High-Resolution Crystal Structure and In Vivo Function of a Kinesin-2 Homologue in *Giardia intestinalis*

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A critical component of flagellar assembly, the kinesin-2 heterotrimeric complex powers the anterograde movement of proteinaceous rafts along the outer doublet of axonemes in intraflagellar transport (IFT). We present the first high-resolution structures of a kinesin-2 motor domain and an ATP hydrolysis-deficient motor domain mutant from the parasitic protist *Giardia intestinalis*. The high-resolution crystal structures of *G. intestinalis* wild-type kinesin-2 (*GiKIN2a*) motor domain, with its docked neck linker and the hydrolysis-deficient mutant *GiKIN2a*T104N were solved in a complex with ADP and Mg²⁺ at 1.6 and 1.8 Å resolutions, respectively. These high-resolution structures provide unique insight into the nucleotide coordination within the active site. *G. intestinalis* has eight flagella, and we demonstrate that both kinesin-2 homologues and IFT proteins localize to both cytoplasmic and membrane-bound regions of axonemes, with foci at cell body exit points and the distal flagellar tips. We demonstrate that the T104N mutation causes *GiKIN2a* to act as a rigor mutant in vitro. Overexpression of *GiKIN2a*T104N results in significant inhibition of flagellar assembly in the caudal, ventral, and posterolateral flagellar pairs. Thus we confirm the conserved evolutionary structure and functional role of kinesin-2 as the anterograde IFT motor in *G. intestinalis*.

INTRODUCTION

The eukaryotic flagellum is comprised of upward of 250 proteins (Luck, 1984; Dutcher, 1995; Ostrowski *et al.*, 2002; Pazour *et al.*, 2005) that are synthesized in the cytoplasm. For accurate flagellar assembly, these flagellar constituents need to be transported from the cell body and assembled at the distal flagellar tips (Binder *et al.*, 1975). Flagellar assembly and maintenance is achieved through the continuous and bidirectional movement of large proteinaceous particles (rafts) from the base of the flagellum to the distal flagellar tip (originally described in Kozminski *et al.*, 1993 and reviewed recently in Rosenbaum and Witman, 2002 and Scholey,

2003). This mechanism, known as intraflagellar transport (IFT), ensures the delivery of axonemal building blocks to the distal flagellar tips. The first observations of IFT particles moving anterogradely along the flagellum were seen in the unicellular alga *Chlamydomonas reinhardtii* using differential interference contrast (DIC) microscopy (Kozminski *et al.*, 1993). The mechanism and regulation of IFT has since been well characterized in experimental systems such as *Chlamydomonas* and *Caenorhabditis elegans* (reviewed in Rosenbaum and Witman, 2002; Scholey, 2003).

Beyond the well-studied role of IFT in the assembly and maintenance of motile flagella, proper IFT functioning is essential for sensory transduction during mating in *Chlamydomonas* (Pan and Snell, 2002), assembly of primary (9 + 0) cilia in the kidney (Lin *et al.*, 2003), vision (Pazour *et al.*, 2002), and chemosensory behavior in metazoans (Perkins *et al.*, 1986; Starich *et al.*, 1995). Recently identified links between proper flagellar function and human ciliary diseases such as polycystic kidney disease (Pazour *et al.*, 2000; Haycraft *et al.*, 2001; Lin *et al.*, 2003) and Bardet-Biedl syndrome (Li *et al.*, 2004; Snell *et al.*, 2004) highlight the importance of understanding both the components and cellular control of flagellar length dynamics (Sloboda, 2002).

The kinesin-2 heterotrimeric complex, which includes two kinesin-2 homologues as well as the kinesin-associated protein (KAP; Wedaman *et al.*, 1996), powers the anterograde movement of IFT proteinaceous rafts along the outer doublet of axonemes. The retrograde movement of rafts toward the cell body is mediated by cytoplasmic dynein 1b (Signor *et al.*, 1999). The kinesin-2 heterotrimeric complex was initially identified biochemically from sea urchin eggs (Cole *et al.*, 1993; Wedaman *et al.*, 1996), and its role in axoneme assembly has since been confirmed in diverse flagellated

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Abbreviations used: *GiKIN2a*, *Giardia intestinalis* kinesin 2a homologue; *GiKIN2b*, *Giardia intestinalis* kinesin 2b homologue; *GiKIN2a*T104N, ATP hydrolysis-deficient mutant strain of *GiKIN2a* gene; IFT, intraflagellar transport; DIC, differential interference contrast microscopy; KAP, kinesin-associated protein; GASP, novel *Giardia* coiled-coil protein; OSM-3, "osmotic avoidance-3" *C. elegans* kinesin-2 homologue that functions as a homodimer; pET17b, Novagen cloning vector; BL21 (DE3), *E. coli* strain for expression of recombinant proteins; IPTG, isopropyl β-D:1-thiogalactopyranoside; PEG, polyethylene glycol; CCP4, Collaborative Computational Project Number 4; *IFT140*, intraflagellar transport protein, Complex A, subunit 140; *IFT81*, intraflagellar transport protein, Complex B, subunit 81; GFP, green fluorescent protein; qPCR, quantitative PCR; His₆, histone 6-tagged protein; Ni-NTA, nickel-charged affinity resin for purification of His-tagged proteins.

eukaryotes. To illustrate, the disruption of kinesin-2 function has been shown to result in severe inhibition of flagellar or cilia assembly in diverse eukaryotes including *C. reinhardtii* (Walther *et al.*, 1994; Kozminski *et al.*, 1995), *Tetrahymena thermophila* (Brown *et al.*, 1999), and a variety of metazoan lineages and cell types (Shakir *et al.*, 1993; Yamazaki *et al.*, 1995; Morris and Scholey, 1997; Han *et al.*, 2003; Sarpal *et al.*, 2003). Aside from its role in IFT, kinesin-2 has also been suggested to have a role in intracellular transport (Yang *et al.*, 2001), ER–Golgi transport (Stauber *et al.*, 2006), and chromosome segregation (Miller *et al.*, 2005).

Although flagellated protists represent a significant fraction of microbial eukaryotic diversity (Patterson *et al.*, 1999), the role of kinesin motors in basic cellular processes such as flagellar assembly and function has only been studied in depth in a handful of protists, including the green alga *C. reinhardtii*, the trypanosomes (*Leishmania major* and *Trypanosoma brucei*) and the ciliate *T. thermophila* (reviewed recently in Scholey, 2008). One such multiflagellated parasitic protist, *Giardia intestinalis*, is a widespread and understudied parasite of humans and animals (Savioli *et al.*, 2006). The interphase microtubule cytoskeleton of *Giardia* is characterized by eight canonical 9 + 2 axonemes (see Figure 5, a and b) as well as several unique microtubular structures—the median body and the ventral disk (Elmendorf *et al.*, 2003). The “ventral disk,” an overlapping spiral microtubule array, is critical to virulence as it mediates the attachment of *Giardia* trophozoites to the intestinal microvilli (Elmendorf *et al.*, 2003).

Flagellar motility is required for *Giardia* to complete cell division and cytokinesis (Adam, 2001; Elmendorf *et al.*, 2003; Nohynkova *et al.*, 2006; Tumova *et al.*, 2007), and the eight axonemes are distinctive in possessing long, cytoplasmic (non-membrane-bound) regions. All eight axonemes then exit the cell body as conventional membrane-bound axonemes. The eight basal bodies are in close proximity to the two nuclei. Each flagellar pair likely possesses a unique molecular identity based on the unique associations with ancillary structures that remain generally uncharacterized beyond their ultrastructure (Elmendorf *et al.*, 2003). In support, several proteins have been shown to localize to different pairs of cytoplasmic and external portions of axonemes including: GASP-180, a member of a novel family of coiled-coil proteins (Elmendorf *et al.*, 2005), and several α -giardins (Szkodowska *et al.*, 2002; Weiland *et al.*, 2005). It has recently been suggested that during giardial division, daughter flagella undergo a maturation process in which the flagella migrate and transform to different flagellar types (Nohynkova *et al.*, 2006).

