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Kimberly A. P. Mitchell

Liberty University, kamitchell@liberty.edu

Betty C. Gallagher

Gabor Szabo

Angela de S. Otero

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NDP KINASE MOVES INTO DEVELOPING PRIMARY CILIA*

Kimberly A. P. Mitchell, Betty C. Gallagher, Gabor Szabo and Angela de S. Otero[¶]

[¶]Department of Molecular Physiology and Biological Physics
University of Virginia Medical School
Charlottesville, VA 22908

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Send correspondence to:

Angela de S. Otero
Department of Molecular Physiology and Biological Physics
University of Virginia Medical School
P.O. Box 800736
Charlottesville, VA 22908-0736
Phone: (434) 924-5108
Fax: (434) 982-1616

SUMMARY

Immunofluorescence staining of murine NIH3T3 fibroblasts grown at high density shows that conventional nucleoside diphosphate (NDP) kinases A and B localize to a sensory organelle, the primary cilium. Similar results are obtained with *Xenopus* A6 kidney epithelial cells, suggesting that NDP kinases are a universal component of the primary cilium. The translocation of NDP kinase into primary cilia depends on size, taking place only when cilia reach a critical length of 5-6 μm . In mature cilia, NDP kinases are distributed along the ciliary shaft in a punctate pattern that is distinct from the continuous staining observed with acetylated α -tubulin, a ciliary marker and axonemal component. Isolation of a fraction enriched in primary cilia from A6 cells led to the finding that ciliary NDP kinase is enzymatically active, and is associated with the membrane and the matrix, but not the axoneme. In contrast, acetylated α -tubulin is found in the axoneme and to a lesser extent, in the membrane. Based on the tightly regulated translocation process and the subciliary distribution pattern of NDP kinase, we propose that it plays a role in the elongation and maintenance of primary cilia by its ability to regenerate the GTP utilized by ciliary microtubule turnover and transmembrane signaling.

INTRODUCTION

NM23/NDP kinases play a central role in a broad range of cellular functions, including nucleic acid synthesis, lipid and carbohydrate metabolism, through their ability to phosphorylate nucleoside diphosphates utilizing the terminal phosphate of ATP. The most abundant and ubiquitous members of this family of proteins, NDP kinases A and B (NM23-1 and 2), are hexamers of two highly homologous polypeptides of 18 kDa, with characteristics of cytosolic proteins. Nevertheless, both proteins are found in association with plasma membranes in stimulated cells [Gallagher et al., 2003], and in the nucleus [Lacombe et al., 2000; Munier et al., 2003]. In recent years, several other proteins expressed at lower levels in specific tissues or organelles were found to contain consensus sequences that define NDP kinases. Although some of these proteins (e.g., NDP kinase C and D) do have enzymatic activity, and therefore are genuine NDP kinases, others seem to be inactive, and therefore are more appropriately referred to as NM23s [Lacombe et al., 2000]. NM23-5, 7, 8 and 9 are expressed in testis and lung, and diverge considerably from canonical NDP kinases. Thus, NM23-7 and 8 have repeats of the NDP kinase catalytic region, while NM23-8 and 9 contain an additional thioredoxin domain [Sadek et al., 2001; Sadek et al., 2003].

Members of the NM23/NDP kinase family are frequently found in association with microtubular structures. Thus, although NDP kinases A and B do not bind directly to purified tubulin [Gallagher et al., 2003], these proteins bind to microtubules during interphase [Pinon et al., 1999], are associated with membranes that co-pellet with taxol-stabilized microtubules, and co-localize with α -tubulin around cytoplasmic vesicles in fibroblasts [Gallagher et al., 2003]. NDP kinase A is a component of the centrosome and co-immunoprecipitates with γ -tubulin [Roymans et al., 2001], and NDP kinase is part of the nucleotide metabolizing enzymes found in

Chlamydomonas flagella and *Tetrahymena* cilia [Watanabe and Flemin, 1976]. NDP kinases A, B, as well as NM23-5, localize to flagella of spermatozoa [Munier et al., 2003], and NM23-5 and 7 are associated with the isolated axoneme of human respiratory cilia [Ostrowski et al., 2002]. NM23-8 is located in the fibrous sheath, a cytoskeletal structure that envelops the sperm axoneme [Miranda-Vizueté et al., 2003], whereas a splice variant of NM23-9 (Tlx-2) present in axonema from cilia and spermatids binds directly to microtubules [Sadek et al., 2003].

