Flagellar Radial Spokes Contain a Ca^{2+} -stimulated Nucleoside Diphosphate Kinase

Ramila S. Patel-King, Oksana Gorbatyuk, Sachiko Takebe, and
Stephen M. King*

Department of Molecular, Microbial and Structural Biology, University of Connecticut Health Center,
Farmington, Connecticut 06030-3305

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The radial spokes are required for Ca^{2+} -initiated intraflagellar signaling, resulting in modulation of inner and outer arm dynein activity. However, the mechanochemical properties of this signaling pathway remain unknown. Here, we describe a novel nucleoside diphosphate kinase (NDK) from the *Chlamydomonas* flagellum. This protein (termed p61 or RSP23) consists of an N-terminal catalytic NDK domain followed by a repetitive region that includes three IQ motifs and a highly acidic C-terminal segment. We find that p61 is missing in axonemes derived from the mutants *pf14* (lacks radial spokes) and *pf24* (lacks the spoke head and several stalk components) but not in those from *pf17* (lacking only the spoke head). The p61 protein can be extracted from *oda1* (lacks outer dynein arms) and *pf17* axonemes with 0.5 M KI, and copurifies with radial spokes in sucrose density gradients. Furthermore, p61 contains two classes of calmodulin binding site: IQ1 interacts with calmodulin-Sepharose beads in a Ca^{2+} -independent manner, whereas IQ2 and IQ3 show Ca^{2+} -sensitive associations. Wild-type axonemes exhibit two distinct NDKase activities, at least one of which is stimulated by Ca^{2+} . This Ca^{2+} -responsive enzyme, which accounts for ~45% of total axonemal NDKase, is missing from *pf14* axonemes. We found that purified radial spokes also exhibit NDKase activity. Thus, we conclude that p61 is an integral component of the radial spoke stalk that binds calmodulin and exhibits Ca^{2+} -controlled NDKase activity. These observations suggest that nucleotides other than ATP may play an important role in the signal transduction pathway that underlies the regulatory mechanism defined by the radial spokes.

INTRODUCTION

The eukaryotic cilium/flagellum is a highly conserved motile organelle built around nine outer doublet microtubules and a central pair of singlet microtubules that form the 9 + 2 axoneme. Individual flagella provide for the motility of single cells such as mammalian sperm and *Chlamydomonas*, and ciliated epithelia are essential for fluid/mucus transport in many organs. Moreover, nodal cilia are required to define the left-right axis during mammalian development (Supp *et al.*, 1997; Nonaka *et al.*, 1998). Many cells also contain primary cilia that seem to play critical sensory roles during normal tissue function and development (Pazour and Witman, 2003). The power for ciliary/flagellar movement is generated by the inner and outer rows of dynein arms that associate with the A tubule of each microtubule doublet and transiently interact with the B tubule of the adjacent doublet

to produce a linear force vector that results in the microtubules sliding with respect to each other. This sliding motion is then converted to a flagellar bend by other axonemal components, including the radial spoke and central pair microtubule complexes (see Mitchell (2000) for a review of *Chlamydomonas* flagellar structure and function).

In *Chlamydomonas*, control of flagellar waveform is mediated through Ca^{2+} signaling pathways (Bessen *et al.*, 1980; Kamiya and Witman, 1984). Reactivation experiments have revealed that all the machinery necessary for waveform regulation is integrated within the demembranated flagellar axoneme (Bessen *et al.*, 1980), and the central pair microtubule complex and radial spokes are thought to play key mechanochemical roles in this regulatory process (for recent review, see Smith and Yang, 2004). Cyclic AMP-dependent phosphorylation also exerts a regulatory effect on flagellar activity (Hasegawa *et al.*, 1987; Habermacher and Sale, 1996) that involves the radial spoke and inner arm dynein systems. There is also accumulating evidence of a role for redox poise in activating flagellar motility in *Chlamydomonas* and both mammalian and sea urchin sperm (Ogawa *et al.*, 1996; Patel-King *et al.*, 1996; Aitken *et al.*, 1997; Sadek *et al.*, 2001, 2003; Harrison *et al.*, 2002). Furthermore, isolated *Chlamydomonas* flagella have been reported to exhibit two distinct nucleoside diphosphate kinase¹ (NDKase) activities, which catalyze the general reaction $\text{ATP} + \text{NDP} \rightarrow \text{ADP} + \text{NTP}$; at least one of these enzymes has a marked preference for generating GTP (Watanabe and Flavin, 1976). Similarly, NDKase activity has been detected in sea urchin sperm flagella (Yanagisawa *et al.*, 1968; Kobayashi *et al.*, 1976), and there are also several mammalian NDKs (nm23-H5, -H7, -H8, and

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¹Nomenclature: a recent report (Yang *et al.*, 2001) identified 22 distinct components (including calmodulin and the LC8 dynein light chain) within *Chlamydomonas* flagellar radial spokes. These proteins have been termed RSP1-22. In an effort to retain a consistent naming system, we propose that p61, which we demonstrate here is also an integral radial spoke component, be henceforth termed RSP23.

* Corresponding author. E-mail address: steve@king2.uchc.edu.
Abbreviations used: MBP, maltose-binding protein; NDK, nucleoside diphosphate kinase; RSP, radial spoke protein; UTR, untranslated region.

-H9) expressed either predominantly or exclusively in testis; for example, the human nm23-H5 NDK has recently been localized to the sperm flagellar axoneme (Munier *et al.*, 1998, 2003). In sea urchin sperm outer arm dynein, the intermediate chain 1 (IC1) polypeptide consists of an N-terminal thioredoxin unit followed by three catalytic NDK modules (Ogawa *et al.*, 1996); the mammalian homologue (Sptrx-2 [nm23-H8]) that is present in sperm also contains three NDK modules (Sadek *et al.*, 2001). A second human protein (Tx1-2 or nm23-H9) containing both thioredoxin and NDK units has been identified in the cilia of lung epithelia, the spermatid manchette, and sperm flagellar axoneme (Sadek *et al.*, 2003). Together, these observations suggest that generation of nucleotides other than ATP may be of widespread significance for ciliary/flagellar function.

Although the purified outer dynein arm from *Chlamydomonas* flagella does not contain a polypeptide similar to sea urchin dynein IC1 (for recent review, see King (2002)), we (Patel-King *et al.*, 2002) and others (Ikeda *et al.*, 2003) recently identified a *Chlamydomonas* flagellar protein (here termed Rib72) consisting of three DM-10 domains (related to the N-terminal noncatalytic segment of human and rat NDK7 [nm23-H7 and nm23-R7]) followed by two consensus EF-hand motifs that could bind Ca^{2+} . This intriguing molecule is tightly associated with the *Chlamydomonas* axoneme (Patel-King *et al.*, 2002), and forms part of the protofilament ribbons within the wall of the A-tubules of each outer doublet (Ikeda *et al.*, 2003). Because NDKs are the only proteins of known function in which the DM-10 domain has been identified, we suggested previously that Rib72 may represent a Ca^{2+} -controlled regulatory component of a flagellar NTP synthesis system (Patel-King *et al.*, 2002).

Because NDKase activity seems to be important for flagellar function and indeed may play a crucial and previously unconsidered role in motor regulation, we sought to identify NDK catalytic units within the *Chlamydomonas* flagellum. Here, we describe a 61-kDa polypeptide consisting of an N-terminal NDK catalytic module and a C-terminal domain containing three IQ motifs that bind calmodulin. We demonstrate that p61 is an integral component of the flagellar radial spokes, which transmit signals from the central pair microtubule complex to the dynein motors and are essential for the control of microtubule sliding within the flagellar axoneme (Smith and Sale, 1992). We also observe that a Ca^{2+} -stimulated NDKase activity is missing in mutants lacking radial spokes, further suggesting that regulation of flagellar function through this structure may involve nucleotides other than ATP.

