Molecular Biology of the Cell
Vol. 15, 3257–3265, July 2004

Protein Targeting of an Unusual, Evolutionarily Conserved Adenylate Kinase to a Eukaryotic Flagellum

Timothy J. Pullen,* Michael L. Ginger,* Simon J. Gaskell,† and Keith Gull*‡

*Sir William Dunn School of Pathology, University of Oxford, Oxford OX1 3RE, United Kingdom; and †Department of Chemistry, University of Manchester Institute of Science and Technology, Manchester M60 1QD, United Kingdom

Submitted March 15, 2004; Accepted April 26, 2004
Monitoring Editor: Paul Matsudaira

The eukaryotic flagellum is a large structure into which specific constituent proteins must be targeted, transported and assembled after their synthesis in the cytoplasm. Using *Trypanosoma brucei* and a proteomic approach, we have identified and characterized a novel set of adenylate kinase proteins that are localized to the flagellum. These proteins represent unique isoforms that are targeted to the flagellum by an N-terminal extension to the protein and are incorporated into an extraaxonemal structure (the paraflagellar rod). We show that the N-terminal extension is both necessary for isoform location in the flagellum and sufficient for targeting of a green fluorescent protein reporter protein to the flagellum. Moreover, these N-terminal extension sequences are conserved in evolution and we find that they allow the identification of novel adenylate kinases in the genomes of humans and worms. Given the existence of specific isoforms of certain central metabolic enzymes, and targeting sequences for these isoforms, we suggest that these isoforms form part of a complex, "solid-phase" metabolic capability that is built into the eukaryotic flagellum.

INTRODUCTION

Motility is often central to cell feeding, survival, or reproduction in most eukaryotic organisms. Fast cell motility or locomotion of material over the cell surface is apparently best produced by the use of cilia or flagella. Although there are intriguing variations in some cell types and genera, most motile eukaryotic cilia and flagella have the canonical 9 + 2 microtubule structure termed the axoneme. This regular and evolutionarily conserved pattern of the nine doublet microtubules and two central pair singlet microtubules in the axoneme is intimately involved in the production of motility via the dynein arm bridges that form between adjacent outer doublets (Porter and Sale, 2000; King, 2003). The resulting microtubule doublet sliding is constrained by other connections so converting the applied force into a bending motion.

In a variety of microbial eukaryotes and in flagella of certain animal cell types (often sperm), a diversity of extraaxonemal structures occurs along with the microtubule axoneme. For example, in mammalian sperm tails, nine outer dense fibers run along the length of the flagellum, and in the principal piece a second extraaxonemal structure, the fibrous sheath, surrounds the outer dense fibers (Eddy *et al*., 2003). One of the most intriguing of these extraaxonemal structures is the elaborate paraflagellar rod (PFR) found in the kinetoplastid protozoa, the Euglenoids, and the dinoflagellates (Bastin *et al*., 1996b). In the African trypanosome *Trypanosoma brucei*, the PFR is present alongside the axoneme from its point of exit from the flagellar pocket. The *T. brucei* PFR has three zones, each of characteristic ultrastructure, and the whole structure is linked to axonemal microtubule doublets 4 through 7 (Gull, 1999).

There are a number of traditional views on the function of these diverse extraaxonemal structures, often involving suggestions that they may alter the mechanical properties of the flagellum in some advantageous manner. More modern molecular interrogations of such structures have revealed that they are important for motility. For example, in the mouse a knockout of the major component of the fibrous sheath prevented normal flagellum development and the resulting sperm were incapable of progressive motility (Miki *et al*., 2002). Likewise, in *T. brucei* RNA interference (RNAi) knockdown of the major paraflagellar rod component *PFRA* resulted in trypanosomes that lacked the major portion of the PFR structure, and although viable, were paralyzed (Bastin *et al*., 1998, 2000a).

Whereas we have some molecular knowledge of the major structural components in the PFR and fibrous sheath, there is a paucity of data on less abundant proteins, which may nonetheless contribute to important regulatory, signaling, or enzymatic functions in flagella. Here, we have used the PFR minus, paralyzed mutants of *T. brucei* to address issues of what biochemistry an extraaxonemal structure contributes to the eukaryotic flagellum. We have isolated flagella from the *snl*-2 mutant of *T. brucei*. This mutant is an inducible RNAi mutant. In the noninduced state, it presents a normal PFR and is actively motile (Bastin *et al*., 2000a). When switched to conditions where RNAi is induced, the trypanosomes lack a full PFR and are paralyzed, yet grow well. Isolated flagella were subjected to two-dimensional (2D) gel analysis and mass spectrometry to identify protein compo-

Article published online ahead of print. Mol. Biol. Cell 10.1091/mbc.E04–03–0217. Article and publication date are available at www.molbiolcell.org/cgi/doi/10.1091/mbc.E04–03–0217.

‡ Corresponding author. E-mail address: keith.gull@pathology.ox.ac.uk.

Abbreviations used: Ap5A, diadenosine pentaphosphate; PFR, paraflagellar rod; RNAi, RNA interference.

nents present in the flagellum that exhibits a PFR, but which are then absent from flagella with no PFR. This proteomic comparison has allowed the identification of a novel set of adenylate kinase proteins that localize to the extraaxonemal structure and are targeted to the flagellum by an N-terminal extension to the protein. We show that the N-terminal extension is both necessary and sufficient for targeting of a GFP reporter protein to the flagellum. Moreover, these N-terminal extension sequences are conserved in evolution, and we find that they allow the identification of novel adenylate kinases in the genomes of humans and worms. These observations reveal a "solid-phase" enzymatic biochemistry for adenine nucleotide homeostasis that is built into the structure of a eukaryotic flagellum.

MATERIALS AND METHODS

Trypanosome Strains, Growth, and Transformation

Procyclic *T. brucei* was maintained at 27°C in SDM-79 medium supplemented with 10% (vol/vol) heat-inactivated fetal calf serum as described previously (Brun and Schonenberger, 1979). Proteomics and analysis of Ty-epitope–tagged *Tb*ADKA and *Tb*ADKA were investigated using the *snl*-2 mutant (Bastin *et al*., 2000a). RNAi experiments were carried out using the 29-13 cell line (Wirtz *et al*., 1999). Transformations were carried out essentially as described previously, by using ZPFM buffer and electroporation of 3 × 100-ms pulses at 1.5 kV with an Electro Square Porator (BTX) (Wickstead *et al*., 2003).

Trypanosome Fractionation

Cells were harvested by centrifugation (850 × *g*, 10 min, 20°C) and washed twice with phosphate-buffered saline. Cytoskeletons were isolated by extraction of 10^8 cells ml^{-1} in 1% (vol/vol) Nonidet-P40 in 0.1 M PIPES, 2 mM EGTA, 1 mM $MgSO_4$, 0.1 mM EDTA, pH 6.9, and containing 5 $\mu g\ ml^{-1}$ pepstatin A, 10 $\mu g\ ml^{-1}$ leupeptin, and 10 $\mu g\ ml^{-1}$ phenylmethylsulfonyl fluoride. After centrifugation (3400 × *g*, 10 min, 4°C), the detergent-insoluble cytoskeletal pellet was extracted twice in 0.1 M PIPES, 1 M NaCl, 2 mM EGTA, 1 mM $MgSO_4$, 0.1 mM EDTA, pH 6.9, plus protease inhibitors (ice, 10 min). Depolymerization of the subpellicular microtubules was visualized by light microscopy, and flagella were subsequently isolated by centrifugation (16 000 × *g*, 15 min, 4°C). The flagella pellet was washed once (0.1 M PIPES, 2 mM EGTA, 1 mM $MgSO_4$, 0.1 mM EDTA, pH 6.9) and resuspended in 20 mM Tris-HCl, 5 mM $MgCl_2$, 1% (vol/vol) Nonidet-P40 and incubated on ice (15 min) with 2.5 μg of RNase, and 8.75 μg of DNaseI. Sixty milligrams of electrophoresis grade urea and 100 μl of a solution containing 2% (vol/vol) Nonidet-P40, 2% (vol/vol) ampholines, pH 3.5–10, 5% (vol/vol) β-mercaptoethanol, and 9.5 M urea were then added, and the samples stored at −80°C before analysis.

