

Mutational Analysis of Centrin: An EF-Hand Protein Associated with Three Distinct Contractile Fibers in the Basal Body Apparatus of *Chlamydomonas*

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Abstract. Centrin, a 20-kD phosphoprotein with four calcium-binding EF-hands, is present in the centrosome/basal body apparatus of the green alga *Chlamydomonas reinhardtii* in three distinct locations: the nucleus–basal body connectors, the distal striated fibers, and the flagellar transition regions. In each location, centrin is found in fibrous structures that display calcium-mediated contraction. The mutant *vfl2* has structural defects at all of these locations and is defective for basal body localization and/or segregation. We show that the *vfl2* mutation is a G-to-A transition in the centrin structural gene which converts a glutamic acid to a lysine at position 101, the first amino acid of the E-helix of the protein's third EF-hand. This proves that centrin is required to construct the nucleus–basal body connectors, the distal striated fibers, and the flagellar transition regions, and it demonstrates the importance of amino acid 101 to normal centrin function. Based on immunofluorescence analysis using

anti-centrin antibodies, it appears that *vfl2* centrin is capable of binding to the basal body but is incapable of polymerizing into filamentous structures. 19 phenotypic revertants of *vfl2* were isolated, and 10 of them, each of which had undergone further mutation at codon 101, were examined in detail. At the DNA level, 1 of the 10 was wild type, and the other 9 were pseudorevertants encoding centrins with the amino acids asparagine, threonine, methionine, or isoleucine at position 101. No ultrastructure defects were apparent in the revertants with asparagine or threonine at position 101, but in those with methionine or isoleucine at position 101, the distal striated fibers were found to be incomplete, indicating that different amino acid substitutions at position 101 can differentially affect the assembly of the three distinct centrin-containing fibrous structures associated with the *Chlamydomonas* centrosome.

THE small calcium-binding protein centrin (also known as caltractin) belongs to the superfamily of calcium-binding proteins that includes calmodulin, parvalbumin, and troponin C. Centrin is found in three locations in the *Chlamydomonas* nucleo–basal body apparatus: 1) the two nucleus–basal body connectors that link the basal bodies to the nucleus, 2) the distal striated fiber that connects the basal bodies to each other, and 3) the flagellar transition regions between the basal bodies and their flagella (Wright et al., 1985; Schutze et al., 1987; Salisbury et al., 1988; Sanders and Salisbury, 1989). Within the transition region, centrin is thought to reside in the stellate fibers that link the doublet microtubules to the central transition cylinder.

The stellate fibers and the nucleo–basal body connectors have been shown to undergo calcium-dependent contraction in vitro, both in isolated nucleo–flagellar apparatuses and in detergent-extracted cell models (Salisbury et al., 1984; Salisbury et al., 1987). Such contraction also occurs in vivo in conjunction with flagellar shedding, a phenomenon that is dependent upon calcium in the external medium (Lewin and Lee, 1985; Sanders and Salisbury, 1989; Jarvik and Su-

han, 1991). In addition, the nucleus–basal body connectors have been shown to contract in a cell cycle-dependent manner, shortening dramatically in early prophase to bring the basal bodies and the nucleus into close apposition (Salisbury et al., 1988; Wright et al., 1989).

The nucleo–basal body apparatus represents the *Chlamydomonas* centrosome (Gaffal, 1988), and so in this organism centrin is clearly a centrosomal protein. But the association of centrin with the centrosome is by no means confined to *Chlamydomonas*; in fact, on the basis of immunological cross-reactivity, centrin-like proteins have been identified in the centrosomes of every algal, protozoan and metazoan cell in which they have been sought (Salisbury et al., 1986; Melkonian et al., 1988; Baron et al., 1991). In mammalian (PtK₂) cells the antigen shows a punctate peri-centrosomal distribution which moves in conjunction with the centrosome throughout the cell cycle. This antigen was initially reported to be ~165 kD in molecular mass (Baron et al., 1991), but more recent analysis has shown that vertebrate cells possess a centrin homolog of standard (~20 kD) size (Salisbury, J., personal communication). The yeast *Sac-*

charomyces cerevisiae also possesses a centrin homolog—the product of the *CDC31* gene—that shows 50% amino acid sequence identity to *Chlamydomonas* centrin (Baum et al., 1986; Huang et al., 1988). In the absence of this protein, the spindle pole bodies, which are the centrosome equivalents in yeast, enlarge but fail to separate into two distinct daughter organelles. Thus, it appears that centrin or a centrin homolog is a regular component of the centrosome in all, or nearly all, eucaryotic cells.

Our understanding of the cellular role of centrin in *Chlamydomonas* has benefited greatly from the analysis of the variable flagella number mutant *yf2*. *yf2* cells lack centrin-containing structures and possess reduced cellular quantities of centrin (Kuchka and Jarvik, 1982; Wright et al., 1983; Hoops et al., 1984; Adams et al., 1985; Wright et al., 1985; Wright et al., 1989; Jarvik and Suhan, 1991). Because *yf2* cells are defective in properly localizing their basal bodies and/or segregating them to daughters at mitosis (Kuchka and Jarvik, 1982; Wright et al., 1985; Wright et al., 1989), we have inferred that centrin-containing structures play significant roles in centrosome function. On the other hand, *yf2* cells are not defective for flagellar autotomy (Jarvik and Suhan, 1991), a process that involves microtubule scission in the transition region, and so we have inferred that the stellate fibers of the transition region, which are absent in *yf2*, are not required for autotomy (Jarvik and Suhan, 1991).

In the study described here, we show that the *yf2* mutation is in the centrin structural gene, changing a glutamic acid to a lysine at position 101, the canonical first glutamic acid of the third of the protein's four calcium-binding EF-hands. We describe a number of pseudorevertants whose centrins have different amino acid replacements at position 101, and we show that some of these revertants are differentially affected with respect to the assembly and function of the three known centrin-containing fibrous structures associated with the nucleo-basal body apparatus.

Materials and Methods

Strains and Culture Conditions

All wild-type strains: *Chlamydomonas reinhardtii* 137c NO *mt*⁺, 137c NO *mt*⁻, and *Chlamydomonas smithii* (Hyams and Davies, 1972) were obtained from the Chlamydomonas Culture Collection, Duke University, Durham, NC. *cwl5* *mt*⁺ was obtained from Dr. D. Kirk. *yf2-220* (referred to throughout this paper simply as *yf2*) was obtained by UV mutagenesis in our laboratory (Kuchka and Jarvik, 1982). All cells were grown under constant illumination in (Sagar and Granick, 1953) medium I (M media), usually supplemented with 0.1% yeast extract, 0.1% proteose peptone, and 0.01 M sodium acetate (YE media). Crosses between *yf2-220* and *C. smithii*, as well as between *cwl5*, *yf2-220* revertants, and *yf2-220* were done by standard methodology (Harris, 1989).

Genomic DNA Isolation

Genomic DNAs from progeny of the *yf2-220* by *C. smithii* cross were isolated on cesium chloride gradients by an adaptation of the method of Weeks et al. (1986). Cells grown on M medium 1.5% agar plates were scraped into centrifuge tubes and washed with 10 ml sterile distilled H₂O. The cells were resuspended in 2 ml water, and 312 μ l of 20% SDS was added with gentle mixing. 454 μ l 5× extraction buffer (345 mM Tris-HCl, pH 8.0, 1.38 M NaCl, 138 mM EDTA, pH 8.0) was then added and the mixture was gently mixed again. The cell suspension was incubated at 50°C for 1 h. 2.66 g CsCl and 157 μ l ethidium bromide (10 mg/ml) were added to the mixture. It was then loaded into 13 × 32 mm quick seal ultracentrifuge tubes and centrifuged in a TL100 ultracentrifuge for 8 h at 75 k rpm (Beck-

man Instrs. Inc., Fullerton, CA). DNA bands were removed with a 1-cc syringe through an 18-gauge needle. The ethidium bromide was removed by repeated extractions with water-saturated N-butanol, and the DNA was ethanol precipitated overnight with 0.3 M sodium acetate (pH 5.2). DNA was then collected by centrifugation, washed twice with 70% ethanol, air dried, and resuspended in 100 μ l TE (10 mM Tris-HCl, 1 mM EDTA), pH 7.5.

