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The kinesin-immunoreactive homologue from *Nicotiana tabacum* pollen tubes: Biochemical properties and subcellular localization

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Abstract. In plant cells, microtubule-based motor proteins have not been characterized to the same degree as in animal cells; therefore, it is not yet clear whether the movement of organelles and vesicles is also dependent on the microtubular cytoskeleton. In this work the kinesin-immunoreactive homologue from pollen tubes of *Nicotiana tabacum* L. has been purified and biochemically characterized. The protein preparation mainly contained a polypeptide with a relative molecular weight of approx. 100 kDa. This polypeptide bound to animal microtubules in an ATP-dependent manner and it further co-purified with an ATPase activity fourfold-stimulated by the presence of microtubules. In addition, the sedimentation coefficient (approx. 9S) was similar to those previously shown for other kinesins. Immunofluorescence analyses revealed a partial co-distribution of the protein with microtubules in the pollen tube. These data clearly indicate that several properties of the kinesin-immunoreactive homologue are similar to those of kinesin proteins, and suggest that molecular mechanisms analogous to those of animal cells may drive the microtubule-based motility of organelles and vesicles in plants.

Key words: Kinesin – *Nicotiana* – Organelle movement – Pollen tube

Introduction

Kinesin is a microtubule-based, ATP-dependent motor protein thought to play an important part in certain eucaryotic cell functions, such as the translocation of membranous organelles and vesicles (Vale et al. 1985; Schroer et al. 1988; Pfister et al. 1989; Brady et al. 1990; Hollenbeck and Swanson 1990; Hirokawa et al. 1991; Rodionov et al. 1991; Wright et al. 1991; Leopold et al. 1992) and the organization of the endoplasmic reticulum (Dabora and Sheetz 1988; Hollenbeck 1989; Houliston and Elinson 1991). An additional role in anaphase chromosome movement was initially proposed on the basis of immunolocalization analyses (Scholey et al. 1985; Leslie et al. 1987; Neighbors et al. 1988), even though some recent observations have challenged this possibility (Pfister et al. 1989; Saxton et al. 1991; Wright et al. 1991). Kinesins have been identified in many different tissues and organisms mainly by means of their mechanochemical property to move microtubules or to translocate isolated organelles and-or anionic beads towards the plus end of microtubule tracks. Initial observations showed that kinesin molecules are usually composed of two heavy chains with a molecular weight of 105–130 kDa and one or two light chains of 60–80 kDa (reviewed by Vallee and Shpetner 1990). The native molecular weight of kinesin was estimated to be approx. 380 kDa by both analyses of sedimentation equilibrium (Kuznetsov et al. 1988) and calculations of sedimentation and diffusion coefficients (Bloom et al. 1988). Electron-microscopical and biochemical investigations showed that kinesins are molecules approx. 80 nm long, with two globular heads containing the ATP- and microtubule-binding sites, and a stalk region terminating in a feathered tail probably involved in connecting cytoplasmic organelles (Amos 1987; Ingold et al. 1988; Hirokawa et al. 1989; Kuznetsov et al. 1989; Scholey et al. 1989; Yang et al. 1990). Recently, molecular genetic investigations in different organisms and many cell types have led to the identification of polypeptides showing an extensive degree of homology with the kinesin heavy chain and collectively called kinesin-like proteins. Such homology is particularly high in the ATPase motor domain of the polypeptides, but much lower in the rest of the molecules. These proteins seem to participate in a wide array of phenomena, but most of them are involved in cell division processes, including separation of spindle pole bodies, segregation of chromosomes, and fusion of nuclei. Because of the partial homol-

Abbreviations: AE-LPLC = anion-exchange low-pressure liquid chromatography; AMPPNP = 5'-adenylylimidodiphosphate; PKH = pollen kinesin homologue; SDS-PAGE = sodium dodecyl sulfate polyacrylamide gel electrophoresis

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ogy of the amino-acid sequences and the distinct functions they show, kinesins and kinesin-like proteins are grouped into a supergene family (for review see Bloom 1992; Epstein and Scholey 1992).

The movement of organelles and vesicles in plant cells has been usually correlated with the presence of actin filaments and the activity of myosin-related motor proteins (Emons et al. 1991). Plant microtubule translocators are also beginning to be characterized, as in the phragmoplast of cultured tobacco cells (Asada et al. 1991). In pollen tubes, cytoplasmic movement has been associated with the presence of myosin polypeptide(s), whereas the role of microtubules in the motility process, as well as the eventual molecular mechanisms of motility, has been ambiguous (Pierson and Cresti 1992). More details are available about the organization of the microtubular cytoskeleton in the angiosperm pollen tube, where it has been described, mainly by using immunofluorescence and electron microscopy, as an array of filamentous structures departing from the grain and extending the entire length of tube. Microtubules have not always been observed in the pollen-tube apex, where a diffuse staining indicated the presence of a tubulin-subunit pool (reviewed by Tiezzi 1991; Pierson and Cresti 1992).

It was previously reported that a monoclonal antibody (k71s23) to the heavy chain of bovine brain kinesin specifically reacted with two polypeptides at approx. 100–108 kDa in cytosolic extracts from *Nicotiana tabacum* pollen tubes (Tiezzi et al. 1992). The polypeptides were immunolocalized in the pollen-tube apex with an organelle-like staining pattern which was removed by Triton X-100 treatment before fixation. Furthermore, like many kinesins, the polypeptides (mainly those of 100 kDa) bound to animal microtubules after treatment with adenylylimido-diphosphate (AMPPNP). Consequently it was thought that the pollen tube contained a membrane-residing immunoreactive homologue of mammalian kinesin (from now on referred to as the pollen kinesin homologue [PKH]). Although this was the first evidence for the presence of a potential microtubule-dependent organelle translocator in plant cells, the involvement of the protein in tube growth and development remained to be established.

In order to provide further information about the structure and function of PKH, we developed a procedure for the purification of the protein from tobacco pollen tubes. In this work, using several approaches, we show that PKH has biochemical, and functional properties typical of the kinesin class, indicating that it could participate in translocation of cytoplasmic organelles and-or vesicles.

Materials and methods

Materials. Plants of *Nicotiana tabacum* L. were grown in greenhouses at the Botanic Garden, University of Siena, Italy. After anther dehiscence, pollen was dried over desiccant and stored at −20°C. Electrophoretic and immunoblotting reagents and dithiothreitol (DTT) were obtained from BioRad Laboratories (Richmond, Cal., USA); GTP was obtained from Boehringer (Mannheim, FRG). All other chemicals (unless mentioned in the text) were purchased from Sigma (St. Louis, Mo., USA). Taxol was a generous gift from Dr. Matthew Suffness of the National Cancer Institute (Frederick, Md., USA).

Electrophoresis and immunoblotting. Sodium dodecyl-sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of polypeptides collected in various fractions was done according to the method of Laemmli (1970). Proteins were separated on 4–16% gradient gels (3% for stacking) and stained with Coomassie Blue R250. The SDS-PAGE of the binding assay experiment was done on 7.5% linear gels (0.75 mm thick) in a Mini-PROTEAN II System (BioRad, Milano, Italy). Molecular-weight standards, including rabbit muscle myosin heavy chain (200 kDa), *E. Coli* β-galactosidase (116 kDa), rabbit muscle phosphorylase b (97 kDa), bovine serum albumin (BSA; 66 kDa), ovalbumin (42 kDa), were purchased from BioRad.

