Bld10p, a novel protein essential for basal body assembly in *Chlamydomonas*: localization to the cartwheel, the first ninefold symmetrical structure appearing during assembly

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How centrioles and basal bodies assemble is a long-standing puzzle in cell biology. To address this problem, we analyzed a novel basal body-defective *Chlamydomonas reinhardtii* mutant isolated from a collection of flagella-less mutants. This mutant, bld10, displayed disorganized mitotic spindles and cytoplasmic microtubules, resulting in abnormal cell division and slow growth. Electron microscopic observation suggested that bld10 cells totally lack basal bodies. The product of the BLD10 gene (Bld10p) was found to be a novel coiled-coil protein of 170 kD. Immunoelectron microscopy localizes Bld10p to the cartwheel, a structure with ninefold rotational symmetry positioned near the proximal end of the basal bodies. Because the cartwheel forms the base from which the triplet microtubules elongate, we suggest that Bld10p plays an essential role in an early stage of basal body assembly. A viable mutant having such a severe basal body defect emphasizes the usefulness of *Chlamydomonas* in studying the mechanism of basal body/centriole assembly by using a variety of mutants.

Introduction

The centriole, present at the core of the centrosome in animal cells, and basal bodies, structures that nucleate the assembly of cilia and flagella, are homologous organelles with nine triplet microtubules arranged in rotational symmetry. In *Chlamydomonas reinhardtii*, the basal body functions as the centriole during mitosis, and it subtends the flagellum in interphase cells. These organelles have long attracted attention from many researchers because of their elaborate structure and complex mechanism of assembly, which involves semi-conservative duplication once per cell cycle (for review see Dutcher, 2003).

The process of centriole/basal body assembly has been extensively studied by EM (Stubblefield and Brinkley, 1967; Dippel, 1968; Allen, 1969; Cavalier-Smith, 1974). The assembly process consists of several distinct steps: (a) an amorphous ring-like structure appears by the side of the mother organelle (Dippel, 1968); (b) a cartwheel structure consisting of a central hub and nine spokes is formed on the ring-like structure (Cavalier-Smith, 1974); (c) the A-tubule of the triplet microtubule is placed at each tip of the cartwheel spoke; (d) the B- and C-tubules are then added to the preexisting A-tubule; and (e) a cylinder consisting of nine triplet microtubules elongates to the mature length (Allen, 1969; Cavalier-Smith, 1974). Because the cartwheel structure displays ninefold symmetry, as does the mature basal body, this structure has been suggested to be the organizing center for the assembly of the microtubule wall (Anderson and Brenner, 1971; Gavin, 1984). However, no direct evidence has been reported for the importance of cartwheels in centriole/basal body assembly.

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Little is known about the molecular mechanism controlling the assembly of centrioles/basal bodies. Molecular dissection of the assembly process is complicated by the fact that isolation and biochemical analysis of basal bodies is very difficult. Such a complex process, not amenable to biochemical analysis, is best studied by the analysis of mutants affecting the process. Recently, genetic approaches using unicellular organisms, such as Chlamydomonas and Paramecium, have identified several proteins crucial for the assembly of basal bodies and their associated structures (Taillon et al., 1992; Ruiz et al., 1999, 2000; Silflow et al., 2001). In Chlamydomonas, two basal body-defective mutants, bld2 and uni3, have led to the discovery of ε-tubulin and δ-tubulin, respectively, and elucidation of their roles in basal body assembly (Dutcher and Trabuco, 1998; Dutcher et al., 2002). The bld2 mutation causes cells to assemble severely truncated basal bodies composed of singlet microtubules. The uni3 mutation causes cells to assemble incomplete basal bodies comprised of doublet, not triplet, microtubules. Therefore, the minor tubulin species ε- and δ-tubulin are thought to function in adding B- and C-tubules of basal bodies, respectively. These works demonstrate the usefulness of Chlamydomonas mutants for the molecular dissection of basal body assembly (Dutcher, 2003).

Here, we produced a collection of flagella-less mutants by insertional mutagenesis and isolated a new mutant, bld10. This mutant is defective in mitosis, frequently shows aberrant mitotic spindles and disorganized microtubule organization within the cell, and grows significantly slower than wild type. Surprisingly, EM failed to detect any basal body-like structures in this mutant. We show that the gene responsible for the bld10 mutation codes for a novel coiled-coil protein that localizes to the cartwheel, which is a structure that is present at the proximal end of the basal body and may function as a scaffold essential for basal body assembly.

