The *bld1* mutation identifies the *Chlamydomonas osm-6* homolog as a gene required for flagellar assembly

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Insertional mutagenesis procedures in *Chlamydomonas* [1] have facilitated the identification and characterization of dozens of genes required for the assembly and motility of flagella in *Chlamydomonas* [2–6]. Many of these genes have been found to have homologs in animal systems. Here we describe a new gene required for flagellar assembly. Null mutants at the *BLD1* locus assemble no flagella, and the flagellar membrane abuts the end of the transition zone distal to the basal body. Unlike mutants with basal body ultrastructural defects, such as *bld2* [7], *bld1* mutants have normal basal bodies and cytoplasmic microtubule rootlets. The wild-type *BLD1* gene was cloned by using DNA flanking the site of insertion of plasmid DNA in an insertional mutant; the cloned gene rescues the *bld1* mutant phenotype upon transformation. The predicted *BLD1* gene product is a 50.4 kDa protein with extensive regions of sequence similarity to the *osm-6* gene of *Caenorhabditis elegans* [8] whose product is necessary for the assembly of a set of sensory cilia [9]. The protein product of the *BLD1* gene corresponds to IFT52 [10], a protein component of "raft" particles shown to undergo rapid transport up and down *Chlamydomonas* flagella between the flagellar membrane and the axoneme in a process known as intraflagellar transport (IFT) [11, 12]. The *BLD1* RNA transcript is upregulated upon flagellar amputation, as observed for many other genes encoding flagellar proteins [13]. These results demonstrate that the function of the IFT52 protein in *Chlamydomonas* is essential for the assembly and/or maintenance of the flagella.

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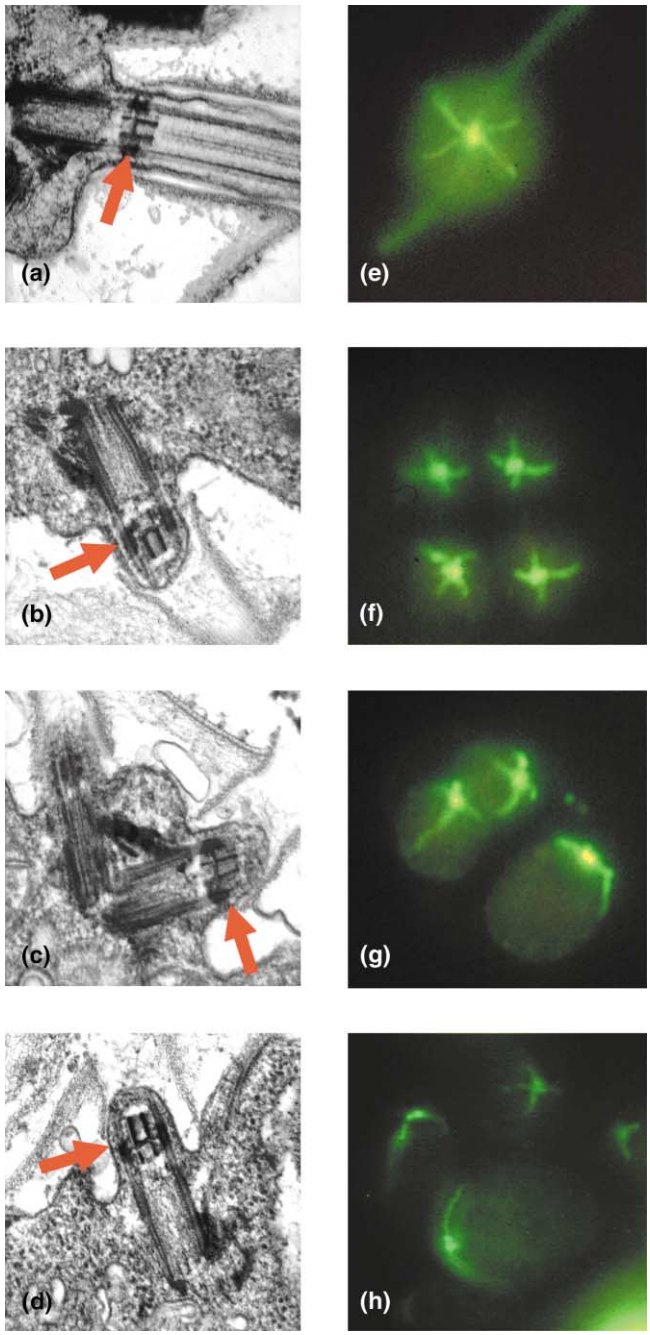
Results and discussion