For immunoblotting, unstained duplicate gels were electrophoretically transferred to nitrocellulose sheets (Schleicher and Schuell, Dassel, FRG, pore size 0.45 μm) as described by Towbin et al. (1979). Membranes were air-dried and then blocked for 1 h with BTBS [2% BSA in Tris-buffered-saline (TBS: 20 mM Tris-HCl, pH 7.5; 150 mM NaCl)]. After incubation for 1 h with the first antibody, k71s23 (Tiezzi et al. 1992; dilution factor 1:5), or a monoclonal antibody to α-tubulin [Amersham International, Amersham, UK] (dilution 1:2,000), both in BTBS, two 15-min washes with the BTBS solution were done. The second antibody (goat anti-mouse Ig, horseradish-peroxidase-conjugated; Cappel Laboratories, Durhan, N.C., USA) was then added at a dilution of 1:4,000 in BTBS and incubated for 1 h. Strips were washed twice (30 min) with the BTBS solution and then twice (10 min) with TBS. The peroxidase reaction was developed in TBS with 0.6 mg · ml^{-1} 4-chloro-1-naphthol and 0.4 μl · ml^{-1} of 30% H_2O_2. The reaction was stopped by rinsing the membranes several times with H_2O. Controls were made by omitting the first antibody. In that case, no staining was detected.

Gels and blots were photographed with a Nikon camera using 35-mm Technical Pan films at 50 ASA (Kodak, Rochester, N.Y., USA).

Protein concentration. The BioRad protein assay reagent, based on the protein-dye binding method of Bradford (1976), was used to quantify protein concentration; BSA was used as the protein standard.

Purification of the PKH. Pollen grains of *Nicotiana tabacum*, hydrated for 2 h, were cultured for 4 h at room temperature (20°C) in BK medium containing 15% sucrose (Brewbaker and Kwack 1963). Germinated pollen grains were collected with a 7-μm filter and then washed thoroughly in HEM buffer [20 mM Na-N-(2-hydroxyethyl)piperazine-N'-2-ethansulfonic acid (Hepes), pH 7.5; 1 mM ethylene glycol bis (β-amino-ethylether)-N,N,N',N'-tetraacetic acid (EGTA); 2 mM $MgCl_2$] containing 15% sucrose. Pollen tubes were subsequently disrupted on ice using a homogenizer (Potter-Elvehjem, Zurich, Switzerland). The extraction buffer was HEM supplemented with 1 mM DTT and protease inhibitors [1 mM phenylmethylsulfonyl fluoride (PMSF); 10 μg · ml^{-1} leupeptin; 0.1 mg · ml^{-1} *p*-tosyl-L-argininemethyl-ester (TAME)] (modified from Neighbors et al. 1988). The homogenate was spun at 15,000 · *g* for 20 min at 4°C in a SS-34 rotor (Sorvall, Bad Nauheim, FRG), the pellet was discarded and the supernatant ultracentrifuged at 175,000 · *g* for 90 min at 4°C in a Sorvall T-865 rotor. The resultant high-speed supernatant (total protein amount: approx. 30 mg) was treated for 30 min at room temperature with hexokinase (0.41 mg · ml^{-1}) and D(+)glucose (50 mM) to deplete the endogenous ATP, then used immediately or stored at −80°C for no more than a week.

The pollen tube extract was then fractionated by anion-exchange low-pressure liquid chromatography (AE-LPLC), performed using fast protein liquid chromatography (FPLC) instrumentation (Pharmacia, Uppsala, Sweden) and an Econo Pac-Q anion-exchange cartridge (BioRad). Proteins were eluted with a linear gradient of 0–0.5 M NaCl in HEM buffer (from now on supple-

mented with 0.1 mM PMSF and 0.5 mM DTT, unless otherwise mentioned). Milli-Q water (Millipore, Roma, Italy) was used in the preparation of the buffers, which were subsequently filtered (pore size 0.22 μm) and then degassed for 30 min under vacuum before use. Porcine thyroglobulin and BSA were used each time to standardize the cartridge. The high-speed supernatant was exchanged into HEM buffer by using a PD-10 column prepacked with Sephadex G-25 (Pharmacia) and then freed of precipitates by centrifugation. The chromatographic run was done at room temperature (20°C) with a flow rate of 2 ml · min^{-1} and monitored by absorbance at 280 nm. Fractions of 1 ml were collected, immediately put on ice to minimize proteolytic cleavage and then assayed by immunoblotting with the k71s23 anti-kinesin antibody.

Immunolabeled fractions were pooled (approx. 8 ml) and concentrated to 1.5 ml at 4°C in a Centricon 30 microconcentrator (Amicon Corp., Beverly, Mass., USA). The sample was subsequently loaded onto linear gradients of 5–25% sucrose (0.3 ml of sample for each gradient) prepared according to the method of Baxter-Gabbard (1972) by three cycles of freeze-thawing 4.5 ml of 15% sucrose in HEM buffer. The linearity of the sucrose gradients was monitored by using 2,6-dichlorophenol-indophenol. After sedimentation at 83,000 · *g* for 15 h at 4°C in a Sorvall AH-650 rotor, samples were collected from the bottom of the gradients into 20 fractions of approx. 0.25 ml each. Porcine thyroglobulin (19.1 S), rat liver catalase (11.3 S), barley β-amylase (8.9 S), BSA (4.4 S) and chicken egg white lysozyme (2.2 S) were used as standards of known sedimentation coefficient ($\times 10^{-13}$, according to Martin and Ames 1961). Fractions were immediately assayed by immunoblotting with the k71s23 antibody and the peak fractions of the PKH were pooled (1.5 ml).

Immunolabeled sedimentation fractions were further fractionated at 4°C by gel filtration chromatography with a Bio-Gel A-1.5m column (45 cm long, 1 cm i.d.; BioRad) equilibrated in HEM buffer and standardized by running phenol red (included) and blue dextran 2000 (excluded). A flow rate of 0.06 ml · min^{-1} was precisely maintained by using an EP-1 Econo Pump from BioRad. Fractions of 0.75 ml were collected and monitored by absorbance at the wavelength of 280 nm. Fractions were again assayed by immunoblotting with the k71s23 antibody. To estimate the molecular weight of the PKH, we used a combination of the sedimentation ($S_{20,w}$) and diffusion ($D_{20,w}$) coefficients (Bloom et al. 1988), respectively determined by sucrose density gradient centrifugation and gel filtration. Markers of known diffusion coefficient ($\times 10^{-7}$ · cm^2 · s^{-1}) were porcine thyroglobulin (2.6), barley β-amylase (5.77) and egg white lysozyme (11.2).

Preparation of microtubules and kinesin from bovine brain. Microtubules were assembled from purified bovine brain tubulin (Fellous et al. 1977) by three cycles of temperature-dependent polymerization and depolymerization. Microtubule-associated-proteins (MAPs) were separated by a cation-exchange chromatography on P11 phosphocellulose (Whatman, Maidstone, UK) with a stepwise gradient of NaCl. Tubulin-containing fractions were made 1 mM in GTP and then subsequently stored at −80°C. Prior to use, tubulin was thawed and incubated at room temperature for 30 min with 30 μM taxol. The sample was centrifuged at 20,000 · *g* for 30 min at 25°C. The supernatant was discarded and the pellet resuspended in a GTP-free buffer, then used.

