs (41).

There is evidence to suggest that the 20-kD CaBP may be a structural component of the SFRs in algal cells. The homologous protein in Tetraselmis was identified as the major protein component of isolated SFRs from the cell, representing >60% of the protein mass (40). This observation led the authors to suggest that contraction of the SFRs occurs through a calcium-induced shortening of 3–5-nm-diam filaments composed of the 20-kD protein. Although there is no direct evidence that the 20-kD CaBP is present in the algal SFRs in a filamentous form or that the protein can assemble filaments in vitro, there is another calcium-sensitive contractile system in which a low molecular mass calcium-binding protein has been identified as the major structural component. In peritrichous and heterotrichous ciliates, contraction of the cell body or a differentiated stalk region of the cell have been shown to be associated with an ATP-independent, calcium-induced shortening of contractile fibers composed of 3–5-nm-diam filaments (for reviews see references 2, 18). In peritrich ciliates, such as Vorticella, the contractile fiber within the stalk of the cells has been shown to be composed primarily of a calcium-binding protein with an M, of 20 kD, termed “spasmin” (2). Although the biochemical relationship of spasmin and the algal 20-kD SFR protein has not been fully established, preliminary evidence has been reported that the two proteins are antigenically related (40).

In addition to its localization to the SFRs, the 20-kD CaBP was found to be associated with a small fiber extending between the basal bodies of a pair. It has been demonstrated by immunoelectron microscopic studies with an antibody raised against the homologous Tetraselmis protein that the protein is specifically localized to the distal striated connecting fiber in the algal flagellate Spermatozopsis (33). In this system the distal connecting fiber has been shown to be, like the SFRs, a calcium-sensitive contractile fiber. Contraction of the fiber has been correlated with a change in the orientation of the basal bodies which occurs in response to an appropriate photostimulus (33).

The antibody raised against the 20-kD CaBP was also observed to weakly, but consistently, stain the entire length of Chlamydomonas interphase flagella. Similar observations were reported in the studies of Wright et al. (52). We recently have obtained direct biochemical evidence that the 20-kD CaBP is a minor component tightly associated with the flagellar axoneme (results to be published elsewhere). Although the precise ultrastructural localization of the protein in the axoneme remains to be determined, we have observed that it is solubilized from isolated axonemes by high salt extraction and corresponds to a component previously shown to copurify with a 12.5-S dynein ATPase isolated from Chlamydomonas (37).

In Chlamydomonas cells fixed in mitosis, the 20-kD CaBP was also found associated with the duplicated basal bodies at the poles of the mitotic spindle. However, in contrast to interphase cells in which the protein was localized to discrete basal body–associated fibers, the 20-kD CaBP appeared to be more focally distributed around the mitotic basal body complexes. At the present time we have not determined how the distribution of the 20-kD CaBP changes at the transition of interphase and mitosis, or how its interphase fiber organization is reestablished at the end of mitosis. More detailed studies on synchronized mitotic cells are needed to capture these events at the immunofluorescence level. The function of the 20-kD CaBP as a component of the mitotic spindle poles in Chlamydomonas is not presently known. An obvious role would be the maintenance of the association of the duplicated basal body complexes with the nucleus during mitosis and/or an involvement in the process of splitting and migration of the daughter complexes to the spindle poles, and ultimately into the daughter cells.

The focal pattern of localization of the 20-kD CaBP to the mitotic spindle poles in Chlamydomonas resembles the centrosomal staining in animal cells which has been observed with human autoimmune antibodies (5). These antisera have been shown at the electron microscopic level to recognize antigenic determinants found in the pericentriolar material surrounding the centrioles (5). These observations are particularly relevant since Salisbury et al. (39) have reported a similar pattern of immunofluorescence staining of the centrosomes in cultured mammalian cells with a polyclonal antibody raised against the Tetraselmis 20-kD SFR protein. If further studies establish that there has been an evolutionary conservation of the association of proteins homologous to the algal 20-kD CaBPs with microtubule-organizing centers of divergent structure, it would suggest that the proteins are of fundamental importance in the function of these major determinants of cell polarity and reproduction.

As an approach toward gaining further information about the structure–function relationship of the 20-kD CaBP, we have undertaken the isolation of cloned cDNA for the protein. In the accompanying manuscript (19), experiments documenting the isolation and characterization of a cDNA clone containing the entire coding region of the Chlamydomonas 20-kD CaBP are described.

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