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## Direct involvement of the isotype-specific C-terminus of $\beta$ tubulin in ciliary beating

Julia Vent<sup>1,\*</sup>, Todd A. Wyatt<sup>2</sup>, D. David Smith<sup>1</sup>, Asok Banerjee<sup>3</sup>, Richard F. Ludueña<sup>3</sup>, Joseph H. Sisson<sup>2</sup>, and Richard Hallworth<sup>1‡</sup>

<sup>1</sup>Department of Biomedical Sciences, Creighton University, Omaha, Nebraska 68178, USA

<sup>2</sup>Department of Internal Medicine, Section of Pulmonary, Critical Care and Sleep Medicine, University of Nebraska Medical Center, Omaha, Nebraska, USA

<sup>3</sup>Department of Biochemistry, University of Texas Health Science Center at San Antonio, San Antonio, Texas, USA

### Summary

In previous studies in *Drosophila*, Nielsen et al. hypothesized that the  $\beta$  tubulin C-terminal axonemal motif 'EGEFXXX', where X is an acidic amino acid, is required for ciliary function and assembly (Nielsen et al., 2001, *Curr. Biol.* 11, 529–533). This motif is present in some but not all mammalian  $\beta$  tubulin isotypes. We therefore investigated whether this motif is important in ciliary function in mammals. In a preparation of isolated, ATP-reactivated bovine tracheal cilia, we found that monoclonal antibodies directed against the C-terminus of  $\beta_{\text{I}}$ ,  $\beta_{\text{IV}}$  and  $\beta_{\text{V}}$  tubulin blocked ciliary beating in a concentration dependent manner. Antibodies against other epitopes of  $\beta$  tubulin were ineffective, as were antibodies against  $\alpha$  tubulin. Peptides consisting of the axonemal motif and motif-like sequences of these isotypes blocked ciliary beating. These results suggest that the axonemal motif sequences of  $\beta_{\text{I}}$ ,  $\beta_{\text{IV}}$  and  $\beta_{\text{V}}$  tubulin are essential for ciliary function. Peptides consisting of corresponding C-terminal sequences in  $\alpha$  tubulin isotypes were also ineffective in blocking ciliary beating, which suggests that the C-terminus of  $\alpha$  tubulin is not directly involved in cilia function in mammals.

### Keywords

Axonemal function;  $\beta$  Tubulin isotype; Cilia; Ciliary beat frequency

### Introduction

Microtubules are the main structures of cellular functions, such as the spatial distribution of organelles and cell motility. In addition, they constitute the scaffold within cilia and flagella. Microtubules are formed by protofilaments of  $\alpha\beta$  tubulin heterodimers stacked head to tail at 8 nm intervals (Meurer-Grob et al., 2001). The sequences of  $\beta$  tubulin isotypes are highly conserved in evolution and even isotypic differences are conserved (Ludueña, 1998). Their main differences lie in the C-terminal structure after helix H12 composed of 18 amino acid residues. Based on experiments with the testis-specific *Drosophila*  $\beta 2$  tubulin isotype, Nielsen et al. (Nielsen et al., 2001) suggested that a specific sequence in the C-terminus [termed here 'the axonemal motif' EGEFXXX, where X is either glutamic (E) or aspartic (D) acid] is required for correct axonemal function and assembly, assayed by fertility and electron

\* Author for correspondence (e-mail: hallw@creighton.edu)

‡ Present address: University Hospital of Cologne, Department of Otolaryngology, Head and Neck Surgery, Kerpener Straße 62, 50924 Cologne, Germany

microscopy. In mammals, this sequence is found in the C-terminus of  $\beta_{IV}$  tubulin at positions 433–439, but axonemal motif-like sequences are also found in  $\beta_I$ ,  $\beta_{II}$  and  $\beta_V$  tubulin in the same positions (see Table 1). In recent studies,  $\beta_I$  and  $\beta_{IV}$  tubulin were found in all mammalian axonemal structures tested, including the cilia of vestibular hair cells, olfactory sensory neurons and ciliated epithelial cells of the nose, trachea, ependyma, fallopian tube and testis (Jensen-Smith et al., 2003; Perry et al., 2003; Renthal et al., 1993; Woo et al., 2002). In olfactory neurons, other isotypes ( $\beta_{II}$  and  $\beta_{III}$  tubulin) were also present (Woo et al., 2002). Recently,  $\beta_V$  tubulin has been found sporadically in axonemal structures, but is apparently not required for ciliary function and assembly (R. Hallworth and R. F. Ludueña, unpublished data). The only isotypes common to all cilia appear to be  $\beta_I$  and  $\beta_{IV}$  tubulin, and their C-termini likely play a key role in ciliary function.

There are seven known isotypes of  $\beta$  tubulin in mammals. Their presence and composition are tissue-type specific, but no functional correlation has been made to date (Hallworth and Ludueña, 2000; Ludueña, 1993). To investigate the functional significance of the C-termini of  $\beta$  tubulin isotypes in mammals, we used a preparation of isolated, de-membrated ATP-activated beating bovine cilia (Hastie et al., 1986; Wyatt et al., 2005). We measured the effect on ciliary beat frequency (CBF) of isotype-specific monoclonal antibodies directed against the C-termini of tubulin and monoclonal antibodies directed against other epitopes of  $\beta$  tubulin, as well as monoclonal antibodies against various epitopes of  $\alpha$  tubulin, using a new, rapid digital video motility analysis method (Sisson et al., 2003). In addition, we examined the effect on CBF of peptides containing (1) the C-terminal amino acid sequences of  $\beta$  tubulin isotypes against which the antibodies were raised (C-terminal tail peptides, CTT peptides) and (2) the peptides containing the amino acid sequences of the isotype-specific axonemal motif or the closest equivalent. This is the first study to investigate the isotype-specific differences of tubulin isotypes in mammalian cilia in a functional, motility-based assay.

## Materials and Methods

### Preparation of cilia

Isolated bovine tracheal cilia were prepared from fresh tracheas by the method of Hastie et al. (Hastie, 1995; Hastie et al., 1986). Tracheas from bovine lungs were obtained from a local abattoir, dissected, cleaned from debris and rinsed in cold PBS (pH 7.4). The orifices were clamped and the tracheas were then incubated with approximately 200 ml of a Triton X-100-based cilia extraction buffer under continuous shaking for 90 seconds [after Hastie, containing Tris HCl (20.0 mM), NaCl (50.0 mM),  $\text{CaCl}_2$  (10.0 mM), EDTA (1.0 mM), 2-mercaptoethanol (7.0 mM), Triton X-100 (100.0 mM) and dithiothreitol (DTT, 1.0 mM) pH 7.4]. The fluid containing cilia was filtered through micromesh (pore size 0.45  $\mu\text{m}$ ). After centrifugation at 10,000 g at 4°C for 7 minutes, the supernatant was discarded and the pelleted cilia were resuspended in resuspension buffer [Tris HCl (20.0 mM), KCl (50.0 mM),  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$  (4.0 mM), EDTA (0.5 mM), DTT (1.0 mM), Soybean Trypsin Inhibitor (10.0 mM), pH 8.0]. The protein concentration was determined using a Bradford-dye assay. The cilia preparation was frozen in a 25% (by weight) sucrose solution in a concentration of one mg/ml in 100  $\mu\text{l}$  aliquots and stored at  $-80^\circ\text{C}$ .