Kinesin was purified as previously described (Tiezzi et al. 1992), with the following exceptions. After ATP-extraction, kinesin was further purified by gel filtration chromatography using a Bio-Gel A-1.5m column (45 cm long, 1 cm i.d., BioRad). The sample was eluted with HEM buffer (see below for composition) containing 0.5 mM DTT and 0.1 mM PMSF. Positive fractions were collected and mixed.

Assay of ATPase activity. Hydrolysis of ATP was measured using the colorimetric method of Gonzalez-Romo et al. (1992), with some minor modifications. For the assay, 25 μl of HEM buffer were added to 100 μl of each sample fraction. The reaction was started by adding 25 μl of 12 mM Mg-ATP. After incubation at 25°C or 37°C for 45 min, the reaction was stopped by adding 150 μl of 12% SDS. Color development was started with the addition of 300 μl of phosphate reagent (6% ascorbic acid, 1% ammonium molybdate in 1 N HCl) to the reaction mixture. After 5 min at room temperature, 450 μl of 2% sodium citrate, 2% sodium *m*-arsenite in 2% acetic acid were added. Color development, due to the formation of a phosphomolybdate complex, was measured after 20 min at room temperature at 850 nm with a spectrophotometer (Shimadzu, Kyoto, Japan). The amount of liberated phosphate (expressed as nmol · min^{-1}) was calculated by comparing the absorbance values to those of a standard curve of inorganic phosphate at known concentrations.

To determine the stimulation of the Mg-ATPase activity, 25 μl of taxol-stabilized bovine brain MAP-free microtubules (final concentration: 1 mg · ml^{-1}) were pre-incubated with the samples for 15 min at room temperature before adding ATP. The specific ATPase activity of microtubules used in the assay (about 0.1 nmol Pi · min^{-1} · ml^{-1}) was subtracted from the reported activities.

Microtubule-binding assay. The immunoblot-positive peak fraction from the gel filtration step was assayed for microtubule binding. Briefly, 75 μl of the gel filtration PKH was mixed with 7.5 μl of 6 mg · ml^{-1} taxol-stabilized MAP-free bovine brain microtubules (final concentration: 0.5 mg · ml^{-1}) and 7.5 μl of either HEM buffer, 60 mM AMPPNP, or 120 mM ATP (final concentration: 5 mM and 10 mM, respectively) to give a volume of 90 μl. Finally, 2 μl of 500 μM taxol was added to stabilize the microtubules. Samples were incubated for 30 min at room temperature (25°C) and then centrifuged at 20,000 · *g* for 30 min at 25°C in a Sorvall SS-34 rotor. Supernatants and pellets (completely resuspended in 92 μl of HEM buffer) were processed for SDS-PAGE. In some experiments, the buffer- and AMPPNP-pellets were washed in 92 μl of HEM buffer containing 10 mM ATP and 10 μM taxol, then incubated for 30 min at room temperature. After centrifugation (conditions as above), the ATP supernatant and pellet were analyzed by SDS-PAGE.

As a control, 7.5 μl of 6 mg · ml^{-1} microtubules was added to 84.5 μl of HEM buffer and subjected to the same treatment without incubation with the PKH.

Immunofluorescence microscopy. Immunofluorescence microscopy on pollen tubes was performed as previously described for the labeling of the PKH (Tiezzi et al. 1992), with the only exception that cellulase (Sigma, Deisenhofen, FRG) was used instead of cellulysin. To visualize the microtubular cytoskeleton, a monoclonal antibody to α-tubulin (Amersham) was utilized at a final dilution of 1:150. Secondary antibody was a fluorescein-isothiocyanate (FITC)-conjugated goat anti-mouse IgG (1:80, Cappel). For double labeling, a rabbit antiserum to *Hibiscus* tubulin (a kind gift of Dr. R. Cyr, Pennsylvania State University, Pa., USA) was used at a dilution of 1:50. The specificity of this antiserum had been previously tested by immunoblotting on tubulin preparations from *Nicotiana* pollen tubes (Tiezzi et al. 1987). The second antibody was a Texas Red-labeled goat anti-rabbit IgG (Cappel) at a dilution of 1:50. All antibodies were diluted in phosphate-buffered-saline (PBS; 0.137 mM NaCl, 2.7 mM KCl, 1.47 mM KH_2PO_4, 8.1 mM Na_2HPO_4), pH 7.2. Coverslips were mounted in Citifluor (Agar Aids, Essex, UK) and observed with a Zeiss (Oberkochen, FRG) Axiophot conventional epifluorescence microscope (using a 100× Plan NEOFLUAR oil-immersion lens) and with MRC-500 and 600 Confocal Laser Systems (BioRad) mounted on a Nikon Labophot fluorescence microscope (a 60× PlanApo oil-immersion lens was used). Photographs were taken directly from the monitor with a Nikon camera using 35-mm TMAX 100 film (Kodak). Control experiments were performed without the first antibodies or by labeling the mouse primary antibodies with the goat secondary antibody. Only a faint non-specific background staining was detected.

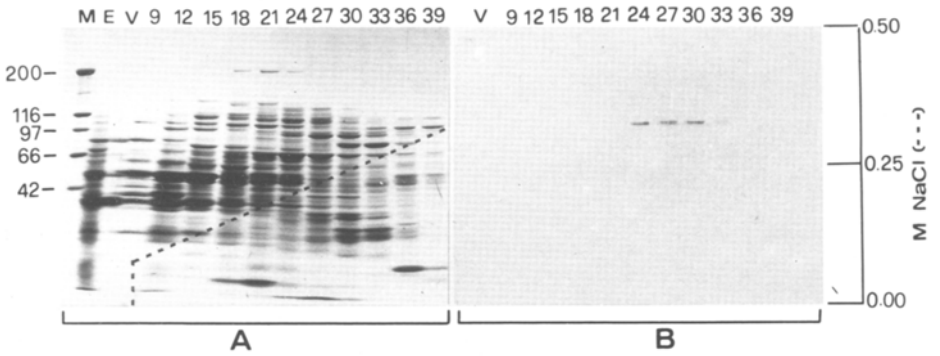

Fig. 1A,B. First step of PKH purification. **A** SDS-PAGE of fractions obtained by eluting a cytosolic extract of *Nicotiana tabacum* pollen tubes (*lane E*) through an Econo Pac-Q cartridge. Only representative fractions are shown, the numbers of which are given at the top of the gel. Unbound proteins (*lane V*) were eluted with five bed volumes of buffer A. Molecular weight of markers (*lane M*) is indicated in kDa on the *left*. *Dotted line* (---) represents the 60-ml linear gradient of 0–0.5 M NaCl used for the protein elution. Aliquots of 0.1 ml were loaded into each well except *lane E* (15 µl) and *lane V* (30 µl). The gel is stained with Coomassie Blue. **B** Nitrocellulose blot of a gel identical to that shown in **A** probed with the k71s23. The antibody reacts with a 100-kDa polypeptide in fractions 24–33, corresponding to approx. 0.25 M NaCl

Results

It was previously shown that the kinesin-immunorelated homologue (PKH) from *Nicotiana* pollen tubes bound weakly to mammalian microtubules with AMPPNP treatment (Tiezzi et al. 1992). In order to increase the yield of the purification procedure, we isolated the PKH using a protocol consisting of an AE-LPLC step followed by sucrose gradient centrifugation and gel filtration chromatography. We took advantage of the k71s23 anti-kinesin antibody to label the polypeptide fractions during each step by immunoblotting techniques. Microtubule affinity binding was used as the final phase of the purification procedure.