Electrophoresis and Mass Spectrometry of Flagellar Proteins

Samples were separated in the first dimension by isoelectric focusing and in the second dimension by SDS-PAGE, and proteins were visualized by silver staining as described previously (Burland *et al*., 1983). In-gel trypsin digestion of proteins (Shevchenko *et al*., 1996) was followed by peptide mass fingerprinting, by using matrix-assisted laser desorption/ionization-time of flight mass spectrometry (Voyager DE-STR; Applied Biosystems, Warrington, United Kingdom), and peptide sequencing by using quadrupole-time-of-flight tandem mass spectrometry (Q-ToF; Waters/Micromass, Manchester, United Kingdom). Peptide sequences obtained from tandem mass spectrometry were used to search the *T. brucei* genome database (http://www.genedb.org/genedb/tryp/index.jsp) and the identity of candidate proteins confirmed by matching the peptide masses obtained by matrix-assisted laser desorption/ionization-time of flight mass spectrometry with those obtained from in silico digestion of the candidate (PeptideMass, http://www.expasy.org).

Plasmid Construction

Construction of plasmids used for 1) expression of Ty-epitope–tagged *Tb*ADKA and *Tb*ADKB from their endogenous RNA polymerase II-transcribed loci, 2) inducible overexpression of *Tb*ADKA::Ty and $Tb\text{ADKA}^{\Delta(1-43)}$::Ty from a rDNA locus by using the RNA polymerase I-transcribed procyclin promoter, and 3) RNAi of flagellar adenylate kinases are all described previously (Pullen, 2002). Briefly, for expression of tagged proteins from their endogenous loci the tagged open reading frames were located between the endogenous 5′ untranslated region (UTR) and an aldolase 3′ UTR. In the overexpression experiments, tagged open reading frames were cloned into pHD430, in which trans-splicing and polyadenylation processing signals are provided by a procyclin 5′ UTR and aldolase 3′ UTR, respectively (Wirtz and Clayton, 1995). The parent vector for the RNAi experiments was pZJM (Wang *et al*., 2000). The expression of $Tb\text{ADKA}^{\Delta(55-260)}$-GFP was achieved after polymerase chain reaction amplification of sequence that encodes the first 55 amino acids of *Tb*ADKA by using the primer combination ADKAN5′ (5′-ATATTTGTGGTACTGCGACC-3′) and ADKAN3′ (5′-TGACATC*CATGG*CAATGAATATTTGCAAAGG-3′) and Herculase DNA polymerase (Stratagene, La Jolla, CA). The resulting polymerase chain reaction product was digested with *Nco*I (site italicised in ADKAN3′, and an additional site spans the start codon of *ADKA*) and cloned into pGAD8-VSG-G4 (Wickstead *et al*., 2003) that had been digested with *Nco*I across the start codon of the *GFP* gene. The resulting plasmid was digested with *Bam*HI for stable integration into a minichromosomal locus.

Adenylate Kinase Assay

Detergent-insoluble cytoskeletons and flagella (5×10^7 cell equivalents) were assayed in a final volume of 1 ml in 0.03 M Tris-HCl, pH 7.75, 1.2 mM $MgSO_4$, 5 mM glucose, 1 mM $NADP^+$, 10 U of hexokinase, and 6 U of glucose-6-phosphate dehydrogenase. The rate of NADPH formation was followed at 340 nm.

Immunofluorescence and Immunoblotting

Immunofluorescence using BB2, which recognizes the Ty-epitope, and ROD-1 monoclonal antibodies, and immunoblotting by using BB2 were carried out using previously described methods (Bastin *et al*., 1996a,b).

RESULTS

Proteomic Analysis of the PFR

The *snl*-2 mutant of *T. brucei* is an inducible RNAi mutant that possesses a PFR and is motile when the RNAi is not induced, but becomes PFR minus and paralyzed when RNAi is induced (Figure 1, A and B) (Bastin *et al*., 2000a). Loss of the PFR structure does not affect viability, and populations of cells can therefore be grown in both states: with or without the PFR. The induction of RNAi ablates the expression of only one protein (PFRA), but this is sufficient to compromise the construction of the major portion of the PFR. We conjectured that many flagellar proteins will be synthesized but not assembled in the flagellum when the PFR is missing. This provided an opportunity to isolate the axoneme and PFR complex from the *snl*-2 mutant in both the RNAi induced and noninduced states, and use 2D gels to compare their protein profiles. We performed this analysis and detected a number of spots clearly missing from the flagellum of the paralyzed, induced RNAi population in comparison with the noninduced, motile population of the *snl*-2 strain (Figure 1, C and D). Four specific spots were subjected to mass spectrometry and sequences derived from the genome database. Two were trypanosome specific and are likely to represent PFR structural proteins. However, intriguingly, two sequences were putative adenylate kinases, which we termed *Tb*ADKA and *Tb*ADKB (Figure 2).

Given the role of adenylate kinases in adenine nucleotide homeostasis and the requirement for such adenine nucleotide balance for dynein motor protein activity in the flagellum (Yagi, 2000), the finding of two adenylate kinases in the assembled flagellum was intriguing. We therefore asked whether we could detect the corresponding biochemical activity in the flagellum. We used the classic approach of detergent extraction of whole cells to produce cytoskeletons (the subpellicular microtubule cortex and the flagellum), followed by salt extraction to depolymerize the subpellicular microtubule cortex, yielding purified flagella (Schneider *et al*., 1987; Robinson *et al*., 1991) and assayed this flagella preparation for adenylate kinase activity. Figure 3A shows that adenylate kinase activity could be detected in flagella preparations from noninduced *snl*-2, but not in paralyzed flagella isolated from RNAi-induced *snl*-2. This activity could be inhibited by diadenosine pentaphosphate (Ap5A) indicative of bona fide adenylate kinase activity (Figure 3A).