Results
Mitotic defects in bld10
To isolate mutants defective in basal body assembly, we screened ~10,000 mutants generated by insertional mutagenesis and analyzed those mutants that did not assemble flagella (Tam and Lefebvre, 1993; Matsuura et al., 2002). Of 74 flagella-less mutants isolated, one mutant (2H4) showed abnormal cell division (see next paragraph). Because the basal body-deficient mutant bld2 displays abnormal cell division (Ehler et al., 1995), we anticipated that mutant 2H4 might also have some defects in the basal body. The mutant 2H4 was distinct from bld10 in size in cell to cell. Bar, 5 μm. (B) Histograms showing the size variability among the four daughter cells soon after division in bld10 and fla10. Ordinate: the number of mother cells counted. Abscissa: the SD of the size of the four daughter cells. The size of each cell was assessed by measuring the area of two-dimensionally projected microscope images. (C) Growth rates. bld10 grows more slowly than wild type (WT). In contrast, the growth rate of bld2-1 is indistinguishable from that of wild type.

We next observed mitotic spindles in wild-type and bld10 cells by indirect immunofluorescence microscopy using anti-α-tubulin antibody to determine whether bld10 cells have defects in mitotic spindles. As shown in Fig. 2 A, bld10 cells frequently formed aberrant spindles. Nuclear segregation during cell division in bld10, as in bld2, often fails (Table I), probably as the result of abnormal mitosis.

Cytoskeletal disorganization in bld10
Basal bodies/centrioles are known to serve as microtubule-organizing centers in interphase cells. In wild-type Chlamydomonas, basal bodies are associated with four microtubules forming the pericentriolar material (PCM). The PCM is the site where the basal body is assembled. It has been reported that the PCM is disorganized in many basal body-defective mutants (Maier et al., 2001; Ruiz et al., 1999, 2000; Silflow et al., 2001). In this study, we have identified several proteins crucial for the assembly of basal bodies and their associated structures (Taillon et al., 1992; Ruiz et al., 1999, 2000; Silflow et al., 2001). In Chlamydomonas, two basal body-defective mutants, bld2 and uni3, have led to the discovery of ε-tubulin and δ-tubulin, respectively, and elucidation of their roles in basal body assembly (Dutcher and Trabuco, 1998; Dutcher et al., 2002). The bld2 mutation causes cells to assemble severely truncated basal bodies composed of singlet microtubules. The uni3 mutation causes cells to assemble incomplete basal bodies comprised of doublet, not triplet, microtubules. Therefore, the minor tubulin species ε- and δ-tubulin are thought to function in adding B- and C-tubules of basal bodies, respectively. These works demonstrate the usefulness of Chlamydomonas mutants for the molecular dissection of basal body assembly (Dutcher, 2003).

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Figure 1. Defects of bld10 in cell division. (A) DIC images of bld10 and fla10 cells. The mutant fla10 lacks flagella but divides normally into four equal-sized daughter cells. In contrast, the daughter cells of bld10 differ in size from cell to cell. Bar, 5 μm. (B) Histograms showing the size variability among the four daughter cells soon after division in bld10 and fla10. Ordinate: the number of mother cells counted. Abscissa: the SD of the size of the four daughter cells. The size of each cell was assessed by measuring the area of two-dimensionally projected microscope images. (C) Growth rates. bld10 grows more slowly than wild type (WT). In contrast, the growth rate of bld2-1 is indistinguishable from that of wild type.
bulle bundles called the rootlet microtubules, which play an important role in determining the position of the cleavage furrow (Ehler et al., 1995). To examine the organization of the rootlets in \textit{bld10}, cells were stained with an antibody to acetylated \(\alpha\)-tubulin (Fig. 2 B). Wild-type cells show a cruciform pattern of rootlet microtubules radiating from the vicinity of the basal bodies. In \textit{bld10} cells, however, the number of the rootlet microtubule bundles was frequently less than or greater than four. The arrangement of those bundles was also aberrant.