### Activation of cilia and measuring CBF

A thawed aliquot of frozen demembrated cilia was mixed with resuspension buffer and cAMP (in resuspension buffer, final concentration 1.0 mM) in a 1:2:1 dilution (by volume). 20  $\mu\text{l}$  of the solution containing 0.25 mg/ml protein were then plated into wells of a 48 well culture plate (Corning Inc., Corning, NY). The plate was centrifuged at 400 g for 2 minutes at room temperature to attach the cilia to the bottom of each well. A first measurement was taken (time  $t=0$ ) and 20  $\mu\text{l}$  of ATP in resuspension buffer (Sigma, MO) (final concentration 1.25

mM) were added to activate ciliary beating, typically at a frequency of 8–10 Hz. Test solutions containing antibodies or peptides in resuspension buffer were added at time  $t=3$  minutes, after three readings of activated cilia. Measurements of CBF were made at intervals of 1 minute for 15–20 minutes. ATP depletion occurred at approximately 25 minutes after ATP addition. Reactivation of cilia was possible with further ATP addition, but was performed for control reasons only. For CBF measurements, antibodies and peptides were diluted in resuspension buffer.

CBF was measured using the rapid automated digital analysis system described earlier (Sisson et al., 2003). Cilia were visualized using phase contrast microscopy (Olympus IMT-2 inverted phase-contrast microscope, Olympus America Inc., Melville, NY) directly connected to a digital camera (Kodak Megaplug ES 310 analog/digital video camera; Eastman Kodak Motion Analysis System Division, San Diego, CA) and a PC workstation (Dell Inc., Round Rock, TX). CBF was determined using Fourier analysis of the entire field of view from the digitized video.

### Generation of antibodies against $\beta$ tubulin isotypes

Antibodies against the C-termini of  $\beta$  tubulin isotypes were raised in mouse hybridoma cells and purified as previously described (Banerjee et al., 1990; Banerjee et al., 1992; Banerjee et al., 1988; Roach et al., 1998). Each monoclonal antibody was prepared to an epitope unique to the C-terminus of that isotype. The C-termini of  $\beta_{IVa}$  and  $\beta_{IVb}$  are identical in amino acid sequence and therefore the antibody against  $\beta_{IV}$  tubulin was unable to discriminate between them. All antibodies used in this study were purified monoclonal IgG class 1 antibodies except for anti  $\beta_{II}$  and  $\beta_{III}$  tubulin, which were IgG2b (Table 2). The antibody against the  $\beta_V$  tubulin C-terminus has not been previously reported (A.B. et al., unpublished). The monoclonal antibody SHM 12G11 specific for mouse  $\beta_V$  tubulin was generated by immunizing mice with the C-terminal peptide EEEINE. The peptide, coupled to keyhole limpet hemocyanin (KLH), was used to immunize mice. The antibody was purified from the hybridoma supernatant using a protein-G-sepharose column. Initial immunization was performed with peptide-KLH while the subsequent immunizations were performed with peptide coupled to BSA, according to Banerjee et al. (Banerjee et al., 1988)

Other antibodies were commercially obtained. The details are summarized in Table 2. Antibodies were used at a concentration of 20  $\mu\text{g/ml}$ , which was within the range of concentrations used in several previous studies that employed antibodies to interfere with ciliary and flagellar beating (Bré et al., 1996; Audebert et al., 1999; Cosson et al., 1996; Gagnon et al., 1996). Controls to assure specific antibody binding to axonemal tubulin were performed by immunohistochemistry and immunoblotting.

### Visualization of antibody-labeled bovine cilia

Isolated cilia were plated onto concanavalin A-coated glass microscope slides (Sigma), allowed to settle for 1 hour and fixed using a solution of 1% paraformaldehyde in PBS. No permeabilizing step was necessary since the cilia are already demembranated. Bound antibodies were visualized using goat anti-mouse secondary antibodies conjugated to Alexa 488 (Molecular Probes, Eugene, OR). Specimens were viewed using an Axioskop II microscope (Carl Zeiss, Jena, Germany) equipped with 40X and 100X objectives and captured using a Spot RT digital camera (Diagnostic Instruments, Sterling Heights, MI). Images were prepared for presentation using Photoshop (Adobe Systems, San Jose, CA). Negative controls were performed by omitting the primary antibody. Positive controls for the isotype-specific  $\beta$  tubulin antibodies were performed by staining organ of Corti tissue. The results seen were in concordance with previous observations (Hallworth and Ludueña, 2000; Hallworth et al., 2000; Vent et al., 2004) (see supplementary material Fig. S1).

## Immunoblotting

Immunoblots were performed under standard conditions with all antibodies used in the above experiments. A uniform amount of homogenized axonemal proteins (15  $\mu$ g) was loaded onto each lane of a 10% polyacrylamide gel (Biorad, Hercules, CA). The protein content had been determined in a Bradford dye assay prior to aliquoting the extracted cilia using a microplate reader and protein (BSA) standards (MPM III 1.080, microplate reader, Biorad). The lanes were run at 100 V and 250 mA for 1 hour in sodium dodecyl sulfate electrophoresis running buffer. Proteins were then transferred from the gels to nitrocellulose sheets at 100 V and 100 mA for 1 hour in a transfer buffer containing Trizma base and glycine (Sigma). The nitrocellulose sheets containing the transferred protein lanes were then cut into strips and were exposed to one of the primary antibodies overnight at 4°C. After thorough rinsing with 1% milk in PBS, the secondary antibody (anti-mouse IgG linked to biotin, Cell Signaling Technology, Beverly, MA), was added and the strips were incubated on a rocker for 1 hour at room temperature. The protein strips were then rinsed three times in 1% milk in PBS for 10 minutes and twice in PBS for 10 minutes. They were then treated with SuperSignal West Pico Chemiluminiscent Substrate (Pierce Biotechnology, Inc., Rockford, IL) for 3 minutes and exposed to a CL-XPosure blue X-ray film (Pierce Biotechnology, Inc., Rockford, IL) for three seconds.

