Centrin deficiency in *Chlamydomonas* causes defects in basal body replication, segregation and maturation

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Summary

Centrin, a 20 kDa calcium-binding protein, is a constituent of contractile basal body-associated fibers in protists and of various centrosomal structures. A construct inducing centrin RNAi was used to study the effect of centrin deficiency in *Chlamydomonas*. Transformants contained variable amounts of residual centrin (down to 5% of wild-type) and lacked centrin fibers. They displayed a variable flagellar number phenotype with mostly nonflagellate cells, suggesting that centrin is required for basal body assembly. Furthermore, basal bodies often failed to dock to the plasma membrane and to assemble flagella, and displayed defects in the flagellar root system indicating that centrin deficiency interferes with basal body development. Multiple basal bodies caused the formation of additional microtubular asters, whereas the microtubular cytoskeleton was disordered in most cells without basal bodies. The number of multinucleated cells was increased, indicating that aberrant numbers of basal bodies interfered with the cytokinesis of *Chlamydomonas*. In contrast to wild-type cells, basal bodies in centrin-RNAi cells were separated from the spindle poles, suggesting a role of centrin in tethering basal bodies to the spindle. To test whether an association with the spindle poles is required for correct basal body segregation, we disrupted centrin fibers in wild-type cells by over-expressing a nonfunctional centrin-GFP. In these cells, basal bodies were disconnected from the spindle but segregation errors were not observed. We propose that basal body segregation in *Chlamydomonas* depends on an extranuclear array of microtubules independent of the mitotic spindle.

Key words: Centrosome, Centriole, Flagella, RNAi, Spindle pole

Introduction

Basal bodies (bbs) are cylindrical microtubular organelles that form the base of eukaryotic flagella and are structurally similar to centrioles (Tassin and Bornens, 1999). Two pathways of bb/centriole assembly can be distinguished: the de novo pathway and the templated pathway (Marshall et al., 2001). The latter term refers to a process in which new bbs form once per cell cycle in the proximity of pre-existing ones; during cell division, pairs of bbs/centrioles are then distributed semi-conservatively (Kochanski and Borisy, 1990). Thus, controlled assembly of bbs and their proper segregation during mitosis are prerequisites to maintaining a constant number of this cell organelle. Centrioles/bbs are usually surrounded by a more-or-less structured matrix known as the pericentriolar material, and both together form the centrosome, which is the dominant microtubule-organizing center (MTOC) in eukaryotic cells. It has been assumed that centrioles serve to organize the centrosome and that centriole replication controls duplication of the centrosome (Bobinnec et al., 1998; Marshall and Rosenbaum, 2000). Centrioles are present at the poles of the spindle apparatus in most metazoan cells, and aberrant numbers of centrioles/centrosomes are often accompanied by malformation of the mitotic apparatus (Lingle and Salisbury, 2000). Multiple or structurally defective centrosomes were often observed in certain cancer cells and may contribute to chromosome loss and aneuploidy. Thus, control of centriole number also seems to be important for genome stability (Winey, 1996).

Several mechanisms can explain aberrant bb/centriole numbers. Centriole duplication usually occurs once per cell cycle at the transition from G1- to S-phase (Pennisi, 1999) and failed duplication or overproduction would lead to deviant numbers. Too many centrioles can also be the result of failed cell division (Meraldi et al., 2002). Furthermore, abnormal numbers of bbs can be the result of errors during mitotic segregation. Several studies suggest that centrin is involved in bb/centriole assembly and centrosome duplication. This calcium-binding protein is present in centrosomal structures over a broad range of species (Salisbury, 1995). Centrin is located in the lumen of mammalian centrioles, in the half-bridge of the yeast spindle pole body (spb), and in various contractile fibers associated with the bbs of protists. Knockout of the gene encoding Cdc31p, a centrin homologue in Saccharomyces cerevisiae, is lethal and cells fail to duplicate the spb (Huang et al., 1988; Spang et al., 1995). Silencing of the centrin gene in the water fern Marsilea effectively inhibits the formation of motile cells (Klink and Wolniak, 2001). Moreover, silencing of human centrin 2 impairs centriole duplication in HeLa cells, emphasizing that centrin is required for the replication of bbs and centrioles (Salisbury et al., 2002). A point mutation in the single-copy centrin gene of the biflagellate green alga *Chlamydomonas* causes the vfl2 phenotype (for ‘variable flagellar number’), suggesting that
centrin is also involved in the segregation of bbs (Wright et al., 1989). In *Chlamydomonas*, centrin is located in the stellate structure of the flagellar transition region, which represents the border between the bb and the axoneme, in the distal connecting fiber (dCF) between bbs, and in the two nucleus-bb connectors (NNBCs), which link the bbs to the nucleus (Salisbury, 1995). These structures are defective in *vfl2* cells and it has been assumed that the missing link between bbs and the nucleus causes mistakes in bb segregation during cell division (Wright et al., 1989). Thus, centrin seems to be involved in both the production of bbs and their distribution to daughter cells, raising the question of how centrin performs these multiple tasks.

Recently, a method to repress gene expression based on RNAi has been established in *Chlamydomonas* (Fuhrmann et al., 2001; Lechtreck et al., 2002). Constructs consisting of a partial genomic DNA with introns linked to the corresponding intron-free DNA in antisense orientation are thought to cause the formation of hairpin RNA molecules that effectively repress homologous genes (Smith et al., 2000). Here, we used this method to suppress centrin and obtained strains containing only about 5% of the amount of centrin present in wild-type cells. Centrin deficiency resulted in a high number of cells without bbs or flagella, as well as additional defects such as elevated numbers of binucleated cells, mistakes in bbs segregation during mitosis, and removal of bbs from the spindle poles. Over-expression of centrin-GFP in wild-type cells disrupted the NBBCs and removed bbs from the spindle poles but did not cause a bbs segregation defect. We assume that centrin deficiency interfered with bb maturation as indicated both by the frequent absence of flagella and the defects in the flagellar root system that, in consequence, cause segregation errors.