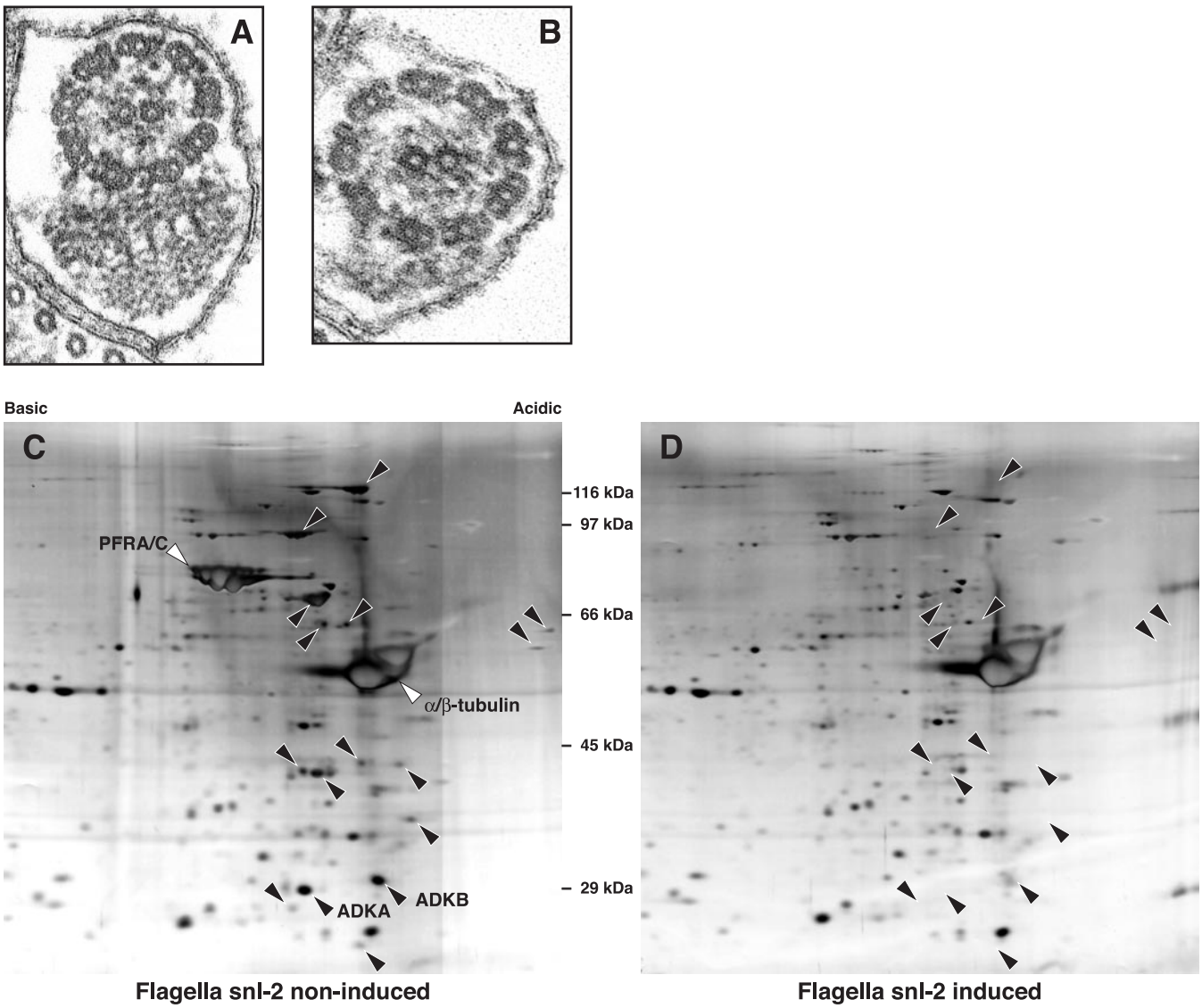

Figure 1. Composition of the PFR can be determined by comparative proteomics of an inducible RNAi mutant. Transmission electron micrograph of thin sections through the flagellum show the wild-type organization of the axoneme and PFR in the noninduced *snl*-2 mutant (A) and the absence of the PFR in paralyzed, induced *snl*-2 (B). Proteomic analysis of flagella purified from induced and noninduced *snl*-2 indicates that the PFR is composed of at least 18 proteins (C and D). The adenylate kinases identified in this study are indicated as ADKA and ADKB in C.

As expected, adenylate kinase activity was also present in the cytoskeletal fraction of the noninduced *snl*-2 mutant. However, this activity was absent from cytoskeletons of the induced *snl*-2 mutant (Figure 3B). Because there is no difference between the cytoskeletons from these two induction states, other than the lack of PFR in the flagellum of induced *snl*-2, we can conclude that adenylate kinase activity is only associated with the flagellum, and not with other areas of the cytoskeleton. The difference in levels of activity between the cytoskeletal and flagellar preparations is likely to arise as a consequence of the salt extraction used to depolymerize the subpellicular microtubules. The use of stringent detergent and salt extractions shows that the adenylate kinase activity must be intimately associated with the flagellum. This demonstration of activity reflected precisely the results of our proteomic investigation.

Localization of Adenylate Kinase along the PFR

These results raised the question of where the adenylate kinases are located in the flagellum. We expressed an epitope-tagged version of *Tb*ADKA from its endogenous locus and used the tag to ascertain whether this adenylate kinase was associated with the detergent-insoluble cytoskeletal fraction. Figure 4A shows that this was indeed the case in *snl*-2 in its noninduced state. However, when PFRA RNAi was induced and the population displayed the paralyzed, PFR minus phenotype, the tagged *Tb*ADKA was located in the detergent-soluble fraction and was absent from the cytoskeletal fraction (Figure 4A). Immunofluorescence images of noninduced cells showed that the *Tb*ADKA protein was located specifically in the PFR and not in the axoneme (Figure 4, B–D). The axoneme extends from the flagellar basal bodies, which are physically connected to the kineto-

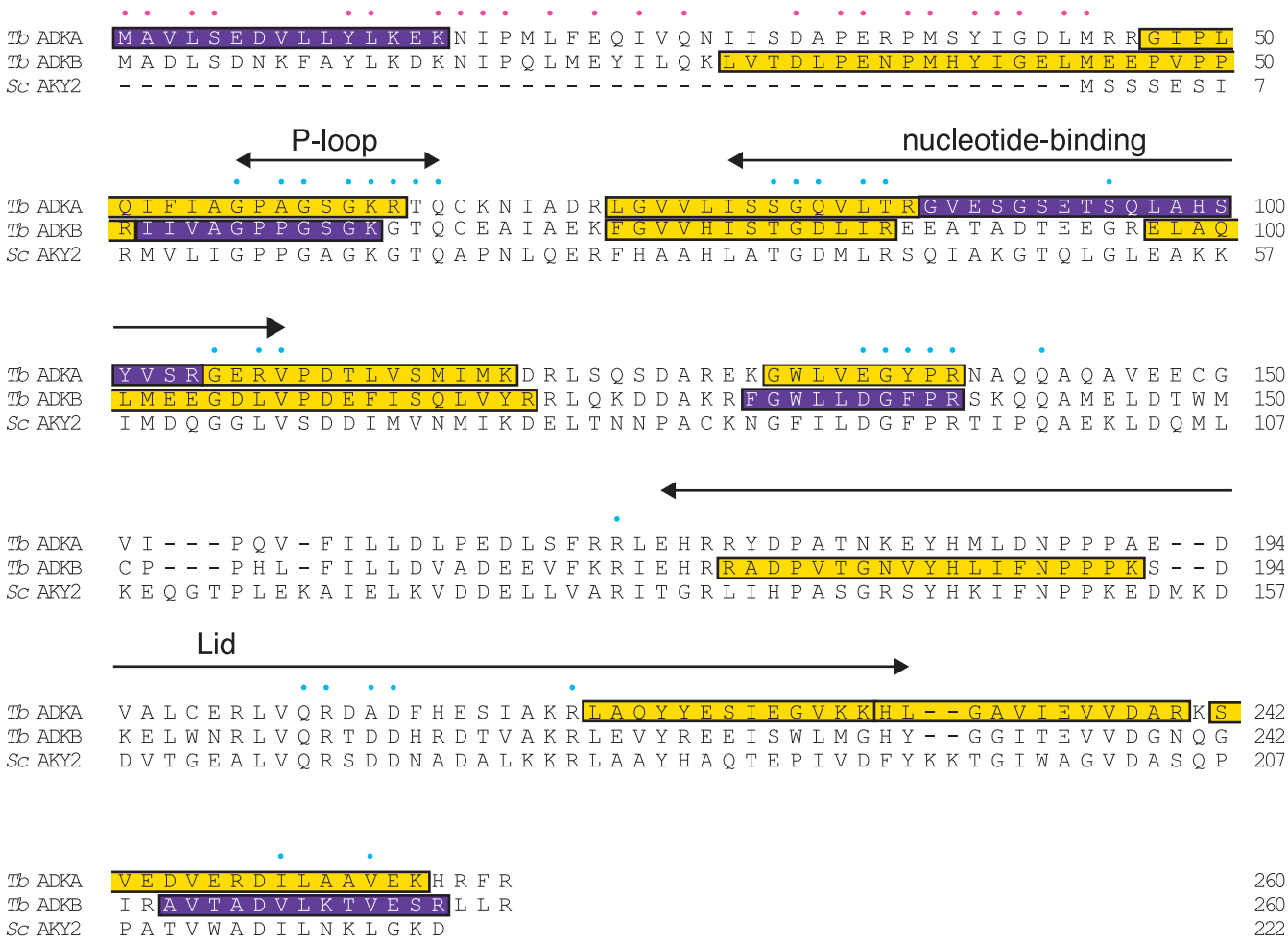

Figure 2. Identification of two flagellar adenylate kinases. Sequences of the two adenylate kinases identified in this study, *Tb*ADKA and *Tb*ADKB, and *S. cerevisiae* adenylate kinase (accession no. AAA66319) were aligned using ClustalW. The gene identities for the *T. brucei* adenylate kinases are Tb927.2.5660 (*Tb*ADKA) and Tb10.70.7330 (*Tb*ADKB); there is 43% identity between the two trypanosome enzymes. Blue dots highlight conserved residues that are important for catalysis or enzyme structure (Schricker *et al.*, 1995; Sanchez and Muller, 1998). Domains involved in the binding of ATP and AMP substrates, and a third characteristic region, known as the LID domain which changes conformation after substrate binding (Fukami-Kobayashi *et al.*, 1996; Miura *et al.*, 2001), are indicated by double-ended arrows. The red dots highlight residues that are identical in the N-terminal extensions of *Tb*ADKA and *Tb*ADKB. Purple shading indicates peptide fragments that were identified by tandem mass spectrometry sequencing; yellow shading indicates peptides identified on a basis of their mass only.

plast (Ogbadoyi *et al.*, 2003) to the end of the flagellum, but the PFR is only present from the point where the flagellum exits the cell body (Gull, 1999). Thus, PFR localization was confirmed by the gap in the immunofluorescence between the kinetoplast and the start of the PFR (Figure 4B). Moreover, the *Tb*ADKA immunofluorescence was entirely coincident with an antibody that specifically detects the PFR (Figure 4C). Finally, the *Tb*ADKA signal lags behind that of the axoneme as the latter extends, exactly as one would expect for a PFR protein (Figure 4D). The same results were observed for *Tb*ADKB (our unpublished data).