## Generation of peptides

Axonemal motif peptides were synthesized using a previously published solid phase method (Taylor et al., 2005). Briefly, N- $\alpha$ -butyloxycarbonyl (Boc)-amino acid derivatives (Bachem Biosciences Inc., King of Prussia, PA and ChemImpex, Wood Dale, IL) were coupled to methylbenzhydrylamine resin (Bachem Biosciences Inc., King of Prussia, PA) using the coupling reagent 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate in excess base. All coupling yields were >99% as determined by the quantitative ninhydrin method (Sarin et al., 1981). Boc groups were removed in trifluoroacetic acid (TFA, Acros Organics, Pittsburgh, PA). Peptides were cleaved from the resin using a mixture of trifluoromethanesulphonic acid/TFA/ethanedithiol/thioanisole (1/9/0.5/1, v/v) and immediately desalted on a BioGel P6 column (90X2.5 cm) (Bio Gel P6, Bio Rad, Hercules, CA) in 5% aqueous acetic acid. The desalted material was loaded on to a Vydac C18 HPLC column (25X2.5 cm) (Vydac C18 column, The Sep/a/rations Group, Hesperia, CA) previously equilibrated with a mixture of water and acetonitrile (9/1, v/v) (Fisher Scientific, Pittsburgh, PA) containing 0.1% TFA. The concentration of acetonitrile in the eluent was increased from 10 to 40% over 50 minutes to elute the peptides from the column. Fractions containing the desired peptides were pooled and lyophilized. Peptides were judged to be >95% pure by analytical RP-HPLC and possessed satisfactory amino acid compositions.

The peptides containing the sequences EGAEAAA, EGEAAAA, EDEDEGE and GEMYEDD were custom made peptides purchased from Bachem Biosciences Inc. (King of Prussia, PA). The C-terminal tail peptides were the peptides against which the  $\beta$  tubulin isotype specific antibodies were raised (see Table 1 and Table 2 for sequences). Their synthesis is described elsewhere (Banerjee et al., 1990; Banerjee et al., 1992; Banerjee et al., 1988; Renthal et al., 1993) (A.B. et al., unpublished). Unless otherwise indicated, chemicals were purchased from Sigma-Aldrich Co. (St Louis, MO).

## Results

### Antibodies against $\beta$ <sub>I</sub>, $\beta$ <sub>IV</sub> and $\beta$ <sub>V</sub> tubulin block ciliary beating

We hypothesized that the C-terminus of  $\beta$  tubulin has functional importance in ciliary beating. Thus, blocking this domain with antibodies should interfere with ciliary beating in isolated bovine cilia. On application of ATP solution (final concentration 1.25 mM) after the initial

reading at time  $t=0$ , isolated cilia began to beat continuously at about 10 Hz. The CBF reached a typical steady state value by time  $t=3$  minutes, at which time solutions containing the antibody were added at a final concentration of 20  $\mu\text{g/ml}$  (equivalent to 0.33  $\mu\text{M}$ ). Axonemes maintained a steady CBF until ATP was depleted, typically after 25 minutes. Fig. 1A shows the effect of application of antibodies on CBF. Each data point is plotted as the mean and one standard error of the mean for at least six experiments. Antibodies against  $\beta_{\text{I}}$ ,  $\beta_{\text{IV}}$  and  $\beta_{\text{V}}$  tubulin reduced CBF to zero within 8–10 minutes. By contrast, antibodies against  $\beta_{\text{II}}$  and  $\beta_{\text{III}}$  tubulin, as well as the buffer only controls (negative control), did not affect CBF. These results indicate that the isotype-specific antibodies against  $\beta_{\text{I}}$ ,  $\beta_{\text{IV}}$  and  $\beta_{\text{V}}$  tubulin interfere with the motility-determining structures of microtubules, and that only those isotype-specific antibodies raised against the C-terminal sequences of  $\beta_{\text{I}}$ ,  $\beta_{\text{IV}}$  and  $\beta_{\text{V}}$  tubulin are able to block ciliary beating.

### The effect of the antibodies on CBF is concentration dependent

We hypothesized that the inhibition of ciliary beating by antibodies would be specific and thus concentration dependent. To test this hypothesis, we examined the effect of antibodies at different concentrations. When the concentration of antibodies against  $\beta_{\text{I}}$ ,  $\beta_{\text{IV}}$  and  $\beta_{\text{V}}$  tubulin was decreased in a logarithmic manner, the time taken for reduction of CBF to zero was increased. Fig. 1B shows the effect of the antibody against  $\beta_{\text{I}}$  tubulin on CBF at varying concentrations (20  $\mu\text{g/ml}$ , 2  $\mu\text{g/ml}$  and 0.2  $\mu\text{g/ml}$  final concentrations, equivalent to 0.33  $\mu\text{M}$ , 0.033  $\mu\text{M}$  and 3.3 nM, respectively). Comparable results were obtained with antibodies against  $\beta_{\text{I}}$  and  $\beta_{\text{V}}$  tubulin (data not shown). Reduction of antibody concentration by at least two orders of magnitude from the initial concentration was required to reduce the effect of the antibodies to control levels.

### Preincubation of axonemes with antibodies inhibited reactivation

As a further test of the specificity of the antibody effect, we pre-incubated cilia with a molar excess (10  $\mu\text{M}$ ) of antibody for 1 hour on ice. The high concentration was chosen to assure all epitopes would be bound by antibody. After equilibration to room temperature, ATP was added to activate ciliary beating. Normal activation to a CBF of 10 Hz was achieved in all samples except the ones containing antibodies against  $\beta_{\text{I}}$ ,  $\beta_{\text{IV}}$  and  $\beta_{\text{V}}$  tubulin, which did not activate (see supplementary material Fig. S2).

### Antibodies against other epitopes of $\beta$ tubulin, or against $\alpha$ tubulin, have no effect on CBF

We hypothesized that the effects of the antibodies against the C-termini of  $\beta_{\text{I}}$ ,  $\beta_{\text{IV}}$  and  $\beta_{\text{V}}$  tubulin on CBF were specific to the C-terminus of tubulin isotypes. To test this hypothesis, we applied monoclonal antibodies against other epitopes of  $\beta$  and  $\alpha$  tubulin. Fig. 1C shows the effect on CBF of an antibody against a C-terminal, non-isotype-specific epitope of  $\alpha$  tubulin (DM1A) and an antibody against a non-isotype-specific epitope (within position 281–446) of  $\beta$  tubulin (TUB 2.1). The antibodies had no effect on CBF at the same concentration as the maximum concentration used in the above described studies. Even higher concentrations (200  $\mu\text{g/ml}$  or 0.66  $\mu\text{M}$ ) did not affect CBF (data not shown). Fig. 1D shows the effect of two monoclonal non-isotype specific antibodies raised against the C- and N-termini of  $\alpha$  tubulin, respectively. They had no effect on CBF at comparable concentrations. These experiments further support the inference that the effects of the antibodies against  $\beta_{\text{I}}$ ,  $\beta_{\text{IV}}$  and  $\beta_{\text{V}}$  tubulin were specific to the binding sites of those antibodies, and that axonemal motility is dependent on the C-terminal epitope of  $\beta$  tubulin isotypes or its direct proximity against which the antibodies are directed. Furthermore, the results indicate that there is no steric hindrance by ineffective antibodies, even against the C-terminus of  $\alpha$  tubulin, to the dynein binding site. They also exclude any non-specific inhibitory effect of the antibodies.