Because of the evolutionary divergence of *Giardia*, it is important to study the structure, as well as the in vitro and in vivo functions of giardial kinesins. Within the kinesin gene family, kinesin-2 homologues are perhaps the most conserved kinesin motors within their group (Wickstead and Gull, 2006) and are present in the majority of eukaryotes that have a flagellum during their life cycle (Richardson *et al.*, 2006; Wickstead and Gull, 2006). The *Giardia* genome contains all components of the kinesin-2 heterotrimeric complex including two kinesin-2 homologues (Wickstead and Gull, 2006) and one KAP homolog (Briggs *et al.*, 2004; Morrison *et al.*, 2007); however, the *Giardia* genome does not contain homologues of the homodimeric OSM-3 complex found in both metazoans and ciliates (Wickstead and Gull, 2006). IFT has been inferred from phylogenomic analyses (Briggs *et al.*, 2004; Morrison *et al.*, 2007), but has not been empirically demonstrated in *Giardia*. Yet, in principle, IFT would only be required to maintain the lengths of mem-

brane-bound, or noncytoplasmic, portions of axonemes. Although many IFT raft proteins and motors are conserved across diverse taxa, some eukaryotes have cytoplasmic axoneme assembly that is IFT-independent, including both the malaria parasite *Plasmodium falciparum* (Avidor-Reiss *et al.*, 2004) and *Drosophila* sperm (Han *et al.*, 2003; Sarpal *et al.*, 2003). Given the central importance of flagellar biology to *Giardia*'s pathogenic life style, flagellar proteins, such as kinesin-2, may make excellent targets for anti-giardial drugs.

In this study, we have solved the crystal structures of the catalytic core of a kinesin-2 homolog (*GiKIN2a*) and its hydrolysis-deficient mutant from *G. intestinalis*. These are the first high-resolution structures of a kinesin-2 motor. The structural fold of the kinesin motor domain is remarkably conserved and provides insights into the nucleotide coordination within its active site. We have also confirmed the ancient and conserved role of kinesin-2 as a plus-end-directed IFT motor in *Giardia*. To determine whether giardial kinesin-2 function is conserved in vivo, we overexpressed a green fluorescent protein (GFP)-tagged kinesin-2 rigor construct (*GiKIN2a*T104N), demonstrating that the majority of cells had dramatic shortening of external axoneme length. Thus, as in other organisms, this kinesin-2 rigor mutant also acts as a dominant negative and supports our inference that IFT-mediated assembly and maintenance of axoneme length is an ancient and conserved process. Cytoplasmic axoneme length was unaffected in this mutant, and we propose that cytoplasmic regions of giardial axonemes do not require kinesin-2 for their assembly.

MATERIALS AND METHODS

Strains and Culture Conditions

G. intestinalis trophozoites, strain WBC6, were maintained at 37°C in modified TYI-S-33 medium with bovine bile (Keister, 1983) within 13-ml screwcap tubes (Fisher Scientific, Pittsburgh, PA). For the analysis of tetracycline-inducible mutants, strains were incubated without doxycycline (EMD Chemicals Inc., San Diego, CA) for 24 h to induce the dominant negative kinesin-2 phenotype.

Bacterial Expression and Protein Purification

The sequence corresponding to the motor domain (1–1053 nt) of *GiKIN2a* (XP_001064328) was amplified from *Giardia* genomic DNA by PCR using KOD polymerase (Novagen, Madison, WI). Two oligonucleotide primers were used for PCR (forward 5'-gactaccatattgtcagcgacaacatcaagggtat-3' and reverse 5'-gactacggtagctacctaattgttgatggtgatggtgcgaatctgcgcattcttcgggtct-3'), producing a fragment with encoded 6xHis-tag and unique restriction sites NdeI (5'-end) and KpnI (3'-end). After digestion and DNA purification (Qiagen, Chatsworth, CA), the fragment was cloned into the pET17b vector (Novagen) and subsequently verified by sequencing. The vector-containing insert was transformed into BL21 (DE3) competent cells (Novagen) and grown in 12L of 2xTY, containing 100 μ g/ml ampicillin. The cell culture was initially grown at 37°C and expression was induced at OD₆₀₀ 0.6 with 0.5 mM IPTG; after induction the incubation temperature was changed to 22°C. The expressed protein contained 357 amino acids and had a molecular weight of 39.6 kDa.

Cells were harvested and frozen in liquid nitrogen. Lysis of cells was achieved by thawing cell pellets in lysis buffer, with subsequent sonication; lysis buffer: 50 mM PIPES, pH 7.5, 150 mM NaCl, 3 mM dithiothreitol (DTT), 2 mM MgCl₂, 1 mM ATP, protease inhibitor tablets (1 per 70 ml; Roche Diagnostics, Alameda, CA), 0.1 mg/ml lysozyme (Sigma, St. Louis, CA), 40 μ g/ml DNaseI (Sigma). The supernatant was loaded onto two consecutive 5-ml HisTrapHP columns (GE Healthcare, Waukesha, WI). After an extensive wash with 50 mM PIPES, pH 7.5, 150 mM NaCl, 3 mM DTT, 2 mM MgCl₂, 1 mM ATP, and 20 mM imidazole, the protein was eluted with 300 mM imidazole, 50 mM PIPES, pH 7.5, 150 mM NaCl, 3 mM DTT, 2 mM MgCl₂, and 1 mM ATP. Peak fractions were loaded onto MonoQ-column (GE Healthcare), the column was washed with buffer C (50 mM PIPES, pH 7.5, 3 mM DTT, 2 mM MgCl₂, and 1 mM ATP), and the protein was eluted with buffer C with 150 mM NaCl added. Peak fractions were pooled and concentrated before being loaded onto a 16/60 Sephacryl S-200 column (GE Healthcare) pre-equilibrated in buffer C with 100 mM NaCl and 1 mM Na₂S₂O₃. The protein was eluted as a single peak and was concentrated at 4°C in Vivaspin concentrators (10,000 MWCO, Vivascience, Westford, MA) to 12 mg/ml before storage in liquid nitrogen at –70°C.

Table 1. Crystallographic data for GiKIN2a and GiKIN2aT104N

Crystal	λ (Å)	Resol. (Å)	I/ σ I ^a	Rm ^b	Multi. ^c	Compl. ^d (%)
GiKIN2a ^e	0.9393	1.6	9.8 (2.2)	0.054 (0.356)	2.0	95.9 (95.9)
GiKIN2aT104 ^e	0.9393	1.8	6.3 (1.8)	0.06 (0.295)	2.0	96.4 (96.4)

^a The signal-to-noise ratio for merged intensities, with highest resolution bins in parentheses.

^b $R_m = \sum h \sum i |I(h, i) - \langle I(h) \rangle| / \sum h \sum i I(h, i)$, where $I(h, i)$ are symmetry-related intensities and $\langle I(h) \rangle$ is the mean intensity of the reflection with unique index h .

^c Multiplicity for unique reflections.

^d Completeness for unique reflections; values in parentheses are the highest resolution bin.

^e GiKIN2a: space group P1, cell dimensions: $a = 44.8$ Å, $b = 48.2$ Å, $c = 72.0$ Å, $\alpha = 89.3^\circ$, $\beta = 87.5^\circ$, $\gamma = 84.3^\circ$ and GiKIN2aT104N*: space group P1, cell dimensions: $a = 45.3$ Å, $b = 48.6$ Å, $c = 71.9$ Å, $\alpha = 89.9^\circ$, $\beta = 87.3^\circ$, $\gamma = 84.6^\circ$.