Unusual N-Terminal Extensions Characteristic of the Flagellar Adenylate Kinases

Examination of the *Tb*ADKA and *Tb*ADKB sequences and comparison with many other known adenylate kinase sequences revealed the conservation of residues known to be important for catalysis and adenylate kinase structure in the trypanosome proteins (Figure 2; Schricker *et al.*, 1995; Sanchez and Muller, 1998). A most unusual feature, however, was the presence of a 55-amino acid-long N-terminal extension, upstream of the conserved P-loop on both *Tb*ADKA and *Tb*ADKB (Figure 2). The N-terminal extensions of the two flagellar enzymes showed some sequence conservation. Interestingly, two lines of evidence indicated that neither extension was cleaved after translation of *Tb*ADKA or *Tb*ADKB. First, the predicted molecular weights and isoelectric points of the full-length proteins matched the values observed when each protein was resolved by 2D electrophoresis (Figure 1C). Second, and more directly, mass spectrometry of both proteins included peptide mass and sequence data in the N-terminal regions (Figure 2).

The presence of the N-terminal extensions clearly raised the issue of whether they are responsible for the unusual location of *Tb*ADKA and *Tb*ADKB in the flagellum. To address this issue, we constructed a deletion of the N-terminal region of *Tb*ADKA [ADKA$^{\Delta(1-43)}$::Ty] and produced cell lines expressing the epitope-tagged versions of either *Tb*ADKA or the truncated *Tb*ADKA from a strong promoter (Figure 5A). We subjected these cell lines to the same deter-

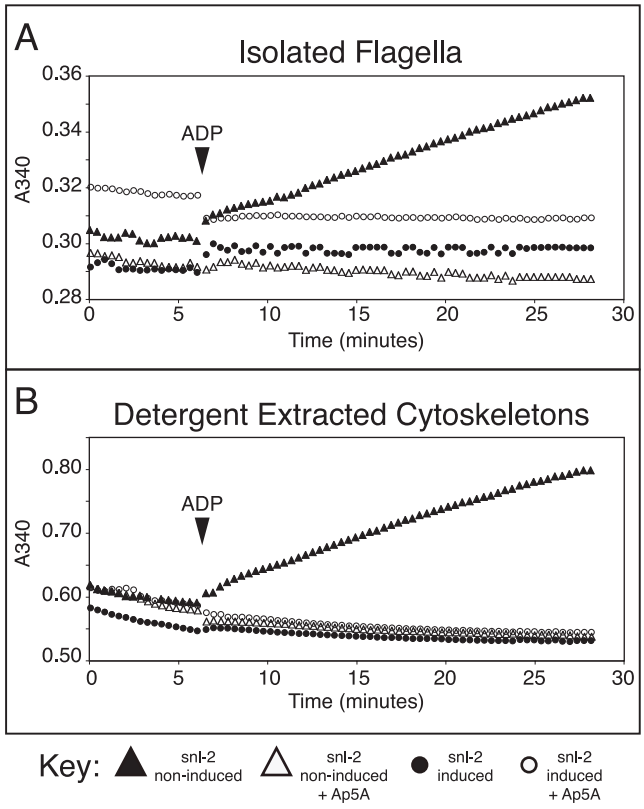

Figure 3. Adenylate kinase activity is associated with purified flagella (A) and cytoskeletons (the subpellicular microtubule and flagellar fraction (B). Activity occurs after the addition of ADP to the equilibrated assay mixture (solid arrowhead).

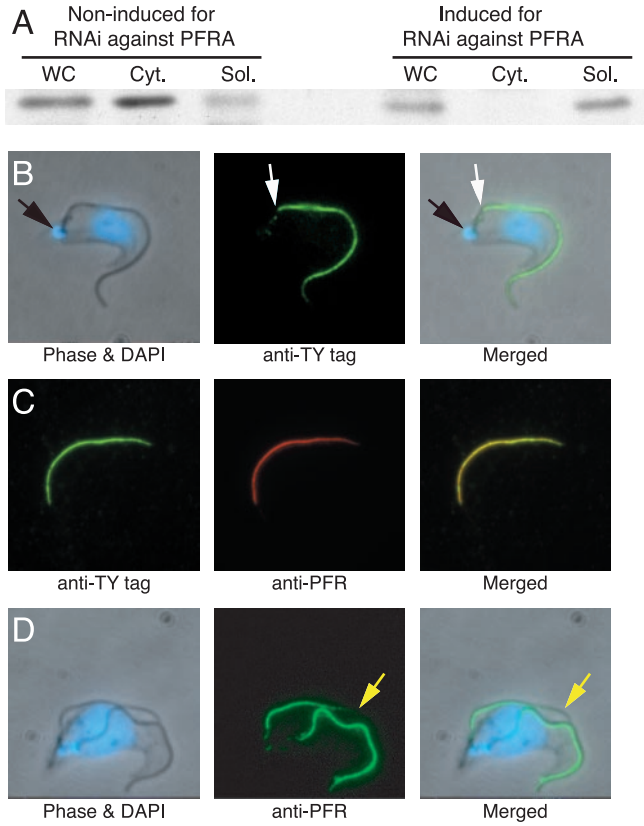

Figure 4. *Tb*ADKA is a PFR-associated protein in motile parasites, but it is not present in the flagella of the induced *snl*-2 mutant. (A) Immunoblot analysis of *Tb*ADKA tagged with the Ty-epitope at its C terminus by using the monoclonal antibody BB2. Cell equivalents (10^7) were loaded per lane. ADKA, expressed from its endogenous locus, partitions into the detergent-insoluble cytoskeletal fraction (Cyt.), except under conditions where the PFR is not assembled (*snl*-2 induced mutant), when it is found only in the detergent-soluble cytoplasmic fraction (Sol.). WC, whole cell extract. (B) Immunolocalization of ADKA::Ty to the flagellum by using BB2. The immunofluorescence pattern indicates that ADKA is present from the point where the flagellum leaves the cell body (white arrow) to its distal tip, but not at the point where the axoneme is nucleated from the basal body that is attached to the mitochondrial genome (black arrow; Ogbadoyi *et al.*, 2003). The positions of the nuclear and mitochondrial (kinetoplast) genomes are revealed by the blue 4′, 6-diamidino-2-phenyindole stain (DAPI). (C) Colocalization of ADKA::Ty with the monoclonal antibody ROD-1, which recognizes a large molecular weight component of the PFR. (D) Incorporation of ADKA into the new flagellum (yellow arrowhead) lags behind the elongation of the axoneme. Cells in C and D were processed as described for B.

gent extraction as used earlier and found that although a significant amount of soluble protein was produced, due to expression from a strong promoter (Figure 5, B–D), only full-length *Tb*ADKA, and not ADKA$^{\Delta(1-43)}$, could be targeted to the flagellum (Figure 5D). The overexpression of these proteins emphasized that even though large amounts of the N-terminal–truncated protein were produced in the cytoplasm, the system was unable to concentrate such protein in the flagellum compartment. We also engineered cell lines that expressed either GFP, or GFP fused at its N terminus to the N-terminal 55-amino acids of *Tb*ADKA. Fluorescence analysis of these cell lines showed that GFP on its own localized to the cell body with insignificant amounts in the flagellum (Figure 5H), whereas GFP carrying the N-terminal adenylate kinase extension localized to the flagellum (Figure 5J). This signal was not seen in cytoskeletal preparations (detergent-extracted cells; our unpublished data), presumably because it was either in a soluble state within flagella before detergent extraction, or, if the extension also provides structural features contributing to PFR incorporation, was only loosely associated with the PFR. Overall, therefore, our data demonstrated that the N-terminal extension present on *Tb*ADKA, and presumably the extension present on *Tb*ADKB, too, are both necessary and sufficient for targeting proteins to the trypanosome flagellum. However, it also seems that once in the flagellum there are additional structural determinants that are needed to anchor the flagellar adenylate kinases within the PFR.