### Specific peptides reduce CBF

Nielsen et al. (Nielsen et al., 2001) hypothesized that a certain amino acid sequence, the axonemal motif, is required for ciliary function and assembly. Because of our antibody findings, we tested the axonemal motif hypothesis using synthesized heptapeptides containing the axonemal motif or amino acid sequences in corresponding positions of mammalian  $\beta_I$ ,  $\beta_{II}$ ,  $\beta_{III}$ ,  $\beta_{IV}$  and  $\beta_V$  tubulin, hypothesizing that the peptides would mimic the C-terminus of  $\beta$  tubulin and thus competitively inhibit beating. We compared their effects to the effects of the C-terminal tail peptides against which the antibodies were raised.

Fig. 2A–E and Table 3 show the results of these experiments. Each plot shows the effect on CBF of the axonemal motif peptide for each isotype (open red triangles), compared to the effect of the corresponding C-terminal tail peptide (open green circles), at the same molar concentrations of peptide (final concentration 0.33  $\mu$ M) as was used for the antibodies. Each plot also shows the average of a series of negative control experiments (filled squares). The amino acid sequences of the C-terminal peptides are given in Table 1 and Table 3.

The results of these experiments are shown in Fig. 2A–E and are summarized in Table 3. In general, the axonemal motif peptides were effective in reducing CBF while the C-terminal tail peptides were much less so. For example, the  $\beta_{IV}$  and  $\beta_V$  tubulin axonemal motif peptides completely abolished ciliary beating in 15 minutes or less, while the corresponding C-terminal tail peptides had only weak effects on CBF (Fig. 2D,E). Both the axonemal motif and C-terminal tail peptide of  $\beta_{III}$  tubulin were ineffective in reducing CBF (Fig. 2C), as expected since  $\beta_{III}$  tubulin is not present in the bovine cilia preparation. However, the axonemal motif peptide of  $\beta_{II}$  tubulin, which is also not present in bovine tracheal cilia, was effective in abolishing ciliary beating, whereas the corresponding C-terminal tail peptide had only marginal effect (Fig. 2B). Further, both  $\beta_I$  tubulin peptides were effective in reducing CBF (Fig. 2A).

It was observed that the content of acidic amino acids (E and D) accounts for much of the inhibitory effect on CBF. The axonemal motif-like peptides generally had five acidic amino acids (with the exception of the peptides of  $\beta_{II}$  tubulin and  $\beta_{III}$  tubulin that have four), while the C-terminal tail peptides have four acidic amino acids (with the exception of the peptides of  $\beta_I$  tubulin and  $\beta_{III}$  tubulin that have five and one, respectively). The C-terminal tail peptide of  $\beta_I$  tubulin (438EEAEEEE) was noted to be equally effective in reducing CBF as its axonemal motif peptide (433EEDFGEE).

### The effect of the axonemal motif peptide is concentration dependent

We hypothesized that the effect of the  $\beta_{IV}$  tubulin axonemal motif peptide EGEFEEE is specific and therefore concentration dependent. To test this, the peptide was added to the activated cilia preparation at concentrations of 0.33  $\mu$ M, 0.033  $\mu$ M and 3.3 nM. The highest concentration of peptide reduced CBF the fastest, while the lowest concentration was essentially ineffective (Fig. 2F).

### The central phenylalanine is important in the axonemal motif sequence

We observed that the axonemal motif peptides that reduced CBF contained, in addition to several acidic residues, a central F at position 436. Also, F436 is not present in the axonemal motif peptide of  $\beta_{III}$  tubulin, which did not reduce CBF. To test the hypothesis that F436 is important, we replaced F436 in the axonemal motif sequence (EGEFEEE) with alanine, resulting in the sequence EGAEAAA. As shown in Fig. 3A, this peptide had no effect on CBF, demonstrating the importance of F436.

## Axonemal motif-like sequences in the C-terminus of $\alpha$ tubulin are not involved in ciliary beating

An E-rich sequence similar to the axonemal motif is present in the C-termini of several  $\alpha$  tubulin isotypes between positions 441 and 447, but these sequences lack the central F (Table 4). To test the hypothesis that the C-terminus of  $\alpha$  tubulin is not involved in ciliary beating, we synthesized two peptides that represented the sequences of four  $\alpha$  tubulin isotypes: EGEGEEE ( $\alpha_{II}$ ,  $\alpha_{III}$ ) and EDEDEGE ( $\alpha_I$ ,  $\alpha_{IV}$ ) (Ludueña and Banerjee, 2005a; Ludueña and Banerjee, 2005b; Ludueña and Banerjee, 2005c; Ludueña and Woodward, 1975). Neither peptide affected ciliary beating at comparable concentrations to previous experiments, supporting the antibody observations that the C-terminus of  $\alpha$  tubulin may not be directly involved in ciliary beating (Fig. 1C,D and Fig. 3B).

## Antibodies are specific for axonemal tubulin

We hypothesized that the effects of antibodies against  $\beta_I$ ,  $\beta_{IV}$  and  $\beta_V$  tubulin on ciliary beating were specific for binding at the  $\beta$  tubulin isotype specific epitopes and are not due to unspecific inhibition of beating. To test this hypothesis, all antibodies were applied to fixed preparations of bovine cilia and processed for immunofluorescence as described in the Materials and Methods. Antibodies against  $\beta_I$ ,  $\beta_{IV}$  and  $\beta_V$  tubulin, which blocked ciliary beating, labeled cilia (Fig. 4). The same was observed for the antibodies against the C- and N-termini of  $\alpha$  tubulin, as well as for antibodies against conserved, non-isotype-specific epitopes in the  $\alpha$  and  $\beta$  tubulin protein (DM1A and TUB 2.1). However, they did not inhibit ciliary beating, making steric hindrance as a reason for CBF inhibition by anti  $\beta_I$ ,  $\beta_{IV}$  and  $\beta_V$  tubulin antibodies unlikely. As expected, antibodies against  $\beta$  tubulin isotypes not present in tracheal cilia (anti  $\beta_{II}$  and anti  $\beta_{III}$  tubulin) did not label isolated cilia in the described preparation (Fig. 4A,B).

Immunoblots of purified de-membrated cilia with all of the above mentioned primary antibodies indicated that labeling is restricted to a single band of molecular mass close to 55 kDa (Fig. 4B), the expected molecular mass of  $\beta$  tubulin (Ludueña, 1998). The amino acid sequence difference between  $\beta$  tubulin isotypes is so small that the isotypes are indistinguishable by molecular mass alone. Strong labeling appeared with anti  $\beta_I$ , DM1A and Tub 2.1 (antibodies against conserved, non-isotype specific epitopes of  $\alpha$  and  $\beta$  tubulin) as well as the C- and N-terminus specific antibodies against  $\alpha$  tubulin. As expected, antibodies against  $\beta_{II}$  and  $\beta_{III}$  tubulin did not label the axonemal proteins at all, because  $\beta_{II}$  and  $\beta_{III}$  tubulins are not synthesized in bovine tracheal cilia. A weak band occurred at 55 kDa with labeling by antibodies against  $\beta_{IV}$  and  $\beta_V$  tubulin. Controls for the well-established antibodies against  $\beta_{II}$  and  $\beta_{III}$  tubulin were performed by staining the organ of Corti (supplementary material Fig. S1).