EXPERIMENTAL PROCEDURES

Molecular Biology

The full-length cDNA clone (AV645300) encoding *Chlamydomonas* p61 was identified in a BLAST search of the *Chlamydomonas* expressed sequence tag database by using the testis-specific nm23-H5 NDK (Munier *et al.*, 1998) as the initial query sequence. This clone was obtained from the Kazusa DNA Research Institute (Chiba, Japan) (Asamizu *et al.*, 1999). The entire 2.3-kb AV645300 clone was sequenced using the molecular core facility at the University of Connecticut Health Center. A 5' section (140–718 base pairs) of this clone encoding the 5'-untranslated region (UTR) and NDK catalytic module were used to probe blots of *Chlamydomonas* genomic DNA and also RNA samples obtained before and 30 min after deflagellation. The 3' portion of the clone has relatively low nucleotide complexity (71.2% GC content), which reflects the Ala-, Pro-, and Glu-rich regions in the C-terminal portion of p61; the use of this region as a probe resulted in unacceptably high backgrounds.

Computational Methods

Sequence searches of the nonredundant database were performed using BLAST. Analysis of the p61 region (scaffold #399) of the *Chlamydomonas*

genome and identification of additional related proteins was performed using the BLAST interface at the Department of Energy Joint Genome Institute (Walnut Creek, CA) (<http://genome.jgi-psf.org/chlr1/chlr1.home.html>). The p61 cDNA sequence was assembled using the GCG suite of software. The repeat structure of p61 was identified and analyzed using COMPARE and DOTPLOT with a window size of 30 and stringency of 25. The COILS program was used to assess the probability of coiled coil regions (Lupas *et al.*, 1991). The multiple sequence alignment was generated with ClustalW and used as the input for phylogenetic analysis. The molecular model for the catalytic domain of p61 was calculated using SWISSMODEL (Peitsch, 1996). The IQ motifs were identified using the Simple Modular Architecture Research Tool (<http://dylan.embl-heidelberg.de>).

Fusion Protein and Antibody Production

Residues 7–199, 7–424, 7–494, 424–494, and 7–586 of p61 were cloned into the pMAL-c2 vector across the *XmnI/XbaI* sites and expressed as C-terminal fusions with maltose-binding protein (MBP). These proteins were purified by amylose affinity chromatography and MBP-p61(7–199) used as the immunogen to inoculate rabbit CT220. The p61(7–199) segment was separated from MBP by digestion with Factor Xa and used to blot-purify a p61-specific antibody fraction from serum by using the method of Olmsted (1986). Fusion proteins begin at residue 7, because we were unable to amplify and subclone the appropriate regions when starting from residue 1.

Chlamydomonas Strains and Isolation of Flagellar Components

The following strains of *Chlamydomonas reinhardtii* were used in this study: cc124 (wild type), *oda1* (lacks outer dynein arms and associated docking complex), *ida1* (lacks inner arm I1), *ida4* (lacks inner arm I2), *pf14* (lacks radial spokes), *pf17* (lacks radial spoke heads), *pf18* (lacks the central pair microtubule complex), and *pf24* (lacks radial spoke heads and several other components; assembles truncated stalks). *Chlamydomonas* strains were grown in R medium (Harris, 1989), deflagellated using dibucaine, and the flagella were isolated by standard methods (King, 1995). Axonemes were obtained by treating flagella with 1% IGEPAL CA-630 (replaces Nonidet P-40) to remove the membrane and either prepared directly for electrophoresis, or further fractionated by extraction with 0.6 M NaCl (King *et al.*, 1986). From the deflagellation step onwards, a protease inhibitor cocktail (P-8340; Sigma-Aldrich, St. Louis, MO) was added to all solutions to yield final concentrations of 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride, 0.8 μM aprotinin, 20 μM leupeptin, 15 μM pepstatin A, and 14 μM N-(N-(L-3-trans-carboxyoxirane-2-carbonyl)-L-leucyl) arginine (E-64). Radial spokes were solubilized from NaCl-extracted *oda1* and *pf17* axonemes by treatment with 0.5 M KI and purified by centrifugation in 5–20% sucrose density gradients as described by (Yang *et al.*, 2001).

Calmodulin Binding Assay

One hundred micrograms each of the MBP-p61 fusion proteins were incubated for 2 h with 100 μl of calmodulin-Sepharose 4B beads (Amersham Biosciences, Piscataway, NJ) in 50 mM Tris-Cl, pH 7.5, 100 mM NaCl, 0.1% Tween 20, 2 mM CaCl_2 . Protein that did not bind was recovered after a brief centrifugation. The beads were then washed six times with buffer and resuspended in 200 μl of 50 mM Tris-Cl, pH 7.5, 100 mM NaCl, 0.1% Tween 20, 2 mM EGTA for 60 min to elute proteins bound in a Ca^{2+} -dependent manner. After an additional wash with EGTA-containing buffer, protein that remained associated with the calmodulin-Sepharose beads in a Ca^{2+} -independent manner was eluted with gel sample buffer.

Electrophoretic Methods

Routine analysis of fusion proteins was performed in 8% SDS polyacrylamide gels. Flagellar components were separated in 5–15% SDS polyacrylamide gradient gels and either stained with Coomassie Blue, or transferred to nitrocellulose and probed with antibody CT220 by using standard methods (Harrison *et al.*, 1998). Polyclonal antibody against radial spoke protein-3 (RSP3) (Williams *et al.*, 1989) was kindly provided by Drs. Dennis Diener and Joel Rosenbaum (Yale University, New Haven, CT). Antibody reactivity was detected using a peroxidase-conjugated secondary antibody and an enhanced chemiluminescent system (Amersham Biosciences).

Nucleoside Diphosphate Kinase Assay

NDKase activity was determined using the bioluminescent method described by Karamohamed *et al.* (1999). The assay is based on the transfer of the terminal γ -phosphate from GTP to ADP, and the subsequent utilization by luciferase of the ATP generated to yield light. A Tricine-based assay buffer system, pH 7.8, containing MgSO_4 , EDTA, dithiothreitol, and bovine serum albumin was used (assay kit FL-AAM; Sigma-Aldrich). ADP and GTP were both added to final concentrations of 0.25 mM, and the sample equilibrated for several minutes to allow any contaminating ATP to be used. The assay was started by addition of axonemes derived from either wild type or the *pf14* mutant strain. Ca^{2+} and EGTA were added to the assay mix as appropriate.

Light output was integrated >15-s intervals by using a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA) and calibrated for each experiment with known amounts of ATP. All assays were performed in triplicate. Specific NDKase activity is expressed as micromoles of phosphate transferred per minute per milligram of protein. Addition of ADP alone generated negligible amounts of ATP, indicating that the axoneme and radial spoke preparations assayed did not contain a flagellar adenylate kinase.

RESULTS

Identification of a Flagellar Nucleoside Diphosphate Kinase

Chlamydomonas flagella have previously been demonstrated to contain an NDKase activity that exhibits a marked preference for generating GTP (Watanabe and Flavin, 1976). A BLAST search of the *Chlamydomonas* expressed sequence tag (EST) database by using the human testis-specific nm23-H5 NDK (NDK5; Munier *et al.*, 1998) as the initial query sequence identified two potential NDK-encoding clones (AV645300 and BG843793). Furthermore, analysis of the recently released *Chlamydomonas* genome (version 1) uncovered two additional NDK genes (in scaffolds #154 and 279). Phylogenetic analysis (Figure 1, a and b) of the sequences available from the databases indicated that the BG843793 clone and the scaffold #279 sequence both encoded NDKs closely related to those in the cytoplasm and chloroplasts of higher plants. Consequently, neither was considered likely to represent a flagellar-specific NDK. In contrast, AV645300 encodes an NDK catalytic module most closely related to the testis-specific mammalian enzymes and to the NDK domains present within the IC1 polypeptide of sea urchin sperm outer arm dynein; the scaffold #154 sequence also groups with these proteins. Because it is present in the EST database and therefore presumably expressed in *Chlamydomonas*, the AV645300 clone was selected as a candidate to encode a flagellar NDK.