During our studies of the intracellular localization of NDP kinases A and B, we found that these proteins localize to yet another microtubule-based structure, namely the primary cilium. Primary cilia are present in most vertebrate cells and originate from the mother centriole [for reviews see Poole et al., 1985, Wheatley et al., 1996, and Pazour and Witman, 2003]. The internal structure of primary cilia differs noticeably from that of motile cilia and flagella: whereas the latter's axoneme is composed of nine peripheral doublet microtubules surrounding two singlet microtubules (9+2 arrangement), primary cilia do not have the core doublet and thus are called "9+0" cilia. Primary cilia also lack other structures linked to motility, such as dynein arms, nexin links and radial spokes, and are thus considered, with the exception of nodal cilia, to be immotile.

Primary cilia were at first thought to be vestigial appendages devoid of function. Subsequent studies [e.g. Gallagher, 1980] noted their widespread presence and conserved ultrastructure, suggesting that these organelles might have an important function in cells. Some hypothesized that primary cilia might have a sensory role (see Poole et al., 1985 and references thereof), but proof of this function was missing. Recent work from several laboratories upholds this idea [reviewed in Pazour and Witman, 2003]. A number of studies have shown that in several cell types elements of signaling pathways such as the polycystins and G protein-coupled

receptors for serotonin (5-HT₆) and somatostatin (sst3) localize specifically to the membrane of primary cilia [Brailov et al., 2000, Schulz et al, 2000, Pazour et al., 2002, Yoder et al., 2002]. However, clear-cut evidence came from the work of Praetorius and Spring [2001, 2003], who demonstrated that primary cilia act as flow sensors in renal cells, responding to bending with increases in intracellular calcium. Notably, mutations that induce defects in primary cilia are connected with a series of pathological conditions, among these renal cystic disease, situs inversus and retinitis pigmentosa [Sloboda, 2002; Pazour and Rosenbaum, 2002].

The primary cilium has been aptly described as the cell's antenna [Pazour and Witman, 2003], which initiates responses to stimuli that control differentiation, development and proliferation, all processes which NDP kinases have been reported to affect by unknown mechanisms [reviewed in Otero, 2000; Kimura et al., 2000; Arnaud-Dabernat et al., 2003]. Our results raise the possibility that the clue to some of these cellular functions of conventional NDP kinases is their localization in primary cilia.

MATERIALS AND METHODS

Materials

Antibodies utilized were: a polyclonal antibody, Ab-1 (Labvision, Fremont, Ca.), specific for NDP kinase A and B [Gallagher et al., 2003], and two distinct polyclonal antibodies to frog NDP kinase, M1 and F1 [Yi et al., 1996; Otero, 1997]. Acetylated α -tubulin was detected with a monoclonal antibody (clone 6-11B-1, Piperno and Fuller [1985]) from Zymed. Secondary antibodies (multiple labeling grade) were from Jackson Immunoresearch Laboratories (Texas Red conjugate) and Molecular Probes (Alexa 488 conjugate). SDS-PAGE gels were from BioRad or Invitrogen.

