Fa1p is a 171 kDa protein essential for axonemal microtubule severing in Chlamydomonas

Rip J. Finst¹,², Peter J. Kim¹, Eric R. Griffis¹ and Lynne M. Quarmby¹,²,*

¹Department of Cell Biology and ²Graduate Program in Biochemistry, Cell and Developmental Biology, Emory University School of Medicine, Atlanta, Georgia 30322, USA
*Author for correspondence at present address: Department of Biological Sciences, Simon Fraser University, 8888 University Drive, Canada V5A 1S6 (e-mail: quarmby@sfu.ca)

SUMMARY

A key event in deflagellation or deciliation is the severing of the nine outer-doublet axonemal microtubules at a specific site in the flagellar transition zone. Previous genetic analysis revealed three genes that are essential for deflagellation in Chlamydomonas. We have now identified the first of these products, Fa1p, a protein required for Ca²⁺-dependent, axonemal microtubule severing. Genetic mapping and the availability of a tagged allele allowed us to physically map the gene to the centromere-proximal domain of the mating-type locus. We identified clones of Chlamydomonas genomic DNA that rescued the Ca²⁺-dependent axonemal microtubule severing defect of fa1 mutants. The FA1 cDNA, obtained by RT-PCR, encodes a novel protein of 171 kDa, which is predicted to contain an amino-terminal coiled-coil domain and three Ca²⁺/calmodulin binding domains. By western analysis and subcellular fractionation, the FA1 product is enriched in flagellar-basal body complexes. Based on these observations and previous studies, we hypothesize that a Ca²⁺-activated, Ca²⁺-binding protein binds Fa1p leading ultimately to the activation of axonemal microtubule severing.

Key words: Microtubule severing, Axoneme, Flagellum, Chlamydomonas

INTRODUCTION

The molecular mechanism and physiological functions of deciliation/deflagellation have received little scientific attention as biological problems. This is in spite of the fact that for nearly half a century, scientists have used deflagellation and deciliation to isolate flagella and cilia from Chlamydomonas and Tetrahymena (Lewin, 1953; Watson and Hopkins, 1962). Although the ecological/physiological roles of deciliation/deflagellation are poorly understood, it is an evolutionarily conserved process. Sea urchin embryos, scallop gills, rabbit oviduct and porcine respiratory tissue, and rat cerebral ependymal cells have all been directly observed to deciliate in response to stress (Auclair and Siegel, 1966; Anderson, 1974; Stephens, 1975; Hastie et al., 1986; Mohammed et al., 1999). A hallmark feature of these processes is the severing of the nine outer-doublet axonemal microtubules at a specific site at the base of the flagellum/cilium, distal to the transition zone. The mechanism of deflagellation has been most thoroughly studied in Chlamydomonas (reviewed by Quarmby and Lohret, 1999).

Chlamydomonas is a well established system for tractable genetic, in vitro, and biochemical studies of the deflagellation behavior (Quarmby, 1996; Finst et al., 1998; Lohret et al., 1998). We have previously demonstrated that the Ca²⁺ sensing mechanism and the machinery of doublet microtubule severing purify with membrane-free preparations of flagellar-basal body complexes (FBBCs; Lohret et al., 1998). Furthermore, the established microtubule severing protein, katanin (Vale, 1991; McNally and Vale, 1993; Hartman et al., 1998), is a component of the FBBC suggesting that it may be a subunit of the microtubule severing machinery. In Chlamydomonas, the 60 kDa subunit of katanin is a single copy gene with high sequence identity to the human p60 ortholog (Lohret et al., 1999). Chlamydomonas p60 is found, among other places, on the inner face of the axoneme at the distal end of the flagellar transition zone where axonemal severing occurs during deflagellation (Lohret et al., 1999). Chlamydomonas p60 is found, among other places, on the inner face of the axoneme at the distal end of the flagellar transition zone where axonemal severing occurs during deflagellation (Lohret et al., 1999). We also showed that exogenously added katanin could sever the complex doublet microtubules of the axoneme. Although, katanin is localized at the proper position and has the necessary biochemical activity to sever axonemes during deflagellation, direct evidence of a role for katanin in deflagellation, as would be provided by mutant strains, is lacking.

In order to directly identify proteins involved in axonemal microtubule severing, we isolated deflagellation-defective Chlamydomonas mutants (Finst et al., 1998). From our analysis of non-essential mutations, we discovered three genes, ADF1, FA1, and FA2, that are necessary for deflagellation (Finst et al., 1998). ADF1 is essential for Ca²⁺ influx whereas FA1 and FA2 are required for shedding of the flagella in response to the Ca²⁺ signal. We did not isolate katanin mutants.
in this screen, suggesting that either it is not involved in deflagellation or it plays other, essential, roles in the cell. We favor the latter explanation (see Lohret et al., 1998) and hypothesize that the products of FA1 and FA2 may be important for the specific action of katin during deflagellation (reviewed by Quarntby and Lohret, 1999).

We report here the cloning of FA1, the first gene required for deflagellation. We isolated a 10.7 kb genomic clone which rescued the deflagellation defect of fa1 mutants. Using RT-PCR we determined that the FA1 CDNA is ~5.7 kb and encodes a product of ~171 kDa with a predicted coiled-coil domain and consensus Ca\(^{2+}\)/calmodulin (Ca\(^{2+}\)/CaM) binding motifs. Northern analysis suggests that the FA1 message is upregulated following deflagellation. As anticipated, Fa1p is enriched in flagellar-based body complexes, but is not detected in the flagella. FBBCs of fa1 mutants fail to undergo axonemal severing following addition of Ca\(^{2+}\). These results suggest that Fa1p plays a role in the transduction of a Ca\(^{2+}\) signal into activation of axonemal microtubule severing.