Restriction Fragment-length Polymorphism (RFLP)¹ Analysis

Genomic DNAs were digested for 4 h at 37°C with BamHI (Boehringer Mannheim Corp., Indianapolis, IN) as per supplier's specifications. The DNAs were run out on 0.8% agarose gels for Southern transfer to nitrocellulose (Maniatis et al., 1982). λ DNA digested with HinDIII was used as a sizing standard. The blots were prehybridized in 0.5 M NaCl, 0.1 M sodium phosphate, pH 7.0, 6 mM EDTA pH 8.0, 1% SDS, and 0.1 mg salmon sperm DNA for 1 h at 68°C. Hybridizations were done for 18 h at 68°C in the same buffer with the addition of random primed labeled probe. The blot was washed three times at 68°C in 2× SSC, 25 mM Na₂HPO₄, 0.1% sodium pyrophosphate, and 0.1% SDS. The blots were further washed in a 50% dilution of this buffer and finally in a 20% dilution. The blots were dried and subjected to autoradiography. The probe was generated from the pCaBP4 (Huang et al., 1988) centrin-containing plasmid. The labeling reaction was done at room temperature for 1 h using 100 ng DNA, 60 ng random hexanucleotide primer (Boehringer Mannheim Corp.), and 10 U Klenow (New England Biolabs, Beverly, MA) in the presence of 7.5 mM Tris-HCl, pH 7.6, 5.5 mM DTT, 5 mM MgCl₂, 25 μ M dNTPs, and 30 mg ³²PdCTP (30 μ Ci) from New England Nuclear/DuPont. The reaction was added to 80 μ l of TE pH 7.6 and the unincorporated nucleotides were removed by Sephadex G-50 spin column (Maniatis et al., 1982).

DNA Sequence Analysis

DNAs were amplified from genomic DNAs by the use of Boehringer Mannheim Corp. *Taq* polymerase in the following conditions: 50 mM KCl, 20 mM Tris-HCl, pH 8.4, 2.5 mM MgCl₂, and 0.1 mg/ml BSA (BRL nuclease-free). 1 U of the polymerase, 50 pmol of each primer, and varying amounts of genomic DNA were also used. The amplification procedure consisted of 30 cycles of denaturing at 95°C, annealing at 55°C, and extending at 72°C in a thermocycler (Perkin-Elmer Cetus Instrs., Norwalk, CT). The reactions were done in 100 μ l final volume under 100 μ l of mineral oil. After the amplification procedure, the mineral oil was removed by CHCl₃/isoamyl alcohol extraction, and the products were separated on a 0.8% agarose gel and visualized with ethidium bromide. Amplification was carried out with primers homologous to sequences in the 5' and 3' noncoding regions of the centrin gene. The primers were as follows: 5' sequence at position 35 5'-CGTCTCTAGCTTCCTAAAGTCG-3'; 3' sequence is at position 1910 5'-CATGAGACCACTCTCACATC-3' (position designations are as given in Lee et al. [1991]). For sequence analysis the double-stranded product was asymmetrically reamplified to obtain single-stranded sequencing template with each of the above primers alone. The reamplification was conducted in an identical manner as the initial amplification. The DNA was purified and analyzed as above, and finally ammonium acetate/ethanol precipitated, washed, and resuspended in TE, pH 7.5. The single-stranded DNA was then used in sequencing reactions with the Sequenase 2.0 sequencing kit (U.S. Biochemical Corp., Cleveland, OH) as per the manufacturer's directions. The sequencing reactions were separated on 6% polyacrylamide/8 M urea gels. The primers used in sequencing the centrin gene included the above primers and the following: position 434 5'-CAGAAT-GTTGCAACGAG-3'; position 1111 5'-ATGCCAGAACGCGAGACATG-3'; position 1124 5'-GAGATTGACAAGGACGGCTC-3'; and position 1179 5'-ATCATGGTCAGGAGGTCTC-3'.

yf2 Revertant Isolation

To obtain revertants of the *yf2-220* mutation, individual clones of a strain carrying the *yf2* mutation in a *cwl5* (cell wall-less) (Hyams and Davies, 1972) background were spread on M plates and mutagenized by UV irradiation to a survival of ~5%. The irradiated plates were placed in the dark for a period of 24 h, after which they were removed and allowed to grow under standard illumination for an additional 4 d. Cells were then scraped from each plate, transferred to 5 ml of M media in 13 × 100 mm tubes, and allowed to grow while illuminated from above. Under these conditions, wild-type cells exhibit positive phototaxis and are found at the meniscus as well as throughout the medium, whereas *yf2* cells settle to the bottom be-

cause of their phototaxis defect. To collect revertants, the upper 2 ml of each culture was removed and transferred to a new 13×100 mm tube containing 3 ml of fresh media and allowed to grow for several additional days, whereupon the transfer and growth protocol was repeated. In all, the process was repeated four times, after which ~50% of the tubes (19 of 36) showed dense growth of phototaxis-competent cells throughout the media and at the meniscus. These cells were streaked on M plates, and single colonies were picked from the plates and retested for phototaxis as well as for flagellar number and swimming. Only one colony from each culture was retained to ensure that each revertant was of independent origin.

Preparation of Cell Ghosts and Nucleo-Flagellar Apparatuses

Extracted cells (cell ghosts) were prepared by incubating a suspension of walled (cw^+) cells in M medium with an equal volume of M medium containing 10 mM dibucaine, and 5% sucrose for 5 min at room temperature to induce deflagellation and nucleus–basal body connector contraction. The suspension was then incubated for an additional 60 min at room temperature with 1/9 volume of 10% NP-40. Nucleo-flagellar apparatuses were prepared from wall-less cells as described previously (Wright et al., 1985). The extraction medium used was 1% NP-40 in NB minus Ca^{++} (25 mM KCl, 10 mM MgCl_2 , 3.7 mM EDTA, 0.67 mM Tris-HCl, pH 7.3).

Electron Microscopy

Cells were collected by centrifugation and resuspended in M media containing 2% glutaraldehyde (Jarvik and Suhan, 1991). After ~60 min at room temperature, the cells were washed several times in media with 2% glutaraldehyde, postfixed in 1% osmium tetroxide, and rinsed twice with water. The samples were en bloc stained for 30 min with uranyl acetate (1%). Dehydration was done with a graded ethanol series and the samples were rinsed in propylene oxide. The samples were infiltrated with Polybed 812 resin under vacuum (10 psi) at 50°C and polymerization was done for 12–24 h. The samples were sectioned on an MT-2B microtome (Sorvall Instruments Div., Newton, CT) using a diamond knife. The silver to gold sections were stained with aqueous uranyl acetate (1%) and Reynold's lead citrate. The samples were examined using an electron microscope (model 300; Philips Electronic Instrs. Co., Mahwah, NJ) at 60 kV (Jarvik and Suhan, 1991).

Indirect Immunofluorescence

Immunofluorescence was performed using monoclonal antiserum 17E10 kindly provided by Mark Sanders and Jeffery Salisbury. The antibody was used at a dilution of 1:200 in PBS. Nucleo-flagellar apparatuses were prepared as described above on 10-well slides (Carlson Scientific, Peotone, IL) coated with polyethylenimine. The slides were blocked for 10 min with calf serum and BSA in PBS before incubation with the antibody. The primary antibody incubation was conducted for 1 h at room temperature. The slides were washed twice in Tween 20 containing PBS and once in PBS. The slides were blocked again and incubated with a 1:32 dilution of RITC-conjugated goat anti-mouse antibody for 1 h. The secondary antibody was washed away as above and the slide was mounted with phenylenediamine containing glycerol, covered with a coverslip, and sealed with nail polish. The slides were examined on an Axio-Phot microscope (Carl Zeiss, Inc., Thornwood, NY) through a $100\times$ Plan Neofluor objective. The images were captured on Hypertech film (Microfluor, Stonybrook, NY). The exposure times as well as printing methods were kept constant for the fluorescent images so that meaningful comparisons between different samples could be made.