Methods

Cell culture- NIH-3T3 fibroblasts were cultured as described (Gallagher et al., 2003). Experiments were performed in cells at passages 4-7 that were 60-80% confluent and serum-deprived for 48-72h, all conditions that increase expression and length of primary cilia in these cells [Tucker et al, 1983, Alieva et al., 1999]. Xenopus A6 cells were grown at 28 °C in 1% CO₂, in a medium composed of 40% Leibowitz L-15, 17% F-12K Nutrient Mixture (Kaighn's modification), 33% water, 8% fetal bovine serum, 1.6 mM L-glutamine, 80 U/L penicillin and 80 μ g/mL streptomycin. Unless otherwise stated, A6 cells were cultured for 7-9 days in complete medium after reaching confluence before staining.

Immunofluorescence- Cells were grown on No. 1 thickness acid-washed glass coverslips, fixed, stained and mounted as described previously [Gallagher et al., 2003]. Primary cilia were isolated as described below; the fraction was placed in a glass coverslip, dried with a stream of nitrogen, fixed and stained. Flow fixation of A6 cells, a procedure that aligns and flattens cilia, was performed essentially as described by Wheatley and Bowser [2000].

Coverslips were examined on a Nikon Diaphot microscope equipped with 40X and 100X oil immersion objectives. Digital images were obtained with a Nikon CoolPix 990 camera. Figures were assembled using Adobe Photoshop software. To ensure that the images of cilia were accurate, in particular for length measurement, 3-5 pairs of through-focus images of acetylated α -tubulin and NDP kinase staining were obtained from each field. Measurements of apparent ciliary length were performed on magnified images using Corel Draw. Results shown are representative of 3-15 independent experiments.

Isolation of primary cilia

Primary cilia were isolated from A6 cells grown in 150 mm. Cells were rinsed gently with PBS, layered with 10 ml PBS and the plates were placed in a rotary shaker at 360 rpm for 4 min at room temperature. To monitor deciliation as well as cell integrity, cells grown on coverslips were shaken under the same conditions, fixed and stained. The supernatants were removed and centrifuged at 1000xg for 10 minutes to remove cells and debris. The cilia were collected by centrifugation at 40,000xg for 30 minutes. Pellets were suspended in 20 mM Tris-HCl pH 8, 50 mM KCl, 4 mM MgSO₄, 1 mM DTT, 0.5 mM EDTA (resuspension buffer, RB; Hastie et al. [1986]) and analysed by immunoblotting. Six to 20 μ g of isolated primary cilia were obtained using four 150 mm dishes. Results shown are representative of 8 preparations.

To localize NDP kinase within primary cilia we developed a method based on isolation procedures for components of intraflagellar transport (IFT) processes [Cole et al., 1998]. To disrupt the outer membrane, pellets obtained at 40,000xg were suspended in 0.5-1 ml of RB buffer, frozen in liquid N₂, thawed and centrifuged at 16,000xg for 30 minutes. The resulting supernatant is the matrix, including soluble proteins. To remove proteins bound to the preparation in a nucleotide-sensitive manner, the pellet, which now contained axonemal and

membrane proteins, was extracted with 10 mM ATP in RB for 10 minutes on ice, and centrifuged as above. Membrane proteins in the ATP-stripped pellet were then solubilized with 0.5 % w/v Triton X-100 in RB on ice for 10 min. The final pellet contained the detergent-insoluble axonemal fraction.

For electrophoresis, TCA-precipitated supernatants and pellets were solubilized in urea sample buffer (4.6% SDS, 8 M urea, 150 mM Tris pH 8.0, 0.1% bromophenol blue, 20 mM DTT) by sonication, alkylated with 60 mM iodoacetamide, and resolved in 15% or 4-20% minigels. Proteins were transferred to nitrocellulose and immunoblotted. Individual band density was quantified using Maxim DL 3.0 (Diffraction Limited). Protein concentration was determined by the BCA assay (Pierce). Enzyme activity was determined in triplicate by the coupled assay as described [Yi et al., 1996].

Electron microscopy

Formvar-coated carbon grids (200-mesh) were floated on drops of a primary cilium suspension for 2 minutes. The grids were stained with 2% uranyl acetate and examined in a Phillips electron microscope at 80kV.

