In Vitro Assays Demonstrate That Pollen Tube Organelles Use Kinesin-Related Motor Proteins to Move along Microtubules

Silvia Romagnoli,1 Giampiero Cai, and Mauro Cresti
Dipartimento Scienze Ambientali “G. Sarfatti,” Università di Siena, via Mattioli 4, 53100 Siena, Italy

The movement of pollen tube organelles relies on cytoskeletal elements. Although the movement of organelles along actin filaments in the pollen tube has been studied widely and is becoming progressively clear, it remains unclear what role microtubules play. Many uncertainties about the role of microtubules in the active transport of pollen tube organelles and/or in the control of this process remain to be resolved. In an effort to determine if organelles are capable of moving along microtubules in the absence of actin, we extracted organelles from tobacco pollen tubes and analyzed their ability to move along in vitro–polymerized microtubules under different experimental conditions. Regardless of their size, the organelles moved at different rates along microtubules in the presence of ATP. Cytochalasin D did not inhibit organelle movement, indicating that actin filaments are not required for organelle transport in our assay. The movement of organelles was cytosol independent, which suggests that soluble factors are not necessary for the organelle movement to occur and that microtubule-based motor proteins are present on the organelle surface. By washing organelles with KI, it was possible to release proteins capable of gliding carboxylated beads along microtubules. Several membrane fractions, which were separated by Suc density gradient centrifugation, showed microtubule-based movement. Proteins were extracted by KI treatment from the most active organelle fraction and then analyzed with an ATP-sensitive microtubule binding assay. Proteins isolated by the selective binding to microtubules were tested for the ability to glide microtubules in the in vitro motility assay, for the presence of microtubule-stimulated ATPase activity, and for cross-reactivity with anti-kinesin antibodies. We identified and characterized a 105-kD organelle-associated motor protein that is functionally, biochemically, and immunologically related to kinesin. This work provides clear evidence that the movement of pollen tube organelles is not just actin based; rather, they show a microtubule-based motion as well. This unexpected finding suggests new insights into the use of pollen tube microtubules, which could be used for short-range transport, as actin filaments are in animal cells.

INTRODUCTION

In eukaryotic cells, the transport of organelles and molecules among different cellular regions occurs along the fibrillar cytoskeleton: microtubules and actin filaments (AFs). Organelle transport is essential for cell viability and is well studied in a variety of cellular processes, such as fast axonal transport in neurons (Mercer et al., 1994) and the transport of pigment granules in melanophores (Rogers et al., 1997). Organelle movement is driven along cytoskeletal filaments by motor proteins: kinesin and dynein are the two families of microtubule-based motor proteins (Hirokawa et al., 1998), whereas myosin is the only family of AF-based motor protein found in eukaryotic cells (Titus, 1993). Motor proteins anchor to the organelle surface and drive them along cytoskeletal filaments by converting into movement the chemical energy released by ATP hydrolysis. In animal cells, microtubules are the primary support for organelle transport; they drive and regulate the positioning of many organelles, including the Golgi apparatus, the endoplasmic reticulum, lysosomes, peroxisomes, and secretory vesicles (Rogers and Gelfand, 2000). Nevertheless, various pieces of evidence have suggested that microtubule- and AF-based motility systems cooperate functionally during organelle transport, such as in dermal pigment cells (Gross et al., 2002), in epithelial cells (Fath et al., 1994), during exocytosis (Bi et al., 1997), and in nerve cells (Coy and Howard, 1994). Cooperation between AF- and microtubule-dependent motility also extends to lower eukaryotes. In the moss Physcomitrella patens, chloroplasts use both the microtubule and AF motility systems, which are regulated differently by two types of photoreceptor (Sato et al., 2001). Therefore, the coordinated activity of microtubule- and AF-dependent motility is likely to be a general principle of organelle transport in eukaryotic cells.

1 To whom correspondence should be addressed. E-mail romagnolis@unisi.it; fax 39-0577-232860.
©Online version contains Web-only data.
Article, publication date, and citation information can be found at www.plantcell.org/cgi/doi/10.1105/tpc.005645.
Studies of cytoplasmic streaming have revealed that organelle transport in plant cells is an AF-dependent process. Dynamic interactions between AFs and membranes are responsible for vesicle movement during secretion, endocytosis, mitochondrial inheritance, the maintenance of an even distribution of subcellular components in large cells, and the asymmetric delivery of membrane material in polarized growing cells (Shimin and Yokota, 1994). AFs are the main scaffold for organelle movement in plant cells, as shown by studies with AF inhibitors (Williamson, 1972), the analysis of organelle movement in cells (Nebenführ et al., 1999), and the association of different myosin classes with plant organelles (Uchida et al., 1999; Yokota, 2000). Microtubules are not likely to be involved in the transport of plant organelles; however, they could be involved in other dynamic processes, such as the positioning of nuclei (Lloyd et al., 1987) and mitochondria (Van Gestel et al., 2002), the transport of chloroplasts (Sato et al., 2001), and vesicle delivery during cytokinesis (Julie Lee et al., 2001). These few examples suggest that microtubules could support the movement of plant cell organelles different from those transported by AFs; alternatively, microtubules could regulate the movement of organelles that translocate along AFs.