Southern blot analysis of *Pst*I-, *Sma*I-, and *Bam*HI-restricted genomic DNA by using the 5' end of the AV645300 EST as a probe resulted in single bands, indicating that *Chlamydomonas* contains only one gene for this NDK (Figure 1c). A search of the *Chlamydomonas* genome also detected only a single 2763 base pair gene consisting of four exons within scaffold #399. Northern blotting provided further evidence that AV645300 indeed encodes a flagellar protein. The 2.54-kb mRNA for this clone was essentially undetectable in *Chlamydomonas* cells that had not been deflagellated, but was highly up-regulated 30 min after flagella excision as is characteristic of integral flagellar components (Figure 1d).

Properties of the p61 Nucleoside Diphosphate Kinase

The entire AV645300 clone of ~2.3 kb was sequenced and found to contain a single open reading frame encoding a 586-residue protein (termed p61) with a mass of 61,364 Da and a predicted pI of 4.48 (Figure 2). The clone contains an in-frame stop codon within the 5'-UTR, a perfect copy of the *Chlamydomonas* polyadenylation signal and a polyA tract at the 3' end. The p61 protein has a NDK catalytic module at the N terminus. This domain (residues 1–200) shares 41% sequence identity (60% similarity) with human testis-specific nm23-H5 NDK (accession no. P56597). Sequence analysis and molecular modeling of residues 2–142 (Figure 3) indicates that this unit contains all the conserved NDK residues, including the active site motif (residues 118–126), that have been demonstrated previously from both structural and mutagenesis studies to be essential for catalysis (Tepper *et al.*, 1994). In addition to multiple putative phosphorylation sites, p61 contains three IQ motifs that could potentially bind calmodulin. These motifs conform precisely to the (I/

V/L)QXXX(K/R)XXX(K/R) consensus (Rhoads and Friedberg, 1997) and are located within the C-terminal domain at residues 362–391, 440–469, and 512–541.

Further sequence analysis (Figure 3) revealed that the entire C-terminal portion of p61 (residues 221–586) is relatively hydrophilic and Ala-rich, containing 25% Ala overall. In addition, the regions encompassing residues 265–349 and 546–586 are Pro-rich (36.5% Pro) and Glu-rich (36.5% Glu), respectively. Because the C-terminal 40-residue Glu-rich domain also contains 32% Ala, this region is very likely helical. However, a high coiled coil prediction was obtained using the COILS program only with a scan window of 14 (but not 21 or 28), and thus this segment is unlikely to form a coiled coil (Lupas *et al.*, 1991). The regions immediately surrounding the three IQ motifs are very similar. Indeed, in the case of IQ2 and IQ3, these motifs are embedded in ~70-residue repeat sequences (residues 424–494 and 496–565) that share 92.8% identity.

To further analyze this presumptive flagellar NDK, the N-terminal region (residues 7–199) of p61 was expressed as a C-terminal fusion with maltose-binding protein and used to immunize rabbit CT220. The resulting serum was blot-purified against p61 (residues 7–199) obtained by digestion of the fusion protein with factor Xa. We also generated a MBP fusion protein containing p61 residues 7–586. This polypeptide has a calculated molecular mass of 103,130 Da, but migrated with $M_r = \sim 135,000$ (Figure 4). On digestion with factor Xa, this fusion protein yielded MBP ($M_r = \sim 40,000$ as expected) and the fusion partner which had a $M_r = \sim 102,000$ (Figure 4a). Thus, recombinant p61 migrates anomalously in SDS-polyacrylamide gels as has been found previously for several other axonemal proteins (e.g., DC1 of the outer arm docking complex; Koutoulis *et al.*, 1997). Furthermore, the MBP-p61(7–586) protein was highly susceptible to proteolysis by endogenous proteases. Indeed, when we examined axoneme samples prepared in the absence of a comprehensive protease inhibitor cocktail, multiple bands ranging from $M_r \sim 102,000$ to 20,000 were detected by the CT220 antibody (our unpublished data). However, when protease inhibitors were added to the isolation buffers, all bands except for those with $M_r = \sim 102,000$ and 40,000 (Figure 4b) were dramatically reduced or absent, indicating that the larger band is highly susceptible to proteolysis. Because recombinant p61 exhibits anomalous electrophoretic mobility (see above), we conclude that the $M_r = \sim 102,000$ band represents native p61. The CT220 antibody was raised against the conserved NDK region of p61, and thus it is likely that the $M_r 40,000$ band represents a second flagellar NDK (most probably the *Chlamydomonas* ortholog of human nm23-H7 that is encoded on scaffold 154 and has a predicted mass of 39.9 kDa).

p61 Is Missing from pf14 and pf24 Axonemes

Fractionation of *Chlamydomonas* flagella revealed that p61 and the $M_r 40,000$ protein are integral components of the axoneme and remained tightly associated with that structure after both detergent treatment to remove the flagellar membrane and soluble matrix components, and extraction with 0.6 M NaCl, which is used to solubilize the dynein arms (Figure 5). In an initial effort to ascertain the axonemal location of p61, we used the CT220 antibody to probe axoneme samples prepared from wild-type *Chlamydomonas* and mutant strains lacking the outer dynein arm and docking complex (*oda1*) or inner arms I1 (*ida1*) and I2 (*ida4*), the radial spokes (*pf14*) and the central pair microtubule complex (*pf18*). The $M_r 40,000$ band detected by CT220 was present in

a

Anthocidaris IC1 #3	1	-----VIKPDAAE-HKEATTEKKEAGFNHSLQRDVELNKLASKLYLEHGGKE-FYENLIDHMSGSLSMVMVLSR-
Human Sptrx-2 #3	1	-----TIGLIKPHATSE-QRQELKIVKEAGFDLTQVRKMPITPEQTEKIPKVTGKD-FYKDLLLEMLSVGFSMVMLTK-
Chlamydomonas p61	1	-----MAETKTPALIKPDAVRAGKAEIMQLIELNGFTTHAKQKLOLTRARAEFFVGEHKGKE-FFPKLVNFMSTSGFIWALVLAK-
Chlamydomonas scaffold 154	1	-----KLSKEAGEFVAVHAGKP-FVDRLLTDFMSSGSRVVAEMLVA-
Rat NDK7 domain #1	1	-----BKTLALIKPDVAIS-KAGEIHEMINKSGFTTITKRRMTLSRKEAADFVDDHHSRP-FYNELIQFITSGFVHAMEILR-
Mouse NDK5	1	1 MVVSMPLPQIYVEKTLALIKPDVVD--KEEIQDILGSGFTTIQRRKRLHSPHCSNFVVEQYQGM-FFPNLTAYMSGSPVAMILAR-
Human NDK5	1	1 MEISMPPPIYVEKTLALIKPDVVD--KEEIQDILRSQFTTIQRRKRLHSPHCSNFVVEQYQGM-FFPNLTAYMSGSPVAMILAR-
Anthocidaris IC1 #2	1	1 -----ALIHPSAKDK-HKDEMLQKIQEAGFEVCLQRMVOLTEDQAEFFYREQGTH-HFEDLLREMTSGSPVAMILAR-
Human Sptrx-2 #2	1	1 -----LTKTLALLRPNLFHE-RKDDVLRI-KDEDFKILEQRQVVLSEKEAQAALCEYENED-YFNKLTENMTSGSPSLALVLLR-
Human Tx1-2	1	1 -----SLAIKPDVAISKKVLEIKRKIKAGFIIEAEHKTVLTETQVNFVSRIADQR-DFEEFVSFMTSGLSYLIVV--
Anthocidaris IC1 #1	1	1 -----CTLAIKPDVAHGHKDEIIMKIQEAGFEILTNEERTMTAEVRLFYQHKAGEE-AFEKLVHVMCSGSPSHLLITLRT
Spinach cytosolic	1	1 -----PKREVTVVLIKPDVAHGHVDSIIAKIEHGFELTTEDKTLTEDEARFFYQHEEEE-HFEVLVTFMASGSPSKILVLTG
Arabidopsis	1	1 -----MEQTFIMIKPDQVQGLVGEIISRFEKKGFSLKALHFFVNVDRPFAEKHVAIDLAKP-FFNGLVVEYIVSGPVVAMVWBG-
Chlamydomonas Scaffold 279	1	1 -----MEQTFIMIKPDQVQGLVGEIISRFEKKGFSLKALHFFVNVDRPFAEKHVAIDLAKP-FFNGLVVEYIVSGPVVAMVWBG-
Chlamydomonas BG843793	1	1 -----BQSYIMIKPDQVQGLVGEIISRFEKKGFSLKALHFFVNVDRPFAEKHVAIDLAKP-FFNGLVVEYIVSGPVVAMVWBG-
Pea	1	1 -----SVRAEISYIMIKPDQVQGLVGEIISRFEKKGFSLKALHFFVNVDRPFAEKHVAIDLAKP-FYKDLVNYIVSGPVVCMVWBG-
Spinach chloroplast	1	1 -----LEQVDQAYIMVFPDQVQGLVGEIISRFEKKGFSLKALHFFVNVDRPFAEKHVAIDLAKP-FFPKLEIYITSGPVVSMWBG-
Rat NDK7 #2	1	1 -----MEQVEEYIMIKPDQVQGLVGEIISRFEKKGFSLKALHFFVNVDRPFAEKHVAIDLAKP-FYKDLVNYIVSGPVVCMWBG-
	1	1 -----CCIIKPHATSGMIGKILIAHRDAGFESATQMFNMDRANVEEFYEVYKGVLSYNDMMTELYSGFCVATEIQQ-