Another intracellular structure associated with the basal bodies is the system of centrin-containing fibers connecting the basal bodies to the nucleus (Huang et al., 1988). We examined these fibers using immunofluorescence microscopy with an anticentrin antibody in wild-type cells and in \textit{bld10} mutant cells (Fig. 2 C). In \textit{bld10} cells we were unable to visualize centrin-containing projections comparable to those seen in wild-type cells. \textit{bld2} cells have similar, severe defects in the assembly of centrin-containing fibers (Ehler et al., 1995).

Electron microscopic observation of basal bodies of \textit{bld10}

We were unable to observe any basal body-like structures in \textit{bld10} cells despite extensive examination of more than 1,000 thin sections. Instead, we very rarely observed structures that resembled flagellar membranes protruding from the cell body without an accompanying basal body nearby (Fig. 3, B and C). Beneath the protrusion, rootlet microtubules and the distal striated fibers, which in the wild-type cells would connect the two basal bodies, were occasionally found, but they were aberrantly positioned. These observations suggest that \textit{bld10} lacks morphologically identifiable basal bodies.

Cloning and characterization of the \textit{BLD10} gene

The \textit{BLD10} gene was cloned using the plasmid tag inserted into the genome of the \textit{bld10} mutant (Tam and Lefebvre, 1993). Southern blots using genomic DNA showed that \textit{bld10} has a single plasmid insertion. Backcrosses of \textit{bld10} to wild-type cells showed Mendelian segregation of the aflagellate phenotype consistent with a mutation caused by the insertion of the nitrate reductase gene into a single genetic locus (unpublished data). By screening a bacterial artificial chromosome (BAC) library using the genomic fragment flanking the inserted plasmid as a probe, we isolated seven clones with inserts ranging from 21 to \(\sim\)100 kb (Fig. 4). All clones were found to rescue the \textit{bld10} phenotype upon introduction of BAC DNA into mutant cells by electroporation. By repeated transformation with those clones after digestion with appropriate restriction enzymes, we found that the region necessary for rescue can be reduced to 13 kb. Analyses of the sequence using the GeneMark and Green-Genie gene prediction programs (Lukashin and Borodovsky, 1998; Kulp et al., 1996) consistently predicted that the fragment contains a single gene consisting of 27 exons extending over 12 kb. The exon–intron boundaries of the predicted gene were confirmed by sequencing DNA fragments produced by RT-PCR using primers designed from the predicted exon regions. Using PCR-based polymorphisms (Kathir et al., 2003) the cloned \textit{BLD10} gene was mapped at the end of Linkage Group X, 6 cM centromere-distal to \textit{PF24i} (unpublished data). No uncharacterized flagellar-assembly mutations have been mapped to this region of the genome, so \textit{BLD10} is a novel genetic locus.

Analysis of the complete cDNA sequence showed that \textit{BLD10} has a single ORF of 4,923 bp that encodes a predicted protein (Bld10p) of 1,640 aa with a predicted molecular mass of 174.6 kD (Fig. 5 A). Bld10p has a high probability of forming coiled coils over its entire length (Fig. 5 B), as predicted using the COILS algorithm (Lupas et al., 1991). A consensus sequence for the myosin-tail domain and two leucine zipper motifs were found in the COOH-terminal half (Fig. 5, A and C). Bld10p as a whole showed no significant homology with any known protein sequence. However, a group of uncharacterized mammalian proteins, including human KIAA0635 and mouse BC062951, are distantly related to Bld10p. Although sharing only 25% identity in a limited region of the sequence, these proteins are predicted to form a coiled-coil structure throughout their length like Bld10p.

While cloning the \textit{BLD10} gene, we were surprised to find that genomic fragments lacking part of the NH\(_2\)-terminal re-
region of the gene rescued the mutation as efficiently as the full-length gene (Fig. 4). Even when we deleted the NH2-terminal 30% of the gene (fragment ΔN), the mutant phenotype was rescued completely, indicating that the NH2-terminal part of Bld10p is not essential for its function.