**MATERIALS AND METHODS**

**Cell strains and culture**

*Chlamydomonas reinhardtii* strains g1 and B214 were obtained from G. Pazour and G. Witman (Univ. of Massachusetts, Medical Center Worcester; Pazour et al., 1995) and mutant strains fa1-3 (Nit1*, mt+) and fa1-4 (Nit1, mt−) were isolated in our lab as previously described (Finst et al., 1998), fa1-1 (previously known as fa-1; Lewin and Burrascano, 1983) and the arg7 strain, defective in the arginosuccinate lyase gene, were obtained from the Chlamydomonas Genetics Center (Durham, NC). A strain expressing hemagglutinin-epitope tagged α-tubulin was obtained from D. Diener and J. Rosenbaum (Yale University, New Haven, CT; Kozminski et al., 1993). Cells were maintained in liquid TAP medium or on plates (1.5% agar; Harris, 1989) at 22°C with constant illumination. All arg7 strains were maintained on medium supplemented with 0.02% arginine (Sigma, St. Louis, MO). Nit1* transformants (see below) were selected by growth on SGII (NO₃) media (Sager and Granick, 1953; Fernandez et al., 1989).

**Isolation of FBBCs and severing assays**

Flagellar-based body complexes were isolated from wild-type and fa1-4 strains; deflagellation was induced as previously described (Lohret et al., 1998).

**Mapping the fa1-3 allele**

Isolation of *Chlamydomonas* genomic DNA and Southern analysis was performed as previously described (Finst et al., 1998). Genomic DNA of strain fa1-3 was individually or doubly digested with endonucleases PstI, SacI, HindIII, BamHI, XhoI, SmaI, and XhoI and size fractionated by agarose gel electrophoresis. Following transfer to and immobilization on nitrocellulose membranes, DNA was hybridized with \(^{32}\)P-labeled probes (Prime-It II kit, Stratagene, La Jolla, CA) generated from either pUC119 or a 1.2 kb fragment from pMS1-7.0 were digested with endonucleases PstI, HindIII, BamHI, KpnI or Sall and fragments ligated into pBluescriptII SK−. Subclones were sequenced by Genomics, Inc. (Augusta, GA) or the Emory University DNA Sequencing Facility (Atlanta, GA). Additional oligonucleotide sequencing primers were synthesized by Integrated DNA Technologies (Coralville, IA).

To identify the defect of the fa1-1 allele, genomic DNA isolated from strains B214 and fa1-1 was used as the template for PCR reactions (conditions described below). Using primers annealing near the 3′ end of the FA1 gene, aberrant products were obtained with fa1-1 DNA as the template. Following DNA sequencing, we identified a rearrangement of this region. To create pW2SElnv, a genomic subclone in which the identified inversion of fa1-1 replaces the corresponding region of the wild-type clone, a series of subcloning steps were used. A 2.5 kb fragment of genomic DNA, isolated by digestion of pW2BE with KpnI and NotI, was ligated to pBluescriptII SK− to produce plasmid pW2KN. A 7.0 kb fragment of genomic DNA, isolated by digestion of MS1 phage DNA with SacI and EcoRI, was ligated to pBluescriptII SK− to create plasmid pMS1-7.0. The 1.6 kb DNA product obtained by PCR of DNA from fa1-1 with primers 8530(+) and 10112(−) (see Table 1) was digested with restriction endonucleases ApaI and NotI. The resulting ~1.3 kb product was ligated to pW2KN that had been similarly digested. This plasmid and pMS1-7.0 were digested with SfiI and NotI and the 1.6 kb and 8.9 kb fragments were ligated yielding plasmid pMS1-7.0Inv. To produce the final plasmid, pMS1-7.0Inv and pW2SE were digested with Ndel and EcoRI and the 4.6 kb and 9.0 kb products, respectively, were ligated yielding plasmid pW2SEInv. DNA sequencing of plasmid pW2SEInv confirmed that the inversion had been appropriately subcloned into the otherwise wild-type sequence.

**DNA sequencing, gene prediction, and fa1 mutant analysis**

In preparation for DNA sequencing of the genomic clone, W2 bacteriophage DNA was digested with Smal or EcoRI and KpnI and fragments ligated into pBluescript II SK−. Bacteriophage DNA of the MS1 clone was individually or doubly digested with EcoRI, SacI, HindIII, BamHI, KpnI or Sall and fragments ligated into pBluescriptII SK−. Subclones were sequenced by Genomics, Inc. (Augusta, GA) or the Emory University DNA Sequencing Facility (Atlanta, GA). Additional oligonucleotide sequencing primers were synthesized by Integrated DNA Technologies (Coralville, IA).

To identify the defect of the fa1-1 allele, genomic DNA isolated from strains B214 and fa1-1 was used as the template for PCR reactions (conditions described below). Using primers annealing near the 3′ end of the FA1 gene, aberrant products were obtained with fa1-1 DNA as the template. Following DNA sequencing, we identified a rearrangement of this region. To create pW2SElnv, a genomic subclone in which the identified inversion of fa1-1 replaces the corresponding region of the wild-type clone, a series of subcloning steps were used. A 2.5 kb fragment of genomic DNA, isolated by digestion of pW2BE with KpnI and NotI, was ligated to pBluescriptII SK− to produce plasmid pW2KN. A 7.0 kb fragment of genomic DNA, isolated by digestion of MS1 phage DNA with SacI and EcoRI, was ligated to pBluescriptII SK− to create plasmid pMS1-7.0. The 1.6 kb DNA product obtained by PCR of DNA from fa1-1 with primers 8530(+) and 10112(−) (see Table 1) was digested with restriction endonucleases ApaI and NotI. The resulting ~1.3 kb product was ligated to pW2KN that had been similarly digested. This plasmid and pMS1-7.0 were digested with SfiI and NotI and the 1.6 kb and 8.9 kb fragments were ligated yielding plasmid pMS1-7.0Inv. To produce the final plasmid, pMS1-7.0Inv and pW2SE were digested with Ndel and EcoRI and the 4.6 kb and 9.0 kb products, respectively, were ligated yielding plasmid pW2SEInv. DNA sequencing of plasmid pW2SEInv confirmed that the inversion had been appropriately subcloned into the otherwise wild-type sequence.