RESULTS

NDP kinase is present in primary cilia of NIH3T3 fibroblasts

Labeling of sub-confluent, serum-deprived NIH 3T3 fibroblasts with an antibody to a conserved region of NDP kinase A and B, Ab-1, produces a strong signal in the nucleus and in the cytoplasm, as previously described [Gallagher et al., 2003]. However, in cells grown at high density, Ab-1 labels an additional, rod-like organelle that varies in length from 1-5 μm . This structure is found near the nucleus of virtually all cells in the specimen (Figure 1A), and has the morphology of a primary cilium. Primary cilia contain stable microtubules that are enriched in a post-translationally modified form of tubulin, acetylated α -tubulin [Piperno et al., 1987]. When we labeled cells simultaneously with Ab-1 and antibodies to acetylated α -tubulin (Fig. 1) we observed overlap of the two labels in this structure (Fig. 1, merged). In addition, the antibody to NDP kinase labels the nucleus, (Figure 1), and acetylated α -tubulin is present in some cytoplasmic microtubules. However, as seen in the merged image (Fig. 1) and in the insets, the co-localization of the antigens is limited to the primary cilia, whose dual labeling is sufficiently well-defined to be readily visible in nearly all cells. These results indicate that NDP kinases A and B are a component of primary cilia of murine fibroblasts. Nevertheless, the cilia in these cells are diminutive, and as a result the details of the distribution of NDP kinases are difficult to discern. In contrast, other cell types such as kidney epithelial lines form polarized monolayers with apical primary cilia that are remarkably long. Therefore, we carried out the next set of experiments in the A6 cell line derived from the distal nephron of *Xenopus laevis*, using affinity-purified antibodies to amphibian NDP kinase to detect its localization.

NDP kinase localizes to primary cilia of A6 kidney epithelial cells

Localization of acetylated α -tubulin and NDP kinase was examined in A6 cells cultured in complete medium for 7-9 days post-confluence. Under these conditions the incidence of primary cilia is high, and they are far longer than those seen in NIH-3T3 cells. Our measurements show that in A6 cells cilia can extend up to 50 μm from the apical surface, with the average length being 23.4 μm (n=53). In flow-fixed preparations (Fig. 2, a,b) NDP kinase and acetylated α -tubulin co-localize along the shaft of the cilia; overlap is also seen in swellings at the tip of cilia (see below). To determine whether the co-localization of acetylated α -tubulin and NDP kinase in renal epithelial cells is indeed restricted to the primary cilium as seen in 3T3 cells, confluent A6 cells already expressing cilia were lifted gently, replated at lower density and allowed to adhere for 2 hours before staining. This approach reduces cell density without removing all primary cilia, allowing examination of the labeling pattern of individual cells. Figure 2(c,d) shows clear overlap of the two labels in the primary cilium. Together with the data on 3T3 cells, these results demonstrate that NDP kinase is a regular component of mature primary cilia.

In images taken at higher magnification, a distinctive feature of the distribution of NDP kinase in cilia becomes evident: differently from acetylated α -tubulin (Fig. 3a), NDP kinase is not evenly distributed inside the cilium. Rather, it is present in discrete spots distributed in an irregular pattern along the length of the cilia (Fig. 3b). Thus, the co-localization with acetylated α -tubulin is not complete, suggesting that NDP kinase is not a component of the axoneme. Also, these images show that NDP kinase is concentrated in the cilium, but not at the area around its base. This localization pattern is similar to those reported for proteins of unknown function that are linked to renal kidney disease, cystin [Hou et al., 2002] and inversin [Morgan et al., 2002].