Localization of Bld10p

The localization of Bld10p in the cell was examined using a polyclonal antibody raised against a bacterially expressed peptide (Fig. 5 A). Fig. 6 A shows the specificity of this antibody by Western blot analysis of proteins in the whole cell extracts. Proteins from wild–type cells yielded a single immunoreactive band with an apparent molecular mass of 170 kD, whereas the extract from bld10 mutants yielded no band. A band of the same size was detected in the extract from bld10 cells rescued with a BAC clone containing the full-length BLD10 gene. A smaller molecular mass band (∼120 kD) was detected in the cells transformed with the DNA fragment truncated at the NH2-terminal end (ΔN). An extract from the bld2 mutant also displayed a band of 170 kD. To determine whether Bld10p is associated with the basal body or its related structures, we analyzed the nucleoflagellar apparatuses (NFAps), i.e., cytoskeletal complexes containing the basal bodies, axonemes, rootlet microtubules, nuclei, and other fibrous structures connected to the basal bodies (Wright et al., 1985). The antibody, while detecting no band in axonemal samples, detected a band in NFAps stronger than in the extract from whole cells (Fig. 6 B), suggesting that the protein resides in the basal body region of the NFAps and not in the axoneme.

Indirect immunofluorescence microscopy confirmed the association of Bld10p with the basal body. When the NFAps from flagellated interphase cells were stained, three or four dots were visible at the position of the basal bodies (Fig. 6 C). The presence of more than two dots suggests that this protein is localized also to the probasal bodies, the precursor of the basal bodies, that are present throughout the G1 phase of the cell cycle (Gaffal, 1988). In mitotic cells, in which flagella are resorbed and the two mother–daughter basal body pairs are located near the spindle poles, Bld10p was found localized at the spindle poles (Fig. 6 C). Thus, Bld10p appeared to be localized to the basal body/centrioles throughout the cell cycle. The mutant bld2 expresses Bld10p at levels comparable to wild-type cells (Fig. 6 A). Bld10p was correctly localized at the position of the basal bodies in bld2 cells, although only one or two dots of Bld10p fluorescence were usually visible (Fig. 6 D). This result suggests that Bld10p is incorporated during assembly of basal bodies at a step before the BLD2 gene product, ε-tubulin.

Immuno-EM

To further define the cellular localization of Bld10p, we next performed immuno-EM on the isolated NFAps. Gold particles conjugated to the secondary antibody were found at the proximal end of the basal bodies in longitudinal sections (Fig. 7 A). In cross sections, particles were found on radial thin filaments in the lumen of the basal body (Fig. 7 B). Bld10p was localized to the structure called the cartwheel, a subcentriolar structure present near the proximal end of the organelle. It is composed of the central tubule and a group of nine filaments radiating from the tubule to each triplet microtubule. Several sets of the nine filaments are usually present in a single centriole/basal body (Gibbons and Grimstone, 1960). The cartwheel makes up a hub and spokes with ninefold symmetry. During the assembly of the centriole/basal body, the cartwheel appears in the earliest stage, when its basement structure is assem-
bled (Anderson and Brenner, 1971). In agreement with this observation, the Bld10p signal was also observed in the probasal body, an immature basal body developing beside the mother basal body (Fig. 7 C).

Discussion

In this paper we show that the BLD10 gene of Chlamydomonas is required for basal body assembly. The BLD10 gene product localizes to the cartwheel, a ninefold symmetrical structure that appears in the early stage of the centriole/basal body assembly.

Extensive electron microscopic observations failed to detect any basal body-like structures in the bld10 cells (Fig. 3). We conclude that this mutant totally lacks basal bodies, although we cannot rule out the possibility that some disorganized basal body-related structure was present and not detected in our electron micrographic analysis. Whether or not some small basal body remnant is present in mutant cells, however, bld10 cells clearly have severe defects in basal body assembly. Some of the defects in bld10 have also been observed in bld2, a mutant that has severely disrupted basal bodies, formed at most of short, singlet microtubules (Goodenough and St. Clair, 1975; Ehler et al., 1995). Both mutants frequently show abnormality in the position of the cleavage furrow, resulting in the production of daughter cells of unequal sizes and containing an abnormal number of nuclei (Fig. 1 and Table I). Both display abnormality in the cytoskeletons associated with the basal body, e.g., rootlet microtubules and nucleus-basal body connectors (Fig. 2 C), and abnormal spindles in mitotic cells (Fig. 2 A). These defects are closely related to each other because the rootlet microtubules function in determining the position of the spindle and cleavage furrow just like astral microtubules in animal cells (Ehler et al., 1995). The deficiencies shared by bld10 and bld2 indicate that both mutants are commonly deficient in some basal body-related structures required for the precise arrangement of cytoskeletal structures.