**RNA isolation, reverse transcription-PCR, and northern analysis**

Total RNA was isolated from vegetative cells following the methods of Wilkerson et al. (1994). Briefly, cells grown in liquid TAP medium
with constant light were harvested by centrifugation and resuspended
in lysis buffer (20 mM Tris, pH 8, 20 mM EDTA, pH 8, 5% SDS, 0.4
mg/ml Proteinase K) at room temperature. Following a 2 hour
incubation, the lysis mixture was phenol/chloroform extracted in the
presence of 0.23 M sodium acetate. Nucleic acid was precipitated with
an equal volume of 4 M LiCl, precipitated overnight at 4°C and collected
washed with 80% ethanol. RNA was resuspended in water and an
equal volume of isopropanol, collected by centrifugation and
incubation, the lysis mixture was phenol/chloroform extracted in the
presence of 0.23 M sodium acetate. Nucleic acid was precipitated with
an equal volume of 4 M LiCl, precipitated overnight at 4°C and collected
washed with 80% ethanol. RNA was resuspended in water and an
equal volume of isopropanol, collected by centrifugation and
incubation, the lysis mixture was phenol/chloroform extracted in the
presence of 0.23 M sodium acetate. Nucleic acid was precipitated with
an equal volume of 4 M LiCl, precipitated overnight at 4°C and collected
washed with 80% ethanol. RNA was resuspended in water and an

For RT-PCR, 500 ng of polyadenylated RNA was reverse
transcribed using 200 U SuperScriptII reverse transcriptase (Life
Technologies, Gaithersburg, MD) and 0.5
M of each primer, 0.5-2.0 mM
MgCl₂, 5% DMSO, 0.3 mM dNTP, 1× Taq DNA polymerase buffer and 0.025 U/μl Taq DNA polymerase (Promega, Madison, WI). The
PCR reaction mixture was denatured at 95°C for 5 minutes, followed by 40 cycles of 95°C for 1 minute, 58-60°C for 1 minute and 72°C
for an additional 8-10 minutes. To identify specific products, a second
round of PCR was frequently performed using 0.2-2% of the first
round product as template and primers predicted to anneal to sites
internal to the first round primers. PCR products were separated by
agarose gel electrophoresis, specific products were excised from the
gel and purified using an Ultrafree-MC filter unit (Millipore, Bedford,
MA), cloned into the pGEM-T Easy vector (Promega, Madison, WI)
and sequenced.

For 3' RACE, we modified the SMART RACE cDNA
Amplification protocol (Clontech, Palo Alto, CA) by designing
primers with high melting temperatures. One-half microgram of dT
Adaptor primer (Table 1) was used for reverse transcription. Each 3'
RACE PCR reaction mixture contained 0.2 μM gene specific, 0.2
μM Long Universal and 0.1 μM Short Universal primers. The reaction
mixture for a second round of 3' RACE PCR was identical except that
0.2 μM nested gene specific and 0.2 μM Nested Universal primers
were used. The RACE reaction mixture was initially denatured at
95°C for 5 minutes followed by 3 cycles of 95°C for 1 minute, 70°C
for 1 minute and 72°C for 5 minutes. Cycles were repeated decreasing the
annealing temperature 2°C every 3 cycles to 58°C. Following 40 cycles at an annealing temperature of 58°C, the reaction mixture was
maintained at 72°C for an additional 12 minutes.

For 5' RACE, we modified the SMART RACE cDNA
Amplification protocol (Clontech, Palo Alto, CA) by designing
primers with high melting temperatures. One-half microgram of dT
Adaptor primer (Table 1) was used for reverse transcription. Each 3'
RACE PCR reaction mixture contained 0.2 μM gene specific, 0.2
μM Long Universal and 0.1 μM Short Universal primers. The reaction
mixture for a second round of 3' RACE PCR was identical except that
0.2 μM nested gene specific and 0.2 μM Nested Universal primers
were used. The RACE reaction mixture was initially denatured at
95°C for 5 minutes followed by 3 cycles of 95°C for 1 minute, 70°C
for 1 minute and 72°C for 5 minutes. Cycles were repeated decreasing the
annealing temperature 2°C every 3 cycles to 58°C. Following 40 cycles at an annealing temperature of 58°C, the reaction mixture was
maintained at 72°C for an additional 12 minutes.

For 5' RACE, the protocol of Eyal et al. (1999) was used with the
following modifications. Reverse transcription was performed as
described above using a 5'-phosphorylated 4058(−) Junction primer.
Following RNase treatment, the reaction mixture was purified with the
QiAquick PCR Purification Kit (Qiagen, Valencia, CA) and eluted with
30 μl dH₂O. After overnight ligation with T4 RNA ligase (New
England Biolabs, Beverly, MA), 5% of the self-ligated cDNA served