As a first step, a high-speed supernatant from *Nicotiana* pollen tubes was fractionated by anion-exchange chromatography. The SDS-PAGE in Fig. 1A shows the polypeptidic content of fractions obtained by eluting approx. 30 mg of cytosolic proteins through an Econo Pac-Q cartridge. The void volume contained several unbound polypeptides, the most prominent migrating at approx. 37 kDa (lane V). Most other bound proteins were eluted between 0.1 and 0.45 M NaCl. Immunoblotting of all fractions showed that one polypeptide at approx. 100 kDa is labeled in fractions 24–33 (peak at approx. 0.25 M NaCl) by the k71s23 anti-kinesin antibody (Fig. 1B). The average protein concentration of the pooled positive fractions was 0.1 mg · ml^{-1}, thus the AE-LPLC eliminated more than 80% of total protein.

After concentration with the Centricon 30 microconcentrator, the sample (protein concentration: 1.0 mg · ml^{-1}) was subsequently subjected to a 5–25% sucrose density gradient centrifugation. Constituent polypeptides of eluted fractions were analyzed by SDS-PAGE (Fig. 2A). By immunoblotting with the k71s23 antibody, the peak of the PKH was identified in fractions 13–14 (Fig. 2B), where large (approx. 150 kDa) and small polypeptides (< 45 kDa) were not observable. Using protein standards for reference, a graph of their sedimentation coefficient versus the fraction number yielded a linear plot, showing that the PKH from *Nicotiana* pollen tubes has a

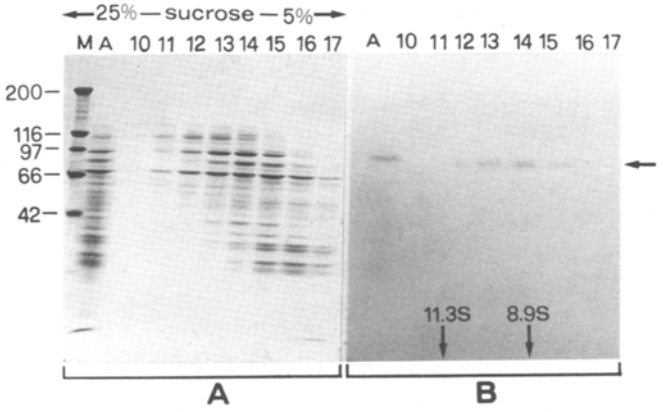

Fig. 2A,B. Second step of the procedure for purification of PKH. **A** SDS-PAGE of representative fractions obtained by centrifugating the AE-LPLC peak of Fig. 1 through a 5–25% sucrose gradient. Aliquots of 0.1 ml were loaded onto each lane. Fraction numbers are given at the top of the gel. *Lane M*, molecular weight markers. *Lane A*, starting material. The gel is stained with Coomassie Blue. **B** Immunoblotting with the k71s23 antibody of the gel fractions shown in **A** transferred to nitrocellulose filters. In fractions *13–14* the antibody recognizes a polypeptide (*arrow*) at approx. 100 kDa which sediments at approx. 9S. The position of sedimentation standards (catalase, *11.3S*; β-amylase, *8.9S*) is indicated

low sedimentation coefficient, around 9 S. Similar results were also observed when the high-speed supernatant was directly fractionated by sucrose gradient centrifugation and then probed by immunoblotting with the k71s23 antibody (data not shown). Hence, the AE-LPLC treatment did not significantly alter the molecular structure of the protein.

Sedimentation fractions 13 and 14 were pooled (protein concentration: approx. 0.2 mg · ml^{-1}) and further fractionated by gel filtration chromatography. The 100-kDa polypeptide was mainly recovered in fractions 27–30 (with a peak in fraction 28) as indicated by SDS-PAGE (Fig. 3A) and correspondent immunoblotting with the k71s23 antibody (Fig. 3B). A 116-kDa polypeptide comigrated in the same fractions, as well as minor amounts of

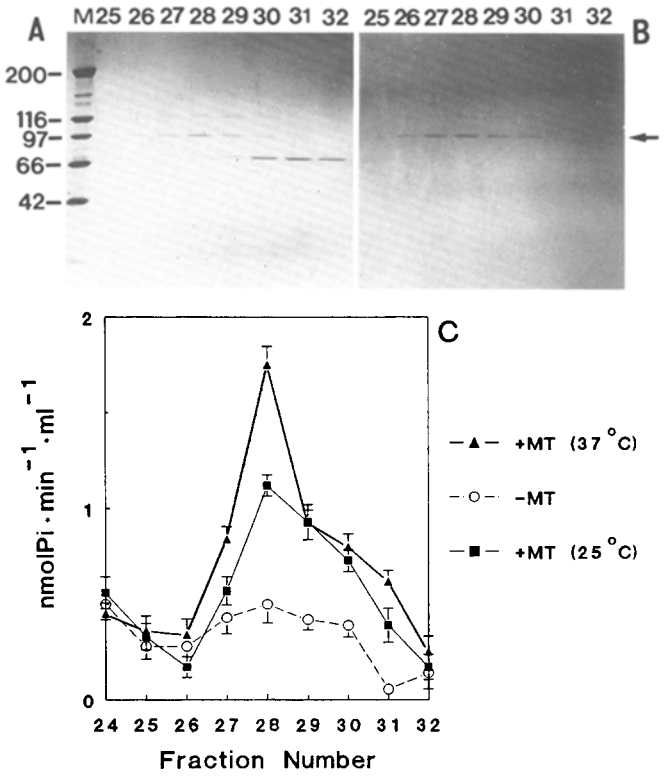

Fig. 3A–C. Third step in the procedure for purification of PKH. **A** SDS-PAGE of representative gel filtration fractions, the number of which is given at the top of the gel. Aliquots of 0.1 ml were loaded onto each lane. Molecular weight markers (*lane M*) are indicated in kDa. The gel is stained with Coomassie Blue. **B** Immunoblotting with the k71s23 antibody of the gel fractions shown in **A** transferred to nitrocellulose membrane. The antibody specifically reacts with a polypeptide at approx. 100 kDa (*arrow*), which peaks in fraction *28*. **C** The ATPase activity of the same fractions is reported, both in the presence (▲ at 37°C, ■ at 25°C) and absence (○) of 1 mg · ml^{-1} microtubules. Values are expressed as nmol Pi · min^{-1} · ml^{-1}. Bars indicate standard errors