**Materials and Methods**

**Strains and cell culture**

The *Chlamydomonas reinhardtii* strain CC-3395 (cwh, arg7.8) was used for all transformations. Cells were maintained in a light:dark cycle of 14:10 hours, at 25°C in TAP [Tris-acetate-phosphate medium (Gorman and Levine, 1965)] with or without 0.02% arginine.

**Plasmid DNA and transformation protocol**

Centrin-GFP: The coding region of centrin including 40 bp of the 5'-UTR was amplified using genomic DNA of *C. reinhardtii* and Cenf1 (5'-GGCGTCTAGACGCGCATCATATTCGCAAGGCCTGCCGCAAGAGGCCGCGGCGAAGAGCGAGCTAGGTCGAATGCCTCGCGGATCTACAGGCAAAGACC-3') as a forward primer containing a XbaI and a Nhel restriction site, and CenR1w (5'-GGCGTCTAGACGCGCATCATATTCGCAAGGCCTGCCGCAAGAGGCCGCGGCGAAGAGCGAGCTAGGTCGAATGCCTCGCGGATCTACAGGCAAAGACC-3') as a reverse primer including a XbaI site and cloned into the pCRGFP (Fuhrmann et al., 1999). The resulting plasmid was cut with XbaI and EcoRI to obtain a fragment consisting of the sense-antisense hybrid DNA and the 3'UTR of pCRGFP, which was ligated into pCB740 as described above. The resulting plasmids contained the arg7 gene, a selectable marker for arginine-requiring strains. Alternatively, the HSP70A/rbcS2 promoter was cloned into pCRGFP in front of the centrin sense-antisense construct resulting in the plasmid pCR-CenAS.

Nuclear transformation was performed as described earlier (Kindle, 1990). In brief, 5x10^6 cells were agitated in the presence of 1-2 μg plasmid DNA linearized with EcoRI, 5% polyethylene glycol 8000, and 0.3 g of 0.5 mm glass beads. Transformants were selected on solid medium without arginine. Individual colonies, visible after 7-10 days, were transferred to liquid media for further analysis. Strains were maintained in liquid medium (50 ml volume), but slowly growing stocks were also prepared in solid TAP medium. As a control, we used a strain transformed with an empty pCB740 (digested with Nhel and EcoRI). Transformation of strains expressing GFP-SFA with pCR-CenAS was performed by co-transformation using the *ble* gene (Stevens et al., 1996) and selection on plates containing zeocin. A description of the N-terminally GFP-tagged SFA will be reported elsewhere (J.S. and K.-F.L., unpublished).

**Western blotting analysis**

Cells from 10 ml culture were pelleted at 500 g for 2 minutes, resuspended in 500 μl MT buffer (30 mM HEPES, 15 mM KCl, 5 mM MgSO4, 5 mM EGTA, 100 μM DTT, pH 7), and lysed by the addition of 500 μl MT buffer containing 3% Triton X-100. After 15 minutes at room temperature, cytoskeletons were pelleted at 18,320 g for 15 minutes at 4°C. The pellets were dissolved in 4x sample buffer, denatured at 95°C for 10 minutes, subjected to SDS-PAGE, and transferred onto PVDF membrane. Western blotting was carried out as described previously (Lechtreck and Geimer, 2000). After transfer, the membrane was fixed by a glutaraldehyde incubation step (Karey and Sirbasku, 1998). Blots were documented using a digital camera and processed using Adobe Photoshop and Illustrator (Adobe Systems, San Jose, CA). The intensity of immunoreactive bands was determined using Metamorph.

**Indirect immunofluorescence**

Cells were pelleted at 500 g for 2 minutes, resuspended in MT buffer, and lysed by an additional volume of MT buffer containing 3% Triton X-100 or 0.5% Nonidet P-40, respectively. After 45 seconds, the cytoskeletons were fixed with 3% paraformaldehyde (final concentration in MT) and were allowed to settle onto poly-L-lysine-treated multiwell slides for 10-15 minutes. Alternatively, cells were concentrated in MT buffer, transferred onto the poly-L-lysine-treated multiwell slide and, after 5-10 minutes, were permeabilized by rinsing in –20°C methanol for 7 minutes (20 seconds for centrin-GFP strains). Immunolabeling was carried out as described (Lechtreck and Geimer, 2000). Images were acquired with a CCD-camera (RT Monochrome spot 2.1.1., Diagnostics Instruments, Sterling Heights, MI) on a Nikon-Eclipse fluorescence microscope and processed using Adobe Photoshop and Illustrator. For measurements of the average fluorescence intensity of the bbs, we used the region statistics tool of Metamorph with the constant region depicted in Fig. 8C.

**Antibodies**

The following antibodies were used for indirect immunofluorescence:
Centrin deficiency in Chlamydomonas

monoclonal anti-centrin BAS6.8 (1:20), polyclonal anti-
Spermatozopsis-centrin (1:200; pCen2), polyclonal anti-
α-tubulin (1:800), monoclonal anti-acetylated tubulin (clone 6-11B-1; 1:600; Sigma), monoclonal anti-α-tubulin (clone DM 1A; Sigma) and monoclonal GT335 (1:1200) (Wolff et al., 1992) [for sources of these and secondary antibodies, see Grunow and Lechtreck (Grunow and Lechtreck, 2001), and references therein]. For western blot analyses, we used a polyclonal anti-Centrin (pCen1) (Salisbury et al., 1984) diluted 1:3000 in blocking buffer, polyclonal anti-GFP 290 (1:3000; Abcam, Cambridge, UK) and secondary antibodies directed against rabbit-IgG conjugated with alkaline phosphatase (Sigma).