### Table 1. Primers for cloning FA1

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Nucleotide sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>3428(+)</td>
<td>5’-GAGGCTTGACTGTTGGAGGTC</td>
</tr>
<tr>
<td>3428(−)</td>
<td>5’-GACCTCACCACGGTCTACGCTTC</td>
</tr>
<tr>
<td>3702(+)</td>
<td>5’-GTCGACAGCGACGCGTACGAG</td>
</tr>
<tr>
<td>4058(−)</td>
<td>5’-CCTGGCGGCTCCACGGCTCATG</td>
</tr>
<tr>
<td>4272(+)</td>
<td>5’-CCACGGACATTCCGGGAACAC</td>
</tr>
<tr>
<td>4319(−)</td>
<td>5’-CTCTGGCCCTTGTGTCATCAG</td>
</tr>
<tr>
<td>4644(−)+BamHI</td>
<td>5’-GGATCTCATGCCTGGCGCATCGCAACATC</td>
</tr>
<tr>
<td>4874(+)</td>
<td>5’-CGAGCGGAGGAGGAGGATTGT</td>
</tr>
<tr>
<td>5162(−)</td>
<td>5’-CTCCTGACAGCGACGCAAGC</td>
</tr>
<tr>
<td>5430(+)</td>
<td>5’-CGGTTGTCGGCGGCGGCGGAGT</td>
</tr>
<tr>
<td>5784(−)</td>
<td>5’-CTGTCGGTCTGCTGCTGTCGG</td>
</tr>
<tr>
<td>6102(−)</td>
<td>5’-CTCTGACAGCTCCTCACGCC</td>
</tr>
<tr>
<td>7032(−)+XhoI</td>
<td>5’-CTCGAGTGTCCATCCTGCTGCGGACGAC</td>
</tr>
<tr>
<td>7037(−)+BamHI</td>
<td>5’-CCCCGGGGGATACGCTGCTGCGGAGATGAC</td>
</tr>
<tr>
<td>7038(+)</td>
<td>5’-GTTGCGGCTGCGGAGGATGAG</td>
</tr>
<tr>
<td>7184(−)</td>
<td>5’-GCCGGCTCTACGCTTGCA</td>
</tr>
<tr>
<td>7484(+)</td>
<td>5’-CCGGCTTCCTGCTGCTGCTGTC</td>
</tr>
<tr>
<td>8530(+)</td>
<td>5’-TGGCCGTGTCGACGATGAG</td>
</tr>
<tr>
<td>9022(+)</td>
<td>5’-CTAACGGACGAGGAGGAG</td>
</tr>
<tr>
<td>9951(−)+EcoRI</td>
<td>5’-CCCGGGGAAATTTCATAGTCAGGTGATCAGGAGCGCGGAG</td>
</tr>
<tr>
<td>9956(−)+BamHI</td>
<td>5’-CCCCGGGGGATACGCTGCTGCGGAGATGAC</td>
</tr>
<tr>
<td>10112(−)</td>
<td>5’-GTTGCTACCTCCATGTAAGCTCC</td>
</tr>
<tr>
<td>10400(+)</td>
<td>5’-CTCTACAAGTGCTGTCAGGAGC</td>
</tr>
<tr>
<td>10657(−)</td>
<td>5’-CCGTCGCGTGGCGGAGTGGT</td>
</tr>
<tr>
<td>11752(−)</td>
<td>5’-CTCGTGACCTCCTGCGTCAACG</td>
</tr>
<tr>
<td>12378(+)</td>
<td>5’-ATCCGGGAGGAGTCTGCGGAG</td>
</tr>
<tr>
<td>12476(−)+NruI</td>
<td>5’-TCGGCACTATCCTGGAAGGAGC</td>
</tr>
<tr>
<td>12497(−)+NheI</td>
<td>5’-GCTAGCTGAGATGCCAGCCGGAGGATTGT</td>
</tr>
<tr>
<td>12499(−)+EcoRI</td>
<td>5’-CCCGGGGAATTCTCATACATGCCGCTGTAACCCAGG</td>
</tr>
<tr>
<td>12777(−)</td>
<td>5’-GTTCAAGCGAGCTCAAGCGG</td>
</tr>
<tr>
<td>12926(−)+XbaI</td>
<td>5’-TCTGACAGCAGGAGGAGAACGCGG</td>
</tr>
<tr>
<td>Long Universal</td>
<td>5’-GCGTCTGAGCTACATCCGGGAGGATGGCAGAGCGG</td>
</tr>
<tr>
<td>Short Universal</td>
<td>5’-GCGTCTGAGCTACATCCGGGAGGATGGCAGAGCGG</td>
</tr>
<tr>
<td>Nested Universal</td>
<td>5’-GGAATGATAGCTGCAATGGCGG</td>
</tr>
<tr>
<td>dT Adaptor</td>
<td>5’-GGAATGATAGCTGCAATGGCGG</td>
</tr>
<tr>
<td>3’RACE/9956(+)</td>
<td>5’-GCUCATCAAATCTGACGGGAGTGGAGT</td>
</tr>
<tr>
<td>3’RACE/10400(+)</td>
<td>5’-CTCTAATCCTGCTGCGTCAACG</td>
</tr>
</tbody>
</table>

For 5' RACE, the protocol of Eyal et al. (1999) was used with the
following modifications. Reverse transcription was performed as
described above using a 5'-phosphorylated 4058(−) Junction primer.
Following RNase treatment, the reaction mixture was purified with the
QiAquick PCR Purification Kit (Qiagen, Valencia, CA) and eluted with
30 μl dH₂O. After overnight ligation with T4 RNA ligase (New
England Biolabs, Beverly, MA), 5% of the self-ligated cDNA served
as template for PCR with primers 3702(+) and 3428(−) (Table 1). Products were separated by agarose gel electrophoresis and specific products were cloned and sequenced as described above.

For northern analysis, 10 μg of polyadenylated RNA was size fractionated on formaldehyde gels and transferred to Zeta Probe GT membranes (Bo-Rad, Hercules, CA) following established procedures (Sambrook et al., 1989). Three fragments of the FAI cDNA, totaling 4.8 kb, were random primed with the High Prime DNA Labeling Kit (Boehringer-Mannheim, Indianapolis, IN) for 3 hours at room temperature using [32P]dCTP and [32P]dATP at 6000 mCi/mmol. Membranes were then hybridized at 65°C for 48 hours in a buffer containing 5× SSC, 2× Denhardt’s solution, 100 mg/ml dextran sulfate, 1% SDS and 0.4 mg/ml sonicated herring sperm DNA.

cDNA sequence analysis

To identify genes related to FA1, searches of the SwissProt and GenBank (Flat File Release 115.0) databases were performed using BLAST and FASTA programs (Altschul et al., 1997). The program COILS (Lupas et al., 1991) was used to predict coiled-coil regions. Calmodulin binding domains were identified by eye using consensus sequences (Rhoads and Friedberg, 1997).

Mutants expressing hemagglutinin-tagged FA1

To facilitate detection of the FA1 product, a genomic construct encoding FA1 was engineered to contain three copies of the hemagglutinin (HA) epitope at the extreme carboxy terminal of the predicted product. First, wild-type genomic DNA was amplified with primers 11752(+) and 12476(−) and the PCR product subcloned into vector pGEM-T Easy. Following digestion with SpeI and NruI, the 700 bp fragment was ligated to vector p3×HA (a plasmid encoding 3 repeats of the 9 amino acid hemagglutinin epitope; generously provided by C. Sillifow, Univ. of Minnesota, St Paul, MN) to create vector p3×HA/11752-12476. Genomic DNA was amplified with primers 12497(+)-NheI and 12926(−)-XbaI and the 500 bp PCR product subcloned into vector p3×HA/11752-12476 following digestion with SalI and NheI to create vector p3×HA/11752-12926. To generate the final construct, vector p3×HAF1A, vector p3×HA/11752-12926 was digested with EcoRI and XbaI and the 1.4 kb fragment ligated to pW2SE.