NDP kinase distribution during primary cilium development in A6 cells

Our next objective was to determine whether NDP kinase is present throughout primary cilia development. To this end, we lifted subconfluent A6 cells that were not yet expressing cilia, plated them at high density, and stained the specimens at intervals with antibodies to acetylated α -tubulin and to NDP kinase. Primary cilia were absent from spreading cells (not shown), and became evident only when cells were 70-80% confluent (Fig. 4a). At this stage just a few cells had cilia; some of these structures were rather short and did not stain for NDP kinase (Fig. 4a, b, arrowhead), in contrast to the longer ones (Fig. 4b, arrow). These small cilia might be the vertebrate equivalent of the "procilia" of sea urchin embryos (Morris and Scholey, 1997), which are thought to be intermediates in the process of ciliary assembly. At 90-95% confluence cells were still dividing (see mitotic spindle in 4c), but the length and incidence of primary cilia had increased considerably, and all contained NDP kinase (Fig. 4d). Confluent cells fixed 9 days after plating expressed very long cilia (Fig. 4e) that stained strongly for NDP kinase (Fig. 4f). Surprisingly, analysis of the data by measuring cilia and determining whether they are positive or negative for NDP kinase shows that the translocation of this protein into primary cilia is abrupt, taking place when cilia reach 5-6 μm (Fig. 5). This observation indicates that NDP kinase is not involved in the early processes of primary cilia assembly, but becomes a part of these organelles as they mature and elongate, and that transport of NDP kinase into cilia occurs at a definite stage of cilium elongation. This suggests in turn that entry of NDP kinase into the cilium is a tightly regulated event. In a previous example of regulated movement of a protein into flagella, Aurora A kinase was rapidly translocated into *Chlamydomonas* flagella in response to rises in cAMP [Pan and Snell, 2000]. However, in contrast to NDP kinase, which appears during cilium formation, Aurora A is recruited to mature, pre-existing flagella.

NDP kinase is present in isolated primary cilia

NDP kinases A and B, as well as the amphibian NDP kinases, are by and large cytosolic proteins. Nevertheless, they are also found in the nucleus, in the plasma membrane of stimulated cells, and in association with the centrosome, cytoskeletal elements, vesicular compartments and sperm flagella (see Introduction). Biochemical approaches based on isolated 9+2 cilia and flagella have been useful in the identification of ciliary components, their localization inside the cilium and the interactions governing their import into these organelles. However, there are no published methods for isolation of primary cilia, most probably because in most cells they are minuscule and thus comprise a minute fraction of total cell volume [Gallagher, 1980]. The feasibility of a successful isolation is higher in A6 cells, which have a high incidence of long primary cilia when cultured for an extended period post-confluence (Figs. 2 and 4). Based on the observation that shear forces can easily lead to deciliation of specimens [Gallagher, 1980], we developed a procedure for isolating primary cilia from A6 cells using brief mechanical agitation in saline followed by differential centrifugation. To prevent damage to the cilia, we avoided detergents and chemicals such as dibucaine, as well as extremes of pH and high calcium. This mild approach also prevented lysis of cells, which remain largely intact after the procedure (data not shown), thus reducing contamination of the primary cilium fraction with proteins from the cell body.

Analysis of the protein composition of the preparation obtained through this method by SDS-PAGE and staining with Coomassie Blue demonstrates that this fraction differs considerably from whole cell extracts. The pattern seen in Figure 6A is very reproducible between different preparations, with the most prominent bands in the whole cilia fraction migrating at 31, 47, 54, 60, 82, 105 and 146 kDa.

Immunoblot analysis of the primary cilium fraction is shown in Figure 6B. Comparison of the signal for the ciliary marker acetylated α -tubulin in equal amounts (10 μ g) of whole cell extracts and primary cilium preparations (Fig. 6B, upper panel) shows a strong band at 54 kDa in the isolated primary cilium, but no detectable reaction with the whole cell extract, as expected from an antigen present at low levels in cells [Piperno et al., 1987]. Thus, the procedure used here yields a fraction highly enriched in a specific ciliary marker. More importantly, isolated primary cilia contain substantial amounts of NDP kinase, although proportionately less than whole cell lysates (70%; Fig 6B, bottom panel). Note that although three NDP kinases have been identified in *Xenopus* [Ouatras et al, 1997], these proteins are virtually identical in terms of amino acid sequence (>96% identity), size and isoelectric point and are not resolved by SDS-PAGE, thus only one band of about 18 kDa is seen in immunoblots.