However, bld10 also displays some defects that have not been observed in bld2. Most strikingly, the apparent generation time of bld10 was much longer than that of bld2, which grows almost as fast as wild type (Fig. 1). In addition, bld10 often forms abnormal spindles with microtubules not tied to the spindle poles, whereas bld2 forms normal, albeit aberrantly positioned, spindles (Fig. 2 A; Ehler et al., 1995). The slow growth of bld10 might be due to the operation of a
checkpoint mechanism that inhibits cell division until spindle microtubules are correctly connected to the kinetochores and spindles. These differences suggest that bld10 basal body defects may be more severe than those in bld2 mutant cells.

Immunoelectron microscopic observations showed that Bld10p localizes to a distinct structure in the centriole, called the cartwheel (Fig. 7). The cartwheel structure has been found in the procentrioles or probasal bodies in many organisms. In vertebrates, it is present in the procentriole but disappears from the mature centriole, whereas in alga and ciliated cells it is retained in the basal body throughout the cell cycle (Alvey, 1986). Previous works described the cartwheel as the first ninefold symmetrical structure that appears in the process of centriole/basal body assembly (Anderson and Brenner, 1971; Cavalier-Smith, 1974; Tamm and Tamm, 1980; Kallenbach, 1982). Thus, the cartwheel has been proposed to function as a scaffold essential for centriole/basal body assembly (Anderson and Brenner, 1971). In the absence of genetic analysis, however, the functional importance of the cartwheel in the centriole/basal body assembly has not been established. The absence of
basal bodies in \textit{bld10} cells and the localization of Bld10p to the centriole/basal body (Fig. 8).

Sequence analysis indicates that Bld10p has a strong tendency to form coiled coils over its entire length, with two leucine zipper motifs and a domain homologous to the myosin tail. Hence, Bld10p is likely to be an elongated protein capable of interacting with other proteins or with itself. Bld10p might well be the major structural component of the cartwheel, which is made up of thin filaments. Rather surprisingly, we found that a Bld10p variant from which the NH\textsubscript{2}-terminal 30\% has been removed can rescue the mutation as efficiently as intact Bld10p. The structure of the basal bodies observed by thin section EM in the rescued cells was indistinguishable from that in the wild-type cells (unpublished data). These observations suggest that the NH\textsubscript{2}-terminal 30\% is not necessary for the putative protein-protein interaction that underlies the cartwheel function. If Bld10p functions through its interaction with itself, this would mean that the interaction does not involve head-to-tail association; rather, the interaction may involve lateral or staggered association between the molecules, like the myosin tails in the thick filaments of skeletal muscle. Determining whether Bld10p interacts with itself or with other proteins, and elucidation of the portions of the molecule important for function awaits future study.

The viability of \textit{bld10} cells despite their severe basal body defect raises the question whether the organelle is essential in \textit{Chlamydomonas}. Based on the genetic analyses of \textit{bld2}, Preble et al. (2001) suggested that the basal body is essential. The original strain of \textit{bld2} that has a truncated gene product (\(\epsilon\)-tubulin) shows a severe basal body defect but it is viable. However, a null allele of \textit{bld2} isolated in a diploid strain is lethal when diploids are sporulated to produce haploids. This result strongly suggests that the \textit{BLD2} gene plays an essential role in assembling the basal bodies and that the complete loss of the organelle should be lethal. On the other hand, the loss of Bld10p, which appears to play an essential role at an earlier step than \(\epsilon\)-tubulin, does not result in lethality. A possible explanation for these inconsistent results is that \(\epsilon\)-tubulin has an essential role not only in assembling the basal body but also in another process essential for viability, for example forming cytoplasmic microtubules. The localization of \(\epsilon\)-tubulin in material surrounding the basal bodies and in spikes protruding along rootlets, may be consistent with this possibility.

In animal cells, it is becoming evident that centrosomes are not essential for mitotic spindle formation (Rieder et al., 2001). In extracts of \textit{Xenopus} oocytes, functional bipolar spindles were assembled in the absence of centrosomes (Heald et al., 1997). Similar results were obtained in vivo as well: when centrosomes are ablated using laser light or a microinjection needle, cells form acentrosomal spindles and complete mitosis (Maniotis and Schliwa, 1991; Khodjakov et al., 2000). Thus, in addition to the normal pathway for spindle assembly, cells have a centrosome-independent pathway, to which certain types of kinesin, dynactin, cytoplasmic dynein, and NuMA are known to contribute (Compton, 1998). Because the orthologues of almost all of these proteins are found in the \textit{Chlamydomonas} genome database (http://genome.jgi-psf.org/chlre2/chlre2.home.html), it is reasonable to speculate that \textit{Chlamydomonas} also has the redundant pathway for spindle assembly.