To determine whether the epitope tagged construct could rescue the fa1 defect, fa1-4 cells were cotransformed with pMN56 and p3×HAF1A, and NIT1 transforming transformants selected by growth on medium containing nitrate. Transformants that were wild-type for deflagellation were designated as 3×HAFA1. To confirm that these rescued mutants retained the nucleotide sequence encoding the epitope tag, genomic DNA was isolated and used as template for PCR with primers 12378(+) and 12777(−). A ~520 bp product demonstrated that the transformed strains retained the nucleotides encoding the epitope tag.

Western analysis

For western analysis, whole cell and flagellar-basal body complex protein was isolated as described (Lohret et al., 1998). Flagella and axonemes were isolated by the dibucaine method (Witman, 1986; King et al., 1986) and purified as described by Smith and Sale (1991). Protein concentration was determined using the Bio-Rad Protein Assay (Hercules, CA). 10 μg of protein was separated on a 5% SDS-PAGE gel and electroblotted to Immuno-Blot PVDF membrane (Bio-Rad, Hercules, CA). The membrane was stained with Ponceau S (Sigma, St Louis, MO) to confirm efficient transfer of all protein samples. The membrane was washed and incubated overnight at 4°C with an anti-HA rat monoclonal antibody (clone 3F10; 200 ng/ml; Boehringer-Mannheim, Indianapolis, IN) according to the manufacturer’s instructions. The membrane was then incubated with anti-rat Ig-POD (200 mU/ml; Boehringer-Mannheim, Indianapolis, IN) for 30 minutes at room temperature and immunoreactive protein identified with BM Chemiluminescence Blotting Substrate (Boehringer-Mannheim, Indianapolis, IN).

RESULTS

FBBC severing assays

Detergent-permeabilized cells deflagellate with addition of Ca2+ suggesting that the components of microtubule severing are localized to the transition zone and associated with the axoneme (Sanders and Salisbury, 1989). Unlike wild-type cells, permeabilized fa1 mutants fail to deflagellate indicating that the FA1 product is important for the formation of a microtubule-severing complex, possibly itself a subunit of the complex (Sanders and Salisbury, 1989). Consistent with this, flagellar-basal body complexes (FBBCs), composed of the two basal bodies, the distal striated fibers which connect the basal bodies and the two axonemes which derive from the basal bodies (Dutcher, 1995), isolated from wild-type cells exhibit Ca2+-dependent axonemal microtubule severing (Lohret et al., 1998; Fig. 1A,B). As shown in Fig. 1C and D, the axonemes of FBBCs purified from fa1 mutants are not severed following addition of Ca2+, suggesting that the fa1 mutants have lost the ability to sense Ca2+ or are unable to transduce the Ca2+ signal into the microtubule severing response.

Mapping of the FA1 gene

The fa1 allele, fa1-3, is a tagged allele that was isolated in a screen for deflagellation mutants following NIT1 insertional mutagenesis (Finst et al., 1998). We created a physical map of the genomic DNA flanking the inserted plasmid in the fa1-3 allele. fa1-3 genomic DNA was digested with various restriction endonucleases and Southern analysis was performed using probes to the 5’ and 3’ ends of the transforming DNA. fa1-3 genomic DNA digested with SalI was hybridized with the pUC vector. As shown in Fig. 2A, the hybridizing product was 4.4 kb. Similarly, genomic DNA digested with BamHI and

![Fig. 1. Flagellar-basal body complexes (FBBCs) isolated from wild-type cells (A) undergo microtubule severing following the addition of Ca2+ (B) resulting in the release of both flagella. As observed previously, more than 95% of the axonemal microtubules were severed (Lohret et al., 1998). In contrast, of the 200 FBBCs from fa1-4 mutants assayed (C), none severed axonemal microtubules after treatment with Ca2+ (D).](image-url)
hybridized with a probe corresponding to the 3' end of \( NIT1 \) yielded a 7.5 kb band. Through this analysis, it was determined that ~2 kb of pUC was deleted, precluding the possibility of using plasmid rescue to clone the flanking DNA. Previous genetic analysis had placed \( FA1 \) within the mating-type locus (Ferris and Goodenough, 1994). The two restriction maps flanking the inserted DNA each matched only a single region in the 1.1 Mb published restriction map of the mating-type locus (Ferris and Goodenough, 1994). The identification of these two flanking sequences was confirmed by probing Southern blots of genomic DNA of wild-type, \( fa1-1, fa1-2, \) and \( fa1-3 \) with a fragment of DNA predicted to be deleted in \( fa1-3 \) (MV2S/Sa in Fig. 2A). A hybridizing band of identical molecular mass is present in the genome of \( fa1-1, fa1-2, \) and wild-type strains (Fig. 2B, lanes 1-3, respectively), but is absent in \( fa1-3 \) (Fig. 2B, lane 4). This result confirmed both the physical position of \( FA1 \) within the centromere-proximal region of the mating-type locus and the presence of a ~36 kb deletion in \( fa1-3 \).

**Rescue of \( fa1 \) mutants and cloning of \( FA1 \)**

A \( fa1-3:arg7 \) double mutant was cotransformed with wild-type DNA from the region of the deletion isolated from individual bacteriophage and \( ARG7 \) as a selectable marker. Transformants were selected by growth on medium lacking arginine and the deflagellation phenotype of individual transformants was determined. Transformation with DNA isolated from two phage, MS1 and W2, completely rescued the deflagellation defect (Fig. 3A). We deduced that the \( FA1 \) gene was located in the 14.7 kb region of overlap between these two phage. Other phage which extend into this region did not rescue the deflagellation defect, suggesting that the \( FA1 \) gene was large. To locate the \( FA1 \) gene within W2, the phage DNA was digested with restriction endonucleases and the resultant fragments were subcloned into plasmids. These plasmids were then cotransformed with a selectable marker, \( NIT1 \), into \( fa1-4 \) mutants. The deflagellation phenotype of transformants that were able to grow on medium containing nitrate as the sole nitrogen source was determined. Transformation with plasmid pW2SE rescued the deflagellation defect of \( fa1-4 \) (Fig. 3A). In contrast, plasmid pW2BE did not complement the \( fa1-4 \) defect. \( FA1 \) was, therefore, contained within the 10.5 kb genomic subclone of plasmid pW2SE.