To identify genetically *Chlamydomonas* gene products required for the assembly of flagella, we performed an insertional mutagenesis screen and isolated flagellaless mutants. *nit1* mutant cells, which are defective in the structural gene for nitrate reductase (NR) [14], were transformed with a plasmid containing the full-length NR gene, and colonies growing on media containing nitrate as the sole nitrogen source were picked into liquid culture [15]. Among these colonies were mutant strains with defective flagella. One of these mutants, Y18, had cells with no flagella when examined by differential-interference contrast microscopy. To determine whether even short flagella were present and to search for possible basal body defects, we examined Y18 cells with transmission electron microscopy (Figure 1). In wild-type cells, the triplet microtubules of the basal bodies provide a template for assembly of the axonemal doublet microtubules beginning just proximal to the H shaped transition region (arrow, Figure 1a–d). The two singlet microtubules of the central pair begin just above the transition region (Figure 1a). In Y18 cells (Figure 1b–d), ultrastructurally normal basal bodies were observed, but no flagella were assembled. The cell membrane was always found in close association with the distal end of the H shaped transition region, indicating that no assembly of flagella occurs in the Y18 mutant.

In *bld2*, a well-characterized basal body mutant of *Chlamydomonas*, defects in the assembly of the basal body lead to a disorientation of the stable rootlet microtubules found at the anterior end of the cell [7]. These microtubules in wild-type cells form a cruciform or "hot cross buns" structure just beneath the basal bodies (Figure 1e). In *bld2* mutant cells, the cruciform structure is disrupted, and the microtubules are present in disoriented tangles. To determine whether the mutation in the Y18 strain disrupts cytoplasmic microtubule arrays, we performed immunofluorescence on wild-type and mutant cells by using a monoclonal antibody to acetylated α-tubulin, which specifically recognizes the stable microtubules of the rootlets [16]. The cruciform rootlet microtubule struc-

Figure 1



The Y18 mutation prevents flagellar assembly without disrupting cytoplasmic microtubule organization. Transmission electron microscopy of thin sections through basal bodies of **(a)** wild-type and **(b–d)** Y18 mutant cells. In Y18 mutants there is no assembly of doublet or central pair microtubules beyond the H shaped transition region (indicated by the arrow). Indirect immunofluorescence microscopy by using an antibody against acetylated α-tubulin in **(e)** wild-type and **(f–h)** Y18 mutant cells. The cruciform array of stable microtubules at the anterior end of the cell is unaffected by the Y18 mutation.

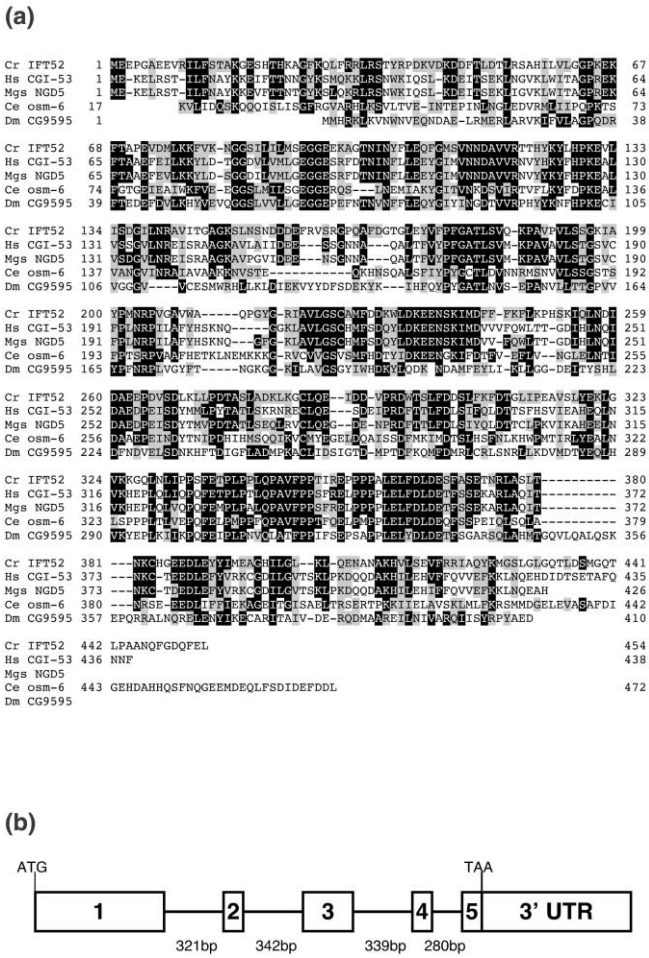
ture in Y18 was indistinguishable from that seen in wild-type cells (Figure 1f–h).