Anthocidaris IC1 #3	71	---EDAVDGRITMGPTDDEYAREHAFESLRALLGKDLVLN-AVHGSSNFEAKTRHERLEFDV-----
Human Sptrx-2 #3	74	---WNAVAEWRRLMGPTDDEEAKLLSFDSEHRAQEGISKLKN-IVHGASNAYPEAKEVNNRLEFEDPEEN-----
Chlamydomonas p61	81	---PGAILAWRALMGPTNVFKARAEQPKCLRALYGTDTGTON-ATHGSDSPISAAREIKFFFTLSGDPITYAEPATAAE-
Chlamydomonas scaffold 154	40	---PGAIRKWRREIP-TNVFKARAEQPKCLRALYGTDTGTON-ATHGSDSPISAAREIKFFFTLSGDPITYAEPATAAE-
Rat NDK7 domain #1	75	---DDAICEWKRLLGCPANSIARSEAPGSRVRLFGDTGIEN-AAHGSDTFESAAREMELEFFSFGG-----
Mouse NDK5	87	---HKAISYWKELMGPSNSIVAKETHFDSLRALYGTDELRLN-ADHGSDNFAASEREIRFMFPVAVIIEPIPIGQAADK--
Human NDK5	87	---HKAISYWKELMGPSNSIVAKETHFDSLRALYGTDELRLN-ADHGSDNFAASEREIRFMFPVAVIIEPIPIGQAADK--
Anthocidaris IC1 #2	72	---ESAIQSWRETIPTTIDEAREKAPDSLRALYGTDTGTON-ATHGSDSPISAAREIKFFFTLSGDPITYAEPATAAE-
Human Sptrx-2 #2	72	---ESAIQSWRETIPTTIDEAREKAPDSLRALYGTDTGTON-ATHGSDSPISAAREIKFFFTLSGDPITYAEPATAAE-
Human Sptrx-2 #1	77	---DNGLOVWKQLDGGRTVEEAIEYFESLCAQFAMDSLFPVNQLYGSDSLETAEREIQHFPPQLQS-----
Human Tx1-2	77	EGFEDVVTWRTVMGPRDENVARRECELSLRALYGTETMPFN-AVHGSRDRREDADRELLALEFSLKFSFSD-----
Anthocidaris IC1 #1	80	DTGEGVSEVRNLDGKDEVAKEEAPDSLRALYGTDTGTON-AMHGADSKETAAREMAFLNPSVPIVPGTG-----
Spinach cytosolic	78	---KGVVAGRKLLIGATNPASE---PGTIRGDFPAIDIGRN-VIHGSDAVDSATKEIALWFPDG-VVHWQSSLSHWIYE-
Arabidopsis	78	---KNVVLGGRKLLIGATNPASE---PGTIRGDFPAIDIGRN-VIHGSDSVESARKEIALWFPDG-PVNWQSSSVHPWVYET
Chlamydomonas Scaffold 279	76	---KGVVAGRKLLIGATNPASE---PGTIRGDFPAIEVGRN-VIHGSDSVESATREIIGLWFPDG-VAQWTPPAKWPWLYE-
Chlamydomonas BG843793	81	---KGVVAGRKLLIGATNPASE---PGTIRGDFPAIEVGRN-VIHGSDSPENGERETAIWFPDG-ITSWQTMETPWLKE-
Pea	81	---VGVVPEARKLLIGATNPLOAE---PGTIRGDFPAVQTGRN-IIHGSDSPENGERETAIWFPDG-ITSWQTMETPWLKE-
Spinach chloroplast	81	---VGVVASSRKLLIGATNPLOAE---PGTIRGDLAVQTGRN-VVHGSDSPDNGKREIGLWKEKEICQWTPAQAPWLR-
Rat NDK7 #2	75	---SNPTKTFREFCQPSDEIARHLRPETLRANFGKTKVGN-AVHCTDLPEDGLLEVQYFEKIL-----

b **c** **d**

Figure 1. NDK phylogeny and Northern/Southern blot analysis of p61. (a) *Chlamydomonas* ESTs AV645300 (encoding p61) and BG843793, the NDKs identified in scaffolds #154 and 279 of the *Chlamydomonas* genome, the NDK modules from the IC1 polypeptide of sea urchin dynein (*Anthocidaris crassispina*), human and murine NDK5, rat NDK7, human Sptrx-2, human Tx1-2, and the cytosolic and chloroplast NDKs from spinach, pea, and *Arabidopsis* were aligned using ClustalW. Residues conserved in $\geq 30\%$ of the sequences were shaded using BOXSHADE. (b) ClustalW alignment was used as the input to generate the neighbor-joining unrooted tree shown. The BG843793 EST and NDK encoded on scaffold 279 clearly group with the NDKs of higher plants. In contrast, AV645300 (p61) and the scaffold #154–9 NDK (p40) are more closely related to the testis-specific mammalian enzymes. (c) Southern blot analysis of *Chlamydomonas* genomic DNA restricted with *Sma*I, *Pvu*II, *Pst*I, and *Bam*HI and probed with the 5' region of the AV645300 EST clone. Single bands were detected in the *Sma*I-, *Pst*I-, and *Bam*HI-digested samples. (d) Northern analysis of *Chlamydomonas* RNA obtained from non-deflagellated cells (NDF) and from cells that had undergone flagella excision and been allowed to regenerate new flagella for 30 min (30' postDF). A 2.54-kb mRNA that is highly up-regulated after deflagellation was detected by the AV645300 probe.

all samples (Figure 6, a–c). In contrast, p61 and its proteolytic breakdown products were completely missing from *pf14* axonemes (Figure 6, a and b). Further analysis revealed that p61 was present in axonemes derived from *pf17*, which

lack only the radial spoke head (Figure 6c), suggesting that p61 is an integral component of the radial spoke stalk. Interestingly, p61 was almost completely absent from *pf24* axonemes; this strain is defective for RSP2 and lacks the