## Discussion

### The axonemal motif is essential for ciliary function

Our findings suggest that the axonemal motif, EGEFEEE, is required for ciliary function and dynein binding. Generally, the results were consistent with the number of acidic amino acids (five E or Ds) determining its effectiveness, with the exception of the axonemal motif-like peptide of  $\beta_{II}$  tubulin, which contains only four acidic amino acids but is equally effective as the axonemal motif. Mizuno et al. (Mizuno et al., 2004) inferred that dynein and kinesin share an overlapping binding site on the tubulin C-terminus. That paper indicated that dynein binds to either  $\alpha$  or  $\beta$  tubulin, but not both and their results were suggestive of  $\beta$  tubulin being the target protein. Skiniotis et al. (Skiniotis et al., 2004) further support the importance of the tubulin C-terminus by its subtilisin digestion. However, we do not exclude the possibility that other regions of tubulin are also required, analogous to the weak and strong binding states of kinesin (Skiniotis et al., 2004). Nevertheless, findings from kinesin cannot definitively be

applied to dynein. Kinesin contains a lysine (K)-rich sequence on its tubulin-binding site, termed the K-loop, which acts as a counterpart to the acidic C-terminus of tubulin (E-hook) (Okada and Hirokawa, 2000). No such sequence exists in dynein (Asai and Koonce, 2001; Gee et al., 1997). Inferences drawn from the interaction of kinesin with tubulin to dynein must be made carefully.

### The axonemal motif sequence pivots around the central F

We found that, in general, C-terminal acidic residues (E or D) are required for ciliary beating. The higher the content of acidic amino acids in the peptide, the stronger was the inhibitory effect on beating. Further, we showed that this region must pivot around the central F436 found in some  $\beta$  tubulin isotypes. However, the study referred to earlier by Okada and Hirokawa did not evaluate dynein and did not focus on the isotype-specific differences in the C-termini of  $\beta$  tubulins (Okada and Hirokawa, 2000). In our study, the further requirement of a central F was established in addition to acidity as a key characteristic for axonemal function. In general, the C-terminal tail peptides, which did not contain F, were less effective than the axonemal motif peptides, with the exception of the peptides for  $\beta_I$  and  $\beta_V$  tubulin, which were equally effective. Our hypothesis is supported by the lack of effect of the two  $\alpha$  tubulin peptides (Fig. 3) which, despite being highly acidic, lack the central F.

### $\alpha$ tubulin is not directly involved in ciliary beating

Our evidence that  $\alpha$  tubulin is not directly involved in ciliary beating is, on first inspection, surprising. Three different antibodies against  $\alpha$  tubulin, two of which were directed against a conserved, non-isotype specific C-terminal amino acid sequence (419–435 and 426–450), had no effect on ciliary function. Furthermore, peptides of the  $\alpha$  tubulin axonemal motif sequence did not affect CBF. Our results are consistent with recent literature that suggests no involvement of  $\alpha$  tubulin in ciliary beating (Audebert et al., 1999; Cosson et al., 1996). Some earlier investigations favoured  $\alpha$  tubulin being important for ciliary function (Gagnon et al., 1996; Hirose et al., 1999). Goldsmith et al. (Goldsmith et al., 1991; Goldsmith et al., 1995) suggest the involvement of both  $\alpha$  and  $\beta$  tubulin for dynein binding (Goldsmith et al., 1991; Goldsmith et al., 1995). The role of  $\alpha$  tubulin might lie in other, yet undetermined functions. For example,  $\alpha$  tubulin may function in binding of microtubule-associated proteins (MAPs) (Rodionov et al., 1990) or interact with dynein for control of microtubule dynamics (Hunter and Wordeman, 2000). However, those studies were conducted on cytoplasmic dynein. Hunter and Wordeman showed that the C-terminus of tubulin is necessary for MAP binding and further suggested a tubulin-binding site of dynein outside the C-terminus. Hoenger et al. describe that each kinesin dimer occupies two microtubule-binding sites (Hoenger et al., 2000). That might be true for dynein also, but has not been investigated so far.

The periodicity of the  $\alpha\beta$  tubulin heterodimer in the microtubule is 8 nm. In cytoplasmic dynein, the step size has been proposed to be dependent on cargo load and to have a minimum of 8 nm (Mallik et al., 2004). If the step size of axonemal dynein is similar, dynein may skip  $\alpha$  tubulin and interact only with the C-terminus of  $\beta$  tubulin.

The sequences of the peptides EDEDEGE<sub>447</sub> (in  $\alpha_I$  and  $\alpha_{IV}$  tubulin) and EGEDEEE<sub>447</sub> (in  $\alpha_{II}$  and  $\alpha_{III}$  tubulin) were derived from Ludueña and Banerjee (Ludueña and Banerjee, 2005a). Due to the absence of isotype specific antibodies for  $\alpha$  tubulins, previous information on the possible distribution of  $\alpha$  tubulin isotypes was obtained by in situ hybridization. The presence of these  $\alpha$  isotypes (I–IV) in the bovine tracheal cilia preparation is plausible but not established (Ludueña and Banerjee, 2005a), thus there is a small possibility that the peptides have no effect due to the absence of these  $\alpha$  tubulin isotypes in bovine cilia. However, antibodies against various non-isotype-specific epitopes of  $\alpha$  tubulin, including the C-terminus, also did not affect CBF.



## Post-translational modifications may alter the secondary structure of the C-terminus

Post-translational modifications (PTM) in tubulins occur mainly at the highly flexible C-terminus (Ludueña, 1998). The most recently discovered PTM, polyglycylation, has been shown to occur in axonemal tubulin from *Paramecium* to sea urchin and mammalian spermatozoa (Bré et al., 1996). Bré et al. also suggested an involvement of polyglycylation in axoneme motility since AXO 49 and TAP 952, monoclonal antibodies against mono- and polyglycylation C-terminal peptides from *Paramecium* axonemal tubulin, specifically inhibited the reactivated motility of sea urchin spermatozoa. Polyglycylation occurs at the C-terminus and is highly variable in its amount (up to 32 glycines on a side chain off the  $\gamma$ -carboxyl chain of glutamic acids E435, E437 and E438). Polyglycylation has not yet been shown to be functionally required, but may affect the secondary structure of the highly flexible C-terminus in tubulin. Gagnon et al. reported that a different PTM, polyglutamylation, in the lateral chain of  $\alpha$  tubulin plays a dynamic role in spermatozoan motility (Gagnon et al., 1996). Possibly due to steric hindrance by the large antibody, Gagnon et al. could not determine the amino acid sequence required for ciliary beating.