To clone the gene affected by the Y18 mutation, we prepared a fragment of the plasmid used for transformation (pMN56) as a hybridization probe to clone fragments of genomic DNA flanking the site of plasmid insertion in the genome of Y18 cells. These fragments, in turn, were used as hybridization probes to identify bacterial artificial chromosome (BAC) clones of wild-type DNA to be tested for rescue of the Y18 phenotype upon transformation. When clone 37l4 was introduced into Y18 cells by transformation, it rescued the flagellaless phenotype, indicating that this clone contained the wild-type gene. Clone 37l4 had been identified previously in our laboratory by using a hybridization probe prepared with PCR to clone the *Chlamydomonas* homolog of the *C. elegans osm-6* gene. *osm-6* mutants show severe defects in the assembly of a set of sensory cilia. Cole et al. [10] previously identified *osm-6* as a homolog of IFT52 in *Chlamydomonas* based on the sequence of three tryptic peptides of IFT52 purified from raft protein complexes.

We designed degenerate primers to isolate the IFT52 gene by using RT-PCR on *Chlamydomonas* poly(A) RNA. An 800 bp RT-PCR product was cloned and its DNA sequence was obtained. The amino acid sequence of the predicted peptide closely matched a region of the *osm-6* protein in *C. elegans*. We used the short PCR product to identify a 2.5 kb clone from a cDNA library prepared by using RNA from deflagellated cells (a gift of John Davies, Exelixis). The sequence of this cDNA clone predicts a protein of 50.4 kDa with similarity to the *osm-6* protein of *C. elegans* over almost its entire length. The proteins are identical at 35% of their amino acid residues, with an additional 22.5% similarity (Figure 2a). As originally noted by Collet et al. [9], the *osm-6* gene shows extensive sequence homology with a predicted protein called NGD5 from a hybrid rodent cell line. The NGD5 transcript is reported to decrease after prolonged treatment of cultured NG108-15 cells with opioid [17]. The predicted protein of the *Chlamydomonas* gene affected by the Y18 mutation is even more closely related to NGD5 than to *osm-6*, showing 49% identity and an additional 21% similarity to the rodent protein. In addition, predicted proteins (from genome sequencing) from *Drosophila melanogaster* (Dm CG9595) and human (CGI-53) show high levels of amino acid identity and similarity to the *Chlamydomonas* protein, indicating that proteins similar to IFT52 are present in many animal genomes.

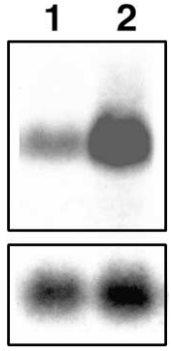
Given that *osm-6* mutants show extensive defects in the assembly of a set of sensory cilia, it seemed likely that the mutant lesion in Y18 that prevents flagellar assembly affected the gene encoding the 52 kDa IFT protein. To study this possibility further, we digested BAC 37l4 into

Figure 2



The *Chlamydomonas* IFT52 protein shows extensive sequence identity and similarity with predicted proteins in many other organisms. **(a)** The *Chlamydomonas* IFT52 protein (GenBank accession number AF397450) was aligned with other predicted proteins using the BLAST algorithm [18]. The following abbreviations were used: Cr, *Chlamydomonas reinhardtii*; Hs, *Homo sapiens*; Mgs, Murinae gen.sp.; Ce, *Caenorhabditis elegans*; and Dm, *Drosophila melanogaster*. Dark shading indicates amino acid sequences identical to those in IFT52, and lighter shading indicates similar amino acid residues. Accession numbers are as follows: *Cr* IFT52, AF397450; *Hs* CGI-53, NP057088; *Mgs* NGD5, AAA96241; *Ce osm-6*, CAA03975; *Dm* CG9595, AAF5240. **(b)** The gene encoding IFT52 has four introns (thin lines) and five exons (boxes), covering a total of 3.5 kb of genomic DNA.

Figure 3



The RNA transcript of the *BLD1* gene is upregulated after flagellar amputation. Total RNA (20 μg per lane) was isolated from cells before (lane 1) or 30 min after (lane 2) deflagellation. The 2.9 kb *BLD1* transcript (top panels) increases in abundance upon deflagellation. The bottom panels are a control for RNA loading, in which a probe for the S14 (small ribosomal protein) gene recognizes a 1 kb transcript which does not increase upon deflagellation.

smaller fragments by using *Sal*I and tested these fragments for rescue of the flagellaless Y18 phenotype upon transformation. A 5.2 kb fragment rescued the flagellaless phenotype of the mutant. The DNA sequence obtained from the 5.2 kb genomic fragment showed that the gene encoding the predicted IFT52 protein covers a region of 3.5 kb and contains four introns (Figure 2b). Southern blot experiments using the full-length cDNA clone as a hybridization probe to examine restriction digests of the BAC clones demonstrated that the cDNA clone was entirely contained within the 5.2 kb fragment (data not shown). We learned from Deane and colleagues (Yale University) that antibodies to the IFT52 protein did not stain *bld1* cells (see accompanying paper by Deane et al. [22]), so we sequenced genomic DNA from the *bld1* mutant. The *bld1* mutant contains a deletion of 117 bp in the gene encoding IFT52, including portions of exon 4 and the following intron (data not shown). We conclude that the Y18 mutant is an allele of *bld1* (now designated *bld1-2*) and that the *BLD1* gene encodes the IFT52 protein.