Assay of the primary cilium fraction for NDP kinase activity shows a specific activity of 1 μ mol/mg/min, about 60% of the activity measured in whole cell lysates, 1.7 μ mol/mg/min, and in agreement with the estimate made by densitometry of immunoblots. Therefore, ciliary NDP kinase is active as an enzyme.

NDP kinase is present in the matrix and membranes of primary cilia isolated from A6 cells

Our next objective was to determine whether NDP kinase is present in primary cilia as a soluble matrix protein, an axonemal component or in association with the surface membrane. To do so, we fractionated isolated primary cilia by a procedure involving sequential freeze/thaw, nucleotide treatment and detergent extraction. Immunoblotting of these fractions indicates that NDP kinase is present in the matrix and membranes, but not in the axoneme-containing pellet (Fig. 6C). The subciliary distribution of NDP kinases in primary cilia differs markedly from to

the axonemal association of NM23-5, 7 and 9 in 9+2 cilia and flagella [Ostrowski et al., 2002, Munier et al., 2003]. Further, this lack of association with the axoneme confirms the observations made in intact cells, where the staining pattern of NDP kinase differs from that of acetylated α -tubulin (Fig. 3). Indeed, the bulk of acetylated α -tubulin was found in the axoneme, with lesser amounts in the detergent extract and only traces in the matrix (Fig. 6C). The presence of acetylated α -tubulin in fractions other than the axoneme was at first unexpected, given that acetylation of α -tubulin takes place after microtubules polymerize [Piperno et al., 1987]. However, Qin et al. [2004] have also found this protein in the membrane+matrix fraction of *Chlamydomonas* flagella, and suggested that soluble acetylated α -tubulin is a by-product of axonemal turnover, being returned to the cell body by retrograde intraflagellar transport (IFT).

Because nucleotides release NDP kinase from intracellular membrane vesicles [Gallagher et al., 2003], we sought to determine whether this is also the case for primary cilia membranes. However, there was no release of NDP kinase when we extracted the membrane + axoneme fraction with 1 mM GTP (not shown) or 10 mM ATP, indicating that distinct mechanisms underlie its binding to ciliary and intracellular membranes.

Appearance of isolated primary cilia

Preparations of primary cilia were examined by microscopy to assess yield and composition. Figure.7A shows that the fraction contains a large number of structures of varying length with the expected tubular shape. Most of these structures have a swelling at one end, which is probably the bulbous tip frequently seen in renal primary cilia (Fig. 7B). In some cases the tubular portion attached to the rounded tip is very short; furthermore, numerous structures have the characteristics of these globular tips, but no visible axoneme. Immunofluorescence

analysis of the isolated primary cilium fraction confirms that the preparation contains not only typical cilia, but also round structures of roughly the size of ciliary tips (Fig. 7B) that stain for both acetylated α -tubulin and NDP kinase (Fig. 7C). Structures that contain only NDP kinase, presumably cell debris, are also visible (Fig. 7C), and constitute an estimated 40-60% of the isolated primary cilia fraction.

Taken together, the data suggest that our isolation protocol causes rupture of the ciliary stem at different positions, and that often this break takes place at the very base of the distal swelling. The resulting ciliary tip is of particular interest, because it is the site of microtubule assembly and disassembly, as well as the area where IFT particles release their cargo, reload, and reverse direction, returning to the base of the cilium [Rosenbaum and Witman,2002].

DISCUSSION

Our data indicate that conventional NDP kinases are present in primary cilia of mammalian and amphibian cells, and are found in cilia from different cell types, namely fibroblasts and epithelial cells. These results were confirmed by the finding of NDP kinase in the matrix and membrane fractions of preparations enriched in primary cilia. Therefore, NDP kinases are universal components of primary cilia. Taken together with the finding that NDP kinases A and B are also observed in sperm flagella [Munier et al., 2003], we expect that these proteins will be eventually found in all cilia and flagella.