Standard EM

Whole cells were resuspended in 0.5 ml TAP and fixed by addition of 0.7 ml TAP containing 5% glutaraldehyde and 1% OsO4 on ice for 30 minutes. To prepare cytoskeletons, cells were harvested by centrifugation and washed once in MT buffer. Cell lysis was carried out by adding MT buffer containing 3% Triton (1:1) and cytoskeletons were immediately prefixed with 2.5% glutaraldehyde (final concentration in MT buffer) and pelleted (5 minutes). After fixation in 0.5% OsO4 and 2% glutaraldehyde on ice for 30 minutes, the cytoskeletons were washed twice, dehydrated and embedded as previously described (Grunow and Lechtreck, 2001).

Results

Centrin deficiency reduced the number of flagella and bbs

Chlamydomonas reinhardtii was transformed with a plasmid containing a sense-antisense hybrid construct of partial centrin DNA under the control of the strong constitutive HSP70A/rbcS2-fusion promoter (Fig. 1A). After selection on solid medium, individual colonies were transferred to liquid medium. Macroscopic examination of transformants showed that about half of the strains (26 of 58 transformants from two transformations) displayed a defect in the motility apparatus in which the majority of cells rested at the bottom of the vessel. Flagellar counts revealed that these strains displayed a vfl phenotype with up to 80% of cells without flagella and up to 3% of cells with three or more flagella. Most strains (15) showed a severe flagellar assembly defect, with an average of fewer than 0.5 flagella per cell (not shown). This effect of the centrin-RNAi construct was confirmed in five independent transformations. Western blotting of transformants revealed that the amount of centrin was reduced in comparison with wild-type. For further analysis, we chose three strains A7, A10 and N41, containing approximately 40%, 28% or 5% of residual centrin, respectively (Fig. 1B). In wild-type cells, centrin is present both in the dCF interconnecting the two flagellar-bearing bbs and in the nucleus-bb connectors (NBBCs) that are surrounded by thin centrin fimbriae and link the bbs to the cell nucleus (Salisbury et al., 1988) (Fig. 1Ca, Fig. 5Aa’). Anti-centrin staining of centrin-RNAi cells revealed only dot-like signals whereas NBBCs and dCFs were absent (Fig. 1Cb-d). Since the centrin dots were located at the base of the flagella they apparently represent bbs. Most cells of A7, a strain chosen from the transformants without severe motility defect, contained two centrin dots (>95%) and possessed two flagella (84.6%); rarely (<5%), a weaker-dotted, centrin staining was present on the cell nucleus (Fig. 1Ce; Table 1). By contrast, A10 and N41 cells were mostly nonflagellate (57% and 74%, respectively), and the average number of centrin dots/bbs per cell was greatly reduced (Table 1). In Fig. 1D, the amount of residual centrin is correlated with the number of bbs or flagella. The data show that decreasing amounts of centrin caused a decline in the number of bbs and flagella and we conclude that centrin deficiency caused a severe defect in bb replication.

Prolonged observation showed that, in the centrin-RNAi

Fig. 1. Expression of a sense-antisense hybrid construct induced centrin deficiency. (A) Design of the sense-antisense hybrid construct of centrin used to induce RNAi in C. reinhardtii. The positions and restrictions sites of the primers are indicated and +1 indicates ATG of the coding region. (B) Western blot of control (lane C) and the RNAi strains A7, A10 and N41. Membrane strips were stained with amido black or developed with anti-centrin (pCen1). (C) Anti-centrin (mAB6.8) staining of methanol-permeabilized control (a) and the centrin-RNAi strains. In the latter, centrin was concentrated at the bbs, and NBBCs were absent. In A10 (c) and especially N41 (d), numerous cells contained no centrin signal. (e) Overlay image of an isolated nucleus of A7 stained with DAPI (black) and centrin (white). Bar, 10 μm.

(D) The average number of flagella (■) and centrin dots (○) was plotted against the amount of residual centrin as determined by western blotting. The data show that bb and flagellar development depend on centrin.
strains, the repression of the centrin gene was not stable (Fig. 2). The loss of the centrin-RNAi effect was accompanied by increasing amounts of centrin on western blots (Fig. 2B), reappearance of the centrin-structures (Fig. 2C), and increasing numbers of biflagellate cells (Fig. 2A). The intensity of centrin-RNAi decreased more rapidly in cultures of N41 in liquid medium, whereas slowly growing N41 stocks maintained in solid media preserved the centrin-deficient phenotype for several months (Fig. 2A). Finally, all isolates of a given strain lost the centrin-RNAi effect, even those maintained in solid medium or raised from isolated nonflagellate single cells. We assume that the transgene itself was silenced, as it has been reported earlier for other transgenes (Wu-Scharf et al., 2000). All experiments depicted in this study were performed while the strains showed a relatively stable RNAi effect, as indicated for N41 in Fig. 2A.

Centrin deficiency interferes with flagellar assembly on bbs

Counts of centrin dots and flagella indicated a discrepancy between the number of centrin dots, presumably representing bbs, and that of flagella in the centrin-RNAi strains (Fig. 1D, Table 1). Double labeling using anti-centrin and anti-α-tubulin showed centrin dots without attached flagella (Fig. 3Aa-c). To test whether these centrin signals represent genuine bbs, we performed immunofluorescence using antibodies to polyglutamylated or acetylated tubulin, which are enriched in bbs (LeDizet and Piperno, 1986; Lechtreck and Geimer, 2000). Both antibodies identified nonflagellate bbs in the centrin-RNAi strains (Fig. 3Ae,f), and also confirmed the absence of bbs from many cells (Fig. 6Ad, not shown). Furthermore, EM analysis of N41 cells showed numerous bbs without or with extremely short flagella revealing that centrin knockdown interfered with flagellar assembly, which has not been reported from vfl2 cells (Fig. 3Bb-e). Some bbs were improperly docked to the plasma membrane or located within the cytoplasm (Fig. 3Bc-e). Similar to earlier observations in the vfl2, a strain with a point mutation in the centrin gene (Jarvik and Suhan, 1991), the transition region of flagellate and nonflagellate bbs of N41 was defective (Fig. 3B; a wild-type flagellum is shown in a) and central pair microtubules were penetrating the bb (Fig. 3Cb).