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1  GCAAGCTTCACGTCATCAATTAACACATTACTTAATAACAATTATAAGTCTTCTATCTGA  60
61  GTTCGGTCCCGCTGAGTTTCGGTCTTGCTTCAAGCCTCCTTAGCTGCTGAGACGTCAC  120
1  M A E L E K T F A L I K P D A V R A G K  20
121  CATGGCAGAGCTAGAAAAGACTTTTCGCGTTAATCAAGCCAGATGCCGTGCGGGCCGGCAA  180
21  A Q E I M Q L I E L N G F T I I A K Q K  40
181  GCGCAGGAAATCATGCAGCTTATCGAGCTGAATGGCTTACGATTATCGCCAAGCAGAA  240
41  L Q L T R A R A E E F Y G E H K G K E F  60
241  GCTACAGTTGACACGGGCCCGCGGAGGAGTTCTATGGCGAGCAACAAGGAAAGGAGTT  300
61  F P K L V N F M T S G P I W A L V L A K  80
301  CTTCCCTAAGCTGGTGAACCTTATGACCAGCGGGCCTATCTGGGCGCTTGTGCTGGCCAA  360
81  P G A I L A W R A L M G P T N V F K A R  100
361  GCCTGGTGCCATTCTGGCTTGGCGTGCGCTGATGGGCCCAACCAACGTGTTAAAGCCCG  420
101  A E Q P K C L R A L Y G T D G T Q N A T  120
421  CGCGGAGCAGCCTAAGTGCTGCGCGCTGTACGGAACGGACCGACCCAGAATGCCAC  480
121  H G S D S P I S A A R E I K F F F F P T L  140
481  CCACGGCAGCGACTCGCCTATCAGCGCGCGCGCGAGATTAAAGTCTTCTTCCCCAGCT  520
141  S G D P T I Y A E P T A A A E Y T I C T K R  160
541  GTCTGGCGACCCGACCATCTACGCGGAGGCCACTGCGCGCGCAGAGTACATTACAAAGCG  600
161  I Q P A L A K A L A L A R E K P S A D  180
601  CATCCAGCCGCTCTGGCCAAGGCGCTGGCTGCGCGCGCAGAAGCGTCCGCGCA  660
181  K F E A I T F V A G Y L L Q N N P N K P  200
661  CAAGTTCGAGGCCATCACCTTCGTGGCGGGCTACCTGCTGCGAGCAACCCCAACAGCC  720
201  K V L M P D E W D P A L M G D D E D D E  220
721  CAAGGTGCTCATGCCGAGCAGAGTGGGACCCAGCGCTGATGGCGCATGACGAGGACGACGA  780
221  A D F I N A R L A A P T G N D G A T K A  240
781  GGCAGACTTCATCAACGCGCGGCTGGCGGCACCCACCGGCAACGACGCGCAACCAAGGC  840
241  E F D A M V E A A K A D T G A C A P P A Q  260
841  CGAGTTCGAGCCATGGTGGAGGCGCCAAGGCGGACACCGCGCGCGCGCGCGCGCGCA  900
261  E F V Y P D S K P T T P V V P A P P A P  280
901  GGAGTTCGTGTATCCCGACAGCAAGCCACACCGGTGGTGGCGCGCGCGCGCGCGCACC  960
281  G S K P P S A S G A R P Q S A R P T S A  300
961  GGGCAGCAAGCCGCGTCCGCGCAGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCG  1020
301  R P P S A S A P P A P L A P V P P P A  320
1021  GCGGCCACCGTCGGCGTCAGCCGCGCCTCCGCGCGCCCTGGCGCGCGGTGCCGCGCGCGCG  1080
321  S S S R P A S G S G R P P S A T A R P P  340
1081  GTCCTCATCCCGCGCGCGCTCGGGCTCAGGGCGCGCGCGCCCTCAGCAACCGCGCGCGCGCG  1140
341  S A T P P P P P P A V E L E E A D D P A  360
1141  GTCGGCCACGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCG  1200
361  Q L D E A A T K V Q A A F R Y Q A R K  380
1201  GCAGCTGGATGAGGCGCGACCAAGGTGCAGGCGCGTTCCTGGGTACCAGCGCGCGAA  1260
381  E V A V M R S E A Q P G E A A A E P E Q  400
1261  GGAGGTGGCGGTGATGCGCAGCGAGGCCAGCCGCTGAGGGCGCGCGCGCGCGCGCGCGCA  1320
401  E A E L Q P E A E P E P Q P E G E Q E P  420
1321  GGAGGCGGAGCTGACGCGGAGGCGGAGCGGAGCGCGCAACCCGAGGGGAGCAGGAGCC  1380
421  Q P Q A S A S S S F L P D G V T E E M A  440
1381  GCAGCCGAGGCGAGCGCTTAGCAGCTTCTACCGACGCGGTGACAGAGGAGATGGC  1440
441  A E A A T R V Q A H M R G H L A R K Q V  460
1441  TGCAGAGGCGCGCAGCGGTGTGACGGCGCACATGCGTGGGCACCTCGCGCGCAAGCAGGT  1500
461  A A I K A Q Q A A P A V A E S S E A L A  480
1501  GCGCGGATTAAGCGCAGCAAGCGCGCGCGCGGTGGCTGAGAGCTCGGAGGCTCTGGC  1560
481  E P E P Q P E A E A E P Q G A S V S S  500
1561  GGAGCCGAGCCCAACCCGAAGCGGAGGCGGAGCGCGAGCCACAGGCGAGCGTCTCTAG  1620
501  S F L P D G V T E E M A E A A T L V Q  520
1621  CAGCTTCCTACCGGACGCGGTGACGGAGGAGATGGCGGACAGGCGGCGGCGGCGGCGG  1680
521  A H M R G H L A R K Q V A A I K A Q Q A  540
1681  GCGCACATGCGCGGGACCTCGCGCGCAAGCAGGTGCGCGGCATTAAAGCGCAGCAAGC  1740
541  A P A V A E S S E A Q A E A Q T E A E  560
1741  GCGCGCGCGGTGGCTGAGAGCTCGGAGGCGCAGGCGGAGGCGGAGCAACCGAGGCGGA  1800
561  A E P Q P D A E A E A G A A E G E A E P  580
1801  GCGGAGCCCAACCGGATGCGGAGGCGGAGGCTGGGCGACCGAGGCTGAGGCCGAGCC  1860
581  E P E A A *  586
1861  GGAGCCGAGGCGCGCTGAGTAAGGCGCAGAGCATGGGGTTCAGGAGCATAGGCCCTTGT  1920
1921  ATACGTGCTGTCTGATTGCTGCTAGCGTAAGGTTATTGCTGACCTGGTGGGTGGCG  1980
1981  ATGGTTTGCAAGGCGCGGTGCGATTGTCAACTAAATATGGTCGACCGTGGCGCGCGCGCG  2040
2041  GTACAAAGTGAAGTCCCGCAGTGAGCAAGCGCGGTGCGGTGAGAGTGTCTCTACATTGG  2100
2101  AAAGCCCTGGATGGAGCGTGGCGCATAGCAGGAGAGCTGGCGTGTGTGAGGTGCCTCT  2160
2161  GTGTCGGTTGCGAAGAGCGCGCGTGTGAGCTGTGACGTGAATGTAAAGGTCAAATGGCA  2220
2221  AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA  2280
2281  AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA  2334

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Figure 2. p61 cDNA sequence. The entire 2.3-kb cDNA clone (AV645300) encoding full-length p61 was sequenced. The p61 protein consists of 586 residues and has a calculated mass of 61,394 Da and a pI of 4.48. The 5'-UTR contains a single in-frame stop codon and the putative polyadenylation signal in the 3'-UTR is underlined. The clone terminates in a polyA tract. This annotated sequence is available under GenBank/European Molecular Biology Laboratory/DNA Database of Japan accession no. AY452667.

spoke head and several other components resulting in a truncated stalk (Huang *et al.*, 1981; Yang *et al.*, 2004). This suggests that p61 associates with the radial spoke stalk near RSP2 in the region distal to the doublet microtubules.

p61 Copurifies with RSP3

To test whether p61 is indeed a radial spoke component, we first treated axonemes from the mutant *oda1* (lacks outer arm

dynein and docking complex) with 0.6 M NaCl to remove additional dynein components. Subsequently, these treated axonemes were extracted with 0.5 M KI to solubilize the radial spokes as described by Yang *et al.* (2001). After dialysis to remove KI, the extract was sedimented in a 5–20% sucrose density gradient (Figure 7, a and b). Both the M_r 40,000 protein and p61 were solubilized under these conditions. The majority of p61 sedimented near the bottom of the