Cilia and flagella are made of 200-250 proteins [Rosenbaum and Witman, 2002], which are synthesized in the cell body. The compositions of the ciliary membrane and matrix are distinct from those of the plasma membrane and cytosol, indicating that strict sorting and targeting mechanisms allow only a limited set of proteins into these structures. This selectivity appears to be accomplished through a "filter" at the base of cilia, as well as through specific interactions with intraflagellar transport (IFT) particles [Rosenbaum and Witman, 2002; Scholey, 2003]. IFT is the process by which motors such as kinesin and dynein move large multimeric complexes of proteins along the axoneme using microtubule tracks. The possibility of a link between NDP kinase and IFT is suggested by the punctate staining pattern of NDP kinase along the ciliary shaft, which is reminiscent of that seen with IFT proteins [Cole et al., 1998, Deane et al., 2001]. Furthermore, the intraflagellar distribution of NDP kinase to the matrix and membrane fractions of primary cilia, but not to the axoneme, is also typical of components of IFT particles and motors [Cole et al., 1998; Qin et al. 2004]. On the other hand, IFT proteins are characteristically concentrated at the base of primary cilia [Cole et al. 1998, Taulman et al.,

2001; Deane et al., 2001], and NDP kinase is not. Hence, we expect that NDP kinase will turn out to be a cargo of IFT, but not an IFT component.

Axonemal length also seems to influence the function of the sensory machinery present in cilia. For example, measurements of the mechanosensory ability of MDCK cells during reciliation show that recovery of the flow-induced Ca^{2+} response takes place only when the growing cilia reach a critical length of 3-4 μm [Praetorius and Spring, 2003], which is comparable to the 5-6 μm threshold we find for NDP kinase translocation into the cilia of A6 cells. Furthermore, Nauli et al. [2003] found that the flow-sensing response of embryonic kidney cells, mediated by polycystins 1 and 2, is absent from cells with few, underdeveloped short cilia. By analogy, the length-dependent translocation of NDP kinase into the primary cilium of A6 cells suggests that it plays a role in the sensory machinery of cilia.

The observation that axonemal microtubules are dynamic structures and are constantly assembling and disassembling [Marshall and Rosenbaum, 2001; Song and Dentler, 2001] suggests a possible role for NDP kinase in cilia elongation and maintenance. Namely, microtubule turnover leads to the hydrolysis of considerable amounts of GTP, but cellular GTP levels are 5-10-fold lower than those of ATP, and diffusion may not be sufficient to provide an adequate GTP/GDP ratio along a thin, long cilium, in particular at the tip. Conventional NDP kinases can regenerate GTP from GDP and ATP at high rates, and are a source of GTP for endocytosis [Krishnan et al., 2001; Palacios et al. 2002]. We suggest that translocation of NDP kinase into growing cilia reflects increased GTP demand during microtubule disassembly, which would become significant only after the cilium reaches a preset length. Transport of NDP kinase into cilia at this stage would provide a source of GTP and allow for accelerated turnover of axonemal tubulin. Additionally, GTP regeneration by NDP kinase might sustain the activity of

ciliary GTPases involved in sensory signaling through G-protein-coupled receptors [Pazour and Witman, 2003] and in IFT (e.g. IFT27, Rosenbaum and Witman [2002]).

In summary, our immunofluorescence localization and biochemical studies provide evidence showing that canonical NDP kinases are standard components of primary cilia in fibroblasts and epithelial cells, and that entry of NDP kinases into the cilium takes place only after a critical length is reached. Furthermore, the development of the first procedure for the isolation of a fraction enriched in primary cilia from A6 cells allowed us to determine that NDP kinases are associated with the membranous and soluble compartments of cilia where transport and signaling processes take place, a location consistent with a role in GTP replenishment.