Design of GiKIN2aT104N Mutant for Structural Analysis

QuickChange site-directed mutagenesis (Stratagene, La Jolla, CA) was performed according to the manufacturer's instructions. The plasmid containing the *GiKIN2a* motor domain was used as the template DNA to obtain the *GiKIN2aT104N* mutant. The mutagenesis primers were as follows: GiNF: 5'-agacagggtcggaagaaatggacgatgggaggtaa-3' and GiNR: 5'-ttacctccatcgctcaattcttccggcaccgtgtct-3'. The altered nucleotides are underlined. The plasmid was sequenced to confirm that only the desired mutation had been introduced.

Crystallization and Data Collection

Purified *GiKIN2a* motor domain protein at 10 mg/ml was crystallized by the sitting-drop vapor diffusion technique. The crystallization buffer for the wild-type and mutant *GiKIN2a* protein was 100 mM bicine, pH 9.0, and 30% (wt/vol) PEG 6000. Before crystallization trials, 1 mM ATP and 2 mM MgCl₂ were added to the protein. Crystals were grown at 18°C and were used when they were between 2 d and 1 wk old. Before data collection, the crystals were harvested in mother liquor with 25% (wt/vol) PEG 400 and frozen directly into liquid N₂.

Data were collected to 1.6 Å resolution on beam-line ID14-3 at the European Synchrotron Radiation Facility (ESRF) in Grenoble (France). Crystals of wild-type and mutant *GiKIN2a* belong to space group P1 and have two molecules in the asymmetric unit; the cell dimensions are given in Table 1.

Structure Determination and Refinement

The collected datasets were processed with MOSFLM and scaled using SCALA from the Collaborative Computational Project (CCP)4 suite (Collab-

orative Computational Project, 1994). The structure of *GiKIN2a* was solved by molecular replacement using PHASER (Storoni *et al.*, 2004) and the *Neurospora crassa* kinesin (PDB entry 1GOJ) as the search model. Crystallographic refinement was carried out using CNS (Brunger *et al.*, 1998) with Engh and Huber (1991) parameters. Water molecules were initially added automatically with the program CNS and subsequently were corrected by visual inspection of the difference map. All manual model building for *GiKIN2a* was carried out using MAIN (Turk, 1992) and for *GiKIN2aT104N* using Coot (Emsley and Cowtan, 2004). The stereochemistry of the model was verified using the program PROCHECK (Laskowski *et al.*, 1993). The parameters of the final models are summarized in Table 2.

Gel Filtration Assay

Native pig brain tubulin was purified by successive cycles of temperature-dependent polymerization and depolymerization as described previously (Weisenberg, 1986) followed by phosphocellulose chromatography (Mandelkow *et al.*, 1985). Two hundred fifty microliters of 10 mg/ml pig brain $\alpha\beta$ tubulin (80 mM PIPES, pH 6.9, 1 mM MgSO₄, 1 mM EGTA, and 1 mM DTT) and 300 μ l of 10 mg/ml *GiKIN2aT104N* were incubated on ice together for 30 min, centrifuged briefly, and injected into a Superose 12 HR10/30 gel filtration column (GE Healthcare). The column was eluted at 0.5 ml/min with 80 mM PIPES, pH 6.8, 1 mM MgCl₂, and 1 mM EGTA, and 500- μ l fractions were collected and analyzed by 12.5% SDS-PAGE. Protein bands were stained with Coomassie Blue. The elution profile of the protein complex was compared with the elution profiles of the individual proteins as well as with known molecular-weight standards (data not shown).

Table 2. Refinement statistics for GiKIN2a and GiKIN2aT104N

Model		
GiKIN2a ADP	GiKIN2aT104N ADP	
2 monomers/ASU	2 monomer/ASU	
2 chains, A and B	2 chains, A and B	
Residues in chain A: 4-40, 55-198, 209-225, 230-247, 263-350	Residues in chain A: 4-40, 55-198, 209-225, 230-247, 263-350	
Residues in chain B: 4-42, 55-198, 209-227, 229-247, 263-350	Residues in chain B: 4-42, 55-198, 209-227, 229-247, 263-350	
2 ADP, 2 Mg	2 ADP, 2 Mg	
615 water molecules	681 water molecules	
Parameters		
Diffraction data	1.6 Å	1.8 Å
R-factor, R-free ^a	0.20 (0.32), 0.22 (0.35)	0.22 (0.31), 0.26 (0.37)
B-factor ^b	22.08 Å	19.21 Å
Geometry ^c	0.005 Å, 1.215°	0.007 Å, 1.066°
Ramachandran ^d	93.1%/0.0%	94.5%/0.0%
Restrained NCS ^d	N/A	N/A

^a Five percent of reflections were randomly selected for determination of the free R-factor, prior to any refinement. R-factors for the highest resolution bins are given in parentheses.

^b Temperature factors averaged for all atoms.

^c The RMS deviations from ideal geometry for bond lengths and restraint angles.

^d Percentage of residues in the "most-favored region" of the Ramachandran plot and percentage of outliers. N/A, not applicable.

Cloning and Transformation of GFP-tagged and Rigor Mutant Strains

The kinesin-2 homolog *GiKIN2a* (XP_001064328), with *AsclI*/*AgeI* sites for subcloning and ~80 base pairs upstream of the start codon to include the native promoter, was amplified from *Giardia* genomic DNA by PCR using the oligonucleotide primers K2aF: 5'ggcgcgccaatgctgcctcaggtgctcagaagaactgcccag3' and K2aR: 5'acccgtagttcataggtgtctatttgagagtagaacaattgtagctgtgt3' (restriction sites are underlined). The PCR amplicon was then subcloned into the pMCS.pac vector (Sagolla *et al.*, 2006) to produce a C-terminal kinesin-2 GFP fusion. To create an inducible dominant negative kinesin-2 construct, *GiKIN2a* was PCR amplified from *Giardia* genomic DNA (using TK2GF: 5'ggcgcgccaatgctgcagcgacaacat-caaggttatgctgctgtgc3' and TK2GR: 5'acccgtagttcataggtgtctatttgagagtagaacaattgtag3') and was cloned downstream of the *ram* promoter and associated tetO elements in pTetGFP.C.pac (Dawson *et al.*, 2007). The kinesin-2 rigor construct *GiKIN2a*T104N was generated by site-directed mutagenesis (Stratagene) of the previous construct using the oligonucleotide primers k2DNF: 5'acaggtggcggaaagaattggagcagtgagg3' and k2DNR: 5'acctcccatgctcaattcttccgccacctgt3' and confirmed by DNA sequencing.

For the other giardial kinesin-2 homolog (*GiKIN2b*) as well as the two GFP-tagged IFT Complex A (*IFT140*) and Complex B (*IFT81*) proteins (see Figure 6), we used a similar strategy as above to PCR amplify genes from the *Giardia* genome and cloned the amplicons into the pMCS.pac vector using the *AsclI*/*AgeI* restriction sites. The two IFT complex proteins were clear homologues in the *Giardia* Genome Database. We used the following oligonucleotide primers for the PCR amplifications of the *GiKIN2b*, *IFT81*, and *IFT140* genes (restriction sites are italicized): K2bF: 5'attagggcgcgctgccaattacac-tgctatctgcacatc3'; K2bR: 5'atcgaaacggtagacgaaacacgcatgcccaggtgtgactttggg3'; *IFT81*F: 5'atcgccgcgcccgtgtttgaaaggttcgacatgtgcggcagctgtcag3'; *IFT81*R: 5'aattaccggt aggttggttattctaatgttgcacatcgcctcggc3'; *IFT140*F: 5'tactggcgcgcccctctatctgt-cataaagcctcagttattg3'; *IFT140*R: 5'gctcccccggaggtgatcgtctttcggctgttagctctatc3'.