## Isotype specificity of $\beta$ tubulin in axonemes

This leads us back to the initial question of the existence of functional correlations of  $\beta$  tubulin isotypes: why do some cells synthesize  $\beta$  tubulin isotypes in one pattern and others synthesize different isotypes? Why, if the isotypes are so similar in amino acid sequence, is there still a requirement for the different isotypes? The answer may lie in the highly variable C-terminus.

It was previously found that  $\beta_I$  and  $\beta_{IV}$  tubulin are synthesized by all ciliated cells types tested in the gerbil (Jensen-Smith et al., 2003). It was inferred that both isotypes are required for axonemal assembly and/or function. However, those observations were based on immunohistochemistry alone.  $\beta_V$  tubulin was recently found to be in some but not all motile cilia (R. Hallworth and R. F. Ludueña, unpublished observations). Therefore,  $\beta_V$  tubulin may be capable of supporting ciliary beating, but may not be absolutely required for ciliary function.  $\beta_{II}$  tubulin has so far not been detected in cilia. However, its axonemal motif-like sequence differs only in the first amino acid from the axonemal motif sequence of  $\beta_{IV}$  tubulin (E433 to Q433). Further, the axonemal motif peptide of  $\beta_{II}$  tubulin was nearly as potent as that of  $\beta_{IV}$  tubulin in blocking ciliary beating. Is  $\beta_{II}$  tubulin incompatible with the correct axonemal assembly or function? A change in only position 433 from glutamate to glutamine is observed, resulting in a removal of one charge in  $\beta_{II}$  tubulin. This may indicate that the secondary structure of the synthetic peptide resembles the axonemal motif peptide more closely than does the corresponding sequence in  $\beta_{II}$  tubulin. It is worth noting that the unusual  $\beta$  tubulin isotypes  $\beta_{II}$  and  $\beta_{III}$  are in some circumstances capable of supporting axonal assembly, if not motility. In the globose basal cells of the nose (the olfactory stem cells)  $\beta_I$ ,  $\beta_{II}$  and  $\beta_{III}$  tubulin are synthesized and incorporated into microtubules. As soon as the cell matures and develops long, immotile sensory cilia,  $\beta_{IV}$  tubulin is also synthesized (Woo et al., 2002). The isotypes do not compartmentalize, that is, all synthesized isotypes are incorporated into all microtubule structures in the cell, including the cilia. Thus  $\beta_{II}$  tubulin and  $\beta_{III}$  tubulin are at least capable of assembling into immotile cilia. It remains to be seen if  $\beta_{II}$  tubulin and  $\beta_{III}$  tubulin are capable of supporting ciliary motility and the assembly into a 9+2 structure, or if they disrupt these.

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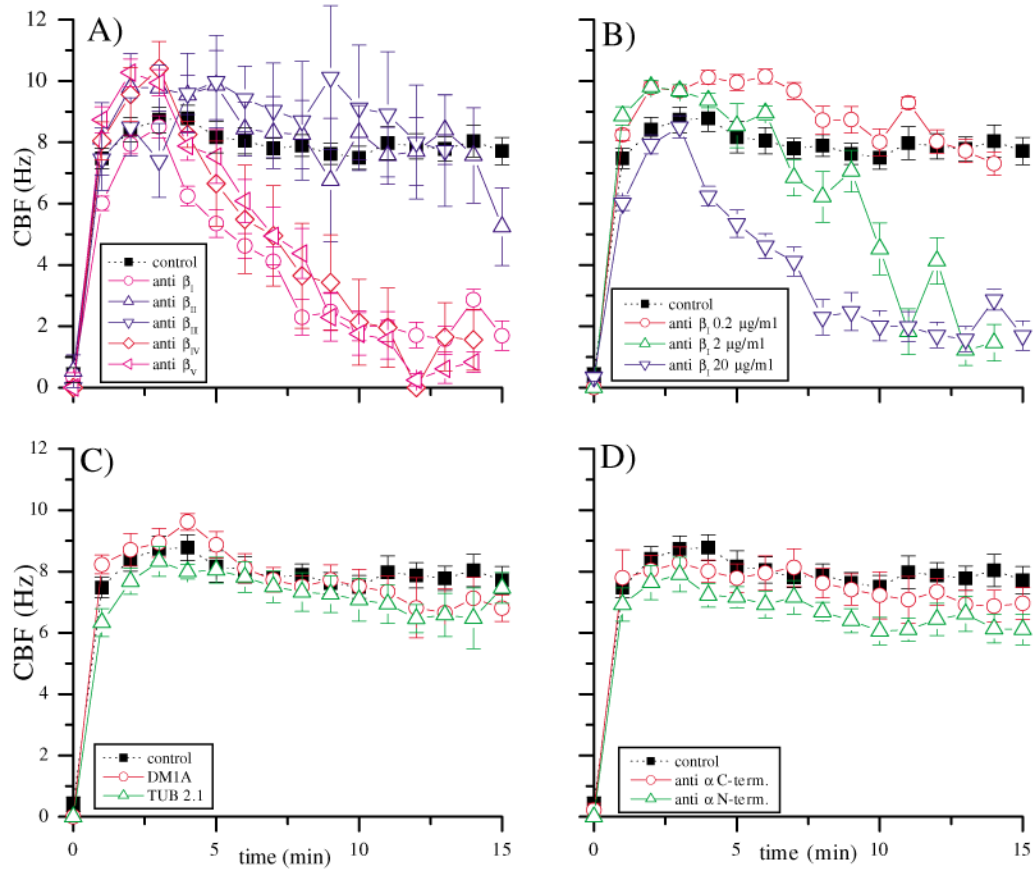
conducted in a facility constructed with support from Research Facilities Improvement Program grant 1 C06 RR17417-01 from the National Center for Research Resources, N.I.H.