In summary, \textit{bld10} lacks a protein that functions in the early stage of basal body formation. The ultrastructural defect in \textit{bld10} cells is more extreme than the defects in any previously studied basal body mutants of \textit{Chlamydomonas}. A simple yet important conclusion from this work is that \textit{Chlamydomonas} mutants that have such a serious defect in the basal bodies are not lethal and can be analyzed by genetic as well as molecular biological means. We may expect that further isolation of basal body-deficient mutants will enable us to identify a variety of new basal body components and deepen our understanding of the mechanism of basal body/centriole assembly.

Materials and methods

Strains and culture conditions

For insertional mutagenesis, strain A54-e18 (ac17 mt1 mt2) (Smith and Lefebvre, 1996) was transformed with the plasmid pMN56 containing the \textit{NIT1} gene (Fernandez et al., 1989). A flagella-less mutant, 2H4, generated during transformation was chosen for further analysis. Strains L5 (\textit{int1}, \textit{apn1}, mt1) and L8 (\textit{int1}, \textit{apn1}, mt1) were used in backcrosses to analyze the linkage of the \textit{bld10} mutation in strain 2H4 to the site of plasmid insertion (Tam and Lefebvre, 1993). Strains CC-478 (\textit{bld2}; mt1) CC-479 (\textit{bld2}; mt2), and CC-503 (cv92; mt1) were obtained from the \textit{Chlamydomonas} Genetics Center. A null allele of \textit{fla10} with an aflagellate phenotype (Matsuzura et al., 2002) was used as a control for cell division experiments. For phenotypic rescue experiments, a double mutant \textit{bld10}arg7 was constructed by crossing \textit{bld10} with an arg7 mutant strain lacking arginosuccinate lyase; Debu et al., 1989). Phenotypically rescued strains \textit{bld10} and \textit{bld10}:\textit{NAR} were transformed with the wild-type \textit{BLD10} gene and the gene lacking the NH\textsubscript{2}-terminal 30\% of the predicted protein, respectively. Culture media used were SGII, SGII-NO\textsubscript{3} (Sager and Granick, 1953; Kindle, 1990), TAP medium (Gorman and Levine, 1965), and TAP medium supplemented with 0.005\% arginine. Cells were grown at 24\degree C on solid agar medium under constant illumination or in liquid medium on a 12 h/12 h light/dark cycle with constant aeration. Growth media was prepared as described previously (Tam and Lefebvre, 1993). Conditions for gamete production and mating were as described previously (Harris, 1989).

Transformation and phenotypic rescue experiments

For production of insertional mutants, A54-e18 cells were transformed by vortexing cells with glass beads (Kindle, 1990; Tam and Lefebvre, 1993). For rescue experiments, a \textit{bld10}arg7 double mutant strain was transformed by electroporation (Shimogawara et al., 1998) using a mixture of the plasmid \textit{PARC7.8} carrying the arginosuccinate lyase gene (Debu et al., 1989) and different fragments of genomic DNA. After transformation, cells were suspended in liquid TAP medium in test tubes and cultivated for 5 d. Rescue of the \textit{bld10} phenotype was judged by observing swimming cells at the menisci of culture tubes.

Identification of the genomic region containing the \textit{BLD10} gene

A genomic DNA fragment adjacent to the plasmid insertion site was isolated by plasmid rescue after digestion of genomic DNA using Sphi (Tam and Lefebvre, 1993). Using the isolated DNA fragment as a probe, a \textit{Chlamydomonas} BAC library (Clemson University Genomics Institute, Clemson, SC) was screened.