Results

RFLP Analysis: Linkage of the *vfl2* Mutation to the Gene Encoding Centrin

To test for genetic linkage between the *vfl2* mutation and the centrin structural gene, we took advantage of a BamHI RFLP between *Chlamydomonas reinhardtii* and *Chlamydomonas smithii* that is detected by the full-length centrin cDNA sequence pCaBP4 (Huang et al., 1988). A cross was performed between *C. smithii* and a *C. reinhardtii* strain car-

rying the *vfl2* mutation as well as *nit1* and *nit2* mutations. Both of these *nit* mutations result in an inability to utilize nitrate as a sole nitrogen source. Several dozen random meiotic progeny were isolated from this cross, and the flagellar number phenotypes of these strains were determined by light microscopic inspection. Strains were scored as *Vfl⁻* if they showed variable numbers of flagella per cell, aberrant swimming patterns, and defective phototaxis. 18 progeny strains from the cross were chosen for further analysis: 9 with *Vfl⁻* phenotypes and 9 with *Vfl⁺* phenotypes. To confirm that these strains were truly meiotic products, they were also scored for their *Nit* phenotypes and for mating type, the determinants of which were found to segregate as expected. Southern blots of BamHI-digested genomic DNA from *C. reinhardtii*, *C. smithii*, and 17 of the 18 strains were probed with pCaBP4. The *C. reinhardtii* control DNA yielded a 7.5-kb fragment, and the *C. smithii* DNA yielded an 8.5-kb fragment. All progeny strains with a *Vfl⁻* phenotype showed the 7.5-kb fragment, and all *Vfl⁺* strains showed the 8.5-kb fragment. These results demonstrate genetic linkage between the *vfl2* mutation and the centrin structural gene, and they suggested to us that the *vfl2* mutation was likely to be in the centrin gene itself.

DNA Sequence Analysis: the *vfl2* Mutation Is a G-to-A Transition That Converts a Glutamic Acid to a Lysine at Position 101 of the Centrin Polypeptide

To directly identify the *vfl2* mutation, genomic DNA was prepared from a strain harboring the *vfl2* mutation, and a DNA species that included the entire centrin coding sequence was amplified by means of the polymerase chain reaction (PCR) using primers that lay within the 5' and 3' noncoding regions of the gene 1,838 bp apart (see Materials and Methods for primer sequences and PCR conditions). Electrophoresis of the reaction products on 0.8% agarose gels revealed a single amplified band of ~2 kb. Single-stranded DNA suitable for sequence analysis was then prepared by asymmetric amplification of the PCR product with each of the primers. Additional primers, as indicated in Materials and Methods, were used to obtain sequence data representing the entire coding sequence of the gene. Only one departure from the wild-type sequence was observed in the *vfl2* DNA: a G-to-A transition at position 1214. To be sure that this transition did not arise as a result of *Taq* polymerase infidelity in the PCR reactions, and to confirm that the mutation cosegregates with the *Vfl⁻* phenotype, sequencing was performed on DNAs prepared from four additional *vfl2* strains representing independent segregants from a *vfl2* x *wild-type* cross; like the first DNA analyzed, these DNAs all had adenine at position 1214. We therefore conclude that the *vfl2* mutation is a G-to-A transition at position 1214 of the centrin structural gene. This mutation leads to a glutamic acid to lysine substitution at position 101 of the centrin polypeptide. Additional genetic evidence that this substitution is responsible for the *vfl2* phenotype will be provided below.

1. Abbreviations used in this paper: PCR, polymerase chain reaction; RFLP, restriction fragment-length polymorphism.

Table I. Nucleotide Changes at Codon 101 and the Corresponding Amino Acid Substitutions

| Strain | Codon 100 | Codon 101 |
|-----------------|-----------|-----------|
| Wild type | GAG (Glu) | GAG (Glu) |
| <i>vfl2</i> | — | AAG (Lys) |
| <i>vfl2 R13</i> | — | GAG (Glu) |
| <i>vfl2 R1</i> | — | ATT (Asn) |
| <i>vfl2 R8</i> | — | AAC (Asn) |
| <i>vfl2 R5</i> | — | ACG (Thr) |
| <i>vfl2 R11</i> | — | ATG (Met) |
| <i>vfl2 R23</i> | GAA (Glu) | ATG (Met) |
| <i>vfl2 R29</i> | — | ATG (Met) |
| <i>vfl2 R31</i> | — | ATG (Met) |
| <i>vfl2 R10</i> | — | ATT (Ile) |
| <i>vfl2 R19</i> | — | ATT (Ile) |

Nucleotide and amino acid changes are shown in bold type. Codon 100 is included because one mutant carries a silent mutation there.

Isolation and DNA Sequence Analysis of Phenotypic Revertants of *vfl2*: Four New Mutant Species of Centrin

19 independent biflagellate phenotypic revertants of *vfl2* were isolated as described in Materials and Methods. When cell lysates of the revertant strains were observed by differential interference contrast microscopy to identify nucleoflagellar apparatuses, it was found that each strain released such apparatuses, indicating that all were revertant with respect to the presence of nucleus–basal body connectors. Nine of the revertants (*vfl2 R11*, *vfl2 R13*, *vfl2 R14*, *vfl2 R15*, *vfl2 R16*, *vfl2 R18*, *vfl2 R23*, *vfl2 R32*, and *vfl2 R34*) were crossed to wild-type, and a minimum of 50 progeny from each cross were scored for their Vfl phenotypes to determine whether any of them carried an unlinked suppressor of the *vfl2* mutant phenotype. All of the progeny were Vfl⁺, indicating that none of the revertant strains carried the *vfl2* mutation along with an unlinked suppressor. It therefore appeared likely that reversion had occurred within the centrin structural gene itself.

To look for the mutations responsible for reversion of the *vfl2* phenotype, DNA sequence analysis of the centrin gene was performed on 14 of the 19 revertants. Four were found to retain lysine at codon 101; these will be described in a separate publication and will not be discussed further here. The other 10 had sustained new mutations at codon 101. These revertants provide unambiguous genetic proof that the G to A transition at codon 101 is responsible for the *vfl2* phenotype. These revertants, and their DNA sequences at and around codon 101, are listed in (Table I). In the table, the mutants have been clustered together on the basis of their amino acid substitutions at position 101 and the mutated nucleotides are highlighted in bold type. One revertant (*vfl2 R13*) has a wild-type DNA sequence and therefore represents a true revertant. The other nine are pseudorevertants whose nucleotide sequences are different from both wild-type and the *vfl2* parent. Six distinct new centrin gene mutations are represented and together they encode four new amino acids at position 101. Thus, including wild-type and the *vfl2* parent, the set of strains analyzed here encode centrin poly-