The Unusual N-Terminal Extensions Identify Novel Conserved Adenylate Kinases in Nematode and Human Genomes

We considered the possibility that the N-terminal extensions of *Tb*ADKA and *Tb*ADKB, or motifs contained within these extensions, might be used to target other proteins to the axoneme or PFR. Whereas a bioinformatic search of the trypanosome genome database identified a number of proteins that share some sequence identity with the N-terminal regions of *Tb*ADKA and *Tb*ADKB, respectively, these do not include known flagellar proteins. Nor could we, by comparison with known flagellar components, identify motifs within the primary sequences of *Tb*ADKA and *Tb*ADKB that

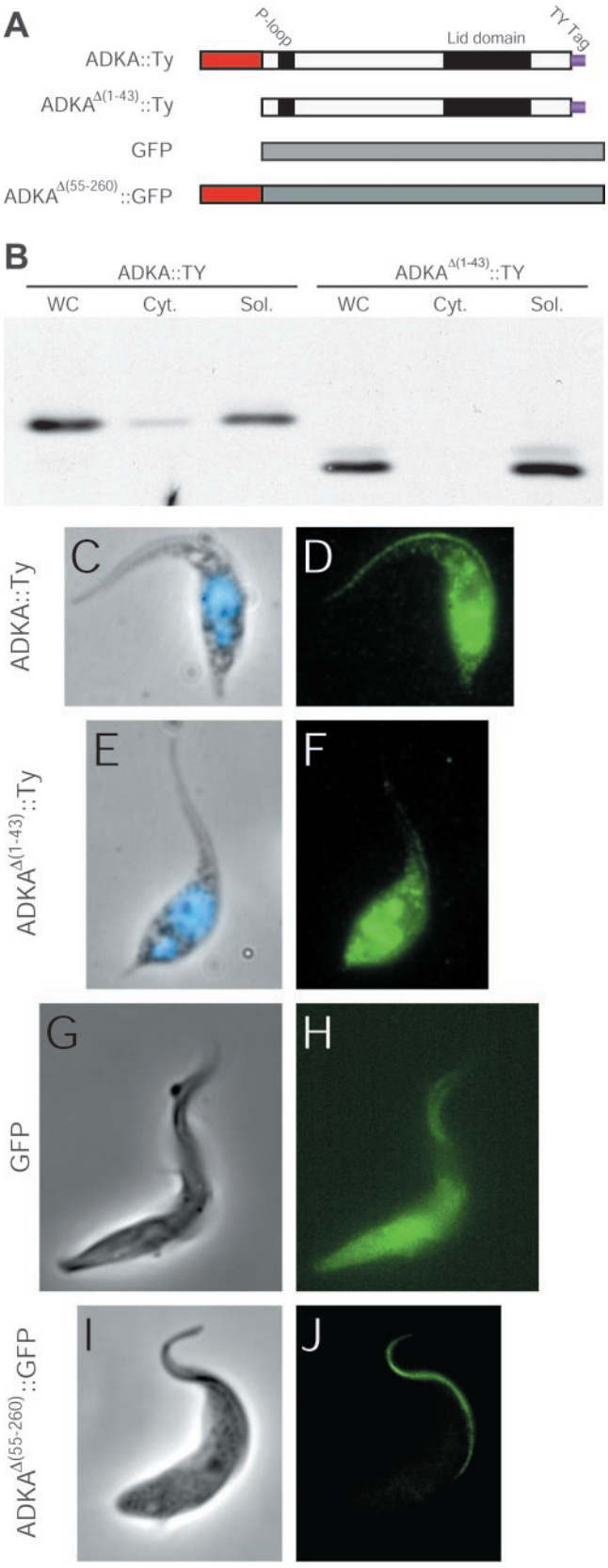

Figure 5. N-terminal extension of the flagellar adenylate kinases is an essential targeting determinant. (A) Cartoon showing the structures of the N-terminal truncation [ADKA$^{\Delta(1-43)}$::Ty] and the chimeric GFP [ADKA$^{\Delta(55-260)}$::GFP] used to obtain the results shown in B–J. (B) Immunoblot analysis of overexpressed ADKA::Ty and ADKA$^{\Delta(1-43)}$::Ty with antibody BB2. Cell equivalents (2×10^6) were loaded per lane. Full-length ADKA is present in both detergent-insoluble and -soluble fractions, but the N-terminal truncated protein is present only in the soluble cytoplasmic fraction. (C–F) Immunofluorescence analysis of ADKA::Ty and ADKA$^{\Delta(1-43)}$::Ty. Methanol-fixed trypanosomes (phase and 4,6-diamidino-2-phenylindole shown in C and E) were used for immunofluorescence with BB2 (D and F). Even though an intense signal can be seen in the cytoplasm, overexpression of ADKA$^{\Delta(1-43)}$::Ty still does lead to any of protein being transported to the flagellum (F). (G–J) Fluorescence analysis of GFP and ADKA$^{\Delta(55-260)}$::GFP (H and J) in formaldehyde-fixed cells (phase, G and I).

were suggestive of flagellar targeting sequences common to many flagellar components. However, when the bioinformatic analysis was extended to the genomes of other organisms we were surprised to find that specific blast analyses using only the N-terminal extensions of *Tb*ADKA or *Tb*ADKB identified similar sequences in the *Caenorhabditis elegans* and human genomes that showed unexpectedly high identity (Figure 6A). These human and *C. elegans* sequences turned out to be the N termini of predicted open reading frames that both contained two complete adenylate kinase domains in tandem (Figure 6B). Identification of conserved catalytic or structurally essential residues in these tandem adenylate kinases suggested that both domains would be able to catalyze phosphotransfer between a nucleotide triphosphate donor and nucleotide monophosphate acceptor.

Phenotype Analysis in a Flagellar Adenylate Kinase RNAi Knock-Down Mutant

Given that induced *snl*-2 lacks a full PFR and is paralyzed, is it possible that removal of only adenylate kinase from the structure might be sufficient to phenocopy the original *snl*-2 paralyzed phenotype? We used an inducible RNAi system (Bastin *et al*., 2000a; Wang *et al*., 2000) to address this question, and we were able to successfully ablate the expression of both flagellar adenylate kinases simultaneously (as evidenced by proteomic analysis by 2D electrophoresis and a decrease of >80% in the adenylate kinase activity found in cytoskeletal extracts) using sequences specific to each gene to generate double-stranded RNA. However, when RNAi-induced and control cell lines were analyzed by videomicroscopy no differences in either speed or direction of movement, or waveform or beat pattern were detected between RNAi-induced and RNAi-noninduced cells. We also saw no structural alterations to the PFR by using electron microscopy.