Centrin tethers bbs to the spindle poles

Whereas the average number of bbs decreased with the amount of centrin, we observed a considerable number of cells that contained more than two centrin dots/bbs (Table 1). In A10, for example, 12.7% of cells contained three or more centrin dots/bbs (Table 1). Double labeling using anti-centrin and anti-α-tubulin showed centrin dots without attached flagella (Fig. 3Aa-c). To test whether these centrin signals represent genuine bbs, we performed immunofluorescence using antibodies to polyglutamylated or acetylated tubulin, which are enriched in bbs (LeDizet and Piperno, 1986; Lechtreck and Geimer, 2000). Both antibodies identified nonflagellate bbs in the centrin-RNAi strains (Fig. 3Ae,f), and also confirmed the absence of bbs from many cells (Fig. 6Ad, not shown). Furthermore, EM analysis of N41 cells showed numerous bbs without or with extremely short flagella revealing that centrin knockdown interfered with flagellar assembly, which has not been reported from vfl2 cells (Fig. 3Bb-e). Some bbs were improperly docked to the plasma membrane or located within the cytoplasm (Fig. 3Bc-e). Similar to earlier observations in the vfl2, a strain with a point mutation in the centrin gene (Jarvik and Suhan, 1991), the transition region of flagellate and nonflagellate bbs of N41 was defective (Fig. 3B; a wild-type flagellum is shown in a) and central pair microtubules were penetrating the bb (Fig. 3Cb).

Table 1. Basal body and flagellar number in control and centrin-RNAi cells

|                  | Flagellar number | Number of centrin dots |                  | Flagellar number | Number of centrin dots |
|------------------|------------------|------------------------|------------------|------------------|------------------------|
|                  | 0    | 1    | 2    | 3    | 4    | n  | 0    | 1    | 2    | 3    | 4    | n  |
| Control          | 1.2  | 0.4  | 98.4 | –    | –    | 251| 1.97 | 0.7  | –    | 99.3 | –    | –    | 276| 1.99 |
| A7               | 5.6  | 6.6  | 84.6 | 2.8  | 0.3  | 286| 1.86 | 2    | 1.2  | 94.5 | 2    | 0.4  | –   | 256| 1.98 |
| F11              | 9.9  | 8.9  | 79.7 | 1    | 0.5  | 202| 1.73 | 5.1  | 2.3  | 80.7 | 5.1  | 2.8  | –   | 176| 2.05 |
| A10              | 57.2 | 22   | 19.2 | 1.6  | –    | 318| 0.65 | 20.6 | 6.5  | 60.1 | 10.9 | 1.4  | 0.4 | 276| 1.67 |
| N41              | 74.4 | 18.1 | 7.5  | –    | –    | 227| 0.33 | 67.1 | 19.9 | 7.4  | 4.2  | 0.9  | 0.5 | 216| 0.53 |

Flagellar number was determined by phase contrast microscopy of fixed cells, and bb number by indirect immunofluorescence using anti-centrin. Shown is the percentage of cells containing 0-4 flagella or 0-5 centrin dots; –, not observed. n, number of cells analysed; Ø, average number of flagella/centrin dots per cell.

Fig. 2. Stability of centrin-RNAi in strain N41. (A) The average flagellar number per cell is plotted against the time in days after transformation. Data were recorded from cultures grown continuously in liquid medium (1, ■), or inoculated from stock cultures of the same strain maintained in solid medium after 152 (2, □) and 204 days (3, △). The points of time for western blotting (WB), indirect immunofluorescence (IF) and embedding for EM are indicated. Arrows on abscissa mark the start of different cultures in liquid medium. (B) Western blot corresponding to the experiment shown in A comparing the amount of centrin in control cells (lane C) and the three cultures of N41. (C) Indirect immunofluorescence corresponding to A and B using anti-centrin on control cells and on three cultures of N41. Note the decrease in the intensity of centrin-RNAi with time as indicated for example by the presence of centrin on the nucleus and residual NBBCs in N41-1. Accordingly, increased amounts of residual centrin were detected in the corresponding western blot and the average number of flagella per cell was close to wild-type. By contrast, the centrin-RNAi effect was still strong in N41-3, a culture retrieved from slowly growing stocks about two weeks prior to analysis.
Centrin deficiency in *Chlamydomonas* dots, indicating a bbs segregation defect (Table 1, Fig. 4A). In wild-type cells, centrin and bbs are located at the poles of the spindle which, in *Chlamydomonas*, is intranuclear with fenestrae at the poles (e.g. Johnson and Porter, 1968; Coss, 1974; Wright et al., 1989) (Fig. 4Ba-c). It was assumed that, in *Chlamydomonas vfl2*, proper segregation of bbs fails due to the absence of intact NBBCs (Wright et al., 1989). Thus, we determined the position of bbs with respect to the mitotic spindle in centrin-depleted cells. Indirect immunofluorescence revealed that centrin dots/bbs were not located at the spindle poles in mitotic cells of A10 (7 of 7 cells) and, more surprisingly, of F11 Fig. 3. Centrin-RNAi cells contained additional, nonflagellate basal bodies. (A) Antibodies to centrin (a-c), polyglutamylated (e) and acetylated tubulin (f) were used to demonstrate the presence of nonflagellate bbs in A10 (a-c) and N41 (e,f), a-c', corresponding anti-α-tubulin images. Arrowheads, bbs without attached flagella. GT335 also stained the cell nucleus because nucleosome assembly proteins are polyglutamylated in addition to α-tubulin (Regnard et al., 2000). Bar, 5 μm. (B) EM analysis of wild-type (a) or N41 bbs (b-e) in longitudinal section. (a) Wild-type bb with transition region (arrowhead) and dCF (arrow). (b-e) bbs of N41 showing extremely short (b) or no flagella (c-e). The transition region, in longitudinal section usually appearing as an H-like structure, was defective in N41 (arrowhead in b). Note that bbs in N41 are improperly docked to the plasma membrane (c,d) or reside within the cytoplasm (e), and sometimes contained electron-dense material in the lumen (arrowhead in c). Arrowhead in d, basal feet. Arrowhead in e, attached microtubules. Bar, 250 nm. (C) Wild-type (a) and N41 bbs in cross-section. The stellate structure (a) was absent in the centrin-RNAi strains (b), causing the central pair microtubules to penetrate the bb. Bar, 200 nm.