To create GFP-tagged and inducible kinesin rigor mutant strains, *G. intestinalis* strain WBC6 was electroporated with roughly 50 µg of plasmid DNA (above) using the GenePulserXL (Bio-Rad, Richmond, CA) with previously described conditions (Sagolla *et al.*, 2006). Episomes were maintained in transformants by antibiotic selection using 50 µg/ml puromycin (Davis-Hayman and Nash, 2002). Tetracycline repression of the kinesin rigor mutant was maintained with 10 µg/ml doxycycline (Sigma).

Quantitation of *GiKIN2a* Overexpression Using Quantitative PCR

The de-repression and overexpression of episomal constructs (after removal of doxycycline) was confirmed using quantitative PCR (qPCR) of the GFP-tagged rigor mutant (*GiKIN2a*T104N), the *GiKIN2a* (both native and mutant forms), and actin as an internal relative standard (Supplementary Figure S2). Previously we have observed the maximal induction of transgenes at 1–8 h after removal of doxycycline, and these levels of induction continued after the removal of doxycycline for over 48 h (Dawson *et al.*, 2007). Total RNA was isolated from 6-ml cultures of the uninduced or overexpressed *GiKIN2a*T104N rigor mutant strain using the Cells-to-cDNA kit (Ambion, Austin, TX). Cells were sampled at 0.25, 0.5, 1, 8, 16, 24, and 48 h after induction. For quantitative analysis of expression using qPCR, 1-µl aliquots of the cDNA synthesis reaction were used in actin-specific (actF 5'ctgagggcccggtgaatgtgtgtgg3' and actR 5'gctctctgcccgtctccggagg3'), GFP-specific (GFPF5'gagctgtaccggggtgtgtgcc3' and GFP5'cgggcatgcccggagctgaagaagtgtgtg3'), and *GiKIN2a*-specific (qKIN2aF 5'ggagccagcacatcacacac3' and qKIN2aR 5'ccgacatgtgtgtctcttagctggc3') PCR amplifications in DyNamo HS SYBR Green pPCR Master Mix (Finnzymes, Espoo, Finland). qPCR was performed with the Opticon 2 system (Bio-Rad). To demonstrate that RNA samples were not contaminated with DNA, PCR amplifications were also performed with control cDNA synthesis reactions that lacked reverse transcriptase. *GiKIN2a*T104N overexpression was compared using the relative method of quantification (Livak and Schmittgen, 2001), and GFP expression and kinesin-2 levels were normalized to the actin gene. Overexpression was determined from comparisons of normalized GFP expression in induced time points to uninduced controls (see Supplementary Figure S2).

Immunofluorescence Microscopy and Image Data Analysis

Immunostaining of the GFP-tagged strains was performed as described previously (Sagolla *et al.*, 2006). Briefly, trophozoites were gently fixed in 1% paraformaldehyde and cytoskeletal buffer (PEM) for 15 min, and later permeabilized in 0.1% TritonX-100/PEM before immunostaining. This preserved both native GFP fluorescence and cytoskeletal structure. To measure the membrane-bound regions of axonemes, we coimmunostained microtubules and axonemes with both α -tubulin and α 14-giardin antibodies (see Figure 7). α 14-giardin has been previously shown to localize only to membrane-bound (not cytoplasmic) regions of all eight axonemes (Szkodowska *et al.*, 2002). Images were collected using an Olympus IX70 wide-field inverted fluorescence microscope (Melville, NY) with an Olympus UPlanApo 100 $\times$, NA 1.435, oil immersion objective and Photometrics CCD CH350 camera cooled to -35°C (Roper Scientific, Tucson, AZ). Serial sections were acquired at 0.2-µm intervals (30 total sections on average) and deconvolved using the SoftWoRx deconvolution software (Applied Precision, Issaquah, WA). Two-dimensional

(2D) projections were created from the 3D data sets using SoftWoRx for presentation purposes. Flagellar length measurements (based on immunostaining of axonemes in the *GiKIN2a*T104N strain) were calculated from 3D image stacks using the Imaris software package (BitPlane, Zurich, Switzerland). Over 100 axonemes were measured for each treatment, with roughly 30 axonemes from each flagellar pair type, i.e., caudal, ventral, posteriolateral, and anterior.

Transmission Electron Microscopy and Scanning Electron Microscopy of Trophozoites

To determine the flagellar ultrastructure and prepare samples for transmission electron microscopy trophozoites were first attached to sapphire discs and then were high-pressure frozen as described in with a few adjustments (Sawaguchi *et al.*, 2003). Cells were attached to cleaned sapphire discs at 37°C. Cells were then frozen using the Baltech high-pressure freezer, and freeze substituted using the Leica freeze substitution apparatus (Deerfield, NY). Cells were embedded with Epon resin and serial-sectioned on a Ultratuc E. Sections were stained with uranyl acetate in 70% methanol and lead citrate and viewed on JEOL 1200 transmission electron microscope (TEM; Tokyo, Japan).

For scanning electron microscopy (SEM), trophozoites were first allowed to attach to either to Aclar or track membrane filters and subsequently were fixed for 1 h in 2% glutaraldehyde in cacodylate buffer. Then, cells were postfixed with 2% OsO₄, dehydrated in ethanol, critically pointed-dried, and coated with the MED 020 Bal-tec high-vacuum coating system (Zurich, Switzerland) using iridium. Images were taken using the Hitachi S5000 FESEM (Brisbane, CA) at 10 Kv.

RESULTS

High-Resolution Crystal Structure of the *GiKIN2a* Motor Domain, and the *GiKIN2a*T104N Mutation

Like most flagellated protists (Briggs *et al.*, 2004; Morrison *et al.*, 2007), *Giardia* has two kinesin-2 homologues (*GiKIN2a* and *GiKIN2b*). The *GiKIN2a* motor domain and its mutant *GiKIN2a*T104N were cloned into the pET17b vector (Novagen) and expressed as C-terminal histone 6–tagged protein (His₆)-tagged protein in BL21 (DE3) cells (Novagen). The proteins were purified over a Ni-NTA column, followed by anion exchange chromatography and gel filtration chromatography. Crystals were grown for both proteins from nearly identical crystallization conditions (0.1 M bicine, pH 9.0, 30%, wt/vol, PEG 6000).

The high-resolution crystal structures of *G. intestinalis* wild-type kinesin *GiKIN2a* (at 1.6 Å resolution) and its mutant *GiKIN2a*T104N (at 1.8 Å resolution) in complex with ADP and Mg²⁺ (Table 1) were solved. The crystallographic model for the wild-type *GiKIN2a* was refined with R/R_{free} values of 0.20 and 0.22 and for the model of mutant *GiKIN2a*T104N with R/R_{free} values of 0.22 and 0.26 (Table 2). Both crystals belonged to space group P1 with two molecules in an asymmetric unit (Table 1). The root-mean-square (RMS) deviation of the wild-type *GiKIN2a* structure from the mutant structure was 0.284 Å, indicating that both structures are very similar in overall conformation. The structure of *GiKIN2a* (Figure 1) reveals a kinesin motor domain in the ADP-bound state with eight β -strands and six α -helices characteristic for the kinesin catalytic core (Sack *et al.*, 1999). The active site in the crystal structure of the wild-type *GiKIN2a* motor domain (Figure 2a) shows that the Mg²⁺ ion (a purple sphere) is coordinated in an octahedral geometry by T104, an oxygen from the β -phosphate of ADP, and four water molecules (blue spheres).