DISCUSSION

Insights into Adenylate Kinase Function in the Flagellum

Motility mediated by the activity of axonemal dynein ATPases requires ATP hydrolysis, but this motility is also sensitive to the balance of ATP and ADP concentrations with recent work suggesting that some inner arm dyneins are strongly inhibited under ADP-free conditions (Yagi, 2000). Studies over the last 35 yr or so have indicated that enzymatic activities that could regulate adenine nucleotide concentrations can be detected in flagellar, ciliary, and axonemal preparations. These reports include accounts of adenylate kinase, nucleotide diphosphate kinase, and phosphagen (creatine and arginine) kinase activities in organisms as diverse as *Tetrahymena*, *Chlamydomonas*, sea ur-

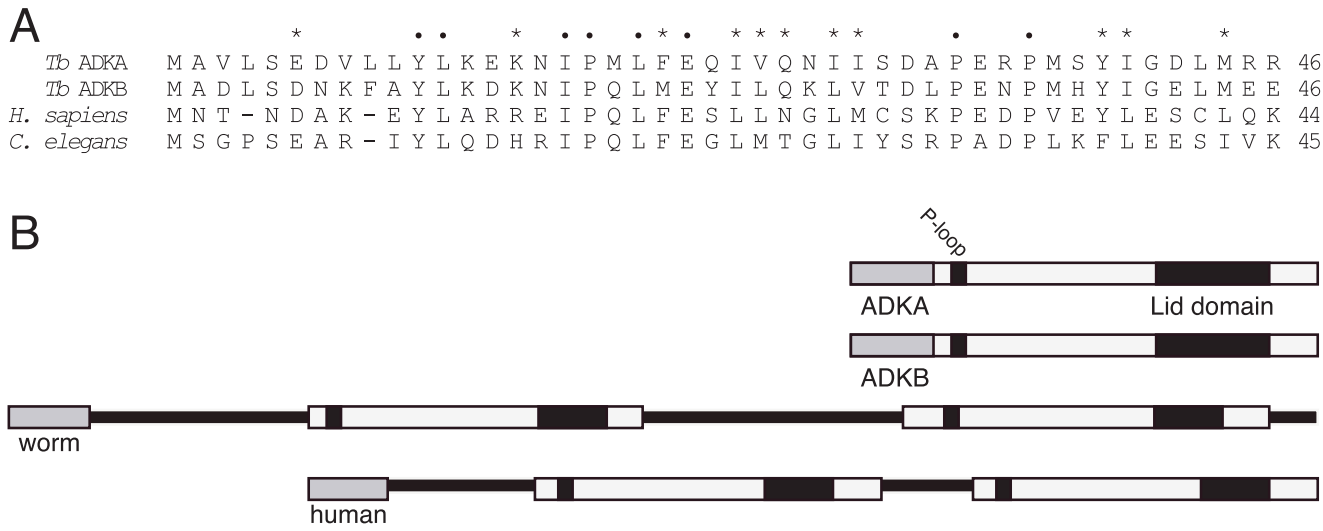

Figure 6. Identification of unusual adenylate kinases in *C. elegans* and *Homo sapiens*. (A) Sequence alignment of the first 46 amino acids of *Tb*ADKA and *Tb*ADKB with two sequences that were identified when these N-terminal regions were used in blast analysis of the genome databases available at National Center for Biotechnology Information. Positions of identity in all four sequences are highlighted using black dots; asterisks indicate positions where functional similarity is conserved. (B) When the two novel sequences were examined more closely (accession nos. CAA15625 [*C. elegans*] and NP_777283 [*H. sapiens*]) both proteins were found to be tandem adenylate kinases in which both putative catalytic domains were downstream of the conserved N-terminal region shown in A.

chin, and mammalian sperm (Gibbons, 1966; Watanabe and Flavin, 1976; Schoff *et al*., 1989; Wothe *et al*., 1990; Ogawa *et al*., 1996; Nakamura *et al*., 1999; Patel-King *et al*., 2002; Miranda-Vizuete *et al*., 2003; Sadek *et al*., 2003). An obvious difficulty, acknowledged by some authors, of biochemical fractionation studies is the definition of the specificity of location of, for example, adenylate kinase activity when this is such a ubiquitous enzyme in the cell (Schoff *et al*., 1989). Our present study now provides direct evidence that particular isoforms of adenylate kinase are associated with the eukaryotic flagellum, and moreover we provide the explanation for the flagellar specificity of these isoforms.

The recognition of the N-terminal extensions on these isotypes provides an explanation for the mechanisms by which unique isotypes of a protein family could be targeted to the flagellum. Adenylate kinases are historically recognized as enzymes of the cytoplasm or mitochondrion in microbes or animal cells. Our discovery of these isotypes has facilitated an initial description of a novel protein sequence necessary and sufficient to endow flagellar localization. This system is distinct from both the targeting signals located within the C-terminal region of the major PFR proteins (Bastin *et al*., 1999a), and the N-terminal sequences important for targeting of certain flagellar membrane proteins (Godsel and Engman, 1999; Bloodgood, 2000; Ignatushchenko *et al*., 2004). A further key point is the biological function of flagellar adenylate kinase activity, and our data provide three important contributions to this debate. The adenylate kinase activity is present in multiple isoforms, they are built into the cytoskeletal structure, and finally they are distributed in a uniform manner along essentially the length of the flagellum. Although multiple isoforms might indicate redundancy or specificity of function, their distributed location does not suggest a local function at either the entry point into the flagellum from the cytoplasm, or the distal tip where assembly of axonemal and PFR components is achieved (Bastin *et al*., 1999a). Also this distribution does not suggest a local function in the initiation of axonemal beating. However, this distribution would indicate a possible function of flagellar adenylate kinase in providing a phosphotransfer relay (Dzeja and Terzic, 2003) that would facilitate a more rapid and efficient movement of adenine nucleotides along the flagellum than would be achieved by diffusional exchange alone. RNAi knockdown of the two flagellar adenylate kinases that we have identified does not obviously affect flagellar motility. It is possible that adenylate kinase activity is required for flagellar motility and that the residual activity (<20% of normal) is sufficient to sustain flagellar motility. Alternatively, adenylate kinase activity is not required under normal culture conditions, or this adenylate kinase system can be bolstered by a redundancy involving yet more homeostatic mechanisms.

The notion of compensatory phenotypes for localized adenylate kinase deficiency may not be so surprising in the light of investigations into the roles played by individual adenylate kinase, and creatine kinase, isoforms in mammals. Transgenic mice deficient in the major cytosolic isoform of adenylate kinase display striking metabolic rearrangements in which wide-scale remodeling of glycolytic and mitochondrial pathway networks are sufficient to sustain high energy phosphotransfer in muscle tissue, except under conditions of metabolic stress (Janssen *et al*., 2000; Carrasco *et al*., 2001; Janssen *et al*., 2003a,b). These observations are relevant when one considers the metabolic plasticity of cultured trypanosomes (Bochud-Allemann and Schneider, 2002; van Weelden *et al*., 2003) and indicate the most direct way to assess whether flagellar adenylate kinases contribute to the economy of energy metabolism will be transmission of null mutants through the natural tsetse fly vector and so explored within the natural nutritional context. This experiment, however, could not be executed with the laboratory-adapted strains currently available.

Our studies clearly locate the flagellar adenylate kinases to the PFR in the trypanosome flagellum. Such extraaxonemal structures are important features of diverse eukaryotic flagella (Santrich *et al*., 1997; Bastin *et al*., 1998; Miki *et al*., 2002). Our data also show that the PFR of trypanosomes is not merely an architectural feature but rather acts as a

matrix into which enzymes such as adenylate kinase and most likely other regulatory proteins can be built. This analysis parallels recent conclusions concerning the biochemical nature of another extraaxonemal structure, the fibrous sheath of mammalian spermatozoa (Miki *et al*., 2002; Eddy *et al*., 2003), and perhaps provides an intriguing example of convergent evolution between phylogenetically distant organisms. Major components of the fibrous sheath have been revealed to be AKAP4, AKAP3, and TAKAP-80, which provide anchoring opportunities for cAMP-dependent protein kinase, and specific isoforms of enzymes such as glyceraldehyde-3-phosphate dehydrogenase and hexokinase (Eddy *et al*., 2003). However, it is important to realize that there is a significant difference between the flagellum of a metazoan sperm and that of a eukaryotic microbe. In essence the sperm lacks cytoplasm, whereas the eukaryotic microbial cell possesses abundant cytoplasm with organelles. Thus, localization of metabolic functions in the flagellum of the latter is best viewed as an isotype differential localization problem, whereas in the former it maybe viewed as more of a cell type-dependent expression phenomenon.