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(18 of 19 cells). The latter strain was similar to A7 with respect to average flagellar and bb number per cell (Table 1), but displayed a somewhat stronger bb segregation defect (19.3% of cells with aberrant numbers of centrin dots compared with 5.5% in A7, which was no longer available for this analysis). Bbs mostly resumed a lateral position with a distance of up to 3 μm to the poles (Fig. 4Bd-j). The data suggest that centrin fibers are needed to tether bbs to the spindle poles. Mitotic A10 often had aberrant bb numbers and spindles without bbs, whereas F11 cells mostly contained two bb pairs that were interconnected by the microtubules of the so-called metaphase band (Johnson and Porter, 1968; Doonan and Grief, 1987; Gaffal and el-Gammal, 1990). This extranuclear system of microtubules present in mitotic cells of *Chlamydomonas* develops from two of the four microtubular roots surrounding the bbs in interphase (Fig. 6Ac). It originates near the bb pairs and consists of two bands, each of four microtubules. The two bands run towards each other, parallel to the spindle and overlap above the nucleus in an antiparallel fashion (arrow in Fig. 4Bd). Then, both bands make a turn and continue down into the cell on the sides of the nucleus perpendicular to the spindle microtubules (arrow in Fig. 4Ba). In A10, the metaphase band seemed unordered or absent (Fig. 4Bh-j). Fig. 4Bk-m show examples of dividing A10 and N41 cells in which bbs were not correctly segregated. Thus, segregation errors contributed to aberrant bb numbers in *Chlamydomonas* centrin-RNAi strains.

Over-expression of centrin-GFP disrupted the NBBCs

Despite the absence of NBBCs, A7 or F11 displayed only a weak *vfl* phenotype (Table 1) and we wondered whether the displacement of bbs from the mitotic poles is sufficient to explain the observed mistakes in segregation. To address this question, we took advantage of an earlier observation (Ruiz-Binder et al., 2002), which showed that expression of GFP-tagged centrin interfered with a system of delicate centrin fibers in the stellate structure of the flagellar transition region. We used the strong HSP70A/rbcS2 fusion promoter to transform control cells with a centrin-GFP construct (Fig. 5). Moderate expression of centrin-GFP (e.g. strain Cen-GFP3) allowed incorporation of the tagged protein in the dCF and the distal parts of the NBBCs (Fig. 5Ab), whereas higher expression levels (e.g. strain Cen-GFP8) disrupted the centrin system of the NBBCs and centrin-GFP accumulated in clumps on the nuclear surface and at the bbs (Fig. 5Ac). Staining of Cen-GFP8 with anti-centrin confirmed the absence of centrin-based NBBCs (Fig. 5Ac'). Furthermore, cytoskeletons isolated from Cen-GFP8 or the centrin-RNAi strain A7 exhibited a reduced stability: about 30% of the nuclei were detached from the basal apparatus, which was not observed with cytoskeletons isolated from Cen-GFP3 or wild-type cells (Fig. 5C,D). Interestingly, bbs were not located at the poles in most mitotic Cen-GFP8 cells (23 of 25) as determined by indirect immunofluorescence using anti-centrin or GT335 (Fig. 5B). In contrast to centrin knockdown cells, some residual centrin was present at the acentric spindle poles (Fig. 5Ba-c). Despite the abnormal mitotic position of bbs, Cen-GFP8 cells were more than 99% biflagellate and contained two bbs. Thus, disruption of the NBBCs via over-expression of centrin-GFP detached the bbs from the spindle poles but did not cause a conspicuous

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**Fig. 5.** Over-expression of centrin-GFP disrupts the NBBCs. (A) Isolated cytoskeletons of control (a), Cen-GFP3 (b) and Cen-GFP8 (c) cells stained with anti-centrin (a’-c’), corresponding GFP images (phase contrast for the control) are shown in a-c. Over-expression of centrin-GFP disturbed the NBBCs (c), whereas moderate expression allowed incorporation into the proximal parts of the NBBCs and into the distal connecting fiber (arrow in b). Bar, 1 μm. (B) Dividing cells of Cen-GFP8 stained with anti-tubulin combined with either anti-centrin (a-c) or GT335 (d,e). (a-c) Small arrowheads, bb pairs; arrows, centrin-GFP clumps; large arrowheads, centrin/centrin-GFP located at the spindle poles. (d,e) Arrowheads, bb pairs detected by GT335 staining. Bar, 5 μm. (C) Phase contrast (a) and corresponding GFP image (a’) of Cen-GFP8 cytoskeletons, which often disintegrated into basal apparatus (arrowhead) and cell nucleus (arrows). (D) Histogram showing the frequency of intact (solid bars) and defect (open bars) cytoskeletons. Data were averaged from three independent experiments. Cytoskeletons of the centrin-RNAi strain A7 and the centrin-GFP-overexpressing strain GFP8 were less stable.
phenotype, indicating that NBBCs are not required to maintain a constant bb number.

