

Chlamydomonas DIP13 and human NA14: a new class of proteins associated with microtubule structures is involved in cell division

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Accepted 21 December 2002

Journal of Cell Science 116, 1449-1462 © 2003 The Company of Biologists Ltd
doi:10.1242/jcs.00337

Summary

We have cloned and characterized a single copy *C. reinhardtii* gene containing an open reading frame of 333 nucleotides encoding a 12.7 kDa protein. The novel protein, DIP13, exhibits 60% identity with two mammalian proteins, human NA14 and an unnamed mouse protein. Homologous sequences are also present in several protozoan, trematode and fish genomes, but no homologs have been found in the completed genomes of yeast, *Drosophila*, *C. elegans* and *A. thaliana*. By using a specific antibody we have localized DIP13 to microtubule structures, namely basal bodies, flagellar axonemes and cytoplasmic microtubules. Anti-DIP13 antibody also specifically recognized human NA14 by

immunofluorescence and stained basal bodies and flagella of human sperm cells as well as the centrosome of HeLa cells. Expression of the *DIP13* open reading frame in antisense orientation in *Chlamydomonas* resulted in multinucleate, multiflagellate cells, which suggests a role for this protein in ensuring proper cell division. Thus, DIP13/NA14 could represent the founding members of a new class of highly conserved proteins that are associated with microtubule structures.

Key words: Basal body, Centriole, Flagella, Microtubule associated proteins, Antisense RNA

Introduction

The simple unicellular biflagellate green alga *Chlamydomonas reinhardtii* is rightfully called a 'green yeast' (Goodenough, 1992), because it is a premier model organism for molecular genetic analyses of, for example, photosynthesis, phototaxis and flagellar assembly and function (Rochaix, 1995; Fuhrmann et al., 2001; Dutcher, 1995a; Rosenbaum et al., 1999). In addition, the organism holds potential for biotechnological applications (Somanchi and Moroney, 1999; Ghirardi et al., 2000; Fukuzawa et al., 2001), not least because a genome project is finally underway (Harris, 2001) and a long-standing problem, the organism's reluctance to transformation with heterologous resistance markers, has been overcome by the introduction of markers against zeocin (Lumbreras et al., 1998), paromomycin (Sizova et al., 2001) and hygromycin antibiotics (Berthold et al., 2002). Moreover, gene function can be studied in *Chlamydomonas* by RNA interference technology (Fuhrmann et al., 2001), as is the case in other organisms.

More recently, *Chlamydomonas* has been gaining importance in the field of basal bodies/centrioles (Dutcher, 1995b; Marshall and Rosenbaum, 2000), because it possesses a well-defined microtubule (MT)-based cytoskeleton (LeDizet and Piperno, 1986; Marshall and Rosenbaum, 2000; Silflow and Lefebvre, 2001) the most prominent structures being two (9×2 +2) flagella and two basal bodies (also called

kinetosomes), which are very similar to the cilia and centrioles, respectively, of animals and humans both in structure and function (Salisbury et al., 1989; Salisbury, 1995; Geimer et al., 1997). Both of these structures are areas of intensive research (Pazour et al., 2000; Yagi, 2000; Deane et al., 2001; Marshall and Rosenbaum, 2001; Silflow and Lefebvre, 2001; Sloboda, 2002). Flagella/cilia consist of about 250 different polypeptides (Dutcher, 1995a), and roughly 200 of these have already been identified both by conventional methods and by a recent proteomic analysis of human cilia (Dutcher, 1995a; Ostrowski et al., 2002). By contrast, basal bodies/centrioles play an important role in mitosis and subsequent cell cleavage (Johnson and Porter, 1968; Ehler et al., 1995; Paoletti and Bornens, 1997; Lechtreck and Grunow, 1999; Marshall and Rosenbaum, 2000) and contain about 200 different polypeptides (Dutcher, 1995b). However, few basal body proteins have been described to date, including centrin in the basal body distal lumen, the microtubule-nucleating protein γ -tubulin in the basal body proximal lumen (Fuller et al., 1995; Marshall and Rosenbaum, 2000) and δ -tubulin (Dutcher and Trabuco, 1998), which is necessary for triplet MT formation.

Since both, flagella/cilia and centrioles consist mainly of MTs, it can be expected that proteins stabilizing these MTs or mediating their interaction with other cellular components will be among the constituents of these organelles still to be found.

Examples of such MT-associated proteins (MAP) that are already known components of human flagella or the mammalian centrosome are RS20 (Whyard et al., 2000) and a protein related to brain MAP1B (Dominguez et al., 1994), respectively.

In this report we describe a new component of the MT cytoskeleton that we call deflagellation-inducible protein of 13 kDa (DIP13). We have found DIP13 and its human counterpart NA14 (Ramos-Morales et al., 1998) associated with MTs both in flagellar axonemes and – obviously more concentrated – in basal bodies/centrioles in both organisms. Reducing the intracellular amount of DIP13 protein in *Chlamydomonas* by RNAi interfered with cell division, resulting in multinucleate, multiflagellate cells. Our current results suggest that DIP13/NA14 is an important general component of the MT cytoskeleton probably with a MT-stabilizing or connecting function.

Materials and Methods

Cell culture

C. reinhardtii wild-type strains CC124 MT⁻ and CC125 MT⁺ obtained from the *Chlamydomonas* Genetics Center (Duke University, Durham, NC) and the double mutant strain *cw15 arg7* MT⁻ from the laboratory of Peter Hegemann (University of Regensburg, Germany) were cultured under aeration in 400 ml of Tris acetate phosphate (TAP) (Harris, 1989) medium (supplemented with 100 µg/ml arginine in the case of *cw15 arg7* MT⁻) in continuous light at 22°C or in minimal medium (M₁) (Sager and Granick, 1953) under a 16:8 hours light/dark regimen at 30°C. *C. reinhardtii* strain *cw15+* (Schlösser, 1994) was cultured in aerated 1 litre flasks (approx. 1 l minute⁻¹) at 16°C under a light-dark cycle of 14:10 hours in WEES medium (McFadden and Melkonian, 1986). Mechanical deflagellation of *C. reinhardtii* CC124 MT⁻ was performed according to Rosenbaum et al. for the purpose of isolating RNA from deflagellated cells (Rosenbaum et al., 1969). Co-transformation of *cw15 arg7* MT⁻ with plasmid pARG7.8 (Debuchy et al., 1989) and *DIP13* antisense construct, pVW1, was performed according to Kindle (Kindle, 1990).

HeLa and COS-7 cells were grown in Dulbecco's modified Eagle medium (Gibco, Life Technologies, Spain) supplemented with 10% fetal calf serum (FCS). The KE37 cell line of T lymphoblastic origin was cultivated in RPMI 1640 medium (Gibco) containing 7% FCS. Both media contained 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were maintained in a 5% CO₂ humidified atmosphere at 37°C.

Flagellar fractionation

To prepare flagellar extracts for immunoblots, experimental deflagellation and collection of flagella followed the dibucaine method (Witman, 1986). Membranes were solubilized from isolated flagella with 2% NP-40, and axonemes collected by centrifugation. The membrane/matrix fraction was removed, and axonemal pellets were resuspended in HMDEK (10 mM HEPES, 5 mM MgSO₄, 1 mM DTT, 0.5 mM EDTA, 25 mM KCl). Concentrated SDS-PAGE sample buffer was added to both fractions prior to electrophoresis.

Nucleic acid procedures

Isolation of DNA, Southern and northern analyses and cloning procedures followed standard protocols (Sambrook et al., 1989). Plasmid DNA from *E. coli*, λDNA from lysates of recombinant phages, and DNA-fragments from agarose gels were prepared using commercially available purification systems (Qiagen, Hilden, Germany). RNA from *C. reinhardtii* was isolated according to Baker

et al. (Baker et al., 1986) and mRNA was isolated from total RNA using the oligotex dT mRNA purification system (Qiagen).

RT-PCR amplification

Reverse transcription of 500 ng *C. reinhardtii* (strain CC125 MT⁺) mRNA isolated from cultures 50 minutes after mechanical deflagellation was accomplished using random hexamer primers and MuMLV reverse transcriptase (United States Biochemical, Cleveland, OH). For the original amplification of a *DIP13* specific probe, PCR was carried out with degenerate 23-mer primers, 5'-GGIGA(G/A)(A/T)(G/C)IGGIGCIGGNAA(G/A)AC (upstream primer) and 5'-GT(C/T)TTI GC(G/A)TTICC(G/A)AAIGC(C/T)TC (downstream primer). Fragments of 140 to 170 bp were cloned after nucleotide fill-in reactions into the *EcoRV* site of pUC BM20 (Boehringer Mannheim, Germany) and individual clones were sequenced. One of these clones, pDIP, carrying an insert of 145 bp, was used as a hybridization probe in northern and Southern analyses as well as for screening DNA libraries. RT-PCR amplification of the 5' untranslated region of *DIP13* was performed after determination of the exon-intron structure. For this purpose, PCR was carried out on reverse-transcribed mRNA using a downstream primer overlapping exons 1 and 2 (5'-CCT CGA TGC ATT TTA CTA GC; nucleotides 1058-1050 and 946-936 of the *DIP13* genomic sequence) and an upstream primer (5'-GCA CCC AAA GCG ACA TCA TC; nucleotides 227-246) derived from the putative *DIP13* 5' untranslated region. PCR was carried out for 40 cycles (1 minute 95°C, 1 minute 60°C and 1 minute 30 seconds 72°C). Amplification yielded the expected product of ~700 bp, which was sequenced directly.