Results from many systems, including trypanosomes, indicate that the localization of cAMP-dependent signaling pathways to flagella is ubiquitous, and at least in some cases is linked to motility (Inaba, 2003; King, 2003). Key enzymes in this process, adenylate cyclases and cAMP phosphodiesterases, have been localized to flagella in several organisms. Thus, adenine nucleotide homeostasis is likely to require extensive regulation in the flagellar compartment to take account of concurrent motility and signaling phenomena and the maintenance of a balance between them. Redundancy in homeostatic systems may therefore explain the absence of singular phenotypes after adenylate kinase RNAi, but we believe that the structural incorporation of enzymes into extraaxonemal structures is significant in explaining why complete removal (mouse knock-outs for AKAP4 and RNAi of PFR-A in trypanosomes, respectively) of the structure has such dramatic consequences (Bastin *et al*., 1998, 2000a; Miki *et al*., 2002). This provides an alternative to considering a merely physical reduction in the diameter of the flagellum as being influential in explaining the abnormal motility phenotype in trypanosomatid mutants that fail to assemble a PFR (Santrich *et al*., 1997). Although this biophysical explanation remains possible, it is important to note that the diameter of the PFR varies by 100% between *Trypanosoma*, *Crithidia*, and *Leishmania* species (150–300 nm) with no observable concomitant change in the motility of these organisms (Bastin *et al*., 1996b, 2000b). In addition, in trypanosomatid species such as *C. oncopelti* that were reported to lack a PFR there was no discernible difference in their motility in comparison with *Crithidia fasciculata*, which does assemble a normal PFR (Bastin *et al*., 1996b). Moreover, we have recently observed that in fact a very small PFR is present in *Crithidia oncopelti* (Gadelha and Gull, unpublished), so providing further support for our hypothesis that the PFR provides a critical platform into which regulatory or metabolic enzymes are anchored.

Insights into Adenylate Kinase Targeting into the Flagellum

Our analysis of the N-terminal extension of *Tb*ADKA provides the molecular explanation for targeting of these isoforms; the extension is both necessary and sufficient for locating proteins to the flagellum. The N-terminal sequences of *Tb*ADKA and *Tb*ADKB show a conservation of several motifs and are clearly related. However, although these adenylate kinases are built into the PFR structure, as defined by detergent and high salt treatment, N-terminal–tagged GFP is targeted to the flagellum but it is not stably incorporated into the PFR, such that it can withstand such treatments. Thus, some additional feature is likely to influence stable incorporation. Although we have previously described a C-terminal motif important in some proteins for targeting to the flagellum (Bastin *et al*., 1999a; Ersfeld and Gull, 2001) and there are at least two distinct mechanisms for targeting proteins to the flagellar membrane (Godsel and Engman, 1999; Bloodgood, 2000; Ignatushchenko *et al*., 2004), the present N-terminal extension sequences represent additional excellent illustrations of eukaryotic flagellar targeting sequences. Are these sequences widely seen in the *T. brucei* genome, and hence could they facilitate identification of other flagellar proteins? The answer here is that we could recognize a number of putative proteins containing sequences that shared identity with the N-terminal sequences described here. However, if there are certain common motifs that define a whole family of flagellar proteins further experimental work will be needed to confirm such candidates.

Targeting motifs are likely to be used by cargo proteins for transportation into the flagellum by intraflagellar transport (Scholey, 2003) or other systems (such as ones necessary for the targeting of membrane proteins (Godsel and Engman, 1999; Ignatushchenko *et al*., 2004). With this in mind, we were therefore intrigued to find predicted proteins in other genomes, such as human and *C. elegans*, that share identity at their respective N termini with the *T. brucei* adenylate kinases. In particular, a [YLxxxxxIPxLxE] sequence, followed by two conserved prolines was present in both of the examples shown in Figure 6. Moreover, both putative polypeptides contain two apparently complete, but nonidentical, adenylate kinase units downstream of the conserved N-terminal region. Interestingly, we recognize that this tandem organization provides some echoes of the unusual triplet creatine kinase targeted to sperm flagella in sea urchins (Wothe *et al*., 1990) and the triplet nucleotide diphosphate kinases that have been localized to the axoneme in sea urchin and *Chlamydomonas* (Ogawa *et al*., 1996; Patel-King *et al*., 2002) and the fibrous sheath in mammalian sperm (Miranda-Vizuete *et al*., 2003). Given the existence of specific isoforms of certain central metabolic enzymes, and targeting sequences for these isoforms, we suggest that these isoforms form part of a conserved complex, cytoskeletal-anchored metabolic capability that is built into the eukaryotic flagellum.

ACKNOWLEDGMENTS

We thank Flavia Moriera-Leite for the illustrative electron micrographs. Sequence data for the *T. brucei* genome were obtained from The Institute for Genome Research and The Sanger Institute; sequencing of the *T. brucei* genome was accomplished as part of the Trypanosoma Genome Network with support from The National Institute of Allergy and Infectious Diseases (National Institutes of Health) and The Wellcome Trust. This work was supported by grants from the Wellcome Trust and the Royal Society. T.J.P. was supported by a Biotechnology and Biological Sciences Research Council studentship. M.L.G. is a Royal Society University Research Fellow and K.G. is a Wellcome Trust Principal Research Fellow.

REFERENCES

Bastin, P., Bagherzadeh, Z., Matthews, K.R., and Gull, K. (1996a). A novel epitope tag system to study protein targeting and organelle biogenesis in *Trypanosoma brucei.* Mol. Biochem. Parasitol. *77*, 235–239.

Bastin, P., Ellis, K., Kohl, L., and Gull, K. (2000a). Flagellum ontogeny in trypanosomes studied via an inherited and regulated RNA interference system. J. Cell Sci. *113*, 3321–3328.

Bastin, P., MacRae, T.H., Francis, S.B., Matthews, K.R., and Gull, K. (1999a). Flagellar morphogenesis: protein targeting and assembly in the paraflagellar rod of trypanosomes. Mol. Cell. Biol. *19*, 8191–8200.

Bastin, P., Matthews, K.R., and Gull, K. (1996b). The paraflagellar rod of Kinetoplastida: solved and unsolved questions. Parasitol. Today *12*, 302–307.

Bastin, P., Pullen, T.J., Moreira-Leite, F.F., and Gull, K. (2000b). Inside and outside of the trypanosome flagellum: a multifunctional organelle. Microbes Infect. *2*, 1865–1874.

Bastin, P., Pullen, T.J., Sherwin, T., and Gull, K. (1999b). Protein transport and flagellum assembly dynamics revealed by analysis of the paralysed trypanosome mutant *snl*-1. J. Cell Sci. *112*, 3769–3777.

Bastin, P., Sherwin, T., and Gull, K. (1998). Paraflagellar rod is vital for trypanosome motility. Nature *391*, 548

Bloodgood, R.A. (2000). Protein targeting to flagella of trypanosomatid protozoa. Cell Biol. Int. *24*, 857–862.

Bochud-Allemann, N., and Schneider, A. (2002). Mitochondrial substrate level phosphorylation is essential for growth of procyclic *Trypanosoma brucei*. J. Biol. Chem. *277*, 32849–32854.

Brun, R., and Schonenberger. (1979). Cultivation and in vitro cloning or procyclic culture forms of *Trypanosoma brucei* in a semi-defined medium. Acta Trop. *36*, 289–292.

Burland, T.G., Gull, K., Schedl, T., Boston, R.S., and Dove, W.F. (1983). Cell type-dependent expression of tubulins in *Physarum*. J. Cell Biol. *97*, 1852–1859.

Carrasco, A.J., *et al*. (2001). Adenylate kinase phosphotransfer communicates cellular energetic signals to ATP-sensitive potassium channels. Proc. Natl. Acad. Sci. USA *98*, 7623–7628.

Dzeja, P.P., and Terzic, A. (2003). Phosphotransfer networks and cellular energetics. J. Exp. Biol. *206*, 2039–2047.

Eddy, E.M., Toshimori, K., and O'Brien, D.A. (2003). Fibrous sheath of mammalian spermatozoa. Microsc. Res. Tech. *61*, 103–115.

Ersfeld, K., and Gull, K. (2001). Targeting of cytoskeletal proteins to the flagellum of *Trypanosoma brucei*. J. Cell Sci. *114*, 141–148.