Determining the \textit{BLD10} cDNA sequence

Exons in the genomic sequence were predicted using the GeneMark (Lukashin and Borodovsky, 1998) and GreenGenie programs (http://www.cse.ucsc.edu/%7Edkulp/cgi-bin/greenGenie; Kulp et al., 1996). The exon–intron boundaries of the gene were determined by RT-PCR. The first strand cDNA for RT-PCR was synthesized using RNA isolated by CsCl cushion centrifugation (Sambrook et al., 1989). The primer used for cDNA synthesis was a 20-mer oligonucleotide designed from the cloned cDNA sequence. The single strand cDNA was purified by a GLASS MAX DNA isolation spin cartridge system (GIBCO BRL). PCR was performed in the presence of 5\% DMSO using the synthesized cDNA as a template.
primers designed from the putative exon sequences, and the Expand™ High Fidelity PCR System (Roche Applied Science).

Production of anti-Bld10p antibodies
For preparation of pAbs against Bld10p, a GST fusion protein carrying the sequence spanning 109 aa of the protein (Fig. 6) was expressed in *E. coli*. The cDNA sequence coding for the peptide was amplified by PCR using primers SM1 (5'-CGGGATCCCTCGGGGTGTTATACGC-3') and KM2 (5'-CCGAATTCCTGGGGCTGTTACGCT-3') and the cDNA isolated from the library as a template. The PCR product was digested at BamHI and EcoRI sites in the primer sequences (underlined) and subcloned into the BamHI-EcoRI sites of the pGEX2T vector (Amersham Biosciences). Expression of the fusion protein was induced by adding 0.1 mM IPTG to the *E. coli* cell culture and the whole bacterial proteins were analyzed by SDS-PAGE. After the gel was stained with Coomassie brilliant blue, the band of the fusion protein in the gel was cut out and immersed in distilled water for 30 min at RT. The gel was loaded on another SDS-polyacrylamide gel and overlaid with the SDS-PAGE sample buffer. After electrophoresis, an appropriate portion of the gel containing the protein was excised, crushed and dialyzed against PBS, and used for immunizing rabbits. The purified antibodies were affinity purified using the fusion protein blotted on PVDF membranes (Olmedo, 1981).

Fluorescence microscopy
Indirect immunofluorescence microscopy was performed as described by Holmes and Dutcher (1989). The primary antibodies used were affinity-purified anti-Bld10p antibody, antiacetylated α-tubulin (monoclonal 6-11B-1; Sigma-Aldrich) diluted in blocking buffer to 1:50, anti-α-tubulin (monoclonal B-5-1-2; Sigma-Aldrich) to 1:100, and anticientrin antibody (a gift from J.L. Salisbury, Mayo Clinic, Rochester, MN) to 1:50. The secondary antibodies used were FITC-conjugated goat anti–mouse IgG and rhodamine-conjugated goat anti–rabbit IgG (Sigma-Aldrich). To observe the localization of DNA relative to spindle microtubules, cells were simultaneously stained with anti-α-tubulin antibody and 0.1 mg/ml DAPI. The samples were prepared as described by Silflow et al. (2001). Images were recorded using an Axiosplan fluorescence microscope (Carl Zeiss Microimaging, Inc.) with a 63×/1.4 NA objective lens and a CoolSNAP CCD camera (Roper Scientific).

EM
Cells grown to log phase were collected by centrifugation and prefixed in 2% glutaraldehyde at RT for 1–2 h. After three washes in cold buffer (Wright et al., 1983), samples were fixed after in 1% OsO4 in collidine acetate and Reynold's lead citrate, and observed with a JEM100-CX electron microscope. The fixation was performed on ice for 1 h. After one wash in PBS containing 50 mM NH4Cl and two washes with PBS, the NFAPs were incubated in PBS containing 1% BSA (PBS/BSA) for 2 h with gentle agitation at 4°C. The NFAPs were treated with primary antibody in the PBS/BSA solution at a dilution of 1:20 to 4°C for 16 h to overnight. After three washes in PBS/BSA, the NFAPs were incubated with the secondary antibody (goat anti–rabbit IgG conjugated with 10-nm gold particles) in PBS/BSA at a 1:20 dilution at RT. After one wash with PBS/BSA and three washes with PBS, the apparatus were fixed after in 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, at RT for 1 h, and then in 1% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, at 4°C overnight. The samples were fixed after in 1% OsO4 in 0.1 M phosphate buffer, pH 7.4, for 1 h on ice. The pellets were block-stained with 1% uranyl acetate for 1 h on ice, dehydrated, and embedded in Epon812 resin. As a control negative, primary antibody was omitted.

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