Isolation of genomic and cDNA clones

20 *DIP13*-specific clones from a λEMBL3-based *C. reinhardtii* genomic library (Goldschmidt-Clermont, 1986), four cDNA clones from a cDNA library in λZAPII (Wilkerson et al., 1998) and 20 cDNA clones from a cDNA library in λExLox (Paul A. Lefebvre, personal communication) were isolated using the cloned 145-bp fragment from pDIP as a probe.

DNA sequencing

λ-DNA and RT-PCR fragments were sequenced manually with the T7-Sequenase quick-denature plasmid sequencing kit (Amersham Life Science, Cleveland, OH) or the Thermo Sequenase radiolabeled terminator cycle sequencing kit (Amersham Life Science, Cleveland, OH). Plasmid subclones were sequenced by primer walking using gene specific primers and fluorescent dye-terminator sequencing with an ABI Prism 310 automated sequencer (Applied Biosystems, Foster City, CA).

Computer-assisted protein analysis

Theoretical analysis of the *DIP13* amino acid sequence and structure was done using the PredictProtein server of the EMBL Institute in Heidelberg (<http://www.embl-heidelberg.de/predictprotein>). Blast searches (Altschul et al., 1997) were performed on the NCBI server (<http://www.ncbi.nlm.nih.gov/BLAST>), Bethesda, USA.

Production of recombinant DIP13

The complete *DIP13* open reading frame (ORF) was amplified by PCR with two gene-specific primers from a cDNA clone. Upstream (5'-CATAGGATCCATGTCTGCTCAAGGCCAAGCTC) and downstream primers (5'-CTG CAAGCTTTCAAGAGCTGGCC-TGCTTCTTC) were binding at the translational start and stop codons (bold italics), respectively. The first 10 nucleotides of each 32-mer introduced a *Bam*HI (upstream, bold) and a *Hind*III site (downstream,

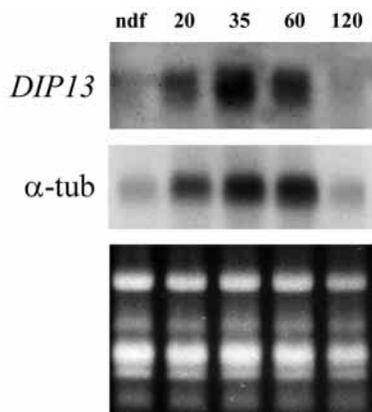


Fig. 1. Analysis of RNA in response to mechanical deflagellation. 20 μ g total RNA per lane isolated before (non deflagellated, ndf) and at defined times (20, 35, 60, 120 minutes) after deflagellation were probed with *DIP13* and α -tubulin cDNA probes under stringent conditions. Exposure times on Kodak Biomax MR films were 1 hour for α -tubulin and 26 hours for *DIP13* at -70°C . An ethidium stain of gel-separated RNA (bottom panel) served as a loading control.

bold) into the 356 bp PCR fragment, allowing to clone the RT-PCR fragment into the His₆ fusion vector pQE30 (Qiagen, Hilden, Germany) via *Bam*HI and *Hind*III giving rise to plasmid pQE30/*DIP13*. Following retransformation of the sequence-verified construct, recombinant *DIP13* was purified from *E. coli* strain M15 pREP4 under denaturing conditions according to the supplier's protocol.

Construction of antisense plasmid pVW1

Plasmid pVW1 is based on cloning vector pIC20R (Marsh et al., 1984) and was constructed for in vivo expression of the *DIP13* open

reading frame (derived from pQE30/*DIP13*) in inverse orientation from a modified *C. reinhardtii* hybrid promoter (M. Fuhrmann, personal communication) (Schroda et al., 2000) containing sequences from the *C. reinhardtii* *Hsp70A* and *RBCS2* gene promoters. Individual sequences were derived from plasmid pMF59 (M. Fuhrmann, personal communication), which in turn is a derivative of plasmid pMF124cGFP (Fuhrmann et al., 1999). In brief, pVW1 contains the following sequences in the order given between the single *Bgl*III and *Kpn*I sites of pIC20R, starting with the hybrid *Bgl*III/*Bam*HI-site (underlined) that originated from cloning: the sequence 5'-AGATCCACTATAGGGCGAATTGGAGCTCCACCGCGGTGGCGGCCGCTCTAGA followed by bases 559 to 826 (accession no. M76725) from the *C. reinhardtii* *Hsp70A* gene promoter. This sequence is followed by the bases 5'-GCTAGCTTAAGATCCCAAT joining *Hsp70A* to *RBCS2* gene bases 935 to 1146 (accession no. X04472). These are followed by the sequence 5'-CTGCAGCTT containing a filled-in partial *Hind*III site (underlined), which in turn is followed by the complete inverse open reading of the *DIP13* gene set free from plasmid pQE30/*DIP13* with *Bam*HI after prior linearization with *Hind*III and fill-in reaction to create a blunt *Hind*III-end for cloning. Consequently, the inverse *DIP13* ORF is immediately followed by the sequence 5'-GGATCCC containing a *Bam*HI site (underlined) followed by bases 2401 to 2626 (accession no. X04472) from the *C. reinhardtii* *RBCS2* gene 3' region, the latter providing a functional polyadenylation signal. The final bases 5'-TAAGCGGGTACC contain the *Kpn*I site at the 3' end of the construct used for cloning into pIC20R. The resulting plasmid, pVW1, is shown in Fig. 7A.

Antibodies

Anti-*DIP13* antiserum was produced commercially (Biogenes, Berlin, Germany) and purified by affinity chromatography using the *DIP13* fusion protein as antigen, as described elsewhere (Page and Snyder, 1992). Anti-NA14 polyclonal antibody has been described previously (Ramos-Morales et al., 1998). The antibody against *C. reinhardtii* α -tubulin used for indirect immunofluorescence has been described (Silflow and Rosenbaum, 1981) and mouse monoclonal antibody B-

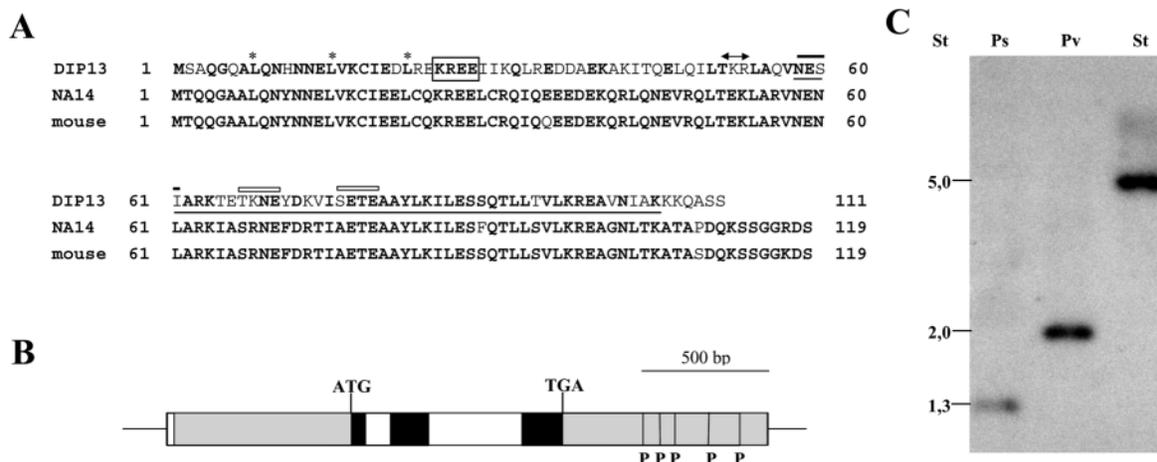


Fig. 2. Comparison of the derived *DIP13* protein with its human and mouse homologs, physical map and Southern analysis. (A) Comparison of the derived *DIP13* protein with two known homologs, human NA14 and a mouse unnamed protein. Residues conserved in at least two of three proteins are shown in bold. The region encoded in plasmid pDIP is underlined. A potential microtubule binding site is boxed, potential phosphorylation sites for casein kinase II (double headed arrow) or protein kinase C (open rectangles), a putative N-glycosylation site (thick black line) and the conserved leucine residues of the leucine zipper-like N-terminal motif (*) are indicated above the sequence. (B) *DIP13* gene structure consisting of three exons (black boxes) and two introns (white boxes). 5' and 3' untranslated regions are shown as grey boxes, positions of translational start (ATG) and stop (TGA) codons as well as five polyadenylation signals (P) are indicated. (C) Southern analysis of *C. reinhardtii* genomic DNA (strain 125 MT+) with a *DIP13* cDNA probe performed under stringent conditions suggests that *DIP13* is a single copy gene. St, molecular size standard; sizes are indicated in kb; Ps, *Pst*I; Pv, *Pvu*II; St, *Stu*I.

Table 1. DIP13 polyadenylation signals

Polyadenylation signal	Nucleotide position in published genomic sequence	Distance*
GGTAA	2003-2007	12
TGTCG	2062-2066	12
TGTAC	2116-2120	13
TGAAC	2243-2247	13
TGCAA	2357-2361	13

*Number of bases between the last base of the polyA signal and the first base of the polyA tail.