Fukami-Kobayashi, K., Nosaka, M., Nakazawa, A., and Go, M. (1996). Ancient divergence of long and short isoforms of adenylate kinase: molecular evolution of the nucleoside monophosphate kinase family. FEBS Lett. *385*, 214–220.

Gibbons, I.R. (1966). Studies on the adenosine triphosphatase activity of 14 S and 30 S dynein from cilia of *Tetrahymena*. J. Biol. Chem. *241*, 5590–5596.

Godsel, L.M., and Engman, D.M. (1999). Flagellar protein localization mediated by a calcium-myristoyl/palmitoyl switch mechanism. EMBO J. *18*, 2057–2065.

Gull, K. (1999). The cytoskeleton of trypanosomatid parasites. Annu. Rev. Microbiol. *53*, 629–655.

Ignatushchenko, M., Nasser, A., and Landfear, S.M. (2004). Sequences required for the flagellar targeting of an integral membrane protein. Mol. Biochem. Parasitol. *135*, 89–100.

Inaba, K. (2003). Molecular architecture of the sperm flagella: molecules for motility and signaling. Zool. Sci. *20*, 1043–1056.

Janssen, E., de Groof, A., Wijers, M., Fransen, J., Dzeja, P.P., Terzic, A., and Wieringa, B. (2003a). Adenylate kinase 1 deficiency induces molecular and structural adaptations to support muscle energy metabolism. J. Biol. Chem. *278*, 12937–12945.

Janssen, E., Dzeja, P.P., Oerlemans, F., Simonetti, A.W., Heerschap, A., de Haan, A., Rush, P.S., Terjung, R.R., Wieringa, B., and Terzic, A. (2000). Adenylate kinase 1 gene deletion disrupts muscle energetic economy despite metabolic rearrangement. EMBO J. *19*, 6371–6381.

Janssen, E., Terzic, A., Wieringa, B., and Dzeja, P.P. (2003b). Impaired intracellular energetic communication in muscles from creatine kinase and adenylate kinase (M-CK/AK1) double knock-out mice. J. Biol. Chem. *278*, 30441–30449.

King, S.M. (2003). Organization and regulation of the dynein microtubule motor. Cell Biol. Int. *27*, 213–215.

Miki, K., Willis, W.D., Brown, P.R., Goulding, E.H., Fulcher, K.D., and Eddy, E.M. (2002). Targeted disruption of the Akap4 gene causes defects in sperm flagellum and motility. Dev. Biol. *248*, 331–342.

Miranda-Vizuete, A., Tsang, K., Yu, Y., Jimenez, A., Pelto-Huikko, M., Flickinger, C.J., Sutovsky, P., and Oko, R. (2003). Cloning and developmental analysis of murid spermatid-specific thioredoxin-2 (SPTRX-2), a novel sperm fibrous sheath protein and autoantigen. J. Biol. Chem. *278*, 44874–44885.

Miura, K., *et al*. (2001). Cloning and characterization of adenylate kinase from *Chlamydia pneumoniae*. J. Biol. Chem. *276*, 13490–13498.

Nakamura, K., Iitsuka, K., and Fujii, T. (1999). Adenylate kinase is tightly bound to axonemes of *Tetrahymena* cilia. Comp. Biochem. Physiol. B Biochem. Mol. Biol. *124*, 195–199.

Ogawa, K., Takai, H., Ogiwara, A., Yokota, E., Shimizu, T., Inaba, K., and Mohri, H. (1996). Is outer arm dynein intermediate chain 1 multifunctional? Mol. Biol. Cell *7*, 1895–1907.

Ogbadoyi, E.O., Robinson, D.R., and Gull, K. (2003). A high-order transmembrane structural linkage is responsible for mitochondrial genome positioning and segregation by flagellar Basal bodies in trypanosomes. Mol. Biol. Cell *14*, 1769–1779.

Patel-King, R.S., Benashski, S.E., and King, S.M. (2002). A bipartite Ca2+-regulated nucleoside-diphosphate kinase system within the *Chlamydomonas* flagellum. The regulatory subunit p72. J. Biol. Chem. *277*, 34271–34279.

Porter, M.E., and Sale, W.S. (2000). The 9 + 2 axoneme anchors multiple inner arm dyneins and a network of kinases and phosphatases that control motility. J. Cell Biol. *151*, F37–42.

Pullen, T.J. (2002). A proteomic investigation of the trypanosome flagellum. Ph.D. dissertation, University of Manchester, Manchester, United Kingdom.

Robinson, D., Beattie, P., Sherwin, T., and Gull, K. (1991). Microtubules, tubulin, and microtubule-associated proteins of trypanosomes. Methods Enzymol. *196*, 285–299.

Sadek, C.M., *et al*. (2003). Characterization of human thioredoxin-like 2. A. novel microtubule-binding thioredoxin expressed predominantly in the cilia of lung airway epithelium and spermatid manchette and axoneme. J. Biol. Chem. *278*, 13133–13142.

Sanchez, L.B., and Muller, M. (1998). Cloning and heterologous expression of *Entamoeba histolytica* adenylate kinase and uridylate/cytidylate kinase. Gene *209*, 219–228.

Santrich, C., Moore, L., Sherwin, T., Bastin, P., Brokaw, C., Gull, K., and LeBowitz, J.H. (1997). A motility function for the paraflagellar rod of *Leishmania* parasites revealed by PFR-2 gene knockouts. Mol. Biochem. Parasitol. *90*, 95–109.

Schneider, A., Sherwin, T., Sasse, R., Russell, D.G., Gull, K., and Seebeck, T. (1987). Subpellicular and flagellar microtubules of *Trypanosoma brucei brucei* contain the same alpha-tubulin isoforms. J. Cell Biol. *104*, 431–438.

Schoff, P.K., Cheetham, J., and Lardy, H.A. (1989). Adenylate kinase activity in ejaculated bovine sperm flagella. J. Biol. Chem. *264*, 6086–6091.

Scholey, J.M. (2003). Intraflagellar transport. Annu. Rev. Cell Dev. Biol. *19*, 423–443.

Schricker, R., Magdolen, V., Strobel, G., Bogengruber, E., Breitenbach, M., and Bandlow, W. (1995). Strain-dependent occurrence of functional GTP:AMP phosphotransferase (AK3) in *Saccharomyces cerevisiae*. J. Biol. Chem. *270*, 31103–31110.

Shevchenko, A., Wilm, M., Vorm, O., and Mann, M. (1996). Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. Anal. Chem. *68*, 850–858.

van Weelden, S.W., Fast, B., Vogt, A., van der Meer, P., Saas, J., van Hellemond, J.J., Tielens, A.G., and Boshart, M. (2003). Procyclic *Trypanosoma brucei* do not use Krebs cycle activity for energy generation. J. Biol. Chem. *278*, 12854–12863.

Wang, Z., Morris, J.C., Drew, M.E., and Englund, P.T. (2000). Inhibition of *Trypanosoma brucei* gene expression by RNA interference using an integratable vector with opposing T7 promoters. J. Biol. Chem. *275*, 40174–40179.

Watanabe, T., and Flavin, M. (1976). Nucleotide-metabolizing enzymes in *Chlamydomonas* flagella. J. Biol. Chem. *251*, 182–192.

Wickstead, B., Ersfeld, K., and Gull, K. (2003). The frequency of gene targeting in *Trypanosoma brucei* is independent of target site copy number. Nucleic Acids Res. *31*, 3993–4000.

Wirtz, E., and Clayton, C. (1995). Inducible gene expression in trypanosomes mediated by a prokaryotic repressor. Science *268*, 1179–1183.

Wirtz, E., Leal, S., Ochatt, C., and Cross, G.A. (1999). A tightly regulated inducible expression system for conditional gene knock-outs and dominant-negative genetics in *Trypanosoma brucei*. Mol. Biochem. Parasitol. *99*, 89–101.

Wothe, D.D., Charbonneau, H., and Shapiro, B.M. (1990). The phosphocreatine shuttle of sea urchin sperm: flagellar creatine kinase resulted from a gene triplication. Proc. Natl. Acad. Sci. USA *87*, 5203–5207.

Yagi, T. (2000). ADP-dependent microtubule translocation by flagellar inner-arm dyneins. Cell Struct. Funct. *25*, 263–267.