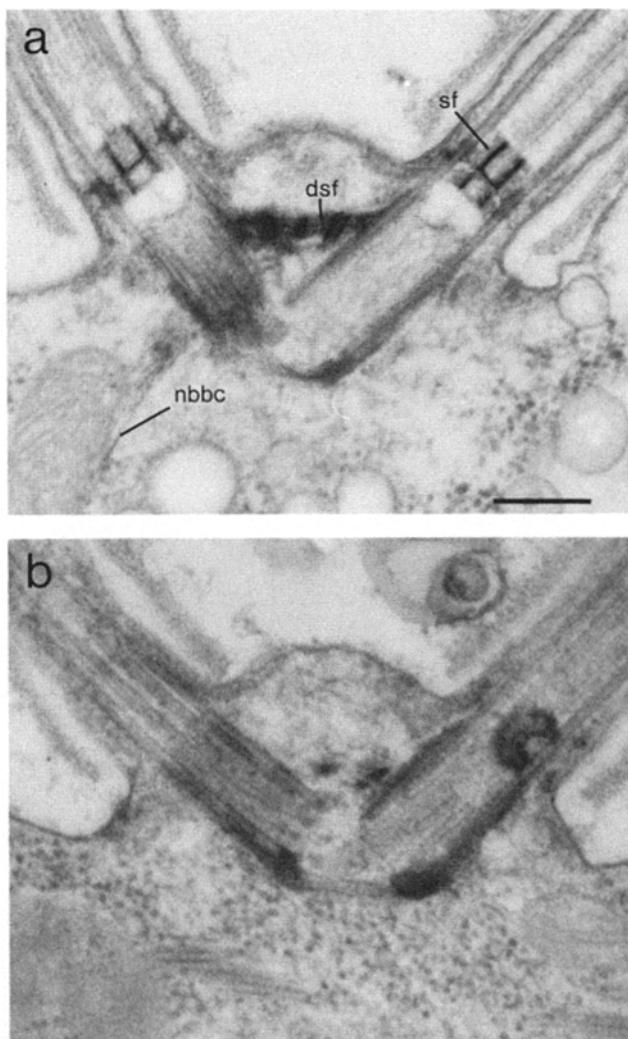


Figure 1. Ultrastructure of basal body apparatus in wild-type (a) and *vfl2* (b). nbbc; nucleus–basal body connector; dsf; distal striated fiber; sf; stellate fiber. Bar, 200 nm.

peptides with six different amino acids at position 101: glutamic acid, lysine, methionine, asparagine, threonine, and isoleucine.

We note that the revertants were isolated after uv mutagenesis, and that the *vfl2* parent carries a pair of adjacent thymines in its antisense strand that could be a target for uv-promoted dimerization and subsequent mutation via excision/repair. Such a mechanism might explain the fact that of the 10 revertants with changes at codon 101, three (*vfl2 R10*, *vfl2 R19*, and *vfl2 R23*) experienced a change of more than one nucleotide.

Electron Microscopy: Not All Revertants Are Fully Wild-Type in Ultrastructure

Nucleus–basal body connectors, distal striated fibers, and stellate fibers are prominent components of the nucleoflagellar apparatus in wild type but are conspicuously absent in *vfl2* (Jarvik and Suhan, 1991). Examples of wild-type and *vfl2* ultrastructure are shown in Fig. 1. All 10 revertants that had experienced mutation at codon 101 were examined by electron microscopy to determine their phenotypes at the ul-

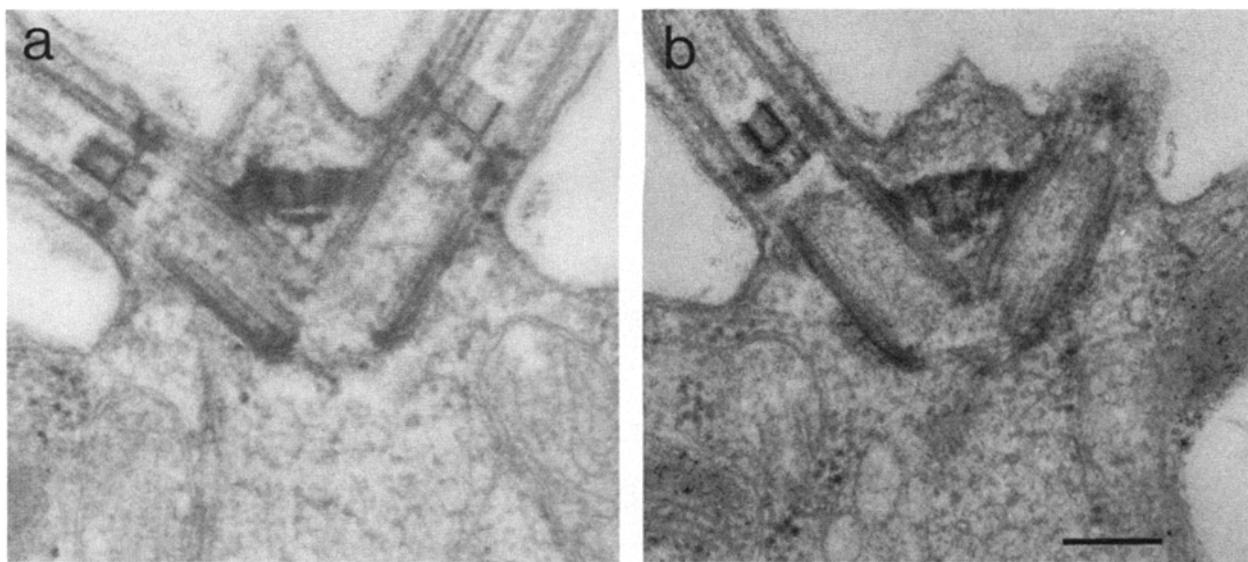


Figure 2. Ultrastructure of basal body apparatus in class 1 revertants. (a) *vfl2 R5*; (b) *vfl2 R8*. Bar, 200 nm.

trastructure level. Based on these examinations, we place them into two phenotypic classes. Class 1 contains four members (*vfl2 R1*, *vfl2 R5*, *vfl2 R8*, and *vfl2 R13*), all of which are fully wild type at the level of resolution afforded by our electron microscopic examination. Examples are shown in Fig. 2. These strains carry either glutamic acid (the wild-type amino acid) or the neutral polar amino acids asparagine or threonine at position 101 of the centrin polypeptide. Class 2 contains six members (*vfl2 R10*, *vfl2 R11*, *vfl2 R19*, *vfl2 R23*, *vfl2 R29*, and *vfl2 R31*), all of which have apparently normal nucleus–basal body connectors and stellate fibers, but carry distal striated fibers that are defective in the central region. Examples are shown in Fig. 3. The class 2 strains carry the hydrophobic amino acids methionine or isoleucine at position 101 of the centrin polypeptide. In the strains with isoleucine at position 101, complete distal striated fibers were never observed; instead, incomplete fibers were observed extending towards each other, but never meeting (Fig. 3, *a* and *b*). The methionine-carrying strains showed a more variable phenotype, with some basal body pairs showing two incomplete distal fibers, and others showing a single fiber whose central region appeared less substantial than in wild type. Examples are shown in Fig. 3, *c*–*e*. An additional defect was occasionally observed in the methionine-carrying members of class 2: electron-dense material was observed just distal to the transition region in a small fraction (<2%) of their flagella (Fig. 3 *f*). While this may reflect a functional defect in the transition region of some class 2 cells, the class 2 transition region typically had a normal ultrastructure that included stellate fibers of normal morphology (Fig. 4).

Localization of Centrin by Indirect Immunofluorescence

Anticentrin mAb 17E10 (Salisbury et al., 1988) stains wild-type nucleoflagellar apparatuses in a distinctive pattern (Wright et al., 1985; Salisbury et al., 1988; Wright et al., 1989). This pattern includes (*a*) intense staining of the nucleus–basal body connectors, (*b*) intense staining in a barlike pattern perpendicular to the connectors at the level of the dis-

tal striated fiber, (*c*) intense staining in perinuclear fimbriae that radiate for several micrometers from the points where the nucleus–basal body connectors contact the nucleus, (*d*) weak staining in additional fimbriae that radiate over much of the nucleus, (*e*) weak staining in the transition region, and (*f*) very weak staining along the length of the axoneme. *vfl2* apparatuses show a much simplified staining pattern, with the strongest signal confined to a single dot associated with each basal body (Wright et al., 1989), and with weaker staining extending into the transition region and along the axoneme. An example is shown in Fig. 5. Measurement of the dot-to-dot distance for a number of *vfl2* basal body pairs gave a distance of 560 ± 45 nm ($n = 13$). Measurement of the length of the bar of staining between the basal bodies in wild type gave a very similar value: 580 ± 50 nm ($n = 10$). These distances are significantly less than the expected distance between the two transition regions, and nearly twice the known end-to-end distance for the distal striated fiber. From these observations, we conclude that the dots of staining in *vfl2* are located on or around the basal bodies themselves (see Discussion).