5-1-2 (Piperno et al., 1987), also directed against α -tubulin and used on immunoblots, was purchased from Sigma. Anti-L23-antibody (McElwain et al., 1993) was kindly provided by Elizabeth Harris (Duke University, NC) and anti-CTR453 antibody (Bailly et al., 1989) was supplied by Michel Bornens (Centre de Genetique Moleculaire-CNRS, Gif sur Yvette, France). Monoclonal anti- γ -tubulin antibody was purchased from Sigma and monoclonal anti-HA antibody was from Amersham Life Science, Cleveland, OH.

Transient transfection of COS-7 cells

The full-length *na14* ORF (Ramos-Morales et al., 1998) was cloned in frame with the HA epitope (Wilson et al., 1984) into the eukaryotic expression vector pECE (Ellis et al., 1986) to obtain an HA-epitope tagged NA14. The resulting plasmid was purified (see below) followed by phenol extraction and ethanol precipitation. COS-7 cells were split 24 hours before transfection so that they were 60-80% confluent for transfection. 2.5×10^6 cells/assay were resuspended in 200 μ l of 15 mM HEPES buffered serum-containing medium, mixed with 50 μ l of 210 mM NaCl containing 10 μ g plasmid DNA and electroporated using a BioRad Gene Pulser. Six hours after electroporation, medium was replaced by fresh medium and cells were processed after 24 hours.

Cell fractionation, immunoprecipitation and preparation of centrosomes

For lysis, COS-7 cells were harvested and washed in PBS. 2×10^7 cells per ml were lysed at 4°C in NP-40 buffer (10 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP-40, 1 mM phenyl methyl sulfonyl fluoride (PMSF) and 1 μ g/ml of pepstatin, leupeptin and aprotinin) for 20 minutes. The extract was centrifuged at 15,000 *g* for 20 minutes and both supernatant (soluble fraction) and pellet (insoluble fraction) were stored at -70°C. For immunoprecipitation experiments, soluble fraction of HA-NA14-transfected cells or recombinant DIP13, as a positive control, were preadsorbed with 10 μ l of preimmune serum on 50 μ l of protein-A-Sepharose and immunoprecipitated with 10 μ l of

anti-DIP13 serum on 50 μ l of protein-A-Sepharose. After incubation and washing, bead pellets and supernatants were analyzed by immunoblotting using anti-NA14 or anti-HA antibodies.

Detergent-soluble and insoluble cell fractions from KE37 cells were obtained by a brief treatment of cells at 4°C with 1% NP-40, 0.5% sodium deoxycholate in TNM buffer (10 mM Tris-HCl pH 7.4, 150 mM NaCl, 5 mM MgCl₂) containing protease inhibitors. Centrosomes from KE37 cells were isolated essentially as described (Bornens et al., 1987).

Electrophoresis and immunoblotting

For analyzing total cells, extracts from 10^6 to 3×10^6 *C. reinhardtii* cells in sample buffer were run per lane on 15% SDS-PAGE gels and electroblotted. Immunoblots were incubated with anti-DIP13 antibody diluted 1:500, anti-L23-antibody diluted 1:500 and anti- α -tubulin antibody (B-5-1-2) diluted 1:2500 respectively, followed by incubation with secondary goat anti-rabbit antibody (or goat anti-mouse antibody in the case of B-5-1-2) conjugated to peroxidase (1:5000; Sigma, St Louis, MO) and detection using the ECL system (Amersham Pharmacia Biotech, Uppsala, Sweden). Densitometric quantification of immunoblots was performed using the software *Optiquant* of Packard Instrument Company (Meriden, CT).

Proteins from mammalian cells were separated on 13.5% SDS-PAGE gels, followed by electroblotting. Nitrocellulose filters were blocked for 1 hour at 37°C in TBST (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Tween 20) containing 5% nonfat dry milk. Then filters were incubated for 1-2 hours at 37°C in the primary antibody diluted in TBST/5% nonfat dry milk, washed in the same buffer, and incubated for 45 minutes at 37°C with secondary anti-rabbit or anti-mouse antibodies conjugated with peroxidase (Amersham Life Science, Cleveland, OH). After washes with TBST, peroxidase activity was revealed using the ECL system (Amersham Life Science, Cleveland, OH).

Indirect immunofluorescence

Indirect immunofluorescence was performed with *C. reinhardtii* strain CC124 MT+ after treatment of cells with autolysin to remove cell walls as described (Kozminski et al., 1993). Anti-DIP13 antiserum was used at a dilution of 1:50 and anti- α -tubulin serum at a dilution of 1:500, respectively. As secondary antibody, Alexa-Fluor488-conjugated goat anti-rabbit IgG (Molecular Probes, Oregon) was used at a dilution of 1:400 for 2 hours. Microscopy was performed under epifluorescence on an Olympus BX60F microscope (Olympus Optical Co., Hamburg, Germany) and images were taken using Kodak TMax 400 or EliteChrome 200 films (Kodak, Stuttgart, Germany). For nucleic acid staining, 0.2 μ g/ml DAPI (Sigma, München, Germany) was used.

Table 2. Proteins with homology to DIP13/NA14

Organism	Comment	Accession number	Residues compared	Identity (similarity) to DIP13 (%)	Identity (similarity) to NA14 (%)
<i>Chlamydomonas reinhardtii</i>	Unicellular green alga	AF131736	111 (full length)	100 (100)	60 (84)
<i>Homo sapiens</i>	Mammalian	Z96932	119 (full length)	60 (84)	100 (100)
<i>Mus musculus</i>	Mammalian	AB041656	119 (full length)	60 (85)	96 (97)
<i>Rattus norvegicus</i>	Mammalian	AW253642	19 (C-terminus)	C-terminus not well conserved	80 (85)
<i>Tetraodon nigroviridis</i>	Fish	AL333725	73 (DIP13) 94 (NA14)	41 (75)	64 (87)
<i>Schistosoma japonicum</i>	Plathelminthes	AA661097	82	43 (73)	41 (67)
<i>Giardia intestinalis</i>	Protozoan parasite	AC072300	103	33 (58)	34 (58)
<i>Leishmania major</i>	Protozoan parasite	AI034895	90	32 (61)	32 (54)
<i>Trypanosoma brucei</i>	Protozoan parasite	AL476620	104	34 (62)	41 (61)
<i>Plasmodium falciparum</i>	Protozoan parasite	AL049183	68	30 (50)	34 (50)
<i>Cryptosporidium parvum</i>	Protozoan parasite	B88451	49 (DIP13) 45 (NA14)	63 (73)	62 (75)

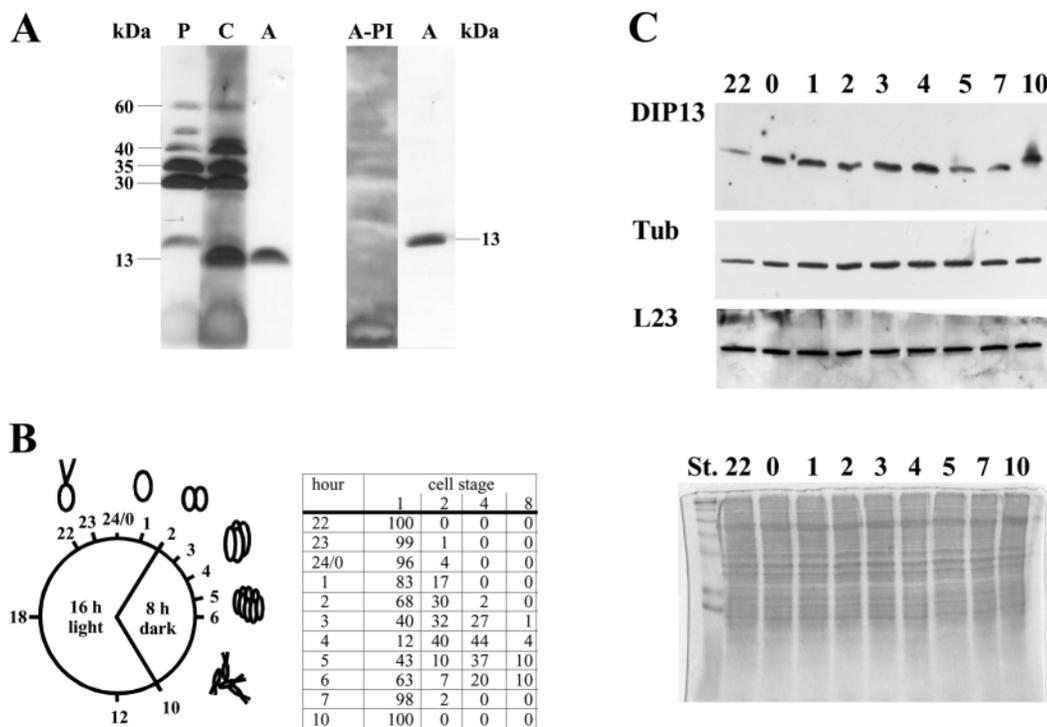


Fig. 3. Specificity of anti-DIP13 antiserum and *DIP13* expression during the cell cycle. (A) Characterization of a polyclonal anti-DIP13 antiserum produced in rabbit. (Left panel) Crude cell extracts from 10^6 *C. reinhardtii* cells per lane were probed with preimmune serum (P; 1:500) as well as crude (C, 1:500) and affinity-purified (A, 1:100) anti-DIP13 antiserum and detected by enhanced chemiluminescence. Sizes of reacting bands are indicated to the left. (Right panel) Crude cell extracts of 10^6 *C. reinhardtii* cells per lane were probed with affinity-purified anti-DIP13 antibody preincubated with 80 μ g recombinant DIP13 per ml incubation buffer (A-PI) or affinity-purified anti-DIP13 antibody (A, 1:100). The size of the reacting band is indicated to the right. (B) The 24 hour *C. reinhardtii* life cycle under a synchronizing 16 hour light/8 hour dark regimen. Every full hour 100 cells from a synchronous culture were analyzed by light microscopy and absolute numbers of cells in the 1-, 2-, 4- or 8-cell stage were noted in the table shown on the right. (C) Western analysis with samples isolated from the same culture as in B during the period of cell division. Equal amounts of protein per lane were probed with affinity-purified anti-DIP13 antibody (DIP13), anti- α -tubulin antibody (Tub) and anti-L23 antibody (L23). Time points of protein sampling correlate with time points in B. The same membrane was incubated successively with the three primary antibodies. Exposure times to film were 1 minute in each case. (Lower panel) Coomassie-stained gel of identical protein samples as a loading control. St, standard lane.