**Identification of the \( FA1 \) cDNA and northern analysis**

In order to identify the \( FA1 \) gene, the DNA of the rescuing clone was sequenced. The GeneMark gene prediction algorithm identified 16 putative exons on one strand whereas the other strand was largely devoid of predicted exons (data not shown).

In order to identify the \( FA1 \) cDNA, we used reverse transcription coupled with polymerase chain reaction (RT-PCR). Polyadenylated RNA isolated from an asynchronous population of wild-type cells was reverse transcribed with a primer designed to anneal to the 3' poly(A) tail of mRNAs. PCR reactions were performed with the primers outlined in Table 1. Following DNA sequencing and subsequent alignment of the genomic sequence with the sequence of cDNA clones, multiple exons were identified, and all introns contained consensus 5' donor and 3' acceptor splice sites (LeDizit and Piperno, 1995).

To clone the 5' and 3' ends of the \( FA1 \) cDNA, Rapid

![Fig. 2](image_url). (A) Physical map of \( FA1 \) and \( fa1-3 \) genomic DNA. The black line represents endogenous genomic DNA, the white line represents a remnant of the pUC119 plasmid and the grey line represents the introduced \( NIT1 \). Relative positions of restriction endonuclease sites in DNA flanking the insertion was determined by digesting DNA of \( fa1-3 \) and probing Southern blots with either a 5' pUC probe or a 3' \( NIT1 \) probe. Using the known restriction sites of the transforming DNA as ‘anchor’ sites, the distance to the respective sites in the flanking DNA could be determined. Tangential lines indicate the relative position of the insertion. Restriction endonuclease sites in the mating-type locus of wild-type cells are as described (Ferris and Goodenough, 1994). Abbreviations for restriction endonuclease sites: B: BamHI, H: HindIII, S: SacI, Sa: SstI, Sm: SmaI, X: XhoI, Xb: XbaI. (B) Southern blot of genomic DNA isolated from \( fa1-1, fa1-2, FA1, \) and \( fa1-3 \) strains digested with \( SmaI \) and probed with MV2S/Sa. The location of this probe is diagrammed in A.
Amplification of cDNA Ends (RACE) was used. For 3' RACE, mRNA was reverse transcribed with dT Adaptor, a primer containing unique 5' sequences for subsequent PCR with primary and nested primers. PCR with primers 10400(+) and Nested Universal resulted in a 1.7 kb product. By comparison of the cDNA and genomic DNA sequence, additional exons were identified as well as a polyadenylation signal (TGTAA) which occurs 19 nucleotides 5' of the poly(A) tail. To clone the 5' end of the FAI transcript, mRNA was reverse transcribed with primer 4058(+) Junction. An inverse PCR approach employed primers 3428(-) and 3702(+) to yield a product corresponding to the 5' end of the FAI transcript. The full-length FAI cDNA, assembled from overlapping cDNA sequences, is 5753 bp (Fig. 3B, Accession Number AF246990).

For northern analysis, poly(A) RNA was hybridized with probes generated from three fragments totaling 4.8 kb and covering 84% of the FAI cDNA. The hybridizing product was ~5.6 kb, consistent with the size of the cDNA obtained by RT-PCR (Fig. 4, lane 2). Lane 3 shows that the FAI mRNA was not detected in a fa1 deletion allele, fa1-4 (see below). The level of expression of a number of flagellar genes is up-regulated following deflagellation (Lefebvre and Rosenbaum, 1986). Lane 1 of Fig. 4 shows that wild-type cells undergoing flagellar regeneration have increased FAI mRNA expression.

Molecular analysis of fa1 mutants

By Southern analysis, the genomic DNA encoding FAI in strains fa1-3 and fa1-4 is deleted (Fig. 2A; data not shown for fa1-4). To identify the defect of fa1-1, a panel of primers spanning the region containing the FAI gene was used for PCR to ‘walk’ across the gene. Most primers produced products of wild-size type. In contrast, a reaction using primers 9226(+) and 10112(+) failed to produce any product. This suggested that fa1-1 carried a mutation near the predicted 3' end. The product of the PCR reaction with primers 8530(+) and 10112(+) was sequenced and a ~1.0 kb inversion was identified (Fig. 3B). Ferris and Goodenough (1994) found that the wild-type MT+ and MT− loci of Chlamydomonas are highly rearranged. These rearrangements, which are rare elsewhere in the genome, include translocations, inversions, duplications and deletions. In order test whether the inversion of fa1-1 was responsible for the deflagellation defect, we constructed a genomic subclone in which the inverted nucleotides of fa1-1 replaced the corresponding wild-type region. fa1-4 cells were cotransformed with the NIT1 gene, as a selectable marker, and a subclone of genomic DNA having either wild-type (pW2SE) or inverted sequences (pW2SEInv). The deflagellation phenotype was determined for both sets of cotransformants. As predicted, 23 of 284 transformants assayed were wild-type for deflagellation following transformation with pW2SE. In contrast, none of the 313 colonies assayed following transformation with pW2SEInv were rescued for the defect. This result demonstrated that the inversion found in fa1-1 is sufficient to impair the function of FAI.

Sequence analysis

The proposed translation initiation site is preceded by two in-frame translation termination codons 45 and 114 nucleotides 5' of the initiation codon. The 3' translation termination codon (nucleotides 5490 to 5492) is 264 nucleotides 5' of the beginning of the poly(A) tail (Fig. 3B). The cDNA encodes 1787 amino acids with a predicted molecular mass of 171.6 kDa and isoelectric point of 7.2 (Fig. 5). Searches of public databases using the amino acid sequence did not identify any proteins with significant sequence similarity. A segment of ~225 amino acids near the amino terminus has a high probability of forming a coiled-coil domain (Lupas et al., 1991; underlined in Fig. 5). In addition, three segments of Fa1p contain consensus motifs for the 1-8-14 type B...
Ca\(^{2+}\)-dependent/calmodulin (Ca\(^{2+}\)/CaM) binding motif (boxed in Fig. 5; Rhoads and Friedberg, 1997). The consensus 1-8-14 type B Ca\(^{2+}\)/CaM binding motif sequence is characterized by a 14 amino acid stretch having hydrophobic residues at positions 1, 8, and 14 and an overall net charge of +2 to +4.