Effect of aberrant bb numbers on the microtubular cytoskeleton

Centrin deficiency caused cells with too many bbs or without bbs, allowing us to study the effect of aberrant bb numbers on the microtubular system. In *Chlamydomonas*, the flagellar basal apparatus with its two flagellar-bearing bbs functions as an MTOC, giving rise to four acetylated microtubular bundles, two consisting of four and two consisting of two microtubules. On these, numerous non-acetylated microtubules originate (LeDizet and Piperno, 1986) (Fig. 6Aa-c). Additional bbs of the centrin-RNAi cells were surrounded by microtubules and were often associated with acetylated microtubular bands (Fig. 6Ae,g, Fig. 3Af). In A10 and N41, we observed numerous cells (21% or 67%, respectively) without bbs. In such cells, we mostly observed an unordered microtubular system (Fig. 6Af), and acetylated microtubules were either present or absent (Fig. 6Ad, cells 3, 4). Also, we observed cells with an incomplete set of microtubular roots (Fig. 6Ad, cell 2, Fig. 3Af). The four microtubular flagellar roots are associated with striated fibers composed predominately of striated fiber assemblin (SFA) (Lechtreck et al., 2002). We transformed cells expressing GFP-SFA with a plasmid inducing centrin-RNAi to study the effect of centrin deficiency on this flagellar root system. In a wild-type background, GFP-SFA uniformly (98.8% of 252 cells) assembled into cross-like structures with prominent fibers attached to the four-stranded microtubular roots and smaller ones associated with the two-stranded roots (Fig. 6Ba). After induction of centrin deficiency, the GFP-SFA system was altered: additional bbs caused aberrant GFP-SFA fiber formation and, in cells without bbs, GFP-SFA fibers were either absent or one or two giant GFP-SFA fibers were formed (Fig. 6Bc-e, not shown). Interestingly, cells with a low amount of centrin but two bbs mostly displayed an altered GFP-SFA system with additional fibers and/or fibers of unusual size (Fig. 6Bb). The data show that bbs are critical to establish the focused microtubular system and that centrin deficiency interferes with the ability of bbs to organize the usually stereotyped flagellar root system of *Chlamydomonas*.

In N41, and to a lesser degree in A10, we observed cells with additional non-wild-type characters that, we assume, were caused by the defects of the microtubular system described above. These include multiple eyespots and pyrenoids (Fig. 7a) indicative for several plastids or incomplete plastid division, a higher variation in cell size and shape, and the occurrence of incompletely divided cells (not shown). Nuclei and pyrenoids were often positioned incorrectly with respect to each other and the cell surface (not shown). Furthermore, we observed that A10 and N41 contained elevated numbers of multinucleated cells (7% and 17%, respectively). Cells with multiple nuclei did not contain elevated numbers of bbs (not shown) and we assume that too many as well as no bbs increased the rate of errors during cell division of *Chlamydomonas* (Fig. 7b-d).

Bbs of the centrin-RNAi strains varied in the amount of associated centrin

In *Chlamydomonas*, bbs duplicate in late mitosis (Gaffal and el-Gammal, 1990). We attempted to analyze when centrin is recruited to the bbs by using
antibodies to acetylated tubulin (6-11B-1), a posttranslational modification acquired early during bb development, and to polyglutamylated tubulin (GT335) which is characteristic for mature, usually flagellate bbs (Lechtreck and Geimer, 2000). Probasal bodies were observed in positions lateral of mature bbs between the four microtubular roots and frequently cells contained aberrant numbers of probasal bodies. These were not stained by anti-centrin (Fig. 8Aa,b), indicating that bbs acquire centrin during their subsequent development. In double-staining experiments with GT335, all centrin dots analyzed were polyglutamylated, but about 5% of the GT335 signals of N41 contained no or only very small amounts of centrin (Fig. 8B). Interestingly, some of these centrin-free bbs had assembled a flagellum (8Bb,c). The data indicate that, under conditions of strong centrin knockdown, some bbs mature, as judged by the presence of polyglutamylated tubulin and axonemes, without acquiring considerable amounts of centrin. In green algae, centrin is not a component of the bbs itself but is restricted to the stellate structure in the flagellar transition region (Schulze et al., 1987). The centrin dots observed at the base of flagella in vfl2 or the centrin-RNAi cells represent remnants of the dCF and NBBCs on the surface of bbs (Taillon et al., 1992).

We measured the intensity of signals obtained by indirect immunofluorescence with monoclonal anti-centrin (Fig. 8C) to analyze whether the amount of centrin at the bbs varied within a strain or between strains. A7 cells showed only small differences (7%) in the staining intensities between the two flagellate bbs of a cell, which presumably reflect developmental differences. Centrin dots in A10 showed about 30-50% of the fluorescence intensity of those in A7. In N41, some dots were of very low intensity (see above) but most bbs showed 10-45% of the intensity in comparison with A7 bbs (not shown). In uniflagellate cells of A10 with two bbs, the signal observed at the base of the flagellum was on average 36% stronger than that at the nonflagellate bb. We assume that a lower centrin content and the absence of a flagellum are indicative for a delay in bb development under conditions of centrin deficiency.

Discussion

In an attempt to refine the analysis of centrin function in Chlamydomonas, we repressed centrin expression by
transforming cells with a sense-antisense hybrid construct. Previous studies have identified *Chlamydomonas vfl2*, a strain comprising a point mutation in the centrin gene that results in the replacement of glutamic acid 101 by lysine (Taillon et al., 1992). *Vfl2* cells display a phenotype of variable flagellar number and structural defects in the centrin fibers similar to those observed in the centrin-RNAi strains. The predominate effects of centrin deficiency, however, were a reduced number of bbs and a failure to assemble flagella onto bbs, which are not apparent in *vfl2* cells. Centrin deficiency resulted in additional, probably secondary defects that were not observed in *vfl2* such as extra-microtubular aster and elevated numbers of multinucleated cells. Our data indicate significant differences in the phenotype of *vfl2* cells comprising a centrin point mutation and the effect of centrin knockdown. *Vfl2* cells contain 20-25% of residual centrin (Wright et al., 1989), whereas strains with down to 5% of residual centrin were identified using centrin-RNAi. In all strains analyzed in detail, the centrin-RNAi effect was lost after prolonged cultivation and wild-type centrin structures re-appeared, excluding the possibility that the observed phenotypes were caused by insertional mutagenesis of other genes. In Cdc31p, the yeast homologue of centrin, certain point mutations affect spb replication while others do not (Ivanovska and Rose, 2001). Accordingly, the point mutation of *vfl2*, which is thought to alter the structure of centrin (Tailon and Jarvik, 1995), might impair some functions of centrin whereas others are not affected. Thus, it remains unclear whether the *vfl2* phenotype is caused by reduced amounts and/or functional modifications of centrin whereas the defects in the centrin-RNAi strain can be fully attributed to reduced amounts of centrin.