The conserved D241 from switch II (DLAGE) is shown hydrogen bonded with one of the water molecules coordinating the Mg²⁺ ion. This rearrangement is in agreement with that of the nucleotide-binding pocket of KIF1A-ADP (Kull and Endow, 2002). As in classic GTPases and other kinesin structures in the ADP state, the *GiKIN2a*'s closed pocket is defined by the characteristic distance (5.86 Å) between P-loop Gly97 and Gly244 of the switch II region. A

Figure 1. Ribbon presentation of the *GiKIN2a* monomer. Red, α -helices; blue, β -sheets; green, neck-linker. ADP is displayed in the active site as ball-and-stick model, and the magnesium ion is shown in purple. The area of the mutated residue on helix $\alpha 2a$ is indicated in yellow. Strands and helices are numbered according to the convention. The figure was made using PyMOL (DeLano, 2002).

typical distance of around 5.7 Å for the closed state has been reported for other kinesins that have been crystallized with bound ADP (e.g., rat kinesin-1, PDB, and 2KIN; Sack *et al.*, 1999).

Contrary to the majority of kinesin structures in the ADP state, a salt bridge between Arg211 of switch I and E246 of switch II, which has been hypothesized to stabilize the closed conformation of the motor domain in analogy to myosin, is not observed in *GiKIN2a* (distance between Arg211 and E246 is 4.3 Å). A similar phenomenon has been described for PDB:KIF1A (Kull and Endow, 2002). Based on results from ATPase activation assays, Yun *et al.* (2001) suggested that this salt bridge between the switch I and switch II region is essential for the ATPase activation of the kinesin motor by microtubules.

As visible in Figure 2b, the mutation T104N did not introduce any major overall conformational changes in the motor domain of *GiKIN2a*T104N and did not cause dissociation or movement in the position of the ADP nucleotide. The most notable difference we observed was in the coordination of Mg^{2+} induced by the mutation, which disrupted an almost perfect octahedral coordination around the magnesium ion because N104 is no longer involved in its stabilization (Figure 2). In the mutant, residue D241 is directly involved in Mg^{2+} coordination, resulting in a shift of its position away from the O atom of the β -phosphate of the ADP nucleotide (2.17 Å in wild type vs. 2.71 Å in the mutant). This shift strengthens the direct interaction be-

a

b

Figure 2. Comparison of the active site of (a) *GiKIN2a* and (b) *GiKIN2a*T104N. The nucleotide ADP is displayed as ball and stick, the Mg^{2+} is shown as a purple sphere, and the coordinating water molecules are shown as blue spheres. Several of the important residues are displayed in ball-and-stick. (a) The Mg^{2+} is coordinated in an octahedral cage by one of the oxygen atoms of the β -phosphate of ADP, the hydroxyl oxygen of T104, and four waters. D241 forms hydrogen bonds with one of the water molecules coordinating Mg^{2+} . The distance between Gly 97 of the P-loop and the Gly244 is 5.86 Å. (b) The mutated residue N104 has moved away from the Mg^{2+} and is no longer involved in its octahedral coordination. The oxygen atoms of D241 are directly stabilizing the Mg^{2+} . The distance between Gly 97 of the P-loop and the Gly244 decreased to 5.75 Å. Dashed lines indicate hydrogen bonds. The figure were made using PyMOL (DeLano, 2002).

tween the magnesium ion and residue D241 and thus inhibits the release of the magnesium ion from the active site.

In the motor domain of the *GiKIN2a*T104N, the salt bridge between Arg211 and E246, with a distance of 3.6 Å, appears to be preserved. Neither Arg211 nor E246 underwent a conformational change in the hydrolysis deficient mutant.

Crystal structures of *GiKIN2a* and *GiKIN2a*T104N (Figure 2, a and b) show that the aromatic ring of Trp105 forms a π -stacking interaction with the adenine ring of the ADP nucleotide. This tryptophan residue is unique to the motor domain of *GiKIN2a* among the kinesin-2 family, as can be

seen in the multiple sequence alignment (Supplementary Figure S1).

The superposition of *GiKIN2a* with monomeric human kinesin, which has two bound sulfate anions as well as ADP (PDB:1MKJ; Sindelar *et al.*, 2002; Figure 3a) shows high overall structure similarity with the RMS deviation of 1.0 Å over 294C^α atoms, despite the evolutionary distance between the proteins. A stretch of twelve amino acids following the catalytic core, referred to as a neck-linker, is suggested to be involved in the kinesin movement and force generation (Chikashige *et al.*, 1997). The docked state of the neck linker is favored for kinesins with ATP bound in the active site, whereas the undocked state is predominant for kinesins with ADP bound (Rice *et al.*, 1999). The neck linker is normally disordered when ADP is bound and is therefore not visible in crystal structures, but the presence of sulfate ions is thought to be responsible for the docked state in 1MKJ (Sindelar *et al.*, 2002). Surprisingly, the ADP-bound *GiKIN2a* also crystallized with the neck-linker in the docked state. Figure 3b displays the close superposition of the neck-linker region of the human (1MKJ) and *Giardia* (*GiKIN2a*) kinesins, branching off at Glu334 of 1MKJ and Asp344 of *GiKIN2a*.

The “road block” activity of the rat kinesin-1 monomer T93N on microtubules has been extensively studied by Crevel *et al.* (2004). The mutated residue T104N in the *Giardia* kinesin-2 homolog corresponds to the T93N mutation of the rat kinesin-1. To examine in vitro the irreversible binding of the kinesin-2 *GiKIN2a*T104N rigor mutant to $\alpha\beta$ -tubulin heterodimers, gel filtration chromatography was used (see *Materials and Methods*). As shown in Figure 4a, $\alpha\beta$ -tubulin eluted from the Superose 12 HR10/30 gel filtration column (GE Healthcare) as a single peak at 13 ml, and the salt appears at 20.5 ml. The profile of $\alpha\beta$ -tubulin was consistent with previous work (Hung *et al.*, 2004), suggesting that $\alpha\beta$ -tubulin predominantly gel filtrated as heterodimer under the conditions used. When a mixture of the *GiKIN2a*T104N kinesin motor and $\alpha\beta$ -tubulin with no added nucleotide was gel filtrated, the kinesin monomer bound tightly to the tubulin heterodimers and eluted as a complex larger than the tubulin heterodimer (Figure 4, a and b). The excess *GiKIN2a*T104N eluted at 14.5 ml and the salt peak trailed at 20.5 ml. The presumed binding stoichiometry is one *GiKIN2a*T104N monomer per microtubule heterodimer (Figure 4b) based on the 1:1 stoichiometry of kinesin-tubulin binding observed with SDS-PAGE (Figure 4b) and reported in previous studies (Crevel *et al.*, 2004).

Ultrastructure of Cytoplasmic and Membrane-bound Regions of the *Giardial* Axonemes

The structure of the eukaryotic motile flagellum—a membrane-bound axoneme consisting of a ring of nine doublet microtubules surrounding a central pair of singlet microtubules—is conserved in evolutionarily diverse eukaryotic microbes (Porter and Sale, 2000; Smith and Yang, 2004; Nicastro *et al.*, 2006), with few exceptions (Schrevel and Besse, 1975; Prensier *et al.*, 1980). Compared with many other flagellated protists, *Giardia* is somewhat unique in that all eight axonemes possess long cytoplasmic regions and exit at specialized structures on the plasma membrane termed flagellar pores (reviewed recently in Elmendorf *et al.*, 2003). We used TEM to confirm the conserved 9 + 2 structure of the giardial axonemes in both cytoplasmic and membrane bound regions, particularly around the basal bodies and flagellar pore region. This ultrastructural analysis indicated that the transition zones of all eight axonemes are restricted to small regions proximal to the

Figure 3. Structural comparison of the human kinesin 1MKJ with the giardial kinesin-2 homolog *GiKIN2a*. (a) Superposition of the C^α atoms of human kinesin structure PDB:1MKJ (blue) and *GiKIN2a* (green; RMS deviation = 1.0 Å over 294 residues). The ADP nucleotide in the active site is shown as red sticks. (b) Superposition of the docked neck linker from PDB:1MKJ (blue) with the docked neck linker of *GiKIN2a* (green). The figure was made using PyMOL (DeLano, 2002).