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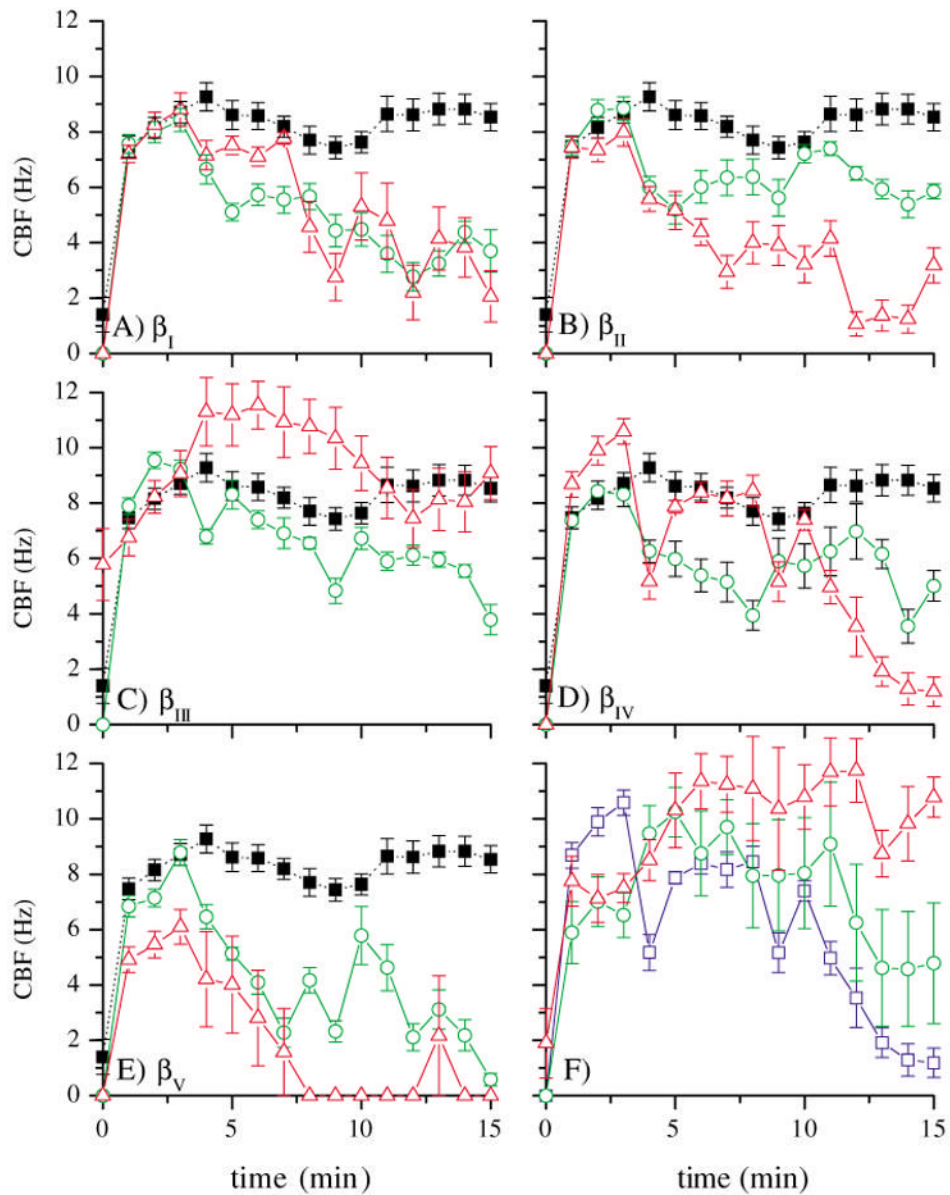
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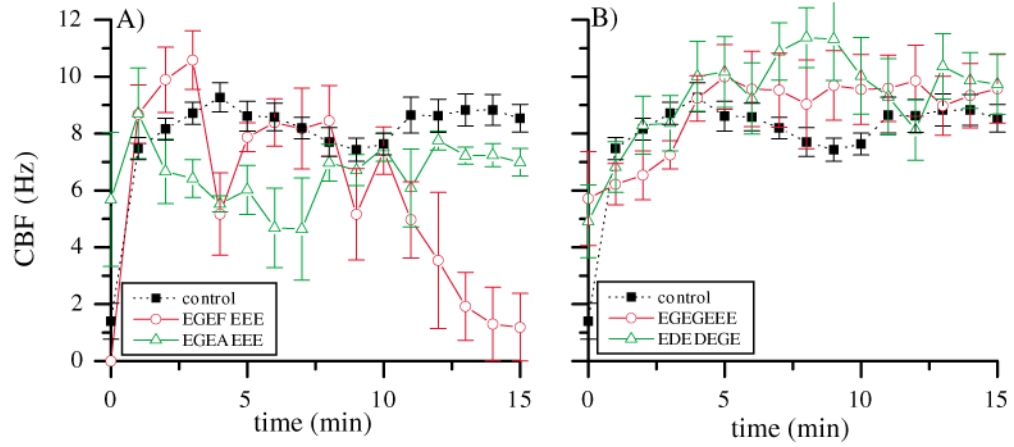
**Fig 1.** (A) Effect of isotype-specific antibodies against  $\beta$  tubulin on CBF. Final antibody concentrations 0.33  $\mu$ M, error bars indicate 1 s.e.m. Filled black squares, control; open pink circle, anti  $\beta_{I}$  tubulin; open upward blue triangle, anti  $\beta_{II}$  tubulin; open downward navy triangle, anti  $\beta_{III}$  tubulin; open red diamond, anti  $\beta_{IV}$  tubulin; open purple triangle, anti  $\beta_{V}$  tubulin. The antibodies depicted in shades of red blocked ciliary beating, the antibodies in blue had no effect on CBF. (B) Concentration dependence of anti  $\beta_{I}$  tubulin; error bars indicate 1 s.e.m. Antibody concentrations: blue triangle, 0.33  $\mu$ M; green triangle, 0.033  $\mu$ M; red circle, 3.3 nM. Similar concentration curves were obtained for anti  $\beta_{IV}$  tubulin and anti  $\beta_{V}$  tubulin. (C) Effect of non-isotype-specific, non-C-terminal antibodies against  $\alpha$  and  $\beta$  tubulin (DM1A, red circle and TUB 2.1, green triangle), error bars indicate 1 s.e.m.; antibody concentrations 0.33  $\mu$ M. (D) Effect of anti  $\alpha$  tubulin C- (red circle) and N-terminal (green triangle), non-isotype-specific antibodies, error bars indicate 1 s.e.m.; antibody concentrations 0.33  $\mu$ M.