Figure 3. Domain structure of p61. A map of p61 illustrating the location of the NDK domain, IQ motifs, and regions rich in specific amino acids is shown. A Kyte and Doolittle hydropathy plot is inserted above the map. Almost the entire C-terminal region is hydrophilic in nature. Bottom, dot plot comparison of p61 to illustrate the repetitive structure containing the three IQ motifs present within the C-terminal region of the protein. The inset at lower right is a molecular model for the p61 NDK domain (residues 2–142) built using SWISSMODEL. Indicated on the model are the residues known to be required for the catalytic activity of other NDKs (Tepper *et al.*, 1994). All these active site residues are completely conserved in p61.

gradient (in fractions 1–3), which is characteristic of isolated radial spokes (Yang *et al.*, 2001); there was also a much smaller fraction of p61 present in fractions 6–8. In contrast, the M_r 40,000 protein was found near the top of the gradient in fractions 11–14. To determine whether p61 comigrated with bona fide radial spoke proteins, we probed the same fractions with an antibody against RSP3 (Williams *et al.*, 1989). The RSP3 peak was found close to the bottom of the gradient as described previously (Yang *et al.*, 2001) and coincided with p61. Based on immunoblot analysis and/or by comparison with the electrophoretic patterns observed by Yang *et al.* (2001), p61 and several other RSP proteins could be readily identified in the peak sucrose gradient fractions (Figure 7a). Densitometric analysis using the RSP3 + RSP4 band as a standard indicates that p61 is present at a ratio of 0.85:1. Thus, p61 seems to be a stoichiometric component of the radial spokes.

To further confirm that p61 is an integral component of the radial spokes, we purified this structure from the mutant *pf17*, which assembles a radial spoke that lacks five head

components (Huang *et al.*, 1981) and consequently sediments more slowly in sucrose gradients (Yang *et al.*, 2001). In these samples, p61 was found in fractions 1–7 (peak was in fraction 4) and again precisely comigrated with RSP3 (Figure 7c). The migration of the M_r 40,000 band from *pf17* axonemes was unaffected, and this protein remained near the top of the gradient in fractions 13–15. Thus, we conclude that p61 is indeed an integral component of the flagellar radial spoke stalk.

p61 Interacts with Calmodulin In Vitro

Sequence analysis (Figure 3) revealed that p61 contains three canonical IQ motifs (IQ2 and IQ3 are identical) that could potentially act to bind calmodulin (Rhoads and Friedberg, 1997). To test this, we examined whether the MBP fusion proteins containing the NDK domain alone [p61(7–199)] or with various IQ motif combinations [NDK+IQ1, p61(7–424); NDK+IQ1+IQ2, p61(7–494); NDK+IQ1+IQ2+IQ3, p61(7–586)] would interact specifically with calmodulin immobi-

Figure 4. p61 migrates anomalously in SDS-polyacrylamide gels. (a) Twenty micrograms of the MBP-p61(7-586) fusion protein was electrophoresed in a 5–15% acrylamide gradient gel, both before and after incubation with factor Xa, and stained with Coomassie Blue. The intact fusion protein has a calculated mass of 103,130 Da but migrated with $M_r \sim 135,000$. The minor bands derive from cleavage of MBP-p61(7-586) by endogenous bacterial proteases. After factor Xa cleavage of the fusion protein, MBP migrated at $\sim M_r$ 40,000 as observed previously, whereas the p61(7-586) segment (calculated mass 60,662 Da) had $M_r \sim 100,000$. (b) Wild-type axonemes ($\sim 100 \mu\text{g}$) prepared in the presence of a protease inhibitor cocktail were electrophoresed in a 5–15% acrylamide gradient gel and either stained with Coomassie Blue (left) or blotted and probed with the CT220 antibody (right). Two prominent bands of M_r 102,000 and 40,000 were obtained. The top band corresponds to p61, which migrates anomalously. The diffuse band located above tubulin is a proteolytic fragment of p61.

lized on Sepharose 4B beads. We found that $\sim 60\%$ of total added MBP-p61(7-586) adhered to the calmodulin resin in the presence of Ca^{2+} and that $\sim 70\%$ of bound protein was eluted after addition of 2 mM EGTA (Figure 8). In contrast, $<10\%$ of the truncated p61(7-199) fusion protein was found associated with the calmodulin-Sepharose beads; all of this presumably nonspecifically bound protein was eluted by washing with EGTA. Because IQ motifs usually bind calmodulin in a Ca^{2+} -independent manner, we next tested whether all motifs within p61 exhibited the same properties. We observed that 87% of a construct containing IQ1 alone bound calmodulin in a Ca^{2+} -independent manner. However, addition of one or both other IQ motifs caused significant amounts ($\sim 50\%$ with IQ2) of the fusion protein to be eluted by EGTA. Furthermore, when IQ2 alone was fused to MBP, the resulting protein bound calmodulin only in the presence of Ca^{2+} and was essentially completely eluted by EGTA. These observations suggest that p61 can bind calmodulin through each of the three IQ motifs. However, whereas association with calmodulin *via* IQ1 is Ca^{2+} -independent, interaction through IQ2 and IQ3 is disrupted upon EGTA addition.

Radial Spokes Contain a Ca^{2+} -regulated Nucleoside Diphosphate Kinase

To assess if radial spokes indeed exhibit NDKase activity in situ and to determine whether that activity is Ca^{2+} stimulated, we first examined the enzymatic properties of flagellar

Figure 5. NDKs are integral axonemal components. *Chlamydomonas* flagella were treated with a nonionic detergent to remove the flagellar membrane and soluble matrix components, yielding microtubule-based axonemes that were subsequently extracted with 0.6 M NaCl. Equivalent samples of each fraction were separated in a 5–15% acrylamide gradient gel and either stained with Coomassie Blue (top) or blotted to nitrocellulose and probed with CT220 antibody (bottom). Both p61 and the M_r 40,000 band are integral axonemal components that are almost completely resistant to extraction by high ionic strength buffers containing 0.6 M NaCl.

axonemes prepared from wild-type *Chlamydomonas* and from the mutant *pf14*. Our assay system tested for the transfer of the terminal phosphate of GTP to ADP and the subsequent utilization of generated ATP by luciferase. Although *Chlamydomonas* flagella contain an adenylate kinase activity, most of this activity is readily solubilized (Watanabe and Flavin, 1976). Direct measurement of ATP production from ADP in the absence of an NTP revealed that the detergent-extracted flagellar axonemes used here contained negligible adenylate kinase activity. We observed that wild-type axonemes in the presence of 1 mM EGTA exhibited a specific NDKase activity of $0.46 \pm 0.03 \mu\text{mol}$ of phosphate transferred/min/mg protein (values given are the mean $\pm$ SD for samples from three separate axoneme preparations). This value is close to the activity of $0.53 \mu\text{mol}$ of phosphate transferred/min/mg protein reported previously by Watanabe and Flavin (1976) for flagella extracted using

Figure 6. p61 is missing in mutant axonemes lacking radial spokes. (a) Axonemes were obtained from wild-type *Chlamydomonas* (WT) and from mutants lacking outer (*oda1*) and inner (*ida1* and *ida4*) dynein arms, radial spokes (*pf14*), and the central pair microtubule complex (*pf18*). After electrophoresis, samples were stained with Coomassie Blue (top) or examined for CT220 immunoreactivity (bottom). The M_r 40,000 protein (p40) was detected in all samples, whereas p61 was absent only in *pf14* axonemes. (b) Axonemes from the *pf14* mutant were prepared in the presence of a comprehensive protease inhibitor cocktail. The sample was electrophoresed and stained with Coomassie Blue (left) or blotted and probed with CT220 (right). These axonemes contain the M_r 40,000 band. However, p61 and its breakdown product migrating just above tubulin (see Figure 4b) were missing. (c) To assess the location of p61 within flagellar radial spokes, axonemes were prepared from wild type, *pf14*, *pf17* (lack the spoke head), and *pf24* (no spoke head and truncated stalk) axonemes. The p61 protein was present in *pf17*, but levels were drastically reduced in *pf24* (upon prolonged exposure, a very weak p61 band was detected in this mutant). This suggests that p61 is located within the distal portion of the radial spoke stalk.

low ionic strength dialysis. Replacement of EGTA with 1 mM Ca^{2+} resulted in a significant increase in axonemal NDKase to 0.81 ± 0.10 μmol of phosphate transferred/min/mg protein. Axonemes prepared from *pf14* had a somewhat lower total specific activity of 0.25 ± 0.08 μmol of phosphate transferred/min/mg protein in the presence of EGTA. This value increased to 0.34 ± 0.06 μmol of phosphate transferred/min/mg protein upon Ca^{2+} addition. Because *pf14* axonemes lack p61, these observations suggest that the p61 NDK accounts for $\sim 45\%$ of axonemal NDKase activity. Furthermore, this activity seems to be stimulated several-fold in the presence of Ca^{2+} , whereas the NDKase activity remaining in *pf14* axonemes was affected to a lesser extent by this cation. We also have observed that in addition to GTP, the axonemal NDKases can use other nucleotides, including CTP, ITP, TTP, and UTP.