For immunofluorescence, HeLa cells were grown on culture-treated slides for 24-48 hours before an experiment. Cells were rinsed twice with phosphate buffered saline (PBS) and incubated in methanol at -20°C for 6 minutes to simultaneously fix and permeabilize the cells. Ejaculated spermatozoa were obtained after an abstinence period of 2-4 days from two fertile donors. Samples were diluted with fresh DMEM medium and incubated for 1 hour at 37°C . Motile spermatozoa were harvested, rinsed twice in PBS and methanol-fixed as above. After methanol treatment, cells were processed as described (Rios et al., 1994). Immunofluorescence analysis was performed on a Leica epifluorescence microscope.

Immunoelectron microscopy

For postembedding immunogold electron microscopy *C. reinhardtii* cytoskeletons (strain cw15+) were isolated as described (Wright et al., 1985) and fixed in MT-buffer (30 mM HEPES, 5 mM Na-EGTA, 15 mM KCl, pH 7.0) containing 2% paraformaldehyde and 0.25% glutaraldehyde for 40 minutes at 15°C . The cytoskeletons were dehydrated to 95% ethanol on ice and infiltrated with LR Gold resin (Plano, Marburg, Germany) at -20°C for 36 hours. For polymerization we used LR Gold resin containing 0.4% benzil and fluorescent light at -20°C . Immunogold labeling of ultrathin sections was performed as described previously (Robenek et al., 1987) with minor

modifications. The anti-DIP13 antibody (1:30 to 1:50) was applied at 4°C overnight and detected with goat anti-rabbit-IgG conjugated to 10 nm or 15 nm gold particles (British BioCell, Cardiff, UK).

Ultrathin sections <80 nm were obtained using a diamond knife (Diatome, Biel, CH) on a RMC MT-6000 microtome (RMC, Tucson, AZ), mounted on Pioloform-coated slot grids and stained with lead citrate and uranyl acetate (Reynolds, 1963). Micrographs were taken with a Philips CM 10 transmission electron microscope using Scientia EM film (Agfa, Leverkusen, Germany).

Results

Identification, cloning and sequence analysis of DIP13

DIP13 specific sequences were first isolated in an RT-PCR-based screen that was in fact intended to identify potential flagellar myosin motor proteins using degenerate primers specific for two conserved peptide motifs in the motor domain of most known myosins, namely the ATP-binding P-loop [GESGAGKT (Saraste et al., 1990)] and a second motif (EAFGNAKT) located about 30-40 amino acids towards the C-terminus. The cloned products in the expected size range of 140 to 170 bp were used as probes for northern analyses,

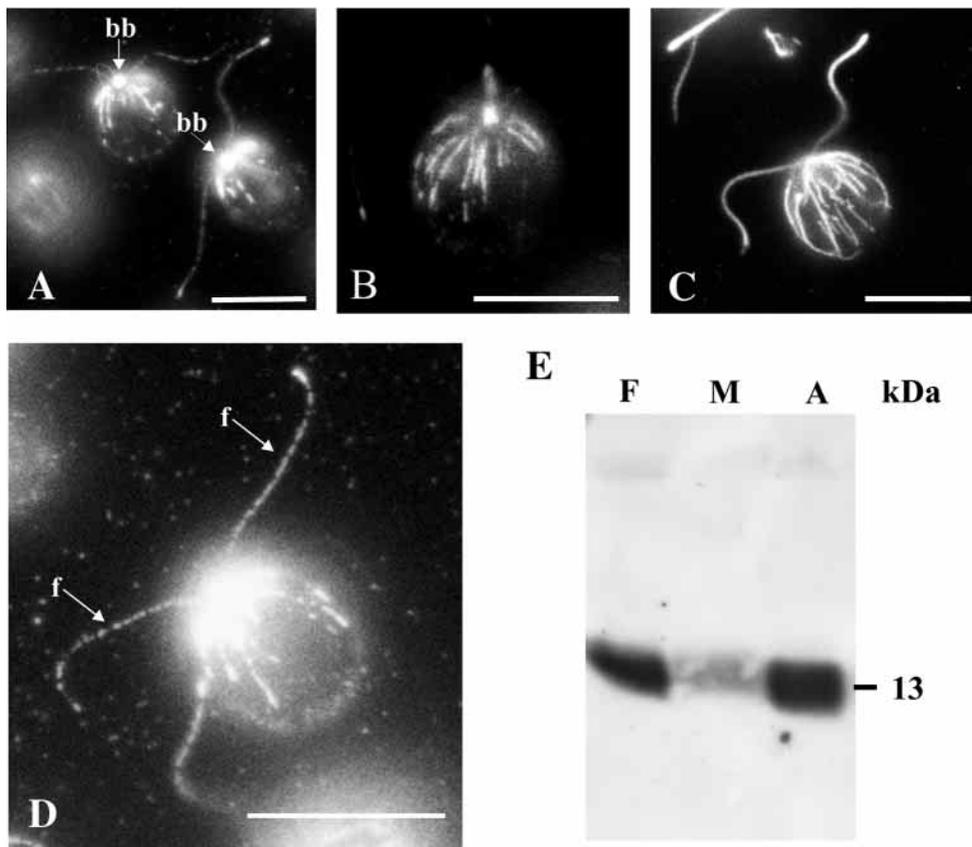


Fig. 4. Indirect immunofluorescence. *C. reinhardtii* wild-type strain 124 MT⁻ was probed using primary antibodies against DIP13 or α -tubulin and a fluorescence-labeled secondary antibody. DIP13 antibody stains basal bodies (A; bb) and the anterior part of the microtubules (B). An overexposed cell is shown in D to point out punctate staining of flagella (f). Flagellar localization was confirmed by immunoblotting of isolated and detergent-extracted flagella (E; F, whole flagella; M, membrane and matrix fraction; A, axonemes). anti- α -tubulin antiserum (C; positive control) shows strong labeling of flagella and cytoplasmic microtubules. Exposure times for photographing indirect immunofluorescence were 12 seconds (A, B) or 30 seconds (D) for DIP13 and 5 seconds for α -tubulin (C). Bars, 10 μ m.

because genes encoding flagellar proteins can be identified after experimental deflagellation (Rosenbaum et al., 1969; Schloss et al., 1984) by a remarkably fast and strong transient accumulation of flagellar RNA caused by transcriptional induction as well as stabilization of flagellar RNA (Baker et al., 1986). One of our probes, pDIP, detected a small mRNA of 1.5 to 1.8 kb (Fig. 1) showing this transient upregulation similar to α -tubulin mRNA (Baker et al., 1986) in response to mechanical deflagellation. Subsequently we found that the newly identified gene encoded a small protein of 13 kDa. Therefore we named this protein *deflagellation inducible protein of 13 kDa*, DIP13.

We then used the pDIP probe to screen a λ EMBL3 genomic library of *C. reinhardtii* (Goldschmidt-Clermont, 1986) and isolated 20 *DIP13*-specific clones. A genomic sequence of 2898 bp was obtained from one of these clones after suitable subcloning into plasmids as described in Materials and Methods. This sequence is available from GenBank under accession number AF131736. The complete *DIP13* cDNA sequence was assembled from several overlapping cDNA clones and RT-PCR fragments. It contains a 333 bp open reading frame (ORF) potentially encoding a 13 kDa protein. Database searches revealed that this protein is very similar to a similar sized protein from humans, NA14 (Fig. 2A) (Ramos-Morales et al., 1998), indicating that the predicted ORF and gene structures are correct. Southern and sequence analyses further showed that *DIP13* is a single copy gene interrupted by two introns (Fig. 2B,C).

The 5' end of *DIP13* mRNA was not determined precisely, but according to the results of several RT-PCR experiments

(data not shown) the 5' untranslated region has a minimal length of 669 bp (Fig. 2B). Five different polyadenylation signals have been identified in the 3' untranslated region (Fig. 2B, Table 1) by sequencing individual cDNA clones. None of these signals, located 308 to 666 bp downstream of the TGA stop codon, has the typical pentanucleotide consensus TGTA normally found in green algae (Schmitt et al., 1992) but each is followed by a polyA tail starting 12 to 13 nucleotides after the last base of the signal. It is likely that all five signals are used at the same time, as indicated by the unusually broad signals obtained on northern blots (Fig. 1).