Figure 4. The *GiKIN2aT104N* mutant kinesin acts as a rigor mutant in vitro. (a) Superose 12 gel filtration profile of purified pig brain $\alpha\beta$ -tubulin and kinesin motor mutant *GiKIN2aT104N*. Key to elution profile line colors: blue, 280-nm absorbance spectrum of unbound $\alpha\beta$ -tubulin; green, 280 nm absorbance spectrum of $\alpha\beta$ -tubulin in complex with kinesin motor mutant *GiKIN2aT105N*; and red, the corresponding 260-nm absorbance spectra of both (b) 12.5% SDS-PAGE showing the elution fractions from 12 to 15 ml in 0.5-ml steps of the Superose 12 gel filtration chromatography of $\alpha\beta$ -tubulin and *GiKIN2aT104N*. The leftmost lane contains the molecular weight marker.

basal bodies (as in other flagellates like *Chlamydomonas*), rather than to the entire cytoplasmic axoneme (Figure 5). Finally, electron-dense structures or “collars” at the points are present where each flagellum exits the cell body (Figure 5, e and f).

GiKIN2a:GFP, *GiKIN2b:GFP*, and IFT Proteins Localize to Cytoplasmic and Membrane-bound Regions of All Eight Axonemes

All components of the heterotrimeric kinesin-2 complex and known components of the IFT complexes A and B required for flagellar growth and assembly are present in the *Giardia* genome (Briggs *et al.*, 2004; Morrison *et al.*, 2007). To determine whether IFT was required for the assembly of all regions of giardial axonemes, we localized both kinesin-2 homologues (*GiKIN2a* and *GiKIN2b*) in trophozoites using C-terminal GFP fusions expressed in cells using their native promoters and fixed under conditions that preserve GFP fluorescence (see *Materials and Methods*). All images are presented as 2D projections of 3D data sets. Although it is possible to distinguish the external and internal axonemes in

Figure 5. Ultrastructure of cytoplasmic and membrane-bound regions of giardial axonemes. The giardial microtubule cytoskeleton is comprised of four main structures (panel a, schematic; panel b, anti-tubulin immunostaining). The eight flagellar axonemes are caudal (cfl), anterior (afl), posteriolateral (pfl), and ventral (vfl), the ventral adhesive disk (vd), the “funis” (fn), and the “median body” (mb; Elmendorf *et al.*, 2003). Basal bodies localize between the two nuclei, with the exception of the two anterior (afl) axoneme basal bodies (a and b). In panel c, transmission electron micrographs demonstrate the conserved ultrastructure of cytoplasmic portions of the axonemes. OD, outer doublet; CP, central pair; BB, basal body; and TZ, transition zone. Scale bar, 200 nm. In panel d, the basal body ultrastructure is presented in further detail. (e) The transition from the cytoplasmic to the membrane-bound regions of the caudal axonemes is shown and illustrates electron-dense material at the plasma membrane (pm). Scanning electron microscopy shows a “collar” region around the exit point of the axoneme (fp, flagellar pore) from the cell body in panel f.

these projections relative to the GFP-labeled IFT proteins, the complex organization and density of microtubules in the peripheral cytoplasm does not permit the discrete visualization of some of the other microtubule structures in the cell such as the funis (see *Materials and Methods*). The *GiKIN2a:GFP* fusion localized along the length of external regions of all eight axonemes (Figure 6, a–h) concentrating at the eight exit points from the cell body or at flagellar pores (Elmendorf *et al.*, 2003) and at all eight distal flagellar tips.

Figure 6. Kinesin-2 and IFT raft complex homologues localize to cytoplasmic regions of axonemes, as well as to external regions including flagellar tips and flagellar pores. In fixed *Giardia* trophozoites, both kinesin-2 GFP fusions (*GiKIN2a* and *GiKIN2b*) localized to cytoplasmic and membrane-bound regions of each of the eight axonemes, concentrating at distal flagellar tips (a–h; see live images in Supplementary Figure S3). In addition, both IFT complex A and B raft homologues (*IFT140* and *IFT81*) localized to similar regions of the axonemes, though both show a pronounced localization to cytoplasmic regions of the posteriolateral axonemes, and the flagellar pores, where the transition from cytoplasmic to membrane-bound regions of the axonemes occurs (i–p). Note localization foci at the distal flagellar tips (arrowheads). The inset (panel l) depicts GFP foci on membrane-bound regions of the right caudal flagella. Red, anti- α -tubulin; green, GFP fusion; blue, DAPI. Scale bar, 2 μ m.

In contrast to other flagellated protists such as *Chlamydomonas* (Vashishtha *et al.*, 1996), we observed no significant localization of kinesin-2 to the region between the two nuclei where the basal bodies are located. Cytoplasmic regions of the posteriolateral and ventral axonemes and each flagellar pore region were particularly enriched in the *GiKIN2a*:GFP fusion. To visualize the localization of proteins of the IFT complex A and B raft complexes, we GFP-tagged giardial homologues of *IFT140* and *IFT81* (*Giardia*DB open reading frames [ORFs] 17251 and 15428, respectively). Both IFT proteins localized to the membrane-bound regions of axonemes, concentrating at the

distal flagellar tips and the flagellar pore regions (Figure 6, i–p, and see inset for localization of foci on membrane bound axonemes). As with the kinesin-2 localization, we observed some cytoplasmic localization of the *IFT81* and *IFT140* GFP fusions, mainly to posteriolateral axonemes.

The *GiKIN2aT104N* Rigor Mutant Acts as a Dominant Negative In Vivo and Disrupts the Assembly of Membrane-bound Regions of All Eight Axonemes

By adapting a common methodology to create kinesin mutants in other organisms (Gelfand *et al.*, 2001; Lin-Jones *et al.*, 2003), we generated an inducible ectopic rigor kinesin-2

Figure 7. The ectopic expression of the rigor *GiKIN2aT104N* mutant results in significantly shortened axonemes. The induced expression of the rigor kinesin-2 transgene (*GiKIN2aT280N*) acts as a dominant negative allele and results in significantly shorter flagella (d–f), than the uninduced control (a–c, and see quantitation of flagellar length in panel g). Axonemes were traced and labeled for ease of interpretation, and these schematics indicate the positions of the eight flagella arranged in four pairs: afl, anterior flagella; pfl, posteriolateral flagella; vfl, ventral flagella; and cfl, caudal flagella. Also, note the increased α -tubulin immunostaining that localizes at the distal tips of axonemes in these representative images of over 200 flagella measured (g). In panel g, various lengths of membrane-bound regions of flagella were quantified before and after induction of the dominant negative *GiKIN2aT104N* mutant for 24 h, using both anti- α -tubulin and anti- α 14-giardin immunostaining (which stains external regions of axonemes). Statistically significant differences based on two-tailed Student *t* tests of induced and uninduced flagellar length measurements (from distal tips to the cell body; **p* < 0.05. Error bars, SD). Numbers above axoneme type (afl, pfl, vfl, and cfl) denote the total number of axonemes measured for each treatment. Red, anti- α -tubulin; green, anti- α 14-giardin; blue, DAPI. Scale bar, 2 μ m.