**Subcellular localization of Fa1p**

Following isolation of the *FA1* cDNA, it was determined that the smallest genomic construct that rescued the deflagellation defect lacked 905 bp of the 3' most exon and intron (pW2SE; Fig. 3A and B). Given that the carboxy-terminal 194 amino acids were apparently dispensable for function, we anticipated that introduction of an epitope tag at the carboxy terminus would not alter the activity of the product. We engineered three copies of the nine amino acid epitope of hemagglutinin (HA) at the carboxy terminus of a *FA1* construct. Following cotransformation of *fa1-4* cells with the nitrate reductase gene, a selectable marker, and the epitope tagged *FA1* construct, the deflagellation phenotype of selected colonies was confirmed. The frequency of rescue of the *fa1-4* mutant phenotype was rescued by the epitope tagged construct, as we predict, this likely explains our inability to detect Fa1p protein, as we infer that introduction of an epitope tag at the carboxy terminus would not alter the activity of the product. We engineered three copies of the nine amino acid epitope of hemagglutinin (HA) at the carboxy terminus of a *FA1* construct. Following cotransformation of *fa1-4* cells with the nitrate reductase gene, a selectable marker, and the epitope tagged *FA1* construct, the deflagellation phenotype of selected colonies was determined. The frequency of rescue of the *fa1-4* deflagellation defect with the HA-tagged construct (p3\·\·HA) was the same as that obtained with an untagged construct (pW2SE). Genomic DNA isolated from cells rescued for deflagellation was used as a template for PCR to confirm that the HA epitope was retained (data not shown).

Monoclonal antiserum recognizing the HA epitope was used for western blots of whole cell, flagellar-basal body complex (FBBC), axonemal and flagellar protein from wild-type, *fa1-4* and *fa1-4* strains in which the mutant phenotype was rescued by the p3\·\·HAFA1 construct (Fig. 6A). The sera recognized a protein of the expected size in FBBC protein from rescued *fa1-4* cells (Fig. 6B, lane 6), but not in either the wild-type or *fa1-4* mutant strains (Fig. 6B, lanes 1-2 and 3-4, respectively). The reactive species is not detected in axonemal or flagellar protein isolated from 3\·\·HFA1 cells (Fig. 6B, lanes 7 and 8). We estimate that ~0.2% of total cellular protein is contributed by the FBBC based on the mass of protein isolated following purification of FBBCs from whole cells. If Fa1p is a transition zone protein, we predict that introduction of an epitope tag at the carboxy terminus would not alter the activity of the product. We engineered three copies of the nine amino acid epitope of hemagglutinin (HA) at the carboxy terminus of a *FA1* construct. Following cotransformation of *fa1-4* cells with the nitrate reductase gene, a selectable marker, and the epitope tagged *FA1* construct, the deflagellation phenotype of selected colonies was confirmed. The frequency of rescue of the *fa1-4* mutant phenotype was rescued by the epitope tagged construct, as we predict, this likely explains our inability to detect Fa1p protein, as we infer that introduction of an epitope tag at the carboxy terminus would not alter the activity of the product. We engineered three copies of the nine amino acid epitope of hemagglutinin (HA) at the carboxy terminus of a *FA1* construct. Following cotransformation of *fa1-4* cells with the nitrate reductase gene, a selectable marker, and the epitope tagged *FA1* construct, the deflagellation phenotype of selected colonies was determined. The frequency of rescue of the *fa1-4* deflagellation defect with the HA-tagged construct (p3\·\·HA) was the same as that obtained with an untagged construct (pW2SE). Genomic DNA isolated from cells rescued for deflagellation was used as a template for PCR to confirm that the HA epitope was retained (data not shown).

Monoclonal antiserum recognizing the HA epitope was used for western blots of whole cell, flagellar-basal body complex (FBBC), axonemal and flagellar protein from wild-type, *fa1-4* and *fa1-4* strains in which the mutant phenotype was rescued by the p3\·\·HAFA1 construct (Fig. 6A). The sera recognized a protein of the expected size in FBBC protein from rescued *fa1-4* cells (Fig. 6B, lane 6), but not in either the wild-type or *fa1-4* mutant strains (Fig. 6B, lanes 1-2 and 3-4, respectively). The reactive species is not detected in axonemal or flagellar protein isolated from 3\·\·HFA1 cells (Fig. 6B, lanes 7 and 8). We estimate that ~0.2% of total cellular protein is contributed by the FBBC based on the mass of protein isolated following purification of FBBCs from whole cells. If Fa1p is a transition zone protein, we predict that introduction of an epitope tag at the carboxy terminus would not alter the activity of the product. We engineered three copies of the nine amino acid epitope of hemagglutinin (HA) at the carboxy terminus of a *FA1* construct. Following cotransformation of *fa1-4* cells with the nitrate reductase gene, a selectable marker, and the epitope tagged *FA1* construct, the deflagellation phenotype of selected colonies was determined. The frequency of rescue of the *fa1-4* deflagellation defect with the HA-tagged construct (p3\·\·HAFA1) was the same as that obtained with an untagged construct (pW2SE). Genomic DNA isolated from cells rescued for deflagellation was used as a template for PCR to confirm that the HA epitope was retained (data not shown).

**DISCUSSION**

From previous work it was established that the components necessary for flagellar microtubule severing are tightly associated with the axoneme (Sanders and Salisbury, 1989; Quarmby and Hartzell, 1994; Finst et al., 1998; Lohret et al., 1998). As expected based on cellular phenotype, we report here that the axonemes of FBBCs isolated from *fa1* mutants are not severed following treatment with Ca\(^{2+}\). Therefore, *fa1* mutants have lost the ability to transduce a Ca\(^{2+}\) signal into axonemal microtubule severing.