The derived DIP13 protein consists of 111 amino acids with a M_r of 12.7×10^3 and an isoelectrical point of 8.39. DIP13 is predicted to be all α -helical (Rost and Sander, 1993; Rost and Sander, 1994) and to our knowledge is the smallest α -helical protein known to date. Remarkably, DIP13 also contains a short sequence (KREE; amino acids 25-28; Fig. 2A) reminiscent of the MT-binding motif KKEE (or KKE^{1/V}) found in the structural MT-associated protein, MAP1B (Noble et al., 1989). The N-terminal region of DIP13 contains a sequence motif reminiscent of a leucine zipper (Fig. 2A; amino acids 8-22), which is known to promote dimerization through α -helical coiled-coil formation. Consistent with this feature we have found during FPLC purification that recombinant DIP13 has a marked tendency to form oligomers (data not shown). Moreover, the PROSITE Dictionary of Protein Sites and Patterns revealed consensus motifs for N-glycosylation, casein kinase II phosphorylation and PKC phosphorylation (Fig. 2A).

Close homologs of *Chlamydomonas* DIP13 are present in humans and mice. While the mouse sequence has only been

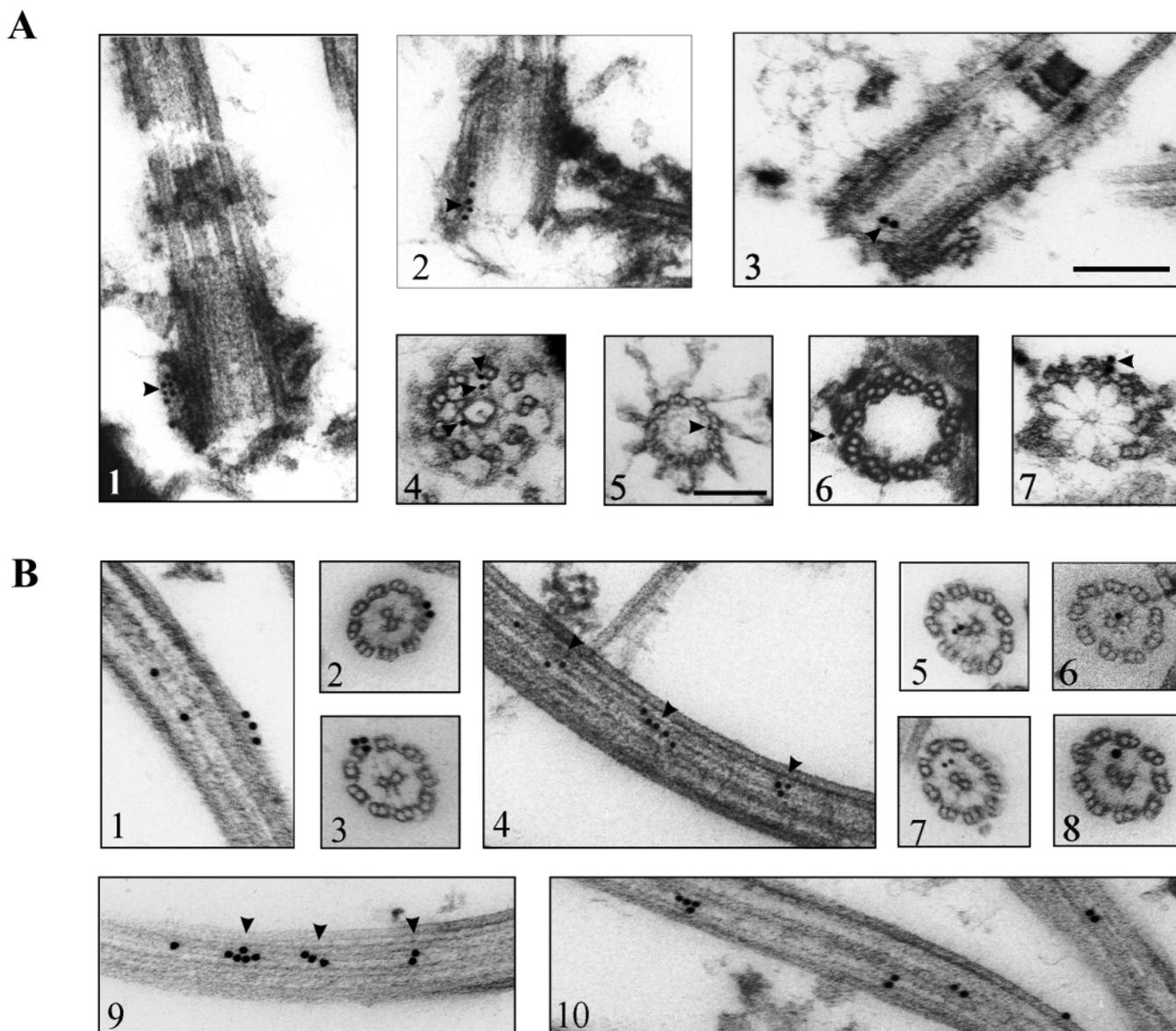


Fig. 5. Immunogold labeling (postembedding technique) of basal bodies and flagella using anti-DIP13 and anti-rabbit-IgG conjugated to 10 or 15 nm gold particles. (A1-3) Longitudinal sections of the basal body region with labeling at the outside of one basal body distal to the neighbouring basal body (A1,2) and labeling of triplet microtubules inside the basal body (A3). (A4-7) Cross-sections from distal to proximal with respect to the cell body demonstrate the presence of DIP13 in several structurally defined zones of the basal bodies. Gold particles are pointed out by black arrowheads. (B) Longitudinal and cross-sections of flagella. Both outer doublet (B1-3) and central pair (B4-6) microtubules are decorated. In some sections (B7-10) no decision can be made. Labeling does not show any periodicity but as indicated in B4 and B9 particles are often found in close proximity (black arrowheads). Bars, 200 nm. Bar shown in A3 applies to all figures except A5.

filed in GenBank (accession number AA274144) as a currently unnamed protein, the human homolog NA14 (accession number Z96932) has been identified by Ramos-Morales et al. as an autoantigen found in patients with Sjögren's syndrome (Ramos-Morales et al., 1998). These two proteins share 60% amino acid sequence identity with DIP13 and have the same overall structural features (Fig. 2A). However, DIP13/NA14 are not restricted to green algae and mammals, as homologous sequences are present in the genomes of several protozoan parasites, a trematode and a fish (Table 2). These predicted proteins share from 30% to 64% identity with DIP13/NA14.

However, no homologs could be detected by database searches in the completed genomes of yeast, *Drosophila*, *C. elegans* and *A. thaliana*.

DIP13 expression during the cell cycle

An anti-DIP13 polyclonal antiserum was raised in rabbit and its specificity was tested on *C. reinhardtii* whole cell extracts (Fig. 3A). As shown in the left panel, both the preimmune and crude sera detected four to six crossreacting bands between 15 and 60 kDa, while affinity-purified anti-DIP13 antiserum