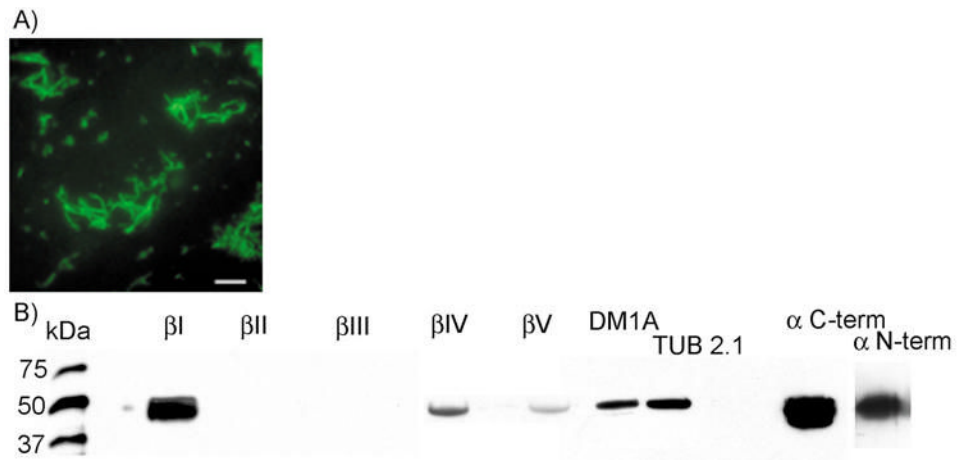
**Fig 2.**

Comparison of the effect of axonemal motif (red triangles) and C-terminal tail peptides (green circles) on CBF and controls (dotted line, black solid squares); error bars indicate 1 s.e.m.; peptide concentrations: 0.33  $\mu$ M. For sequences, see Table 1. (A) sequences derived from  $\beta_I$  tubulin (433EEDFGEE and 438EEAEEEA). (B) sequences derived from  $\beta_{II}$  tubulin (433QGEFEEEE and 440EGEDEA). (C) sequences derived from  $\beta_{III}$  tubulin (433GEMYEDD and 443SESQGPK). (D) sequences derived from  $\beta_{IV}$  tubulin (433EGEFEEEE and 439EAEVEVA). (E) sequences derived from  $\beta_V$  tubulin (433EEAFEDE and 441EEEINE). (F) Concentration-dependent effect of the axonemal motif peptide (EGEFEEE), concentrations: 0.33  $\mu$ M (blue squares), 0.033  $\mu$ M (green circles) and 3.3 nM (red triangles).



**Fig 3.**

(A) Alanine scan of the axonemal motif peptide: when the central F is replaced by an A, there is no more effect of the peptide on CBF, red circles, 433EGEF EEE, green triangles, 433EGEA EEE. Peptide concentrations: 0.33  $\mu$ M. Error bars indicate 1 s.e.m. (B) The effect of  $\alpha$  tubulin axonemal motiflike peptides on CBF, red circles, 441EGEG EEEE, green triangles, 441EDEDEGE. Peptide concentrations: 0.33  $\mu$ M. Error bars indicate 1 s.e.m.



**Fig 4.** (A) Example of a labeling of isolated bovine tracheal cilia by the isotype-specific antibody against  $\beta_{IV}$  tubulin. Scale bar 10  $\mu$ m. (B) Immunoblotting of isolated bovine tracheal cilia by antibodies against  $\alpha$  and  $\beta$  tubulin.

Relationship between the  $\beta$  tubulin C-terminal sequence, the axonemal motif peptide, and isotype-specific  $\beta$  tubulin C-terminal tail peptides, by residue position

**Table 1.**

AA	432	433	434	435	436	437	438	439	440	441	442	443	444	445	446
$\beta_I$	E	E	E	D	F	G	E	E	A	E	E	E	A		
$\beta_{II}$	E	Q	G	E	F	E	E	E	E	G	E	D	E	A	
$\beta_{III}$	E	G	E	M	Y	E	D	D	E	E	E	S	E	S	Q <sup>*</sup>
$\beta_{IV}$	E	E	G	E	F	E	E	E	A	E	E	E	Y	A	
$\beta_V$	G	E	E	A	F	E	D	E	D	E	E	E	I	N	E

The shaded peptides indicate the axonemal motif (in  $\beta_{IV}$ ) or its nearest equivalent (in  $\beta_I$ ,  $\beta_{II}$ , and  $\beta_V$ ). Notice that the axonemal motif and motif-like sequences pivot around F at position 436, indicated in red, whereas  $\beta_{III}$  contains the amino acid 436Y, shown in blue. The underlined peptides are those against which the isotype-specific antibodies used in this study were raised.

\*  $\beta_{III}$  tubulin is 449 amino acids long, and the C-terminal tail peptide is 443SESEQGPK.

AA, amino acid. Data from Ludueña, 1998.

Table 2.

## Epitopes of antibodies

Antibody	Epitope	Ig isotype	Host species	Concentration used for immunoblots	Concentration used for immunohistochemistry	Order number
$\beta_1$ tubulin	EEAEEDA	IgG1	Mouse	1:50,000	1:200	Sigma T7816
$\beta_1$ tubulin	EGEDEA	IgG2b	Mouse	1:500	1:500	Sigma T8535
$\beta_{III}$ tubulin	SESQFPK	IgG2b	Mouse	1:500	1:1000	Sigma T8660
$\beta_{IV}$ tubulin	EAEDEVA	IgG1	Mouse	1:5,000	1:400	Sigma T7941
$\beta_V$ tubulin	EEEEINE	IgG1	Mouse	1:1,000	1:200	Not commercially available
TUB 2.1	Unspecified conserved epitope within AA 281–446 of rat brain $\beta$ tubulin (Matthes et al., 1988)	IgG1	Mouse	1:10,000	1:200	Sigma T4026
DM1A	AA 426–450 of $\alpha$ tubulin (Breitling and Little, 1986)	IgG1	Mouse	1:30,000	1:200	Sigma T9026
$\alpha$ tubulin C-terminus	SEAREDMAALEKDYEEV (AA 419–435 of human $\alpha$ tubulin)	IgG1	Mouse	1:10,000	1:1000	Synaptic Systems # 302 201
$\alpha$ tubulin N-terminus	AA 65–97 of $\alpha$ tubulin	IgG1	Mouse	1:10,000	1:1000	Abcam ab7750



Table 3.

Effects of Peptides on CBF

Axonemal motif peptide (in $\beta_{IV}$ ) or heptapeptide in corresponding position	Effect	C-terminal tail peptide	Effect	Control peptides	Effect
$\beta_{IV}(43)EEDFGEE$	+	$\beta_{I}(43)EAEAEAA$	+	EGEAEAE	-
$\beta_{II}(433)QGEFEFE$	++	$\beta_{II}(440)EGEDEA$	-	EGEGEEE	-
$\beta_{III}(435)GEMYEDD$	-	$\beta_{III}(443)SESQGPK$	-	EDEDEGE	-
$\beta_{IV}(433)EGEFEFE$	+++	$\beta_{IV}(439)EAEVEA$	++		
$\beta_{V}(433)EAEFEDE$	+++	$\beta_{V}(441)EEENE$	++		

-, no effect; +, some inhibitory effect; ++, moderate inhibitory effect; +++, strong inhibitory effect leading to reduction of CBF to 0 Hz

Table 4.

Comparison of  $\alpha$  tubulin sequences

AA	438	439	440	441	442	443	444	445	446	447	448	449	450	451
$\alpha_1$	D	S	Y	E	D	E	D	E	G	E	E	E	E	Y
$\alpha_{II}$	D	S	V	E	G	E	G	E	E	E	G	E	E	Y
$\alpha_{III}$	D	S	V	E	G	E	G	E	E	E	G	E	E	Y
$\alpha_{IV}$	D	S	Y	E	D	E	D	E	G	E	E	E	Y	
$\alpha_{V/II}$	D	S	V	E	A	E	A	E	E	G	E	E	Y	
$\alpha_{V/III}$	D	S	F	E	E	E	N	E	G	E	E	F		

Data from Ludueña and Banerjee, 2005a. Shaded peptides indicate axonemal motif-like sequences.