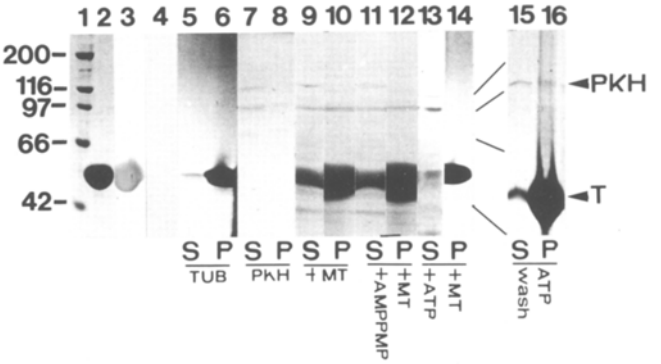

Fig. 4. Binding of partially purified PKH to animal microtubules as shown by SDS-PAGE. Molecular weight markers (*lane 1*) are indicated in kDa on the *left* of the gel. The gel filtration peak fraction (number 28) was incubated with microtubules (0.5 mg · ml^{-1}, *lane 2*) under several conditions. The microtubules were sedimented and the resulting supernatants and pellets were analyzed by SDS-PAGE (*S* and *P*, respectively, indicate each supernatant and pellet). *Lanes 3* and *4* (loaded with fraction 28 and microtubules as for *lane 2*) are, respectively, control immunoblots with a monoclonal anti-α-tubulin and the k71s23 antibody. *Lanes 5* and *6* show that microtubules alone pellet under the conditions used. The PKH alone does not pellet (*lanes 7, 8*). In the presence of microtubules, the 100-kDa polypeptide is essentially found in the pellet (*lanes 9, 10*). Treatment with AMPPNP does not seem to modify the binding affinity (*lanes 11, 12*). If ATP is initially added to the microtubule/PKH mixture, only microtubules are found in the pellet (*lanes 13, 14*). The pellet in *lane 12* was washed with ATP and sedimented again. The final ATP-supernatant and pellet show that the PKH is released by ATP (*lanes 15, 16*). The positions of tubulin (*T*) and the PKH are indicated. Aliquots of 50 µl were loaded in each lane (except *lane 2*, 2.5 µl). The gel is Coomassie-stained

lower-molecular-weight polypeptides (the latter not always observed). A 70-kDa polypeptide was eluted around fraction 31, clearly separated from the PKH. When protein standards were applied and run on the column, $1/D_{20,w}$ plotted against their K_{av} [$= (V_e - V_o)/(V_t - V_o)$, V_e: elution volume, V_o: void volume, V_t: total volume] generated a linear graph, from which the diffusion coefficient of the PKH was estimated to be about 3.7. By using the Svedberg equation (Bloom et al. 1988) the combination of the sedimentation and diffusion coefficients of the protein provided a native molecular mass of approx. 205 kDa.

Gel filtration fractions were evaluated for Mg-ATPase activity at 37°C both in the presence and absence of taxol-stabilized bovine brain microtubules. The purified tubulin fractions used in the enzymatic assay (and also in the binding experiment, protein concentration: approx. 6 mg · ml^{-1}) were shown to be free of any contaminating protein in Coomassie-stained gels (see Fig. 4, lane 2). The identity of the purified protein was also evaluated by immunoblotting with a monoclonal antibody to α-tubulin (see Fig. 4, lane 3). We also tested for the presence of kinesin in the tubulin preparation by immunoblotting with the k71s23 antibody, but no reaction was detected (Fig. 4, lane 4).

The basal level of Mg-ATPase activity increased more than fourfold in the presence of 1 mg · ml^{-1} microtubules and the activation profile clearly corresponded to the elution of the immunolabeled 100-kDa polypeptide (Fig. 3C). At this purification step, the PKH displayed an Mg-ATPase activity of approx. 8 nmol Pi · min^{-1} · mg^{-1}, activated to approx. 35 nmol Pi · min^{-1} · mg^{-1} by bovine brain microtubules. The ATPase activity of microtubules alone was just detectable by the colorimetric assay, but it was subtracted from the calculated activities. Decreasing the assay temperature to 25°C decreased the degree of activation to 62% of the original value (see Fig. 3C). We found a corresponding reduction in the background (and an enhancement of the signal-to-noise ratio) if SDS was used to stop the enzymatic reaction in place of acids (such as trichloroacetic acid-containing solutions). Furthermore, the microtubule concentration was also a relevant factor since best results were obtained by using tubulin at 1 mg · ml^{-1} (final concentration). The sensitivity and stability of the assay allowed us to accurately determine the enzymatic activity of the PKH, although the protein possesses low specific ATPase activity. As a control, the microtubule-dependent stimulation of the ATPase activity of bovine brain kinesin was also tested, yielding a value of 98 nmol Pi · min^{-1} · mg^{-1} in the presence of microtubules

and only 17 nmol Pi · min^{-1} · mg^{-1} in the absence (data quite similar to those obtained by Wagner et al. (1989), at the same purification step).

Immunolabeled gel filtration fractions showing a stimulated ATPase activity contained other polypeptides (mainly at 116 kDa) in association with the PKH. To evaluate if the observed stimulation was dependent only on the 100-kDa polypeptide but not on contaminating proteins, we verified the ability of the polypeptides to interact with microtubules in an ATP-dependent manner. The peak fraction of the gel filtration PKH (number 28, protein concentration: approx. 0.05 mg · ml^{-1}) was added to purified taxol-stabilized bovine brain microtubules (Fig. 4, lane 2). Incubation was carried out in the following conditions: (i) without microtubules, (ii) with microtubules, (iii) with microtubules and 5 mM AMP-PNP, (iv) with microtubules and 10 mM ATP. Microtubules alone form pellets under the conditions used (lanes 5, 6), whereas the PKH alone does not (lanes 7, 8). When microtubules were added, the 100-kDa polypeptide was constantly found in the pellet in association with microtubules (lane 10). On the other hand, all of the 116-kDa polypeptide (and part of the 100-kDa polypeptide) remained in the supernatant (lane 9). The presence of 5 mM AMPPNP did not appreciably enhance the binding of the 100-kDa polypeptide to microtubules (lanes 11, 12). When ATP was added to the initial mixture, both PKH and the 116-kDa polypeptide were found in the supernatant, whereas microtubules were in the pellet (lanes 13, 14). When the AMPPNP pellet (lane 12) was washed with 10 mM ATP and centrifuged again, the 100-kDa polypeptide was detached and found in the supernatant (lane 15), the pellet containing essentially microtubules and traces of the 100-kDa polypeptide (lane 16). After the ATP-extraction step, the 100-kDa polypeptide is the only one detectable by staining the gel with Coomassie Blue (apart from depolymerized tubulin, lane 15). This indicates that the binding affinity of the PKH for animal microtubules is ATP-sensitive, but AMPPNP-insensitive.