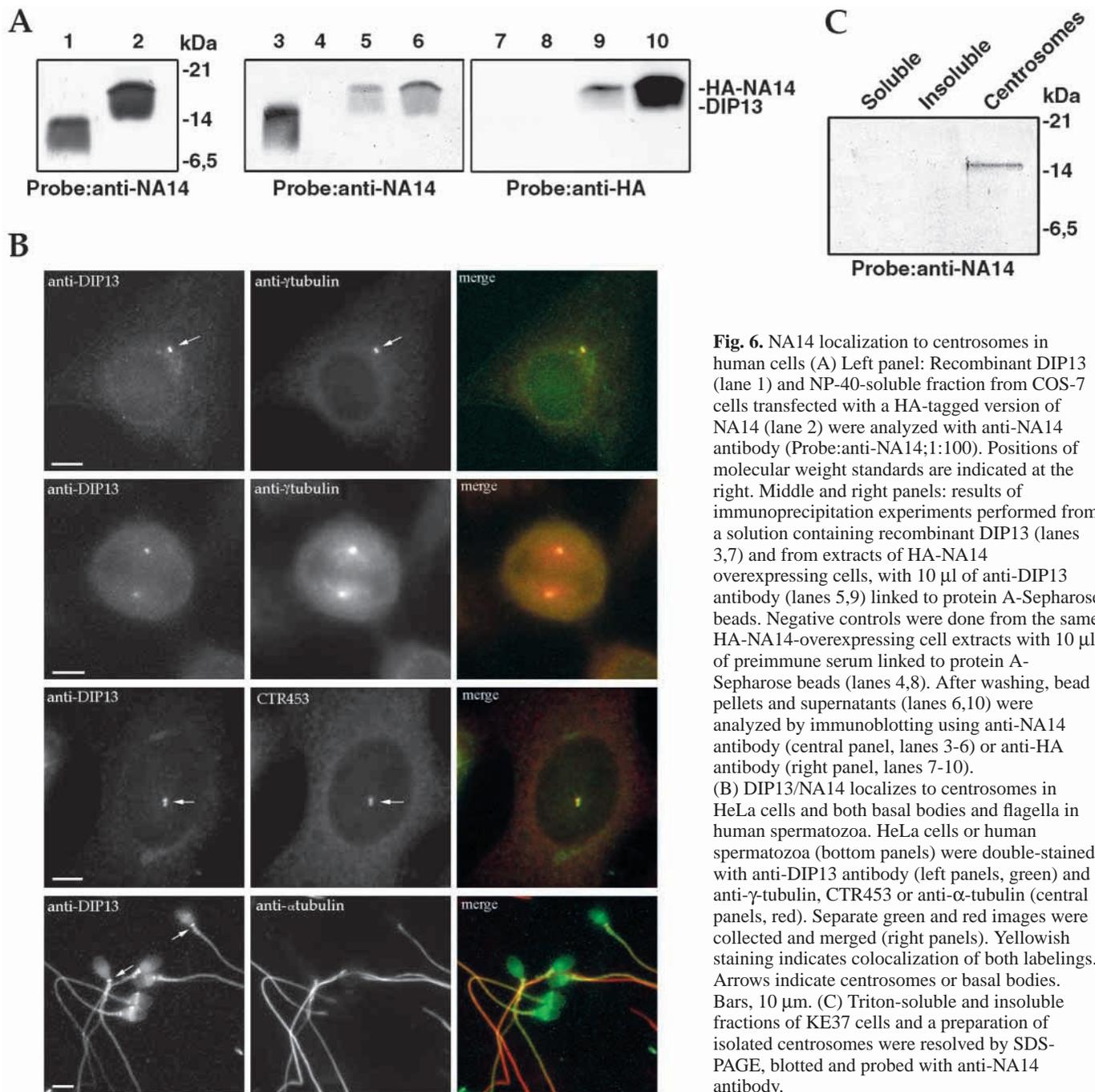


Fig. 6. NA14 localization to centrosomes in human cells (A) Left panel: Recombinant DIP13 (lane 1) and NP-40-soluble fraction from COS-7 cells transfected with a HA-tagged version of NA14 (lane 2) were analyzed with anti-NA14 antibody (Probe:anti-NA14;1:100). Positions of molecular weight standards are indicated at the right. Middle and right panels: results of immunoprecipitation experiments performed from a solution containing recombinant DIP13 (lanes 3,7) and from extracts of HA-NA14 overexpressing cells, with 10 μ l of anti-DIP13 antibody (lanes 5,9) linked to protein A-Sepharose beads. Negative controls were done from the same HA-NA14-overexpressing cell extracts with 10 μ l of preimmune serum linked to protein A-Sepharose beads (lanes 4,8). After washing, bead pellets and supernatants (lanes 6,10) were analyzed by immunoblotting using anti-NA14 antibody (central panel, lanes 3-6) or anti-HA antibody (right panel, lanes 7-10). (B) DIP13/NA14 localizes to centrosomes in HeLa cells and both basal bodies and flagella in human spermatozoa. HeLa cells or human spermatozoa (bottom panels) were double-stained with anti-DIP13 antibody (left panels, green) and anti- γ -tubulin, CTR453 or anti- α -tubulin (central panels, red). Separate green and red images were collected and merged (right panels). Yellowish staining indicates colocalization of both labelings. Arrows indicate centrosomes or basal bodies. Bars, 10 μ m. (C) Triton-soluble and insoluble fractions of KE37 cells and a preparation of isolated centrosomes were resolved by SDS-PAGE, blotted and probed with anti-NA14 antibody.

shows only the one expected band at 13 kDa. This band is not detected by the preimmune serum but is also present in crude serum. In addition, preincubation of affinity-purified anti-DIP13 serum with recombinant DIP13 protein successfully prevents detection of the 13 kDa protein in *Chlamydomonas* extracts (Fig. 3A, right panel). Therefore we conclude that our polyclonal anti-DIP13 antiserum specifically and sensitively detects DIP13 protein.

Preliminary experiments for testing DIP13 expression along the *C. reinhardtii* life cycle (Fig. 3B) were carried out by taking samples from synchronized cultures every 2 hours. Samples were then processed in parallel by western blotting using anti-

DIP13 antiserum or by northern blotting with a DIP13-specific cDNA probe. Two maxima of expression, one during the period of cell division and the other about 6 hours later after the beginning of the light phase following cell division were detected (data not shown). We first focussed on the maximum of DIP13 expression during *C. reinhardtii* cell division. To this end, 100 cells per time point from a synchronized culture were analyzed by light microscopy for their division stage hourly (Fig. 3B). At the same time points, samples were taken from these cultures and identical amounts of total protein were analyzed on immunoblots (Fig. 3C) using three different antibodies, namely anti-DIP13, anti α -tubulin and anti-L23

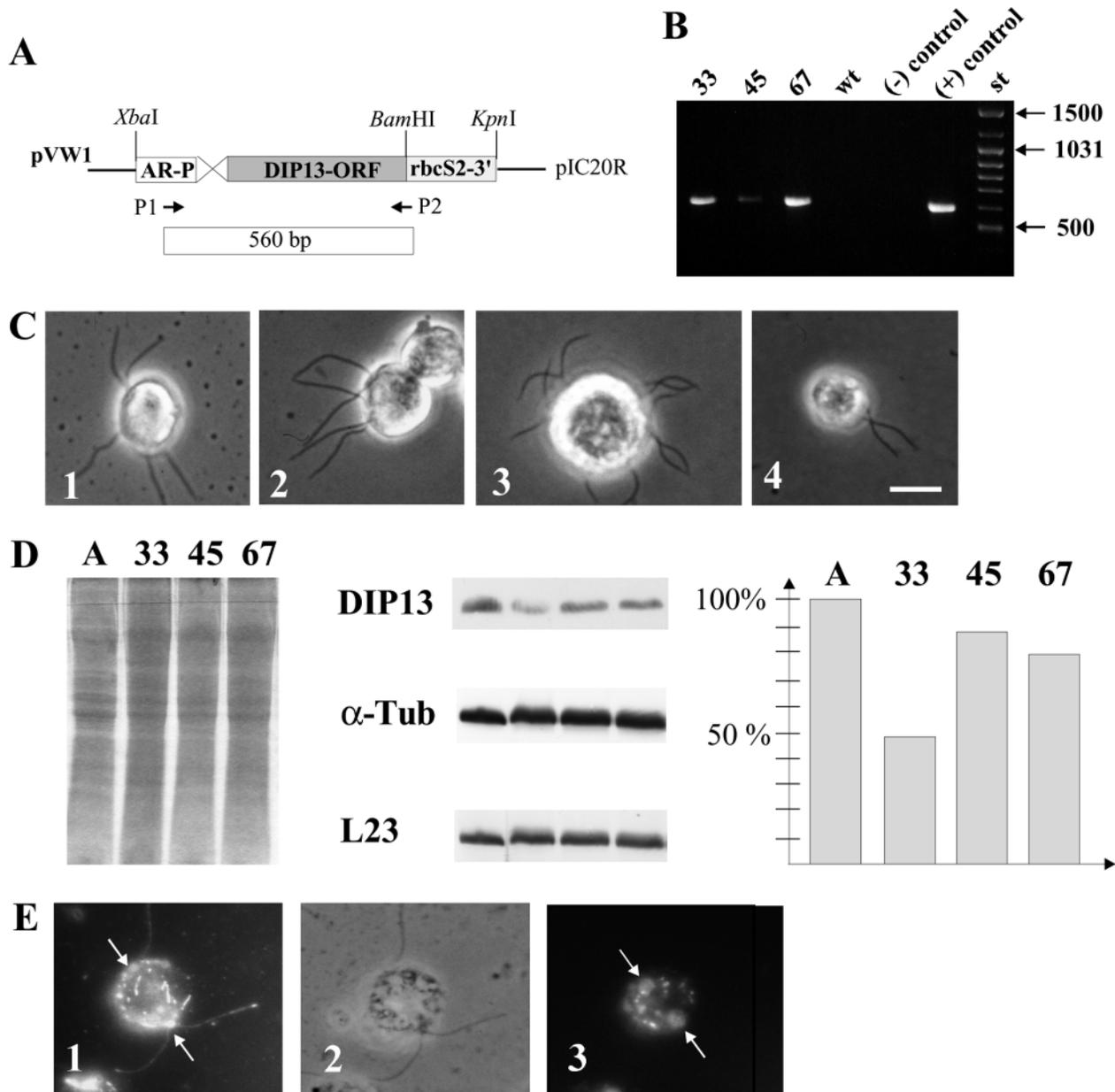


Fig. 7. *DIP13* antisense RNA experiments. (A) pVW1 antisense construct. The AR-promoter (AR-P), *DIP13* inverse reading frame (*DIP13*-ORF) and the *RBCS2* gene 3' sequences (*RBCS2*-3') are indicated. Single restriction sites *Xba*I, *Bam*HI, and *Kpn*I are given for orientation. Primers (P1, P2) for amplification of a specific 560-bp fragment (open box) are indicated at their respective positions. (B) Result of PCR analysis with primers P1 and P2 and genomic DNA of three putative transformants (#33, 45 and 67) and control genomic DNA from the untransformed parent strain (wt). Controls were done without added template (- control) or 1 ng pVW1-DNA (+ control). St, DNA size standard. For orientation, some fragment sizes (in bp) are indicated to the right. (C) Phenotypic comparison of antisense transformants (C1-3) and untransformed cells (C4). Bar, 10 μ m. (D) Analysis of *DIP13* protein reduction in antisense strains #33, 45 and 67. Left panel: Coomassie-stained gel as loading control. Lane A: extract from the untransformed strain. Middle panel: immunoblot with the same amounts of protein per lane of the same four strains probed successively with anti-*DIP13* antibody (*DIP13*), anti- α -tubulin antibody (α -Tub) and anti-L23 antibody (L23). Right panel: result of densitometric analysis of *DIP13* protein levels derived from the immunoblots shown. For standardization of loading, the signals obtained with anti L23 antibody were used. (E) Indirect immunofluorescence with anti-*DIP13* antibody (E1) and DAPI staining (E3) of a transformed cell of strain #45 showing typical labeling (compare Fig. 4) at the two basal body spots (E1, white arrows) and two nuclei (E3, white arrows) at opposite poles. E2, corresponding phase image. Bar in C4 (10 μ m) applies to all microscopic images.