Immunostaining of nucleo-flagellar apparatuses from each of the revertants with antibody 17E10 yielded patterns that were consistent with their phenotypes at the ultrastructural level (Fig. 6). Thus, the class 1 patterns were essentially indistinguishable from that of wild type (Fig. 6, *e* and *f*), and the class 2 pattern showed a reduction in staining intensity between the basal bodies, at the position of the distal striated fibers, that was particularly pronounced in the strains with isoleucine at position 101 of centrin (Fig. 6, *j*–*l*).

All Revertants Exhibit Contraction of the Nucleus–Basal Body Connector and Stellate Fibers

When the *Chlamydomonas* cell sheds its flagella by the active process known as flagellar autotomy, there occurs dramatic calcium-dependent contraction of the stellate fibers and of the nucleus–basal body connectors. Six revertants (*vfl2 R11*, *vfl2 R13*, *vfl2 R14*, *vfl2 R16*, *vfl2 R23*, and *vfl2 R32*) were ex-

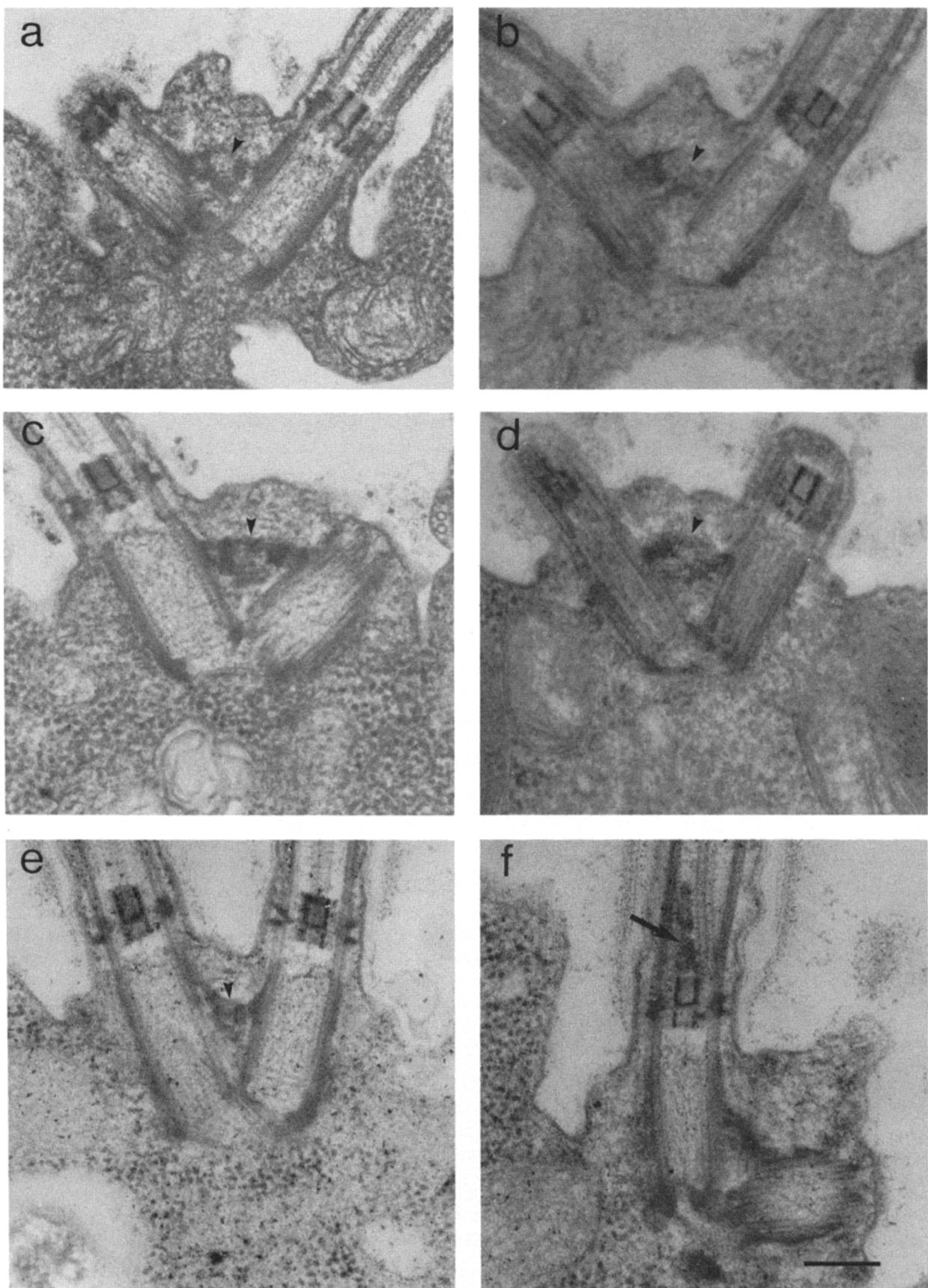


Figure 3. Ultrastructure of basal body apparatus in class 2 revertants. (a) *vfl2 R10*; (b) *vfl2 R19*; (c) *vfl2 R23*; (d) *vfl2 R29*; (e and f) *vfl2 R11*. Arrowheads indicate aberrant central regions of the distal striated fibers. Arrow in f indicates amorphous material distal to the transition region. Bar, 200 nm.

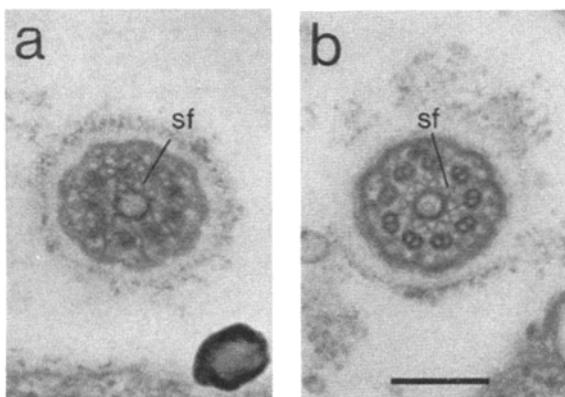


Figure 4. Ultrastructure of the transition region in transverse section in class 1 and 2 revertants showing the presence of stellate fibers. (a) *vfl2 R29*; (b) *vfl2 R19*. sf, stellate fiber. The different appearance of the two sections reflects the fact that they are at different levels within the transition region and does not indicate an intrinsic difference in morphology between the strains. Bar, 200 nm.

amined with respect to these contraction phenomena by treating them with the autotomy-inducing agent dibucaine, extracting with 1% NP-40 to produce cell ghosts, and observing them by thin section transmission electron microscopy. In cells prepared in this way, it is particularly easy to observe the nucleus–basal body connectors because (a) the cytoplasm is largely extracted, providing greater contrast,

and (b) the mitochondria, which normally lie very close to the connectors and can obscure them, are extracted. All of the strains displayed contracted nucleus–basal body connectors. In addition, all exhibited autotomy, and in each case autotomy was accompanied by contraction of the stellate fibers. Examples are shown in Fig. 7. We also observed what appeared to be contraction of the distal striated fiber in these cell ghosts, with the fiber showing a pronounced thickening at its center, and with the angle between the basal bodies becoming more acute than in nontreated cells (cf. Figs. 2 and 3 with Fig. 7, a and b). This angle was not reduced in basal body pairs with incomplete distal striated fibers from the class 2 mutant *vfl2 R23* (Fig. 7 d), a result consistent with the interpretation that contraction of the distal striated fiber is responsible for reducing the angle between the basal bodies.