mutant. After demonstrating that *GiKIN2aT104N* remained bound to tubulin heterodimers in vitro (above), we confirmed that this rigor kinesin-2 mutant acted as a dominant negative in vivo (*Materials and Methods* and Figure 7). After de-repression of this construct by the removal of doxycycline from the medium, we quantified levels of the wild-type *GiKIN2a*, *GiKIN2aT104*, and actin (used as a relative control) using qPCR. Overexpression of the dominant negative construct is minimally 2–5-fold greater than the wild-type *GiKIN2a*, beginning at 15 min and reaching a maximal level of 25-fold overexpression at 24 h (Supplementary Figure S2). Twenty-four hours after the removal of doxycycline repression, we immunostained cells with both anti- α -tubulin to stain all microtubule arrays and anti- α 14-giardin (Figure 7), which localizes and marks only membrane-bound regions of axonemes (Szkodowska *et al.*, 2002). Using these axonemal markers (Figure 7g), we quantified the lengths of external regions of axonemes (from cell body to distal tips) in each flagellar pair (more than 100 flagella measured for each treatment). We found that the majority of cells had flagellar length defects. In particular, there was a clear and statistically significant decrease in the length of membrane-bound regions of axonemes (~30% shorter in caudal flagella and ~15% shorter in both ventral and posteriolateral flagella), relative to uninduced controls (see Figure 7g). We were not

able to detect a significant shortening of the anterior axonemes or shortening of cytoplasmic axoneme region after induction of the dominant negative *GiKIN2aT104N*, however. The anti- α 14-giardin antibody clearly marks all non-cytoplasmic regions of axonemes (Figure 7, a–f). In extreme cases (roughly 5% of cells), the cytoplasmic regions of the axonemes were curled or bent and apparently were unable to exit the cell body (data not shown). We qualitatively monitored flagellar beat in live induced cells and noticed no substantial defects (data not shown); however, a more comprehensive analysis of flagellar beating in the *GiKIN2aT104N* strain (particularly in the ventral or posteriolateral flagellar pairs) would be instructive to compare with prior studies of flagellar beat patterns (Campanati *et al.*, 2002).

DISCUSSION

Understanding the mechanism of control of flagellar and ciliary length has become an increasingly important area of cell biological research, due to links between improper functioning of these organelles and human medical disorders such as polycystic kidney disease and Bardet-Biedl syndrome (Pazour *et al.*, 2000; Snell *et al.*, 2004). The study of *Giardia* flagellar biology has obvious clinical relevance in

terms of parasitology, as well as nonclinical relevance in terms of basic flagellar cell biology and cytoskeletal evolution (Elmendorf *et al.*, 2003). Each pair of giardial axonemes is differentiated, and each pair contributes to various modes of flagellar motility (Campanati *et al.*, 2002). Motility is required for *Giardia* to find suitable sites to attach and detach from surfaces including the intestinal villi (Campanati *et al.*, 2002). Thus, flagellar motility is critical for the completion of *Giardia*'s life cycle in the mammalian host both during interphase and during late stages of cytokinesis.

The Structures of *G. intestinalis* Kinesin GiKIN2a and GiKIN2aT104N

Although the giardial kinesin-2 motor, *GiKIN2a*, has a low sequence identity to other kinesin-2 homologues, there are no striking differences observed in kinesin-2 structure as compared with that of eukaryotes such as metazoans. Similar to the structure of both the rat kinesin K349 and the human kinesin K349 (Sindelar *et al.*, 2002), the new high-resolution crystal structures of *GiKIN2a* and *GiKIN2aT104N* contain ADP in the active site accompanied by a docked neck linker (residues 331–344).

This high-resolution crystal structure of the *GiKIN2aT104N* rigor mutant afforded the opportunity to visualize the mutated residue within the active site and assess the impact of this mutation on nucleotide binding. The overall confirmation of the rigor mutant *GiKIN2aT104N* was the same as for the wild-type motor, yet the coordination of the magnesium ion was disturbed. It is known from previous work (Ma and Taylor, 1997) that in the absence of microtubules the release of ADP from the kinesin active site requires the stripping of the magnesium ion. In the wild-type kinesin the aspartate residue D241 in the switch 2 region (DLAGE) indirectly coordinates the magnesium ion through a water molecule (Figure 2a), whereas the conserved threonine residue T104 (GKTWT) directly coordinates the magnesium ion. In the structure of the *GiKIN2aT104N* rigor mutant (Figure 2b) the asparagine residue N104 is too distant to be involved in the direct coordination of the magnesium ion, which has moved closer to aspartate D241. In case of aspartate D241, the binding affinity of the highly negatively charged oxygen ($pK_a = 3.9$) of its acid group for the magnesium ion is much stronger than the affinity provided by threonine's side chain oxygen ($pK_a = 16$). Therefore, the enhanced stabilization of the magnesium ion through D241 as seen in Figures 2, 3, and 5b most likely prevents the ADP release.

It is possible that even in the presence of microtubules, a similar magnesium coordination could be observed in *GiKIN2aT104N*. It is known from previous work (Ma and Taylor, 1997) that in the absence of microtubules the release of ADP from the kinesin active site requires the stripping of the magnesium ion from the active site with EDTA or apyrase, whereby the magnesium exchange limits ADP exchange. Before obtaining a high-resolution crystal structure of the *GiKIN2aT104N* rigor mutant, it was thought that the mutation would result in a weakly binding magnesium ion that would exchange very quickly and thereby promote the release of the nucleotide. Thus, we expected to obtain a crystal structure of *GiKIN2aT104N* with an empty nucleotide-binding pocket. However, the comparison of wild-type and rigor mutant kinesin structures demonstrated that the magnesium ion in *GiKIN2aT104N* appeared to be held in place through the direct interaction with D241 as seen in Figure 2b. Most likely the altered coordination slows down the magnesium ion and ADP release, thereby keeping the motor bound to the microtubules. However, proof of this hypothesis and a more intricate understanding of the com-

plex interplay of the motor domain, neck-linker and microtubule would require a crystal structure of $\alpha\beta$ -tubulin in complex with the rigor mutant.

Cytoplasmic Regions of Axonemes Are Not Elongated Transition Zones

The observation that the eight giardial axonemes possess long cytoplasmic regions before exiting at flagellar pores (Elmendorf *et al.*, 2003) might indicate that cytoplasmic axonemes are essentially elongated "transition zones." Previous work has demonstrated that cytoplasmic regions of axonemes, particularly the nonmotile caudal pair, have a conserved flagellar ultrastructure and retain radial spokes, dynein arms, and the central microtubule pair (Clark and Holberton, 1988; Elmendorf *et al.*, 2003; Carvalho and Monteiro-Leal, 2004). There has been no prior study that has empirically shown that IFT is required for the assembly of both cytoplasmic and membrane bound portions of the giardial axonemes. It has been suggested, however, that both IFT-mediated and non-IFT-mediated assembly of axonemes can occur simultaneously in the same cell (Han *et al.*, 2003; Briggs *et al.*, 2004).

Using TEM, we confirmed that the 9 + 2 structure of the giardial axonemes is present in both cytoplasmic and membrane-bound regions (Figure 5). Further, the transition zones of all eight axonemes are restricted to small regions proximal to the basal bodies (as in other flagellates like *Chlamydomonas*), rather than to the entire cytoplasmic region (Figure 5, e and f). Electron-dense structures are present at the regions where each flagellum exits; the cell body and SEM imaging also showed "collars" in the vicinity of the flagellar pores (see Figure 5).