To better understand the role of this gene in deflagellation, we have cloned *FA1* and localized the gene product. The *FA1* gene encodes a protein of 1787 amino acids with a predicted molecular mass of 171.6 kDa. Database searches using the deduced amino acid sequence did not identify any orthologs. The secondary structure of Fa1p is predicted to include a coiled-coil domain of ~225 amino acids near the amino terminus (Lupas et al., 1991). Coiled-coil domains are characterized by heptad repeats forming an amphipathic alpha helix and are present in a number of functionally diverse proteins. Hydrophobic interactions between coiled-coil domains result in stable protein-protein interactions. These interactions mediate assembly of both homo- and hetero-oligomeric complexes of varying subunit number (Shoeman and Trab, 1993; Beck and Brodsky, 1998; Heimbuch et al., 1999). A candidate for hetero-oligomeric interactions with Fa1p is the product of *FA2*, a second gene required for microtubule severing (Finst et al., 1998). It should be noted that the primary sequence of Fa1p is highly similar to other predicted prokaryotic and eukaryotic proteins (Weis et al., 1991). The predicted protein is ~18 kDa larger than the wild-type protein, indicating that Fa1p is an integral membrane protein. The predicted protein contains a large signal sequence having sequence similarities to signal sequences of other integral membrane proteins.

To better understand the role of this gene in deflagellation, we have cloned *FA1* and localized the gene product. The *FA1* gene encodes a protein of 1787 amino acids with a predicted molecular mass of 171.6 kDa. Database searches using the deduced amino acid sequence did not identify any orthologs. The secondary structure of Fa1p is predicted to include a coiled-coil domain of ~225 amino acids near the amino terminus (Lupas et al., 1991). Coiled-coil domains are characterized by heptad repeats forming an amphipathic alpha helix and are present in a number of functionally diverse proteins. Hydrophobic interactions between coiled-coil domains result in stable protein-protein interactions. These interactions mediate assembly of both homo- and hetero-oligomeric complexes of varying subunit number (Shoeman and Trab, 1993; Beck and Brodsky, 1998; Heimbuch et al., 1999). A candidate for hetero-oligomeric interactions with Fa1p is the product of *FA2*, a second gene required for microtubule severing (Finst et al., 1998). It should be noted that the primary sequence of Fa1p is highly similar to other predicted prokaryotic and eukaryotic proteins (Weis et al., 1991). The predicted protein is ~18 kDa larger than the wild-type protein, indicating that Fa1p is an integral membrane protein. The predicted protein contains a large signal sequence having sequence similarities to signal sequences of other integral membrane proteins.
also be noted that other factors could interact with Fa1p because our screen for loss-of-function mutations affecting deflagellation would not have identified genes with functions essential for life. The presence of a coiled-coil domain is consistent with our hypothesis that Fa1p is a component of a stable axonemal microtubule severing complex.

As mentioned above, the Ca\(^{2+}\) sensor of deflagellation purifies with membrane-free preparations of flagellar-basal body complexes (Lohret et al., 1998). It is, therefore, of interest that Fa1p is predicted to have three 1-8-14 type B Ca\(^{2+}\)/CaM binding domains. This class of calmodulin-binding motifs is found in a number of proteins including caldesmon and smooth muscle myosin light chain kinase (Rhoads and Friedberg, 1997). Gitelman and Witman (1980) determined that, in *Chlamydomonas*, calmodulin is present both as a detergent-soluble fraction and stably associated with the axoneme and is, therefore, a candidate for the Ca\(^{2+}\) sensor of deflagellation. Another candidate is the calmodulin-family member, centrin, which has been implicated in deflagellation (Sanders and Salisbury, 1989). Associated with the centrosome in most cells, centrin localizes to a variety of subcellular sites in *Chlamydomonas* including the distal end of the flagellar transition zone (Sanders and Salisbury, 1989). Ca\(^{2+}\)-induced contraction of centrin-containing fibers is temporally and spatially correlated with the severing of outer doublet microtubules (Sanders and Salisbury, 1989).

Although Ca\(^{2+}\)-induced contraction of centrin-containing fibers accompanies deflagellation in wild-type cells, the role of centrin in deflagellation is unclear. A mutant strain, *vfl2-1*, has a missense mutation in the first EF-hand Ca\(^{2+}\)-binding domain of centrin (Taillon et al., 1992). In *vfl2-1* cells, the stellate fibers of the flagellar transition zone are poorly organized likely resulting from the inability of centrin to be polymerized into filamentous structures (Taillon et al., 1992). Sanders and Salisbury (1994) reported that detergent-permeabilized cell models of *vfl2-1* treated with Ca\(^{2+}\) in the absence of any external shear forces (such as pipetting) do not deflagellate. However, under normal experimental conditions, *vfl2-1* cells exhibit wild-type deflagellation behavior (Jarvik and Suhan, 1991; Quarmby and Lohret, 1999). In contrast, cells with the Fa\(^{-}\) phenotype do not shed their flagella under any circumstances. Ca\(^{2+}\)-induced contraction of centrin-containing fibers and doublet microtubule severing are defective in detergent-extracted cell models of *fa1-1* cells, yet centrin localization and transition zone ultrastructure in this strain are indistinguishable from wild-type (Sanders and Salisbury, 1989). The organization of the transition zone in the deletion allele, *fa1-3*, also shows no structural defects (P. Beech and R. Finst, unpublished observations). It is clear that even if centrin plays a role in deflagellation, additional factors are important.

To localize the Fa1p product within the cell, an HA-epitope tagged product was created. This approach has been used previously to investigate the distribution of nonacetylatable \(\alpha\)-tubulin in *Chlamydomonas* (Kozminski et al., 1993). The deflagellation defect of *fa1-4* mutant cells was rescued following transformation with a genomic construct of Fa1p having three copies of the HA epitope at the carboxy terminus. Therefore, the epitope tagged Fa1p was expressed, functional and at least partially targeted to the appropriate cellular compartment. Protein isolated from whole cells, FBBCs, axonemes and flagella of rescued mutants was used for immunoblots with a monoclonal anti-HA antibody.

We warmly thank Drs P. Ferris and U. Goodenough (Washington University) for generously providing us with bacteriophage DNA, detailed physical maps of the mating type locus and much guidance. We thank Dr C. Silflow and M. LaVoie (Univ. of Minnesota) for the p3\(x\)-HA plasmid and advice on epitope tagging. We are grateful to the members of the Quarmby lab and to Drs W. Sale, H. C. Hartzell, and M. Powers and members of their labs at Emory for helpful advice and stimulating discussion. This work was supported by National Science Foundation grant MCB-9603716 (L.M.Q.). R.J.F. is supported by a National Institutes of Health predoctoral fellowship (T32 GM-08367).