Discussion

Centrin Is Required to Construct the Nucleus–Basal Body Connectors, the Distal Striated Fibers, and the Stellate Fibers, and It Is Also Required for Accurate Basal Body Localization and/or Segregation

Previously reported analyses of the *vfl2* phenotype led to a number of conclusions about the functions of the various centrin-containing structures in *Chlamydomonas*. Thus, having demonstrated that *vfl2* is defective for the nucleus–basal body connector, we were able to conclude that

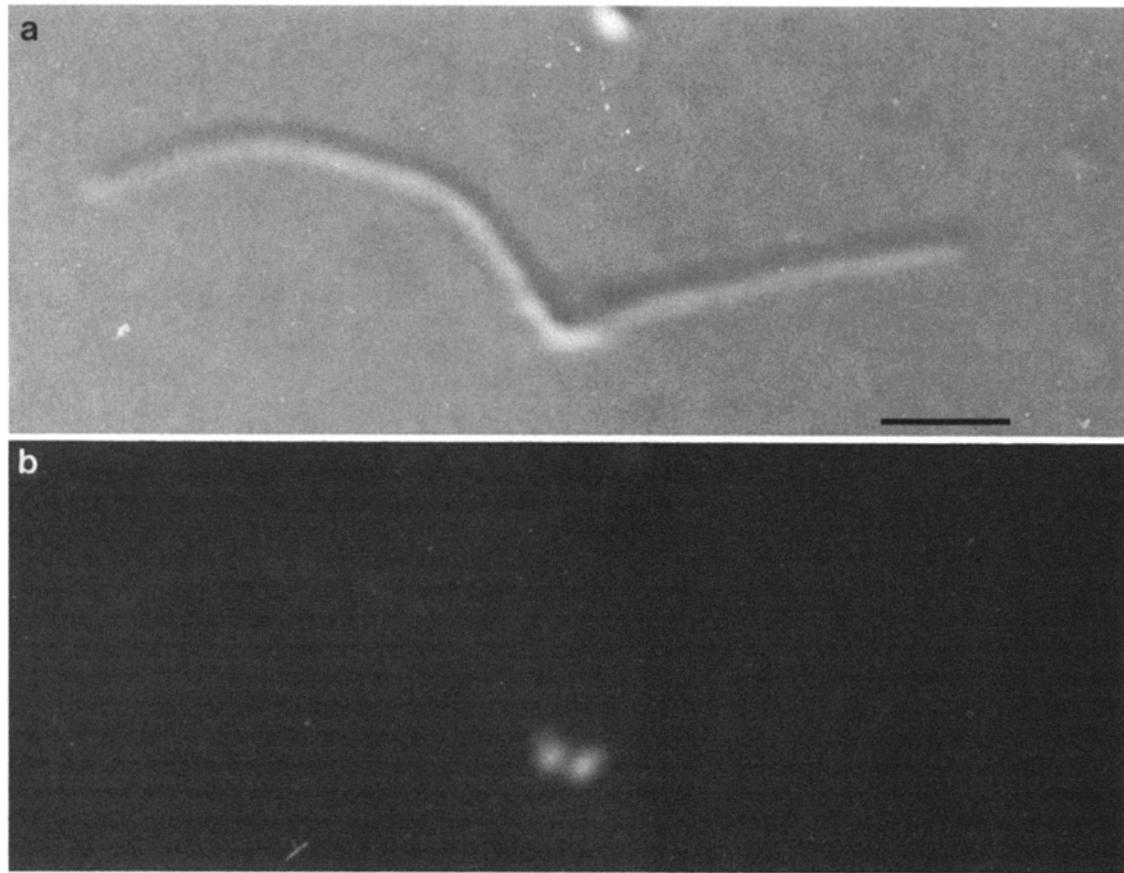


Figure 5. Flagellar apparatus from *vfl2*. (a) Differential interference contrast. (b) Indirect immunofluorescence using anticentrin monoclonal antibody. Bar, 2 μ m.

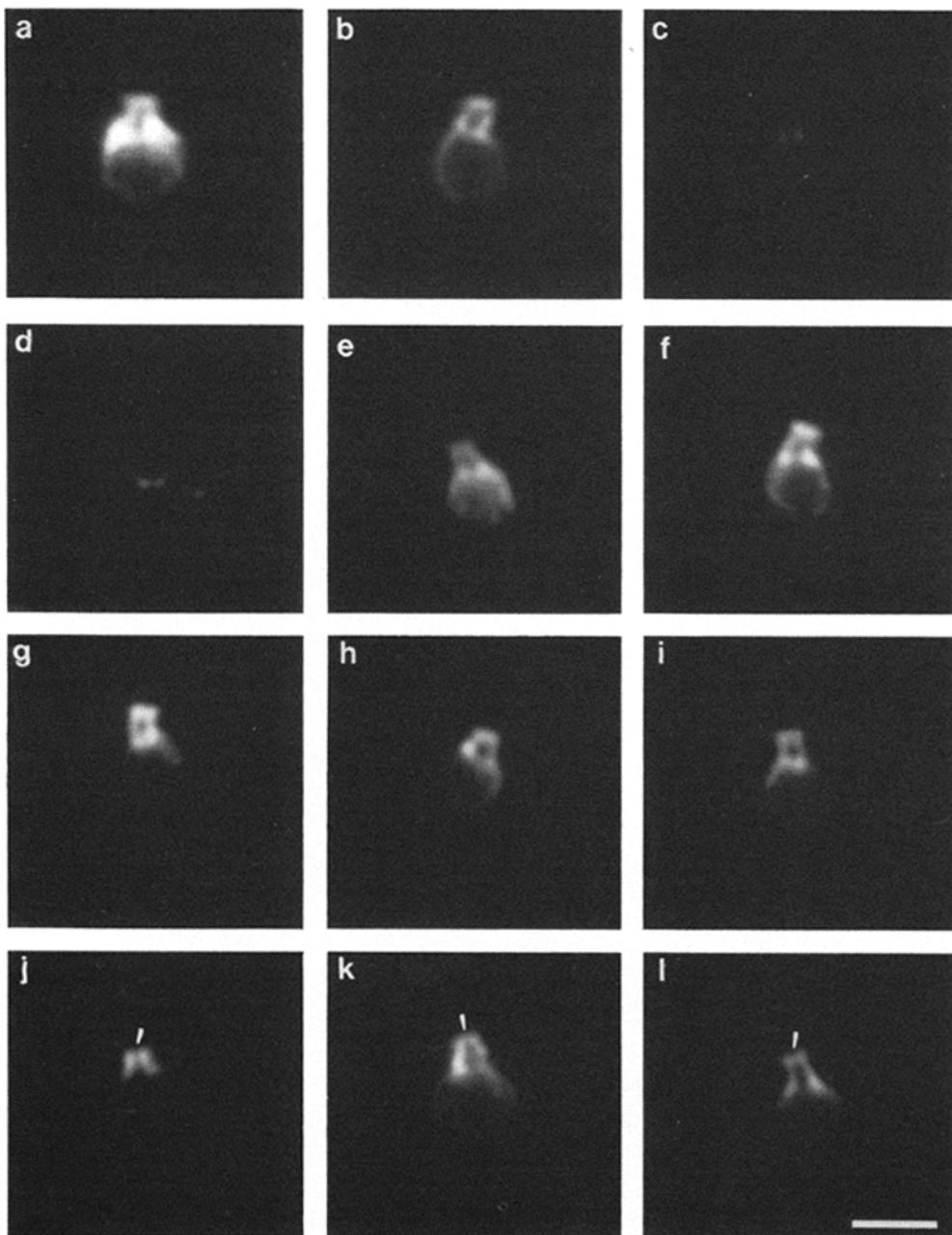


Figure 6. Immunolocalization of centrin in nucleo-basal body apparatuses from class 1 and class 2 revertants. (a and b) Wild-type; (c and d) *vfl2*; (e) *vfl2 R13*; (f) *vfl2 R5*; (g) *vfl2 R1*; (h) *vfl2 R31*; (i) *vfl2 R11*; (j and k) *vfl2 R10*; (l) *vfl2 R19*. Arrows in j–l indicate diminished staining between basal bodies. Bar, 5 μ m.

the connector is not absolutely required for mitosis (Wright et al., 1989). Likewise, having shown that *vfl2* cells lack stellate fibers in the transition region, we were able to conclude that these fibers are not required for flagellar autotomy, a process that involves the severing of the doublet microtubules of the axoneme just above the transition region (Jarvik and Suhan, 1991). The present study provides evidence that the primary defect in the *vfl2* mutant is in the centrin structural gene itself. We can now say with assurance that (a) centrin is required to construct the nucleus-basal body connectors, the distal striated fibers and the stellate fibers, and (b) centrin is required for accurate basal body localization and/or segregation.