The intracellular distribution of the PKH was tested by indirect immunofluorescence and compared with that of tubulin in double-labeling experiments. Through-focus series of images were taken with BioRad MRC-500 and 600 Confocal Systems, also making use of the "XZ-command" and "length" utilities to reconstruct cross-sections of the pollen tube and to measure the fluorescence-intensity profiles. Figure 5 shows a series of 8 images obtained at different focal planes (levels of 2 μm each) through a pollen tube labeled with the k71s23 antibody. Labeling of punctate, organelle-like structures was prominently observed at the apex of the pollen tube (Fig. 5A–D), as shown by Tiezzi et al. (1992). The distribution of such structures was not strictly limited to the apex, but they were also observed in older parts of the pollen tube, even though much less abundantly than in the apex. Also, in this case, a distinct punctate staining was localized in the cortical region of the pollen tube (arrows in Fig. 5E). Some spots of fluorescence were particularly intense (arrows in Fig. 5B,C). The whole series of images was used to reconstruct a cross-section at the

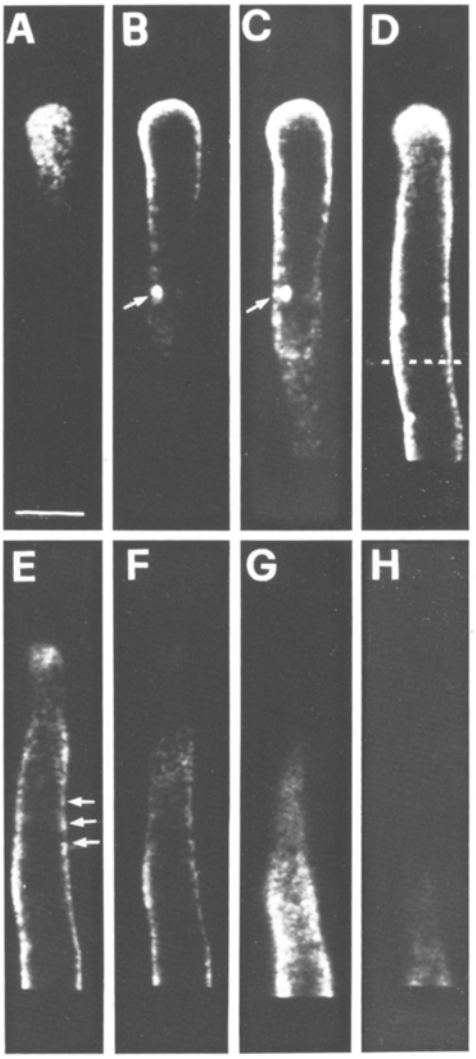

Fig. 5A–H. Immunofluorescence staining pattern of a *Nicotiana tabacum* pollen tube labeled with the k71s23 antibody and examined with a BioRad MRC-500 Confocal Laser Scanning Microscope. Several different focal planes of a tube (2 μm each) are shown. The PKH is mainly localized in the apex (**A–D**), but is also present in older parts of the tube, where the optical sectioning reveals an essentially cortical distribution. *Arrows* in (**E**) indicate the characteristic punctate staining, normally observed and consistent with the labeling of membrane-bounded organelles. Some spots of fluorescence are particularly bright [*arrows* in (**B–C**)]. The dotted line (**D**) indicates the level at which the cross-section of Fig. 6A has been reconstructed. × 200; bar = 10 μm

level indicated by the dotted line in the top-right image and corresponding to approx. 40 μm from the apex. The cortical distribution of the punctate structures is consequently clear, extending for approx. 1 μm into the pollen tube (Fig. 6A). The microtubular array has been extensively characterized in this area (Tiezzi 1991; Pierson and Cresti 1992) and shows a cortical localization resembling the anti-kinesin staining. Figure 6B is a reconstructed cross-section, at the level corresponding to that shown in Fig. 6A, of a pollen tube with the same diameter as that in Fig. 6A but labeled with the anti-α-tubulin antibody. Microtubules are mainly present in the cortex of the tube,

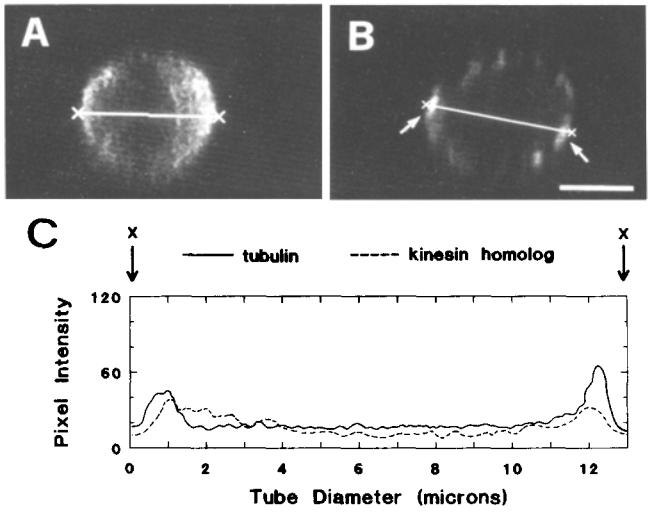

Fig. 6A–C. Co-distribution of PKH and tubulin in the older parts of *Nicotiana tabacum* pollen tubes. **A** Computer-reconstructed cross-section from the focal planes shown in Fig. 5 at the level indicated by the dotted line in Fig. 5D. The distribution of the PKH is clearly cortical. **B** An analogous cross-section at the same level as the picture shown in **A** obtained from a pollen tube labeled with the anti-α-tubulin antibody and having the same diameter as the tube in **A**. Microtubules are essentially distributed in the cortex, sometimes arranged in larger aggregates (*arrows*). ×620; bar = 5 μm. **C** Measurement of the relative fluorescence intensity (expressed as pixel intensity) in the two cross-sections shown in **A** and **B** at the level indicated by the X----X lines. Graphics have been superimposed. The peaks of maximum intensity overlap and show a width of approx. 1 μm in both cases. Data collected during different experiments were very similar to that shown here as an example

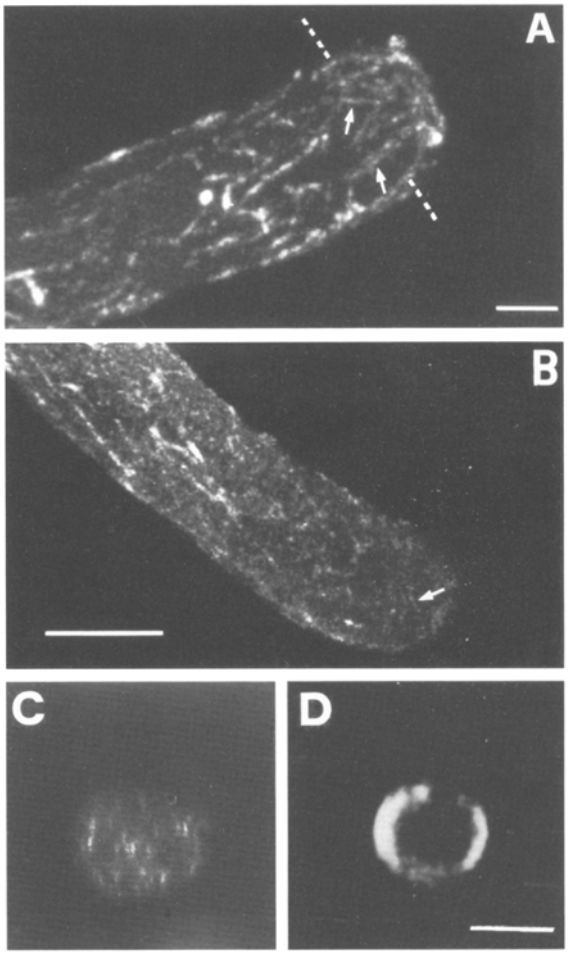

Fig. 7A–D. Localization of microtubules in the tips of tobacco pollen tubes. Immunofluorescence microscopy of pollen tubes using the monoclonal antibody to α-tubulin (**A**, ×690) or the rabbit antiserum (**B**, ×560). Pictures were obtained with the BioRad MRC-500 Confocal System and were generated by the addition of images taken at different focus planes (0.7 μm each). Only the apical region of the tubes has been considered. Thin, disordered filamentous structures are clearly radiating into the tip (*arrows*). The array does not show a constant longitudinal distribution, typical of microtubules in the older parts. **C** Computer-reconstructed cross-section from the image shown in **A** at the level indicated by the *dotted line* (approx. 5 μm from the apex). Microtubules are distributed in the whole cytoplasm, not strictly limited to the cortex (×590). **D** A cross-section at the same level as the picture in (**C**) obtained in a pollen tube labeled with the k71s23 antibody. The PKH maintains the characteristic cortical distribution (×590). Bars = 5 μm (**A**), 10 μm (**B–D**)