(McEwain et al., 1993). The latter detects a ribosomal protein and served as a loading control together with a Coomassie-stained gel also presented in Fig. 3C. As shown, *DIP13* protein levels are clearly elevated between hours 0 (onset of division)

and 4 (mid-division). After 5 hours, when most cells are in the 4- (37 cells), 8- (10 cells) or (new) 1-cell stage (43 daughter cells) and division is close to completion, the amount of *DIP13* is clearly decreased again and stays low until hour 7. Increased

protein levels were also seen for α -tubulin from hour 0 but unlike DIP13 these levels did not go down after completion of division. As expected, the amount of L23 protein stayed constant throughout the experiment. In summary, these data substantiated that *Chlamydomonas* cells need increased amounts of DIP13 during cell division (as is the case for flagellar biosynthesis; see Fig. 1) and were in accordance with the possibility that DIP13 is a structural component of the *C. reinhardtii* cytoskeleton.

DIP13 is localized to centrioles, flagella and cytoplasmic MTs

Affinity-purified DIP13 antibody was used to localize DIP13 in fixed and permeabilized *Chlamydomonas* cells by indirect immunofluorescence. As shown in Fig. 4, DIP13 antibody strongly labeled basal bodies and cytoplasmic MTs (Fig. 4A,B,D). The strong basal body labeling was clearly not paralleled by the control performed with anti- α -tubulin antibody (Fig. 4C). Labeling of cytoplasmic MTs, however, appeared similar with both antibodies, although DIP13 labeling seemed to be restricted to the anterior half of cytoplasmic MTs (compare Fig. 4A,B,D with C). In addition, weaker punctate staining was seen with the DIP13 antiserum along the flagella (Fig. 4A,D). This punctate distribution does not appear to be artefactual since similarly treated cells exhibited an homogeneous staining along the flagella with anti- α -tubulin antibodies. (Fig. 4C).

To determine whether flagellar DIP13 is associated with the axoneme, whole and detergent-extracted flagella from vegetative *Chlamydomonas* cells (strain CC124 MT-) were analyzed by immunoblotting with anti-DIP13 antibody (Fig. 4E). As shown, DIP13 is clearly detectable in flagella and obviously associated with the axoneme.

In order to localize DIP13 in basal bodies and flagella in more detail, we performed immunogold labeling of isolated *C. reinhardtii* cytoskeletons. In the basal body region, DIP13 is located both at the outside (Fig. 5A1-3,6,7) and the inside of the basal bodies (Fig. 5A4,5). Strongest labeling was reproducibly detected at the one side of the basal body distal to the neighbouring basal body (Fig. 5A1,3). In flagella, the protein seemed to be associated both with outer doublet (Fig. 5B1-3) and central pair MTs (MT; Fig. 5B1,4-6) of the axoneme. A count of 214 gold particles from 139 axonemal sections resulted in exclusive outer doublet localization in 51% of the axonemes and in central pair localization in 33% of the axonemes. In about 16% of the cases no decision could be made between outer doublet and central pair labeling (Fig. 5B7-10). It has to be noted that in agreement with the punctate staining seen in immunofluorescence (Fig. 4A,D), immunogold labeling of longitudinal axonemal sections displayed multiple isolated spots with accumulated gold particles along the length of the axoneme (Fig. 5B1,4,9).

NA14 is localized to the centrosome and sperm flagella

NA14 was initially identified as a minor autoantigen recognized by an autoimmune serum from a Sjögren syndrome patient. Neither the autoimmune serum nor a polyclonal antibody raised against recombinant NA14 recognized endogenous NA14 by IF. For this reason, subcellular localization was achieved by expression of an HA-tagged

version of NA14. Under these conditions, exogenous NA14 localizes at the nucleus (Ramos-Morales et al., 1998). NA14 was, therefore, believed to be a nuclear autoantigen. However, since NA14 exhibits a remarkably high sequence similarity to *C. reinhardtii* DIP13, which definitely showed a localization pattern typical for a cytoskeletal protein, we considered it to be necessary to reexamine NA14 localization in human cells and sought to test the possibility that anti-DIP13 antibody could serve to determine the subcellular localization of NA14.

We first wondered whether anti-NA14 antibody (Ramos-Morales et al., 1998) was able to recognize recombinant DIP13 and, vice versa, whether anti-DIP13 antibody detected NA14 on immunoblots. As shown in Fig. 6A (left panel), anti-NA14 clearly recognized recombinant DIP13 (lane 1). An extract from HA-NA14 overexpressing cells was included as a positive control (lane 2). On the contrary, anti-DIP13 antibody did not detect HA-NA14 on immunoblots (data not shown). We next attempted to immunoprecipitate NA14 by using anti-DIP13 antibody (Fig. 6A, middle and right panels). Lysates from HA-NA14-overexpressing cells were immunoprecipitated with the preimmune serum, as a negative control, (lanes 4,8) or with anti-DIP13 antibody (lanes 5,9).

As a control, recombinant DIP13 was also immunoprecipitated under identical conditions (lanes 3,7). Bead pellets (lanes 3-5,7-9) and supernatants (lanes 6,10) were separated by SDS-PAGE and transferred to nitrocellulose filters. Identical blots were incubated with either anti-NA14 (middle panel) or anti-HA antibodies (right panel). As can be observed, anti-DIP13 antiserum immunoprecipitated both purified DIP13 (lane 3) and HA-NA14 (lanes 5,9) indicating that the antibody was able to recognize native NA14. Obviously, purified DIP13 was not recognized by anti-HA antibody (lane 7).

These results prompted us to use anti-DIP13 antibody for IF experiments in human cells. As shown in Fig. 6B (top three panels), anti-DIP13 labeling in methanol-fixed cells strictly colocalized with the centrosomal markers γ -tubulin and CTR453 at the centrosome and mitotic poles of HeLa cells. However, unlike in *Chlamydomonas*, no staining of cytoplasmic MTs was observed. When similar experiments were carried out on HA-NA14-overexpressing cells, anti-DIP13 antibody recognized both endogenous NA14 at the centrosome and exogenous HA-NA14 in the nucleus (data not shown) demonstrating the specificity of anti-DIP13 labeling.

To confirm this centrosomal localization of NA14, immunoblots were performed on triton-soluble and insoluble extracts of KE37 cells and on isolated centrosomes (Fig. 6C). Anti-NA14 antiserum reacted with a centrosomal protein of ~14 kDa. Together these results indicate that NA14 is, in fact, a centrosomal protein in HeLa cells.

Finally, we investigated whether NA14 also localized in complex microtubular structures in human cells. To do this, we double-stained human spermatozoa (Fig. 6B, lower panel) with anti-DIP13 and α -tubulin antibodies. Labeling of these cells clearly resembles that of *Chlamydomonas* cells since strong labeling of the basal body region with weaker labeling of the axonemes (which in turn showed strong labeling for α -tubulin) was observed.

Antisense RNA inhibition of DIP13 expression

It is well-established that expression of antisense RNA within

living cells leads to inhibition of expression of the target gene (Rosenberg et al., 1985; Fuhrmann et al., 2001) and can provide useful insights into the function of the protein of interest. To get a first hint at DIP13 function, we expressed *DIP13* antisense RNA in transgenic *Chlamydomonas* cells. To this end, *DIP13* cDNA was cloned in inverse orientation under the control of the *C. reinhardtii* hybrid AR-promoter (Fig. 7A) [(Schroda et al., 2000) M. Fuhrmann, personal communication] and the construct, pVW1, was stably transformed into *Chlamydomonas* cells. Six transformed cell lines were identified by PCR analysis three of which are shown in Fig. 7B. It was noted that these transformed strains showed significantly slower growth than the untransformed strain (data not shown). Asynchronous cultures of these three strains together with the untransformed control strain were grown under constant light and analyzed by light microscopy for phenotypic abnormalities. Unlike in untransformed control cultures, a small fraction of cells of any given culture had severe defects in cell morphology: cells were enlarged (Fig. 7C3), had multiple pairs of flagella (up to 12 flagella instead of two; Fig. 7C1-3) and unusual cell shapes (Fig. 7C2) among which spindle-shaped cells (Fig. 7E2) with two pairs of flagella at opposite cell poles were most prominent. Using these criteria for defining abnormal cell morphology, cells from five to 10 random visual fields were counted and the number of unusual cells expressed as fraction of total cells. In five independent experiments of this kind with strains 33, 45 and 67 the sum of all abnormal cells in any given culture was always between 5 and 14% of total cells while blind controls with the untransformed strain ranged between 1 and 2%. There were no major differences in numbers of unusual cells between the three different antisense strains.