**Centrin and bb segregation**

In wild-type cells of *Chlamydomonas*, centrin and bbs are located at the spindle poles. Since NBBCs are defective in the *Chlamydomonas* mutant *vfl2*, it has been assumed that these linkers are needed for proper segregation of bbs during cell division (Wright et al., 1989; Marshall et al., 2001). Here, we show that centrin deficiency detaches bbs from the spindle poles, but nevertheless provide evidence against a crucial role of NBBCs in the equal distribution of bbs. Indirect immunofluorescence and EM failed to detect NBBCs in the centrin-deficient strains and we observed a decreased stability of isolated nucleo-flagellar-apparatuses similar to earlier observations on *vfl2* cells. Despite the apparent absence of NBBCs in all analyzed strains, these differed considerably in their ability to maintain a constant bb number (e.g., 94.5% of A7 cells contained bb pairs). It seemed possible that cells suffering from mild centrin knockdown were able to assemble NBBC-like fibers during mitosis that link the bbs to the spindle poles and thereby ensure proper segregation. The analysis of mitotic cells demonstrated that bbs are not at the poles in most cells regardless of whether the strains showed a mild or a strong *vfl2* phenotype. We conclude that centrin and NBBCs are needed to tether bbs to the spindle poles. However, the data also suggested that correct distribution of bbs in *Chlamydomonas* neither requires NBBCs nor an association of bbs with the spindle poles. To test this assumption further, NBBCs were disrupted by over-expressing centrin-GFP, which resulted in a decreased stability of the nucleo-flagellar apparatus complexes. Indeed, bbs were detached from the poles in most cells expressing centrin-GFP, emphasizing the role of the NBBCs in tethering bbs to the spindle poles. Interestingly, these strains were perfectly able to segregate their bbs. We conclude that NBBCs or an association of bbs with the spindle poles are not crucial for segregation of bbs in *Chlamydomonas*. Observations from other green flagellates related to *Chlamydomonas* support this conclusion. In *Polytomella agilis*, NBBCs were not observed (Schulze et al., 1987) and bbs are not at the poles, but nevertheless centriole/bb numbers are maintained constant in these cells. Bbs are not at the poles of the mitotic spindle in *Dunaliella bioculata*, which possesses NBBCs in interphase (Grunow and Lechtreck, 2001). In metazoan cells, the poles of acentric spindles often appeared less focused than those with centrioles, suggesting a role of these microtubular cylinders in spindle assembly or maintenance (Compton, 2000). By contrast, spindles appeared mostly normal in centrin-deficient cells of *Chlamydomonas* despite the absence of bbs at the poles, suggesting a fundamental difference between metazoan centrioles and green algal bbs, the latter apparently not essential for mitotic spindle assembly.

If not the NBBCs, what else might ensure the distribution of bbs to the daughter cells? A study in 1968 (Johnson and Porter, 1968) first reported two bands, each of four microtubules, that persist throughout mitosis and arc over the mitotic nucleus. These ‘metaphase band’ microtubules are identical with the two four-stranded microtubular flagellar roots associated with the bbs during interphase. During bb segregation, each pair of bbs keeps one root and the two roots overlap in an antiparallel fashion. A central overlapping region of the metaphase band is maintained while the roots elongate and the bbs migrate towards the nuclear poles (Gaffal and el-Gammal, 1990; Ehler et al., 1995). This sequence of events has lead to speculations that the metaphase band moves the bbs into both cell halves in a process analogous to that of spindle elongation with a motor situated in the zone where the microtubules overlap (Sluiman and Blommers, 1990). In this study, we established experimental conditions in which bbs are no longer attached to the spindle poles via centrin fibers. Cells were still able to segregate bbs properly and we postulate that the extranuclear spindle-like microtubule system of the metaphase band provides the force and structure for this process. Accurate centriole/bb segregation is fundamental and therefore might be ensured by more than one mechanism, which would explain the centrin-dependent association of bbs with the spindle poles in *Chlamydomonas* and other systems. In *Chlamydomonas*, the two mitotic assemblies of microtubules (spindle and metaphase band) can be distinguished due to the persisting nuclear envelope, but one might speculate that a subpopulation of microtubules in the spindle of metazoan cells serves to separate the pairs of centrioles more than in chromosome movements or spindle elongation.

**Centrin and bb duplication**

A strong reduction in the amount of centrin as in N41 caused a decrease in the average number of bbs. It has been shown that the repression of centrin expression in *Marsilea* inhibits the differentiation of motile cells (Klink and Wolniak, 2001) and, more recently, that siRNAs to human centrin2 inhibit
Centriole replication in HeLa cells (Salisbury et al., 2002). Thus, bb assembly in *Chlamydomonas* and many other systems seems to depend on centrin. What could be the role of centrin during bb/centriole replication? One possibility is that centrin is an essential component of bbs/centrioles. Indeed, centrin is located in the distal lumen of centrioles e.g. in metazoan cells. In green algal bbs, however, centrin was observed only in the stellate structure between the bb and the axoneme but is otherwise restricted to bb-associated structures. Furthermore, we report here that the amount of centrin attached to bbs can be very low under conditions of centrin deficiency, indicating that centrin is not required to maintain bbs. In various systems, centrin has been observed in structures on which bbs subsequently assemble. These include the blepharoplast of *Marsilea* or 'fibrous granules' during the differentiation of ciliated epithelial cells (Laoukili et al., 2000). Similarly, new bbs develop on the end of centrin fibers in the green flagellate *Spermatozopsis similis* (Lechtreck and Bornens, 2001). Centrin structures precede centriole assembly in metazoan cells (Middendorp et al., 1997) and the de novo assembly of bbs in the amoeboflagellate *Naegleria* (Levy et al., 1998). Finally, it is noteworthy that Cdc31p is not a component of the spb itself but of the half-bridge, a structure on which material for the new spindle pole is deposited (Spang et al., 1995; Adams and Kilmartin, 1999). Centrin-containing structures might provide assembly sites for bbs and other centrosomal organelles over a broad range of species (Adams and Kilmartin, 2000). Centrin knockdown seriously reduced the amount of centrin and we assume that many bbs failed to recruit enough centrin to ensure the formation of probasal bodies within one cell cycle. Subsequent cell divisions will then dilute the bbs, resulting in cells without them.