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FOOTNOTES

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†Abbreviations: NDP kinase, nucleoside diphosphate kinase; PBS, phosphate-buffered saline; IFT, intraflagellar transport; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride, RB, resuspension buffer.

FIGURE LEGENDS

Figure 1. NDP kinase localizes to the primary cilium in NIH3T3 fibroblasts

Subconfluent, serum-deprived cells were stained for NDP kinase (NDPK) and acetylated α -tubulin. The boxed areas are magnified in the insets. Arrowheads show primary cilia stained for NDP kinase; N, nucleus. Bar: 25 μ m.

Figure 2. Localization of NDP kinase in primary cilia of A6 kidney epithelial cells

A6 cells were grown in full medium for 7 days post-confluence and either fixed and stained directly (a,b), or lifted, replated at a lower density and stained after 2h (c,d). Acetylated α -tubulin (a,c); NDP kinase (b,d). Arrow in d shows primary cilium. Bars: 20 μ m

Figure 3. NDP kinase stains primary cilia in a punctate pattern.

A6 cells grown in full medium for 7 days post-confluence were fixed, stained with antibodies to acetylated α -tubulin (a) and NDP kinase (b) and photographed at high magnification. Arrows in b point to some of the dots where NDP kinase is concentrated. Bar: 10 μ m.

Figure 4. Localization of NDP kinase during primary cilium development.

Subconfluent A6 cells not yet expressing primary cilia were plated at high density and stained at intervals of 6h (a,b), 24h (c,d) and 9 days (e,f). Acetylated α - tubulin (a,c); NDP kinase (b,d). Arrow in (b) and (d) show some of the primary cilia staining for NDP kinase; arrows in (f) point to exceptionally long primary cilia. Arrowhead in (a,b) indicates a short cilium that stains strongly with anti-acetylated α -tubulin but lacks NDP kinase. A mitotic spindle stained by anti-acetylated α -tubulin is indicated by an asterisk (*) in (c). Bar: 25 μ m.

Figure 5. Translocation of NDP kinase into the primary cilium is length-dependent.

The apparent lengths of cilia were measured in images of cells treated as in Figure 4, fixed after intervals of 6 to 8h post-plating. Each cilium was measured using the image stained for acetylated α -tubulin and then categorized by visual inspection of 3-5 thru-focus images as positive or negative for NDP kinase; the result (NDPK + or -) was plotted against length.

Figure 6. Biochemical characterization of isolated primary cilia.

Primary cilia were isolated as described in Methods. Panels A and B: equal amounts of whole cell extracts (WC) and the fraction enriched in primary cilia were resolved by SDS-PAGE, followed by staining with Coomassie blue (panel A) or immunoblotting with antibodies to acetylated α -tubulin (AcTub) and NDP kinase (NDPK) (panel B). The size in kDa and the migration distance of molecular mass markers is shown between the two panels. The most prominent bands in the primary cilium fraction are indicated by dots. Panel C: Isolated primary cilia were fractionated into matrix, ATP-extractable (ATP) components, detergent extractable components and axoneme as described in Methods. Fractions were resolved by SDS-PAGE and immunoblotted as above.

Figure 7. The primary cilia fraction contains whole cilia as well as isolated ciliary tips.

Panel A: Electron micrograph of a primary cilium preparation negatively stained with uranyl acetate. Panel B: Merged fluorescence microscopy image of A6 cells doubly stained with antibodies to acetylated α - tubulin and NDP kinase. Note that, in order to preserve ciliary tips, these cells did not undergo flow fixation. Panel C: Merged fluorescence microscopy image of a primary cilium preparation stained as in B. Red: acetylated α - tubulin; green, NDP kinase; yellow, overlap regions. Arrows indicate primary cilia, and arrowheads point to ciliary tip-like structures. Bars: (a), 5 μ m; (b,c) 25 μ m.