Inferences about Centrin Structure

Centrin belongs to the superfamily of EF-hand proteins in

which loops that coordinate divalent cations are flanked by short α -helices known as the E and F helices. The EF-hand proteins are diverse in function. Some, such as parvalbumin, are thought to act as calcium buffers in particular tissues or cell types. Others, such as calmodulin and troponin C, interact with other proteins to make their activities sensitive to free calcium levels. We shall not attempt to review the large literature on EF-hand proteins, but instead refer the reader to several recent reviews (Kretsinger and Nockolds, 1973; Roberts et al., 1986; Kretsinger, 1987).

The canonical sequence for the E-helix, based on the known amino acid sequences of more than 150 members of the superfamily, can be represented as En**nn**n—with E being glutamate, n a hydrophobic amino acid, and * any amino acid (Kretsinger, 1987; Kretsinger et al., 1991). As noted by Huang et al. (1988), all four E helices of *Chlamydomonas* centrin conform to this consensus sequence. The resi-

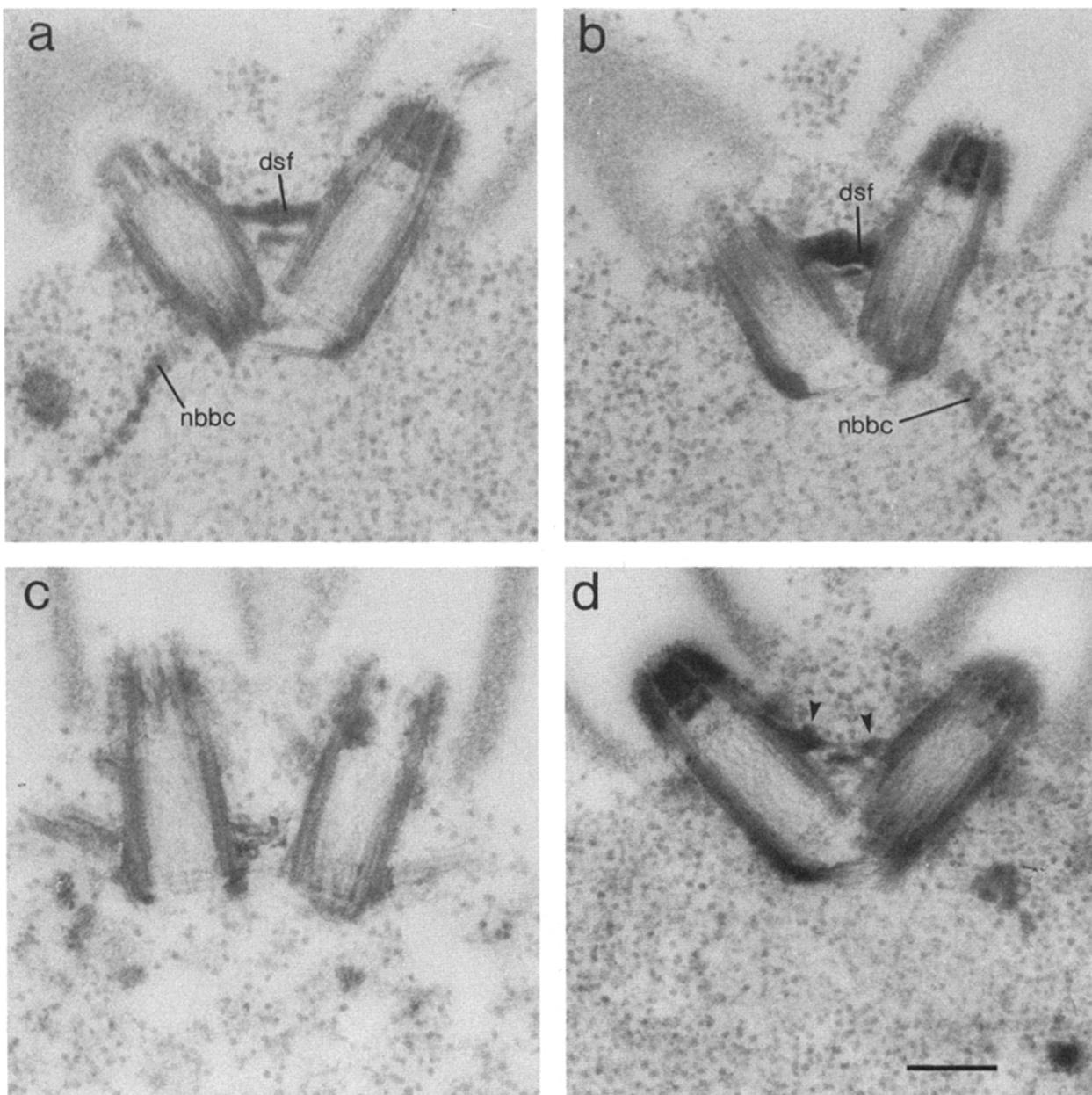


Figure 7. Basal body pairs in detergent-extracted cells. (a and b) *vfl2 R13* (genotypically wild-type revertant); (c) *vfl2*; (d) *vfl2 R23*. Arrowheads in d indicate incomplete distal striated fibers. *dsf*; distal striated fibers. *nbcc*; contracted nucleus–basal body connector. Bar, 200 nm.

due that is changed in *vfl2*, Glu 101, is the NH₂-terminal member of the E-helix of the protein's third EF-hand. The glutamic acid at the start of the E-helix is very highly conserved throughout the entire EF-hand superfamily (Kretsinger, 1987; Kretsinger et al., 1991), and so, on phylogenetic grounds alone, we would expect Glu 101 to be important to proper centrin function.

Structural data for other EF-hand proteins whose size (~20 kD) and organization (four EF-hands) are similar to centrin's (Roberts et al., 1986; Kretsinger et al., 1991), as well as analyses of a variety of mutant proteins produced by directed mutagenesis in vitro (Craig et al., 1987; Persechini and Kretsinger, 1988; Putkey et al., 1988; Persechini et al., 1989; Weber et al., 1989; VanBerkum et al., 1990), suggest that these proteins may be organized into two distinct structural domains joined by a long central helix which may act

as a flexible tether. This central helix is composed of the F-helix from the second EF-hand, the linker between the second and third hands, and the E-helix of the third EF-hand. Since the amino acid substitution in *vfl2* is at the junction of the linker and E-helix, it would not be surprising to find that the *vfl2* mutation has a severe effect on centrin tertiary structure, and that this is responsible for the severe phenotype of *vfl2* at the level of protein function.

Without knowing the structure of wild-type and *vfl2* centrin at atomic resolution, we cannot say with certainty how the change from Glu to Lys at position 101 affects the protein's overall structure. However, given the highly conserved nature of Glu 101, and the dramatic change in charge caused by the substitution of lysine at this position, we venture the speculation that Glu 101 participates in an ionic interaction that stabilizes the structure of the centrin polypeptide. In the

EF-hand protein parvalbumin, it is known from x-ray diffraction analysis that glutamic acid 81, which is equivalent to Glu 101 of centrin, forms a salt bridge to arginine 75 (Kretsinger and Nockolds, 1973). There is an arginine at a nearly equivalent position in *Chlamydomonas* centrin (Arg 96), and so we would not be surprised to find a salt bridge involving Glu 101 and Arg 96 in the wild-type centrin polypeptide. We also know that the steady-state level of centrin in *vfl2* cells is reduced to only ~25% of the wild-type level (Wright et al., 1989). This suggests that *vfl2* centrin is more susceptible than wild-type to intracellular proteolysis, as would be expected if it were present in an incorrect or partially denatured conformation. In all revertants tested, the centrin levels were normal, as would be expected if such a model were the case (data not shown).