Next, cell extracts from cultures with a comparably high (10-14%) fraction of abnormal cells were analyzed on immunoblots. Fig. 7D shows the result of such an experiment. Densitometric analysis of these immunoblots (middle panel) using L23 protein as a loading control indeed showed that DIP13 levels were reduced between 12 and 50% in these particular cultures while tubulin levels were comparable in all four strains.

Finally, DAPI staining of nuclei showed that cells with multiple pairs of flagella contained also multiple nuclei, one nucleus per flagella pair (Fig. 7E). In summary the results of functional analysis available to date suggest that expression of *DIP13* antisense RNA leads to a defect in cell division. These results will be discussed below.

Discussion

DIP13 and NA14 are the first representatives of a new class of proteins associated with MTs that have been found at the same structures, namely basal bodies and flagella in *Chlamydomonas*, as well as centrosomes of HeLa cells and basal bodies and flagella of human sperm cells. RNAi experiments have shown that reducing *DIP13* expression causes defects in cell division and results in multinucleate *Chlamydomonas* cells.

In the light of the DIP13 localization pattern it is conceivable that these proteins can bind to MTs directly, an idea supported by two facts: first, both DIP13 and NA14 contain a motif, KREE, which is similar to the MT-binding sites found in

MAP1B (Noble et al., 1989); and second, DIP13 can be extracted from axonemes under high salt conditions. The latter feature is shared with known MAPs (Suprenant et al., 1993; Maccioni and Cambiasso, 1995). However, to fulfill the biochemical definition of a structural MAP, DIP13/NA14 will have to show copurification with tubulin in subsequent rounds of MT assembly and disassembly (Mandelkow and Mandelkow, 1995).

Interestingly, DIP13/NA14 localization showed slight differences compared with α -tubulin. In particular, they seem to be more concentrated in *Chlamydomonas* basal bodies and in human centrosomes. In addition, anti-DIP13 antibody recognized preferably anterior MTs while anti α -tubulin antibody also stained posterior MTs (compare Fig. 4A,B,D with 4C). Because it is well-known that certain MT subsets present, for example, in *Chlamydomonas* flagellar axonemes and basal bodies (Le Dizet and Piperno, 1986; Piperno et al., 1987) are more stable and contain acetylated tubulin, there is a possibility that DIP13/NA14 are preferentially associated with these more stable MT subsets. This possibility is not ruled out by the fact that no MT labeling was observed in HeLa cells, because those contain very few, if any, acetylated stable MTs (Alieva et al., 1999; Vorobjev et al., 2000). However, immediate attempts to colocalize DIP13 with acetylated MTs in *Chlamydomonas* using monoclonal antibody 6-11B-1 (LeDizet and Piperno, 1986) have not yielded clear results. Therefore, very careful follow-up studies for colocalization of tubulin and acetylated tubulin with DIP13 and NA14 in *Chlamydomonas* and several human cell types will be necessary to confirm or disprove this hypothesis.

In our opinion, DIP13 and NA14 are not highly specialized but rather seem to have more general functions. From our current view the two most likely possibilities are (1) general stabilization of MTs, and (2) linking MTs to motile systems. Both possibilities are not mutually exclusive. Together, immunofluorescence and immunoelectron microscopy revealed strong labeling at the outside of the proximal end of the basal body, a place that could be the attachment point for the *Chlamydomonas* flagellar rootlet MT system (MTR), which consists of two sets of two (2MTR) and two sets of four (4MTR) MTs and is descending into the cell along the cell periphery from the proximal end of the basal bodies. The MTR system plays an important role in basal body positioning before mitosis, and during cell division it is nucleating MTs that in turn initiate (together with the internuclear MTs) the formation of the phycoplast (Ehler et al., 1995).

As a general MT stabilizing and connecting protein, DIP13 could be responsible for MTR anchoring to the basal bodies and stabilization of the 4MTR during cytokinesis. Moreover, it could keep the 4MTR (which are attached to migrating basal body pairs) in contact with each other during pro- and metaphase. Reduced DIP13 protein levels (as a consequence of successful RNAi) could lead to a loss of contact between the basal bodies and the 4MTR and improper elongation or depolymerization of the 4MTR, or loss of contact between two growing 4MTR. Moreover, force production relying on MT polymerization/depolymerization or the action of MT motors could also be compromised in this scenario. The consequence, however, would always be misplaced basal bodies, mispositioned cleavage furrows and, therefore, impaired cell division. This is what we observed.

It is not yet known why only a minor fraction of the cells in cultures of DIP13 antisense transformants showed these particular phenotypes. A possible answer could come from the finding of only minor reductions of protein levels in antisense cultures (Fig. 7D). It is conceivable that in some cells the antisense effect is stronger at a given time, resulting in more reduced DIP13 levels than in other cells. A cell division defect could then be observed only in cells with DIP13 levels below a given threshold concentration. However, this apparent drawback could be an advantage in the end, because it seems conceivable that a DIP13 reduction of 90-100% caused by RNAi could make the cytoskeleton very unstable so that the phenotype would be lethal.

Several mutants with phenotypes similar (but not identical) to *DIP13* RNAi transformants have been described previously. Among these is the *bld2* mutant, which is characterized by multinucleate big cells, but lacks basal bodies and flagella (Ehler et al., 1995). *Vfl2* and *vfl3* mutants, defective in centrin or the distal connecting fiber, respectively, also show random segregation of basal bodies (Wright et al., 1983) and thus have multiple flagella but, in contrast to *DIP13* RNAi transformants, are mononucleate and of normal size. At present, *DIP13* RNAi transformants appear to be similar to *oca1* and *oca2* cytokinesis mutants described previously (Hirono and Yoda, 1997). Cultures of these mutants contain large abnormally-shaped cells with multiple flagella; each pair of flagella is connected to one nucleus. Analyses underway will show whether *DIP13* and *oca* mutants are genetically linked.

DIP13/NA14 have also been found to be associated with flagellar axonemes of *Chlamydomonas* and human cells. The functions they could perform in this organelle are unclear at present but the fact that immunogold staining did not show a discrete staining pattern with a certain periodicity raises the possibility that the function in flagella is a dynamic rather than a static one. We are currently constructing a *DIP13/CGFP* fusion vector employing a modified version of the green fluorescent protein gene codon-optimized for *C. reinhardtii* (Fuhrmann et al., 1999) for in vivo localization (Ruiz-Binder et al., 2002) of *DIP13* to solve the above question.

DIP13, *NA14* and the mouse unnamed protein (AB041656) are the founding members of a new class of small proteins that might share conserved functions. Data from localization experiments are corroborated by the fact that the derived human and mouse proteins display 97% amino acid sequence identity, while *DIP13* and *NA14* still share 60% identical amino acids. As expected, we identified homologous proteins in a variety of eukaryotic organisms including several protozoans, *Schistosoma* and a fish. Unexpectedly, the completed genomes of *C. elegans* (www.sanger.ac.uk), *Drosophila* (www.ncbi.nlm.nih.gov), yeast (<http://genome-www.stanford.edu/Saccharomyces>) and *A. thaliana* (<http://www.arabidopsis.org/blast>) do not appear to contain a member of this protein family. Since yeast and *Arabidopsis* do not have centrioles or flagellated cell stages and they use different modes of cell division, the observed absence of a *DIP13/NA14* homolog is plausible. However, the apparent absence of *DIP13/NA14* is surprising in the cases of *Caenorhabditis* and *Drosophila*. Both organisms possess centrioles and ciliated/flagellated cell types. Maybe these organisms employ proteins with functional but no sequence homology to *DIP13/NA14*.

Taking into consideration all the results presented here, we propose that there is a new class of small proteins that are associated with MTs and that may be of general importance in many eukaryotic organisms of different phyla. Many questions are immediately obvious, among these the presence of *DIP13/NA14* homologs in other eukaryotic phyla, the molecular basis of *DIP13/NA14* interaction with MTs and further elucidation of *DIP13/NA14* function. Experiments to address these questions are underway.

We thank Kurt Wilkerson, George Witman and Paul A. Lefebvre for *Chlamydomonas* cDNA libraries and are indebted to Joel Rosenbaum in whose laboratory the first *DIP13* clone was isolated. Many thanks to Peter Hegemann for *Chlamydomonas* strain *cw15 arg7* MT- and Markus Fuhrmann for plasmid pMF59. We are indebted to Michel Bornens for the anti-CTR453 antibody, to Elizabeth Harris for anti-L23 antibody and to Michel Goldschmidt-Clermont for the *Chlamydomonas* genomic DNA library. We are also grateful to Birgit Scharf for help with FPLC protein purification. We are indebted to Gregory Pazour, David L. Kirk and Ivan Raska for critical reading of the manuscript and for helpful discussions. This work was supported by the Deutsche Forschungsgemeinschaft (SFB 521/B1), Czech grants 304/00/1481, AV0Z5039906 and MSM111100003 and the Ministerio de Ciencia y Tecnología (CICYT, n° PM99-0143), Spain.

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