Giardial IFT Components Localize to Cytoplasmic and Membrane-bound Regions of Axonemes

Homologous components of both the retrograde and anterograde IFT complexes (A and B), and the kinesin-2 motors are present in the genome, yet IFT has not been empirically demonstrated in *Giardia*. Both giardial kinesin-2:GFP fusions (*GiKIN2a* and *GiKIN2b*) localized along the length of axonemes, concentrating in foci at the flagellar tips and the flagellar pore regions of all eight axonemes (Figure 6 and Supplementary Figure S3), the same area where electron-dense structures were observed using electron microscopy (Figure 5, e and f). The cytoplasmic regions of two pairs of axonemes—the posteriolateral and ventral—were also seen to accumulate significant *GiKIN2a*:GFP and *GiKIN2b*:GFP signals, possibly indicating that IFT particles dock on cytoplasmic portions of axonemes and accumulate at flagellar pore regions.

In *Chlamydomonas*, the kinesin-2 homolog FLA10 and plus-end-tracking protein EB1, accumulate both at the flagellar tips and at the flagellar basal bodies (Vashishtha *et al.*, 1996; Pedersen *et al.*, 2003). The basal body/transition zone region has thus been suggested as a docking site for the organization of IFT particles, based on such immunolocalization data of kinesin-2 homologues and IFT proteins to basal bodies (Deane *et al.*, 2001) and the disruption of basal body localization in either kinesin-2 mutants (Vashishtha *et al.*, 1996; Cole *et al.*, 1998) or KAP mutants (Mueller *et al.*, 2005). There was no significant localization of kinesin-2:GFP to the eight basal bodies (localized between the two nuclei), which is similar to *Tetrahymena* IFT localization (Brown *et al.*, 1999, 2003) and is contrary to *Chlamydomonas*, trypanosomes (Absalon *et al.*, 2008), and mammals (Folliet *et al.*, 2006), where the basal body transitional fibers have been shown to

be the docking site for IFT particles (Vashishtha *et al.*, 1996; Brazelton *et al.*, 2001; Deane *et al.*, 2001).

The notion that IFT particles assemble and dock on the cytoplasmic regions of axonemes needs to be confirmed using live analysis of IFT particle movement on both cytoplasmic and membrane-bound regions of axonemes, however. For this initial investigation, we cautiously interpret that the exit point and distal tip regions of giardial axonemes represent the beginning and end points of the IFT pathways; future investigation of IFT particle assembly and movement are required to resolve whether IFT functions in the assembly and maintenance of cytoplasmic axonemes in *Giardia*.

Is IFT Required for the Assembly and Maintenance of Both Cytoplasmic and Membrane-bound Regions of Giardial Axonemes?

Kinesin-2 disruption mutants commonly do not extend axonemes beyond the transition zone of the basal bodies in diverse flagellated organisms (Perkins *et al.*, 1986; Starich *et al.*, 1995; Nonaka *et al.*, 1998; Brown *et al.*, 1999; Marszalek *et al.*, 1999; Takeda *et al.*, 1999; Pan and Snell, 2002; Pazour *et al.*, 2002; Lin *et al.*, 2003). To test that the giardial kinesin-2 rigor mutant had a dominant negative phenotype (as has been shown in other flagellated organisms; Gelfand *et al.*, 2001; Lin-Jones *et al.*, 2003; Betley *et al.*, 2004; Fan and Beck, 2004; Brown *et al.*, 2005), we overexpressed the rigor kinesin-2 mutant (*GiKIN2aT104N*) and monitored flagellar length in each flagellar pair (exit points to distal tips; *Materials and Methods* and Figure 7). The overexpressed kinesin-2 rigor mutant resulted in significantly shorter flagellar lengths in the membrane-bound regions of axonemes (between ~15 and 30% in the caudal, ventral, and posteriolateral flagellar pairs; see Figure 7). Therefore, we propose that *GiKIN2a* is required for IFT-mediated assembly of external or "membrane-bound" regions of axonemes and that the *GiKIN2aT104N* mutant acts as a dominant negative *in vivo*. Although giardial IFT particles might assemble on cytoplasmic regions of axonemes (Figure 6), IFT-mediated axoneme assembly would only be required for the external or membrane-bound regions of axonemes. The lack of a complete inhibition of IFT-mediated axoneme assembly, however, could be due to the fact that only one member of the kinesin-2 heterotrimeric complex was disrupted as has been seen in *Tetrahymena*, where the kinesin-2 complex may be able to function as a homodimer (Brown *et al.*, 1999).

An IFT-independent mode of flagellar morphogenesis, however, may be responsible for cytoplasmic axonemal assembly and possibly for the membrane-bound regions of the anterior axonemes. In contrast to the caudal, posteriolateral, and ventral flagellar pairs in *Giardia*, we found that anterior flagellar length was unaffected in the dominant negative kinesin-2 mutant. This observation is consistent with prior findings that anterior flagellar length is less affected by microtubule drugs than the other flagellar pairs (Dawson *et al.*, 2007). An alternative explanation is that anterior axonemes assemble at a different, likely slower rate than the other axonemal pairs. IFT-independent assembly of axonemes has some precedent in other organisms. For example, IFT is required in *Drosophila* for the assembly and maintenance of sensory neurons, but IFT is not required for the assembly and function of *Drosophila* sperm flagella (Han *et al.*, 2003). Similarly the malarial parasite *P. falciparum* lacks kinesin-2 homologues (Briggs *et al.*, 2004) and *Plasmodium* basal bodies/centrioles form and nucleate axoneme assembly in only the microgamete cytoplasm, rather than in the somatic stages (reviewed recently in Morrisette and Sibley, 2002).

This hypothesis needs to be further confirmed using additional experimental methods for disrupting IFT or kinesin-2 function.

***Giardia* Flagellar Function and the Evolution of Flagellar Assembly Mechanisms**

Despite the extensive cytoplasmic regions of the axonemes, there is no empirical evidence suggesting that the giardial axonemes are aberrant in terms of either molecular architecture or function, compared with more commonly studied axonemes in experimental systems such as *Chlamydomonas*. Further, *Giardia* possesses a full complement of microtubule-associated proteins, such as 24 kinesins from the majority of kinesin families including the kinesin-2 homologues studied here (Wickstead and Gull, 2006). Although prior contentions that *Giardia* is a "derived" or "degenerate" parasite (Knight, 2004), the flagellar assembly mechanisms involving IFT (Briggs *et al.*, 2004) are likely to be as highly conserved as the structure of the giardial axoneme. "Derived" is a relative term, and the ultrastructural state of flagella in *Giardia* would need to be compared with a closely related nonparasitic species for this designation to have any relevance. In support of this notion, we have shown here that the giardial kinesin-2 homologue *GiKIN2a* has no striking differences in its structure compared with other kinesins, and *GiKIN2a* retains a conserved function as the anterograde motor for intraflagellar transport and axoneme assembly. It is clear that mechanisms for eukaryotic flagellar assembly are highly conserved throughout eukaryotic evolution.

Functional and structural studies of cytoskeletal and flagellar proteins in diverse protists (Brugerolle, 1991) provide a unique evolutionary and comparative perspective to cytoskeletal mechanisms in other well-studied experimental systems. For these reasons, the study of giardial flagellar length dynamics could serve as an additional experimental system with which to elucidate fundamental flagellar assembly and maintenance mechanisms in other eukaryotes. *Giardia* has been proposed to be a member of one of the earliest diverging branches of eukaryotes in single or multigene eukaryotic phylogenies when an archaeal outgroup is included (Sogin *et al.*, 1989; Best *et al.*, 2004; Ciccarelli *et al.*, 2006; Morrison *et al.*, 2007). In many cases, the *Giardia* genome contains fewer homologues of cytoskeletal proteins (unlike metazoans or plants), providing perhaps a more ancestral or simpler model for experimental studies of flagellar biology (Morrison *et al.*, 2007). Importantly, the conservation of flagellar assembly mechanisms in *Giardia* illustrates the ancient origins of these mechanisms in the evolutionary history of the Eucarya.

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