To further test whether isolated radial spokes indeed contain a functional NDKase, we determined the enzyme activity present after sucrose gradient fractionation of 0.5 M KI extracts from *oda1* and *pf17* axonemes. We observed that peaks of NDKase activity in the various fractions precisely coincided with radial spoke-associated p61 and also with p40 nearer the top of the gradient (Figure 7, b and c; bottom). The p61 and p40 NDK peaks from the *oda1* KI extract sucrose gradient (fractions 1 and 12) had specific activities of 6.2 and 8.7 μmol of phosphate transferred/min/mg protein, respectively.

DISCUSSION

Studies on both sea urchin sperm and *Chlamydomonas* revealed that demembranated flagella exhibit NDKase activity, suggesting that nucleotides other than ATP may be of functional importance (Yanagisawa *et al.*, 1968; Kobayashi *et al.*, 1976; Watanabe and Flavin, 1976). However, apart from the identification of NDK modules in the IC1 polypeptide from sea urchin sperm outer arm dynein (Ogawa *et al.*, 1996), the intraflagellar location of these enzymes and the role they might play in motility has remained almost completely unexplored. Here, we describe a functional Ca^{2+} /calmodulin-regulated NDK (termed p61 or RSP23)² from

flagella of *Chlamydomonas* that is present in the radial spoke stalk, suggesting that intraflagellar Ca^{2+} signaling involves production of nucleotides other than ATP.

p61(RSP23) Is a Component of the Radial Spoke Stalk

The p61 (RSP23) protein contains a single NDK catalytic module at the N terminus; this region contains all the residues that in other NDKs have been shown to be necessary for catalytic activity (Tepper *et al.*, 1994). Indeed, we observed that radial spokes contain $\sim 45\%$ of the total axonemal NDKase activity. The C-terminal 381-residue section has a high Ala content ($\sim 25\%$ overall) and consists of several distinct regions, including an 85-residue Pro-rich domain adjacent to the catalytic module. This is followed by a triple repeat structure, each section of which contains an IQ motif that could potentially bind calmodulin. The protein terminates in a highly acidic 40-residue region.

We prepared an antibody against the catalytic domain of p61 and found that it detected two bands of M_r 102,000 and 40,000 in flagella. The M_r 102,000 band was identified as p61 after analysis of recombinant protein. The anomalous migration of p61 in SDS gels is likely caused by the Ala-, Pro-, and Glu-rich C-terminal domain because the recombinant catalytic NDK module alone migrated with the predicted M_r . This polypeptide was absent from *pf14* mutant axonemes, which do not assemble the entire radial spoke, and from *pf24* axonemes that lack the distal region of the spoke stalk and the head. However, p61 was not missing in any of the other mutant strains that we analyzed, including *pf17* that only lacks the radial spoke head domain. As is characteristic of the radial spokes, p61 could be extracted from the axoneme only with a strong chaotrope (0.5 M KI) but not with 0.6 M NaCl that is used to solubilize the dynein arms (Yang *et al.*, 2001). Furthermore, after KI extraction from both *oda1* and *pf17* axonemes, p61 copurified in sucrose gradients with the radial spoke cAMP-dependent protein kinase-anchoring protein RSP3. Thus, we conclude that p61 is an integral component of the radial spoke stalk and is likely located in the region distal to the doublet microtubules.

Figure 7. p61 copurifies with radial spoke protein 3. (a) Coomassie Blue-stained lane of isolated radial spokes (from the sucrose gradient shown in b). The p61 protein and several RSP components were identified by immunoblot analysis and/or by comparison with the electrophoretic analysis performed by Yang *et al.* (2001). (b) Axonemes from the mutant *oda1* were treated with 0.6 M NaCl to remove inner arm dyneins, and the radial spokes were subsequently extracted with 0.5 M KI. This extract was then sedimented in a 5–20% sucrose density gradient. Fractions were electrophoresed in a 5–15% acrylamide gradient gel and either stained with Coomassie Blue (top) or blotted to nitrocellulose and probed with CT220 (middle) and the RSP3 antibody (bottom). Most of the p61 and RSP3 proteins were found together in fractions 1–3. In contrast, the M_r 40,000 band detected by antibody CT220 sedimented near the top of the gradient in fractions 11–14. NDKase activity of each fraction is shown in the lower panel, and precisely copurified with p61 near the bottom of the gradient and also with p40 toward the top of the gradient. (c) Radial spokes lacking the spoke head were extracted from *pf17* axonemes and sedimented in a 5–20% sucrose gradient. Both p61 and RSP3 were present nearer to the top of the gradient in fractions 1–7; sedimentation of the M_r 40,000 band was unaffected by the *pf17* mutation. NDKase activity also was shifted up the gradient and again followed the p61 profile.

Interconnection of Ca^{2+} Signaling with NTP Synthesis

Several Ca^{2+} -binding proteins have been identified within the *Chlamydomonas* flagellum. These include a light chain (LC4) of outer arm dynein (King and Patel-King, 1995); an outer arm docking complex protein (DC3) (Casey *et al.*, 2003); centrin, which is associated with a subset of inner dynein arms (Piperno *et al.*, 1992); calmodulin (Gitelman and Witman, 1980; Van Eldik *et al.*, 1980; Witman and Minervini, 1982; for review, see Otter, 1989); and the protofilament ribbon component Rib72 (Patel-King *et al.*, 2002; Ikeda *et al.*, 2003). Identification of Ca^{2+} -binding proteins associated with individual dynein heavy chains, and analysis of the microtubule-binding properties of mutant dyneins lacking individual motor units (Sakato and King, 2003) suggests that Ca^{2+} signals may impinge directly on particular heavy chains. This does not, however, preclude the possibility that

Ca^{2+} also signals through additional mechanisms. Indeed, Yang *et al.* (2001) recently reported that one pool of flagellar calmodulin is present within the radial spokes, although the stoichiometry remains to be ascertained. Furthermore, pharmacological studies support a role for radial spoke calmodulin in controlling dynein-driven microtubule sliding possibly through a calmodulin-dependent protein kinase (Smith, 2002).