Other genes encoding flagellar proteins have been shown to respond to flagellar amputation by increasing transcript levels [13]. We examined the levels of the *BLD1* transcript in wild-type cells by RNA blot analysis. The 2.9 kb *BLD1* transcript is present in nondeflagellated cells, and its level increases by 30 min after deflagellation, consistent with the likelihood that the *BLD1* gene product is a flagellar protein (Figure 3).

In summary, the *Chlamydomonas* homolog of the *osm-6* gene of *C. elegans* is required for the assembly and/or maintenance of flagella. In the *bld1-2* mutant the gene encoding the IFT52 protein is completely deleted, and no flagella are assembled. The flagellar membrane closely abuts the transition zone, indicating that the active movement of raft proteins and their cargo along the flagella is essential for the assembly of any flagellar proteins into the axoneme. The conservation of *BLD1* protein sequence in organisms from *Chlamydomonas* to human indicates that *BLD1* homologs in many systems may be needed to assemble cilia and flagella. The accompanying paper [22]

reports the localization of the *BLD1* gene product to the transition fibers, suggesting that these fibers may play an active role in the assembly process.

Materials and methods

Strains and culture conditions

The *Chlamydomonas* strain used for mutagenesis was A54-e18 [2], which has a deletion in the structural gene for nitrate reductase. Strains were grown in liquid medium and on 1% agar plates in 1/2 R medium, as described [2].

Degenerate primers and RT-PCR

Two 8-fold degenerate PCR primers were designed from the *Chlamydomonas* tryptic peptide sequences p52tr-16 and p52tr-18 [10]: 5′-CTAC TTYCTGGAGCAGTTYGGYATG-3′ and 5′-CTCRTCCAGRTCRAA CAGCTCCAG-3′ where Y = C + T and R = G + A. Reverse transcriptase (RT)-PCR was carried out using the primer derived from p52-tr18 and 1 μg of *Chlamydomonas* poly(A) RNA isolated from deflagellated cells (strain 21*gr*, mating type +) with a 5′ RACE kit (GIBCO-BRL, Life Technologies) according to the manufacturer's instructions for reverse transcription of RNA with high GC content. The cDNA product was used as template for a PCR reaction containing 40 pmol of both the p52tr-16 and p52tr-18 primers. PCR was performed as follows: 1 cycle of 5 min at 94°C and then 10 min at 80°C during which Taq DNA polymerase (Fisher Biotech, Pittsburgh, Pennsylvania, USA) supplied with buffer A was added to the reaction; 36 cycles of 1 min at 94°C, 45 s at 58°C, 2 min at 72°C; and 1 cycle of 10 min at 72°C. The PCR product was cloned with the pCR2.1 vector and INVaF′ cells according to the protocols in the TA cloning kit (Invitrogen, Carlsbad, California, USA).

Library screening and sequence analysis

A probe made from the degenerate PCR product was used to screen a BAC library of *Chlamydomonas* genomic DNA (Incyte Genomics, St. Louis, Missouri, USA). The cDNA clone was obtained by screening a normalized *Chlamydomonas* cDNA library in bacteriophage lambda Zap II (a kind gift of John Davies, Exelixis). A full-length cDNA in a pBluescript II SK-plasmid was excised from the lambda ZAP clones. Genomic and cDNA clones were sequenced at the Advanced Genetics Analysis Center (University of Minnesota, St. Paul, Minnesota, USA), and sequences were analyzed using the GCG programs (Genetics Computer Group, Madison, Wisconsin, USA). GenBank searches were performed using the FASTA and BLAST programs [18].

Chlamydomonas *transformation*

BAC clones and restriction fragments isolated from purified BAC clones were cotransformed with pARG7.8 DNA [19] into *bld8 arg7* cells, as described [15].

Immunofluorescence and electron microscopy

Indirect immunofluorescence was performed following fixation in cold methanol, as described [20]. The monoclonal antibody used in these experiments was 6-11B-1, specific for acetylated α-tubulin (a kind gift of Gianni Piperno, Mount Sinai School of Medicine). Thin-section transmission electron microscopy was performed as described in Dentler and Adams [21].

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