If Glu 101 makes a salt bridge to a positively charged side chain elsewhere in the centrin polypeptide (possibly, but not necessarily, Arg 96), then a lysine at position 101 would not only fail to form the salt bridge but would, by charge repulsion, prevent the polypeptide from assuming its native structure. Alternatively, Lys 101 may cause the protein to assume an inactive conformation by participating in an inappropriate salt bridge that affects the conformation of the polypeptide. Our data are compatible with either of the above models, since in each of them the mutant phenotype derives largely from the presence of lysine rather than from the absence of glutamic acid. Thus, the class 1 pseudorevertants, which have the neutral polar amino acids asparagine or threonine at position 101, produce apparently normal centrin-containing structures. (We cannot, of course, say that these structures are truly normal, only that they appear normal in ultrastructure and, insofar as we can tell, in function.) Neither of these amino acids is charged, and could not restore a salt bridge, but it may well be that the salt bridge stabilizes the protein in its correct conformation but is not strictly necessary for the correct structure to form. In addition, both threonine and asparagine are polar amino acids that should interact in an energetically favorable way with water and thus not hinder the polypeptide from assuming its normal conformation at the start of the third E-helix. A rather different possibility is that the *vfl2* mutation affects the phosphorylation of centrin, and that an aberrantly phosphorylated centrin is responsible for the *vfl2* phenotype. This possibility should not be ignored, since centrin is a phosphoprotein (Salisbury et al., 1984; Wright et al., 1985; Salisbury et al., 1987), and since at ser-98 there is a potential phosphorylation site three amino acids from the site of the *vfl2* substitution.

One Protein/Multiple Fibrous Structures

Chlamydomonas centrin is an unusual EF-hand protein in that (a) it is involved in the formation of fibers, (b) the fibers can have diverse morphologies, and (c) the fibers show calcium-dependent contraction. In each case, centrin is required for the assembly or maintenance of the structure, as demonstrated by the *vfl2* phenotype. While centrin may not be the only protein in any of the structures where it is found, it is clear from our results that it is an essential component of each. It should be added that there exist two additional centrin-containing structures in the cell—the perinuclear fimbriae and an unknown component of the axoneme (which need not be fibrous). We have not considered these structures here because their ultrastructures are not known. It is impor-

tant to add that the 20-kD calcium-binding protein spasmin, which has been identified in a number of ciliates (Ochiai et al., 1988), also forms fibers that show Ca⁺⁺-dependent contraction. Ciliate spasmin appears, on immunological grounds, to be a close relative of centrin (Salisbury, J., personal communication; Buhse, H., personal communication), and so centrin and spasmin may represent a closely related group of EF-hand proteins with a special set of functional properties.

vfl2 Centrin Interacts with the Basal Body

In spite of the fact that no centrin-containing structures are apparent in electron micrographs of *vfl2*, indirect immunofluorescence of flagellar apparatuses from *vfl2* cells shows a distinct dot of staining associated with each basal body. We believe that the centrin is concentrated on or very near the middle of each *vfl2* basal body, and not in the transition regions or between the basal bodies at the level of the distal striated fibers. We base this conclusion on the following two arguments. First, we can rule out localization between the basal bodies on simple geometric grounds: if this were the case, the dots of staining would be displaced relative to the line defined by the faint staining of the axoneme, but in fact no such displacement is apparent (Fig. 5). Second, we can rule out localization in the transition region based on the distance between the dots, which is the same as the end-to-end distance for the bar of staining seen in the wild-type controls. Since it is known that the ends of this bar mark the basal bodies themselves and not the transition regions which are about twice as far apart (Sanders and Salisbury, 1989), it follows that the dots observed in *vfl2* mark the basal bodies as well.

To explain the close association of *vfl2* centrin with the basal body, we favor the interpretation that the structural changes in centrin produced by the substitution of lysine at position 101 do not interfere with the protein's ability to interact with its attachment site(s) on the basal body, but do prevent the appropriate interactions required for polymeric fiber formation.

Differential Requirements for the Assembly of Different Centrin-containing Structures

The fact that centrin is found in fibrous structures of diverse morphologies presents us with the challenge of identifying the features of the protein that provide the specificity for the formation of each structure. Table II, which summarizes our light and electron microscopic observations, indicates that different amino acid substitutions at position 101 differentially affect the assembly of the centrin-containing structures associated with the nucleo-basal body apparatus. The class 2 revertants are particularly interesting in this context, since they assemble apparently normal nucleus-basal body connectors and stellate fibers, but their distal striated fibers are aberrant (Fig. 5). This phenotype is consistent with a mode of morphogenesis in which fiber assembly initiates at each basal body and proceeds outward towards the other. In wild type this would yield two half fibers—one on each basal body—that could then join and fuse to form the complete distal striated fiber (Gaffal, 1988). Such half-fibers have been seen in other *vfl* mutants in which the relative orientations of the basal bodies are abnormal, making fusion impossible

Table II. Correlation between Amino Acid Substitutions at Position 101 and Ultrastructure Phenotypes

| Strain | Amino acid 101 | NBBC | DSF | TR |
|-------------|----------------|------|-----|----|
| Wild type | Glu | + | + | + |
| <i>vfl2</i> | Lys | - | - | - |
| Revertants | | | | |
| Class 1 | Glu | + | + | + |
| | Asn | + | + | + |
| | Thr | + | + | + |
| Class 2 | Met | + | * | + |
| | Ile | + | * | + |

NBBC, nucleus–basal body connectors; DSF, distal striated fibers; TR, transition region. A plus (+) indicates a normal structure; a minus (-) indicates absence of the normal structure (or set of structures in the case of the transition region); and an asterisk (*) indicates a distinctly abnormal structure or structures.

on steric grounds (Wright et al., 1983; Hoops et al., 1984; Adams et al., 1985). In the class 2 revertants, it appears that the half fibers are so aberrant as to be incapable of fusing properly even though they are oriented correctly with respect to one another. The centrin in the class 2 revertants has the hydrophobic amino acids methionine or isoleucine at position 101. These substitutions, and perhaps any hydrophobic amino acid substitutions at this position, are incompatible with the assembly of correct distal striated fibers but do permit the assembly of nucleus–basal body connectors and stellate fibers (Figs. 4 and 5). This represents evidence for differential roles for particular amino acid residues in determining the assembly of the individual centrin-containing structures. There is precedent for such a differential effect of amino acid substitutions. In calmodulin—a related EF-hand protein with multiple functions—substitution of lysines for glutamates 82, 83, and 84 results in differential inhibition of the protein's ability to interact with and modulate the activity of several of its target polypeptides (Craig et al., 1987).

The Nucleus–basal Body Connectors Are Required for Accurate Centriole Duplication and/or Segregation

Our data argue against a necessary role for either the distal striated fibers or the transition region in determining accurate basal body segregation. With regard to the transition regions, it is known that these parts of the nucleo–basal body apparatus are shed by the cell before entry into mitosis (Gaffal, 1988), and thus are not available to play such a role. With regard to the distal striated fibers, the phenotypes of a number of *vfl* mutants, other than *vfl2*, are particularly relevant. Some of these mutants, such as *vfl1* and *vfl3*, have incomplete distal striated fibers but normal nucleus–basal body connectors (Wright et al., 1983; Hoops et al., 1984; Adams et al., 1985), whereas others, such as *vfl2*, lack both distal striated fibers and nucleus–basal body connectors (Jarvik and Suhan, 1991). As reported here, there now exist additional mutants (the class 2 revertants) with normal nucleus–basal body connectors and defective distal striated fibers. In these mutants the basal body/centriole cycle runs normally. Thus, having eliminated the transition regions and the distal striated fiber as the controlling structures needed for accurate basal body segregation, we are left with the nucleus–basal body connectors; and so we conclude, in agreement with previous indications (Wright et al., 1985, 1989),

that the nucleus–basal body connectors are required for proper basal body/centriole duplication and segregation.

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