The C-terminal domain of p61 contains three IQ motifs that are predicted to bind calmodulin (Rhoads and Friedberg, 1997). Indeed, we found that the full-length recombinant p61 protein bound calmodulin-Sepharose, whereas the NDK catalytic domain alone did not. Interestingly, p61 contains two classes of IQ motif that exhibit distinct interactions with calmodulin. IQ1 acts as a canonical Ca^{2+} -independent binding domain. However, IQ2 and IQ3 (which are identi-

Figure 8. p61 binds calmodulin through both Ca^{2+} -dependent and Ca^{2+} -independent mechanisms. Equimolar amounts of the full-length MBP-p61(7-586), the truncated MBP-p61(7-199), MBP-p61(7-424), MBP-p61(7-494) fusion proteins, and a MBP protein to which only the IQ2 region (p61 residues 424–494) was attached, were incubated with calmodulin-Sepharose beads in the presence of 2 mM Ca^{2+} . Protein that did not bind was recovered after a brief centrifugation (lane marked flow through). After several washes, protein bound in a Ca^{2+} -dependent manner was eluted with buffer containing 2 mM EGTA (EGTA eluate). Any protein that remained associated through a Ca^{2+} -independent interaction was obtained by incubating the beads in gel sample buffer (lane marked beads). Samples were electrophoresed in an 8% acrylamide gel and stained with Coomassie Blue. Maps illustrating the domain structure of the various constructs are shown at right.

cal) bound calmodulin through Ca^{2+} -sensitive interactions. Although, IQ motifs are generally considered to represent Ca^{2+} -independent calmodulin binding sites, there have been several previous reports of Ca^{2+} -dependent interac-

tions via these segments (e.g., myosin V; Trybus *et al.*, 1999 and IRS-1; Munshi *et al.*, 1996). Thus, we suggest that at least some of the calmodulin present within the radial spokes is likely permanently associated with p61 through IQ1. This is consistent with our observation that the radial spoke-associated NDK accounted for much of the axonemal Ca^{2+} -stimulated NDKase activity. It is also possible that soluble calmodulin present in the flagellar matrix can bind to the additional IQ motifs within p61 after a Ca^{2+} increase. Because flagellar axonemes contain multiple calmodulin and Ca^{2+} -binding proteins, analysis of reconstituted p61/calmodulin will be required to demonstrate that Ca^{2+} regulation of NDKase activity occurs directly through the p61 C-terminal domain.

Modulation of p61 activity in response to a Ca^{2+} signal would lead to a local increase in GTP (or other NTP) levels (Figure 9). This suggestion further implies that downstream GTP-binding proteins are present in the axoneme that could transduce this signal (see below). This hypothesis does not of course preclude the possibility that calmodulin also associates with other components of this large macromolecular complex. For example, radial spoke protein 2 (RSP2) contains two 1-8-14 calmodulin-binding motifs (see Rhoads and Friedberg (1997) for motif description) and has recently also been shown to bind calmodulin in a Ca^{2+} -dependent manner (Yang *et al.*, 2004).

What Role Might GTP Play in Flagellar Function?

The biochemical studies of (Watanabe and Flavin, 1976) on *Chlamydomonas* flagella revealed the presence of two distinct soluble NDKs (one of which likely derived from cell body contamination) and also an insoluble NDKase activity; these enzymes had a significant preference for generating GTP. Our data support their observation that most *Chlamydomonas* flagellar NDKase activity is tightly associated with the axoneme. The small soluble fraction described by (Watanabe and Flavin, 1976) may have derived from proteolysis of p61,

Figure 9. Model for Ca^{2+} -dependent GTP signaling through the radial spokes. The diagram illustrates the location of structures associated with a single flagellar doublet microtubule. Within the radial spoke stalk, RSP3 is located at the base and is thought to anchor cAMP-dependent protein kinase (PKA), resulting in phosphorylation of inner dynein arm proteins. The p61 protein and associated calmodulin (CaM) are also present in the stalk. Ca^{2+} binding results in activation of the p61 NDK module to generate GTP (or some other NTP), which subsequently could be used either by a GTP-binding switch protein, a specific GTPase or a guanylate cyclase. Intriguingly, the calmodulin-associated RSP2 protein, which contains a GAF domain that might potentially bind cGMP and act as a downstream target for p61-generated GTP, is also a component of the radial spoke (Yang *et al.*, 2004) and is apparently located close to p61. The dynein regulatory complex (DRC) is located near the base of the radial spokes and inner arms.

as we observed a soluble p61-derived fragment ($M_r \sim 20,000$) containing the NDK module in flagella prepared in the absence of a comprehensive inhibitor cocktail (data not shown). Alternatively, it may represent a flagellar pool of the standard cytosolic NDK.

There are several distinct possibilities for what the GTP (or other nucleotides) generated by these enzymes in response to a Ca^{2+} signal might be used for. First, as originally suggested by Watanabe and Flavin (1976), flagellar GTP could be used to recharge tubulin dimers that have dissociated from the doublet and/or central pair microtubules and thus are in a nonpolymerizable state as they contain GDP. Although this remains a formal possibility, the identification of intraflagellar transport as a mechanism for replenishing axonemal components (Kozminski *et al.*, 1993), our observation that p61 is present exclusively in the radial spokes that are arrayed along the entire axonemal length, and the fact that the radial spoke-defective mutants lacking p61 make full-length flagella all argue against this model.

A second more likely possibility is that GTP generated in response to a Ca^{2+} signal is utilized either directly by a GTP-binding switch protein or other specific GTPase, or by a guanylate cyclase which could convert GTP to cGMP and lead to second messenger signaling (Figure 9). Intriguingly, *Chlamydomonas* RSP2 contains a GAF (cyclic GMP, adenylyl cyclase, and FhlA) domain (Yang *et al.*, 2004), some of which are known to bind cyclic nucleotides and act as regulatory modules (for recent review, see Hurley, 2003). Furthermore, because both RSP2 and p61 are missing in the *pf24* mutant, they are likely located close together within the spoke superstructure. Although cGMP-mediated effects on flagellar motility have not previously been reported in *Chlamydomonas*, studies in *Paramecium* have revealed a role for cGMP in control of both swimming speed and direction of the ciliary power stroke (Bonini and Nelson, 1988) and have identified proteins phosphorylated in a cGMP-dependent manner (Ann and Nelson, 1995). Furthermore, cyclic nucleotide-gated channels control Ca^{2+} entry into mammalian sperm (Wiesner *et al.*, 1998).

A third possibility derives from the observation that both sea urchin and mammalian sperm contain modular proteins consisting of thioredoxin modules and catalytic NDK domains (Ogawa *et al.*, 1996; Sadek *et al.*, 2001, 2003); the sea urchin protein is known to be an integral component of the outer dynein arm. This suggests an interconnection between flagellar redox state and NDKase activity. In *Chlamydomonas*, flagellar thioredoxins have only been found associated with the α and β heavy chains of the outer dynein arm (Patel-King *et al.*, 1996). However, it is possible that redox changes, Ca^{2+} signaling and NDKase activity are interconnected through additional flagellar components in this organism. Finally, initial proteomic studies of human cilia have identified at least one enzyme (pyruvate dehydrogenase) normally associated with intermediary metabolism (Ostrowski *et al.*, 2002). Consequently, because GTP plays key roles in several metabolic pathways, it is possible that some of these reactions also occur in the flagellar matrix.

Other *Chlamydomonas* NDKs

In addition to p61, we identified three other NDKs within the *Chlamydomonas* genome. Two of these are single NDK modules with no associated domains and clearly group with the canonical cytosolic and chloroplast enzymes of higher plants. The third NDK (encoded on genome scaffold #154) has a predicted size of 39.9 kDa and is most closely related to p61 and the mammalian testis-specific NDKs. Analysis of the genome sequence indicates that this protein contains an

N-terminal DM-10 domain similar to the three found in Rib72 (Patel-King *et al.*, 2002; Ikeda *et al.*, 2003), followed by two catalytic NDK modules. Although no ESTs have so far been identified for this sequence in *Chlamydomonas*, our antibody against the catalytic domain of the p61 NDK does detect an axonemal component of appropriate size (M_r 40,000), suggesting that this protein also may function in flagella. Furthermore, the apparent mammalian ortholog (nm23-H7) is expressed in a testis-specific manner (Munier *et al.*, 2003).

In conclusion, we have described here a NDK within the *Chlamydomonas* flagellar radial spokes that binds calmodulin and is regulated in a Ca^{2+} -dependent manner. These data suggest a mechanism for integrating intraflagellar Ca^{2+} signaling through the radial spokes with the production of NTPs. Consequently, we predict that *Chlamydomonas* flagella contain signaling protein(s) that specifically bind nucleotides other than ATP. We are currently seeking to identify such axonemal components by photoaffinity labeling with biotinylated nucleotide analogs.

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