where some intense fluorescent spots suggest the local formation of large microtubule bundles (arrows). Furthermore, microtubules are mainly distributed in a 1-μm-thick region that is localized in the same area of the PKH, as revealed by the measurement of the relative fluorescence intensity (Fig. 6C).

The presence of microtubules in the pollen tube apex has always been a matter of controversy (review by Tiezzi 1991; Pierson and Cresti 1992). Confocal Laser Microscopy allowed visualization of a thin network of filamentous structures in the apex of pollen tubes labeled with the monoclonal anti-α-tubulin. These structures (such as those illustrated in Fig. 7A) are perhaps quite fragile, since they could not be clearly detected in all tubes. A quite similar type of staining, but of lower intensity, was obtained using the antiserum to *Hibiscus* tubulin (Fig. 7B). A cross-section at the level indicated by the dotted line in Fig. 7A (approx. 5 μm from the apex) is shown in Fig. 7C. Microtubules seem to be diffusely present throughout the whole apical cytoplasm with no particular preference for the cortex (contrary to what is found in the older parts of tube). On the other hand the anti-kinesin staining reveals a prevalently cortical localization of the antigen (Fig. 7D). Figure 8A–F shows a double staining with the anti-kinesin and the polyclonal anti-tubulin antibody on the same pollen tubes. Samples were observed using conventional epifluorescence microscopy. The generative cell shown in A (asterisk) is not stained by the anti-kinesin antibody (B), but its microtubular cytoskeleton is labeled (C). In the older parts of a tube (D), the anti-kinesin staining provides evidence of single fluorescence spots diffusely distributed (E), whereas the anti-tubulin labeling produces a stronger staining (F). Details of such co-localization can be best appreciated by the use of the Confocal Microscope. As shown in the double-labeling example in Fig. 8G,H, of the cortical area near the tip approximately indicated in the drawing (approx. 3 μm of thickness), the PKH is randomly distributed in a characteristic organelle-like pattern (G). Some elongated structures are also present. The anti-ki-

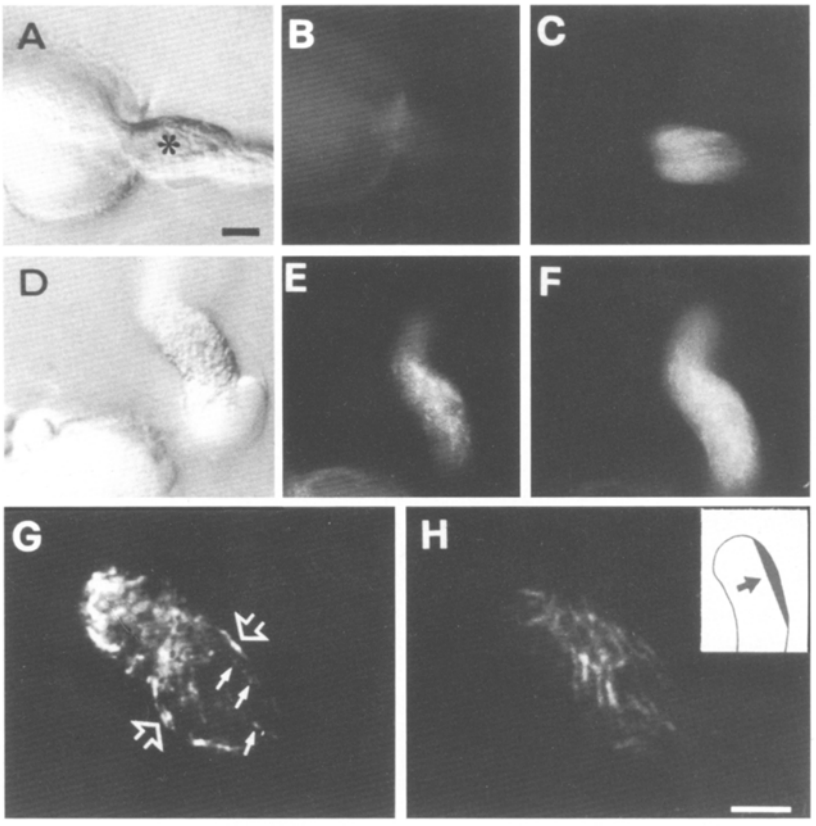

Fig. 8A–H. Double immunofluorescence microscopy of pollen tubes using the k71s23 monoclonal antibody to kinesin and the polyclonal rabbit antibody to tubulin. Samples were observed with the Axiophot epifluorescent microscope (**A–F**) or with the BioRad MRC-600 Confocal System (**G,H**). **A** and **D** are the DIC views of cells stained both with the k71s23 antibody (**B** and **E**) and with the anti-tubulin antibody (**C** and **F**). The generative cell (*asterisk* in **A**) is not stained by the anti-kinesin (**B**), whereas its microtubular cytoskeleton is evident (**C**). In the older parts of the vegetative tube (**D**), k71s23 stains some punctate structures (**E**) distributed within the microtubular network (**F**). In (**G,H**) the field shown by the double labeling is a cortical region near the apex (approx. 3 μm thick) indicated by the black area in the drawing. The tube tip is in the *top left corner*. The k71s23 antibody staining reveals structures sometimes co-aligned along microtubular tracks (*small arrows* in **G**). In other cases the distribution trend of these structures is similar to that of tubulin (*open arrows*). Microtubules are not arrayed longitudinally, but are randomly dispersed (**H**). **A–F** ×260; bars = 10 μm. **G,H** ×790; bars = 5 μm

nesin staining does not seem to co-localize with individual (or bundles of) microtubules. However, the trend of the PKH distribution often resembles that of microtubules, as indicated by the large open arrows in (G). Sometimes single fluorescence spots are seen to co-align with microtubules (small arrows in G). Microtubules are not longitudinally arrayed as in the older parts of the tube, but they form an apparently disordered network beneath the plasma membrane (H).

Discussion

The preliminary identification of the kinesin immuno-reactive homologue from *Nicotiana* pollen tubes represented the first evidence concerning the possible presence of a microtubule-dependent organelle translocator in plant cells (Tiezzi et al. 1992). In the present paper we have analyzed the biochemical and functional characteristics of the PKH by determining additional factors, mainly the ability of the polypeptide to interact actively with microtubules. The protein was purified according to (i) charge (AE-LPLC), (ii) shape and (iii) size (sucrose gradient centrifugation and gel filtration). The k71s23 anti-kinesin antibody was used to label the protein during the purification procedure. Microtubule affinity binding in the absence or presence of ATP was used as a final purification step and, at the same time, to answer the question of whether the purified protein is able to bind to microtubules in an ATP-sensitive fashion.