**Centrin and bb maturation**

We observed bbs without attached flagella in centrin-deficient cells. Nonflagellate bbs can be caused by defects in flagellar assembly (e.g. the bald1 mutation) (Brazelton et al., 2001) or by defects in the bbs that hinder them to template an axoneme (e.g. bald2 or uni3) (Dutcher and Trabuco, 1998). However, in the centrin-RNAi strains, ultrastructural defects in bb triplets, typical for bald2 or uni3, were not observed. All strains assembled at least a few motile, full-length flagella qualified by the observation that N41 (and more rarely A10) often had shorter than wild-type flagella, resulting in a reduced average length (for N41: 9.4 μm±2.85 μm, n=67; control: 12.1 μm±0.98 μm, n=44). It is unlikely that flagellar assembly is blocked by defects of the transition region because these also occurred in flagellate bbs of *vfl2* and centrin-RNAi cells (Taillon et al., 1992). Centrin has been identified in the flagella of *Chlamydomonas* (LeDizet and Piperno, 1995) and *Tetrahymena* (Guerra et al., 2003). Thus, flagellar assembly could be perturbed because centrin is an essential component of the flagellum. Alternatively, many bbs in the centrin-RNAi strains could be in an immature condition not allowing flagellar assembly. Similar to the formation of a primary cilium on the mother centriole in metazoan cells, bbs of *Chlamydomonas* need to have a certain age before flagellar assembly occurs: between the formation of new bbs during the end of mitosis (Gaffal and el-Gammal, 1990) and the assembly of axonemes after the next mitosis, bbs rest in an immature nonflagellate state. Bb maturation involves an increase in size, docking to the plasma membrane, development of a flagellar root system, assembly of the transition region and flagella, accumulation of centrin, and biochemical modifications of bb tubulin. Indeed, several observations suggest improper bb maturation in the centrin-RNAi strains: bbs had a reduced centrin content, some failed to dock to the plasma membrane, and the flagellar root system was often defective (e.g. missing roots and an altered distribution of GFP-SFA, a marker for microtubular roots). We assume that centrin deficiency delays bb development and/or disturbs the pace of steps occurring during bb maturation. It is important to note that improper bb maturation could be related to segregation defects: the metaphase band develops from the microtubular roots and it is reasonable to assume that defects in the flagellar root system will affect the metaphase band and thereby might cause mistakes in bb segregation. However, other scenarios are possible: root microtubules are nucleated not directly on the bbs but at the dCF, which thereby provides a link between bbs and the microtubular cytoskeleton potentially related to segregation. Furthermore, proper segregation of bbs might require a physical link between the new and the old bb of each pair in the form of a centrin-based new dCF ensuring that all bbs, including new ones without roots, are attached to the metaphase band. The metaphase band has also been implicated in the determination of the cleavage plane and the assembly of the cytokinetic apparatus of *Chlamydomonas* (Johnson and Porter, 1968; Ehler et al., 1995). Therefore, defects in the flagellar root system could be responsible for the formation of multinucleated cells. Moreover, probasal bodies of green algae develop in association with the flagellar roots [Lechtreck et al. (Lechtreck et al., 1997); and references therein] and therefore defects in the roots could even interfere with bb replication.

**Differential sensitivity of centrin structures and functions**

The amount of residual centrin varied considerably between transformants, allowing us to analyze the differential sensitivity of centrin-based processes and centrin-based structures. Residual centrin was concentrated near the bbs, which apparently have the highest affinity for centrin. In A7 and other strains, we observed a dotted staining on the cell nucleus, whereas linkers to the bbs were absent, indicating that the fibrillae are less sensitive to decreasing amounts of centrin than the proximal parts of the NBBCs. The strains analyzed in detail are representative of different levels of impaired centrin function: A7 displayed a mild defect, F11 an intermediate segregation defect, whereas bb replication seemed unaffected as indicated by an average number of bbs per cell of near 2. In A10, the number of cells with aberrant bb numbers was increased and the average number of bbs was slightly decreased, suggesting beginning impairment of bb duplication in addition to segregation defects. In strains like N41, bb duplication was strongly disturbed with in average of only 0.53 bbs per cell; segregation defects and bbs without flagella were frequent. Our analysis indicated further that about 40% of the wild-type amount of centrin is sufficient to restore (Fig. 2) or maintain (Fig. 1D) a mostly biflagellate phenotype and that flagellar assembly onto bbs is more sensitive to centrin deficiency than bb assembly. Thus, in a condition of centrin deficiency, flagella number cannot be used to determine bb
number. In a broad view, the various tasks of centrin in Chlamydomonas were affected by decreasing amounts of this protein in the following order: segregation, ability to assemble flagella, replication of bbs. This differential sensitivity seemed unrelated to the presence or absence of certain centrin-based cytoskeletal elements: NBBCs and the dCF were defective in wild-type centrin but over-expressing centrin-GFP, which supported by the analysis of strains having normal levels of the known centrin-based structures like the NBBCs, a view cytoskeletal elements: NBBCs and the dCF were defective in flagella, replication of bbs. This differential sensitivity seemed protein in the following order: segregation, ability to assemble

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