The PKH seems to be composed of a polypeptide with an apparent molecular weight of approx. 100-kDa, similar to that of kinesin heavy chains from different organisms (Vallee and Shpetner 1990). A previous paper (Tiezzi et al. 1992) reported the presence of another related polypeptide at 108-kDa, identified by immunoblotting in pollen tube extracts. When such extracts were fractionated by AE-LPLC, the localization of the 108-kDa polypeptide was uncertain and not constant, therefore no further investigations were carried out. The presence of associated proteins has not been determined, since no particular polypeptides are seen to co-elute with the PKH during all the purification steps. However, it must be noted that the light chains of kinesin from sea-urchin eggs are obtained in variable amounts during different purification procedures (Buster and Scholey 1991). In addition, light chains have not yet been reported for *Acanthamoeba* kinesin (Kachar et al. 1987) or for *Dictyostelium* kinesin (McCaffrey and Vale 1989). A 116-kDa polypeptide is seen to co-elute with the PKH during the first purification steps, but it is unlikely that the two polypeptides are part of the same molecular complex, since they show a dissimilar behavior in the presence of microtubules.

The PKH exhibits a sedimentation coefficient of approx. 9S on sucrose density gradients. This is in agreement with former data on animal kinesins (Scholey et al. 1985; Bloom et al. 1988; Kuznetsov et al. 1988; McCaffrey and Vale 1989) and thus it may suggest the possibility that the protein is a long, thin molecule with an apparent

morphology suitable to connect cytoplasmic organelles to microtubules. Calculations of diffusion and sedimentation coefficients of the PKH allowed a native molecular weight of approx. 205 kDa to be determined by using the Svedberg equation. These data raise the possibility that PKH is a dimer of the 100-kDa polypeptide. However, it must be considered that erroneous estimations of the protein's molecular organization could be generated by the approximate values of molecular weight obtained by the Svedberg method. Furthermore, dimerization of kinesin-like polypeptides has been only presumed on the basis of analogy to conventional kinesin (De Cuevas et al. 1992), and a single motor domain of kinesin has been determined to be sufficient to generate in vitro microtubule motility (Yang et al. 1990). Some kinesin-like proteins could therefore exist also as a single polypeptidic chain.

The protein also co-purifies with an ATPase activity four-fold stimulated by microtubules. This is a characteristic property of microtubule-based motor proteins, even though the degree of activation and the values of specific ATPase activity reported here (approx. 35 nmol Pi $\cdot$ min^{-1} $\cdot$ mg^{-1} in the presence of microtubules at 37°C, 20.2 nmol Pi $\cdot$ min^{-1} $\cdot$ mg^{-1} at 25°C) are substantially lower than those shown for other kinesins (Kuznetov and Gelfand 1986; Murofushi et al. 1988; Saxton et al. 1988), and only partially comparable to the activity of sea urchin egg kinesin (Cohn et al. 1987). It must be also noted that, although pollen tubes are routinely cultured at room temperature (20–25°C), the highest enzymatic activity was obtained at 37°C. Stimulation of the ATPase activity of kinesins has been reported to depend on the protein source and the experimental conditions, including the purification procedures, the buffer composition and, to a certain degree, the microtubule concentration (Kuznetov et al. 1988; Wagner et al. 1989). Furthermore, in our case the use of bovine brain microtubules could result in a low stimulation of the ATPase activity of the PKH. In fact, although tubulins are members of a highly conserved gene family, plant tubulins show some peculiarities when compared to those of animals, such as different electrophoretic mobility and lower degrees of sensitivity to drugs such as colchicine (for review see Fosket and Morejohn 1992). Since bovine brain microtubules could have binding sites which are not exactly recognized by a plant kinesin, we are currently trying to purify tubulin from pollen of *Nicotiana* or other pollen species (data not shown) in order to verify if bovine brain microtubules constitute a poor substrate.

The microtubule-activated ATPase activity of the PKH also reflects on the specific binding of the polypeptide to animal microtubules. In fact the 100-kDa polypeptide can bind to microtubules in an ATP-dependent manner, although its binding capacity is not enhanced by the presence of AMPPNP, contrary to that reported for other kinesins. Similar results had been also obtained directly, starting from a high-speed supernatant of pollen tubes in the presence of AMPPNP (Tiezzi et al. 1992). However, the enzymatic and binding assays suggest that the association between the PKH and microtubules is regulated by ATP, as in the case of "conventional" kinesins and kinesin-like proteins, and consequently favors the role of the PKH as a cytoplasmic microtubule-based motor.

The intracellular distribution of the PKH has already been described (Tiezzi et al. 1992). However, new data have been obtained by double-immunofluorescence analyses with anti-tubulin and anti-kinesin antibodies. The resulting observations can be summarized as follows: (i) the PKH is distributed not only in the apex, but also cortically in the older parts of the tube; (ii) the generative cell is not stained by the anti-kinesin antibody; (iii) the anti-kinesin staining of the apex indicates an organization of structures sometimes co-distributing with microtubules but not precisely located on them. In older parts of the tube, microtubules and the PKH are concentrated in the same cortical region, corresponding to approx. 1 μm of thickness. The immunofluorescence staining in this area is not as bright as that obtained in the apex, suggesting a lesser abundance of the polypeptide. The identity of the labeled organelle-like structures is not known. The immunofluorescence staining pattern in the tube tip suggests the labeling of secretory vesicles, while the punctate structures in the older parts could represent larger organelles, aggregates or elements of the cortical endoplasmic reticulum known to be closely associated with microtubules and actin filaments in the pollen tube (reviewed by Hepler et al. 1990). The reported immunofluorescence observations are largely in agreement with those already obtained by other workers. Several reports described particle-like components, probably of membranous origin, diffusely distributed in the cytoplasm of many cell types and mostly concentrated in microtubule-enriched regions (Neighbors et al. 1988; Hollembeck 1989; Pfister et al. 1989; Hollembeck and Swanson 1990; Houliston and Elinson 1991; Wright et al. 1991). A precise co-localization of kinesin with microtubules has not yet been reported (except for some sporadic alignment), suggesting that kinesin is essentially a cytoplasmic component linked to membrane-bounded organelles rather than to microtubules.

Our data clearly support the conclusion that the 100-kDa polypeptide from *Nicotiana tabacum* pollen tubes is the main component of a protein functionally related to the kinesin class. Even though an actomyosin-based organelle movement has been reported in pollen tubes (Khono et al. 1992), our data indicate that in higher plants the translocation of organelles and-or vesicles could be also associated with the microtubular system, with biochemical modalities similar to those already described in animal cells. To further verify this hypothesis, other types of analysis will be necessary to understand the protein structure and to determine the relationships between the PKH and kinesins. Since our observations are not able to show structural homologies, but provide indications of biochemical, functional and localization properties common to kinesins and kinesin-like proteins, the PKH could be classified in the category of the "kinesin-related polypeptides" (as proposed by Cole et al. 1992).

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