In fungal hyphae and in tip-growing plant cells (root hairs and pollen tubes), AFs and microtubules participate in the distribution of organelles according to the cell growth rate. The relative contributions of AFs and microtubules generally are understood in fungal hyphae and root hairs, but data are controversial for pollen tubes (Geitmann and Emons, 2000). The angiosperm pollen tube acts as a cellular channel for delivering sperm cells from the pollen grain to the ovary during fertilization. Membrane trafficking in the pollen tube occurs in both directions and determines the even distribution of organelles and molecules along the tube (except in the apical and subapical regions) (Pierson et al., 1990). Although AFs and microtubules are equally abundant in the pollen tube, current models of organelle transport indicate that AFs are the main tracks along which organelles are transported. This hypothesis is based on inhibition studies (Mascarenhas and Lafountain, 1972; Lancelle and Hepler, 1988), motility assays with isolated organelles (Kohno and Shimmen, 1988), and the localization of myosins on the organelle surface (Heslop-Harrison and Heslop-Harrison, 1989; Tang et al., 1989; Miller et al., 1995; Tirlapur et al., 1995). On the other hand, microtubules most likely correlate with the movement of both the generative cell and the vegetative nucleus (Astrom et al., 1995; Miyake et al., 1995). Other possible roles of microtubules involve pulsatory growth (Geitmann et al., 1995) and the accumulation of vacuoles in the pollen tube tip (He et al., 1995).

The relationships between microtubules and organelle trafficking in the pollen tube are not clear, because microtubule inhibitors do not produce particular effects on both cytoplasmic streaming and pollen tube growth (Heslop-Harrison et al., 1988; Geitmann et al., 1995). On the other hand, a series of microtubule motor proteins has been identified and characterized in the pollen tube. A pollen kinesin homolog (Tiezzi et al., 1992) and two dynein-related polypeptides (Moscatelli et al., 1995) have been identified in the vegetative cytoplasm of pollen tubes and localized in association with unclassified membrane structures (Cai et al., 1993; Moscatelli et al., 1998). More recently, a 90-kD kinesin-related protein (90-kD ATP-microtubule-associated protein [ATP-MAP]) showing motor activity was found in association with organelles and microtubules in the cortical region of pollen tubes (Cai et al., 2000), which suggested that the 90-kD motor protein translocates organelles along microtubules in tobacco pollen tubes. Microtubule-based motors identified to date in the pollen tube have different distributions. The pollen kinesin homolog is localized in the apex and flanks of the tube (Cai et al., 1993), whereas dynein-related polypeptides have a more homogenous distribution (Moscatelli et al., 1998). By contrast, the 90-kD ATP-MAP is localized in the cortical regions but is absent from the apex (Cai et al., 2000). The different distribution of microtubule-based motors could imply that these proteins have distinct functions. Additional evidence for the role of microtubule motors in Arabidopsis pollen tubes has come from the genetic analysis of double mutants, which suggested that a kinesin-like protein is involved in pollen tube growth and vesicle transport (Krishnakumar and Oppenheimer, 1999).

The presence of organelle-associated motors and evidence from mutant analysis suggested that pollen tube organelles interact dynamically with and translocate along microtubules, likely through the activity of motor proteins. Because inhibition studies indicated that the contribution of microtubules to cytoplasmic streaming and pollen tube growth is likely to be poor, the result of any dynamic interactions between microtubules and membranes should be found elsewhere. According to the model of functional cooperation between AF- and microtubule-dependent motility described in other cells, we can hypothesize that pollen tube microtubules cooperate with AFs in the fine-tuning of organelle and vesicle transport or, alternatively, that microtubules could be necessary for the local movement of specific membrane-bound organelles (Cai et al., 2001).

Biochemical, cytological, and genetic analyses have indicated that pollen tube microtubules possess the appropriate protein machinery to move organelles. However, these data do not explain the correlations between microtubules, motor proteins, and membrane-bound organelles and do not clarify the movement of pollen tube organelles along microtubules. In addition, unequivocal evidence of pollen tube organelles that move along microtubules still is missing. In the present study, we have used the microtubule-organelle motility assay to establish that pollen tube organelles exhibit clear motor activity along microtubules and to define some crucial aspects of this movement. The attachment of microtubule tracks to a substrate is a powerful tool with which to evaluate the motor activity of undefined or purified membrane-bound organelles under defined experimental conditions (Urrutia et al., 1993). The aim of the present work is to
analyze the conditions under which the movement of pollen tube organelles occurs along microtubules and to investigate the molecular machinery that supports this activity. Pollen tube organelles are shown to move along microtubules under different experimental conditions in an ATP-dependent manner in both the presence and the absence of pollen tube cytosol. This finding suggested that the motor proteins responsible for organelle motility are bound to the organelle surface. Organelle movement is cytochalasin independent, which suggested that AFs are not required. Because cytosol is not necessary for organelle movement, we removed peripheral proteins from the organelle surface and showed that the extracted proteins promote the movement of latex beads along microtubules. An ATP-dependent microtubule binding assay was used to identify putative motors from the pool of peripheral organelle proteins. We have isolated a kinesin-related protein that is able to translocate microtubules in in vitro motility assays and that cofractionates with a microtubule-enhanced ATPase activity. These results provide clear evidence that organelles from a higher plant cell (the pollen tube) actively move along microtubules by means of motor proteins and challenge the notion that organelle transport in the pollen tube is exclusively an AF-dependent process.

RESULTS

Components of the in Vitro Motility Assay

In this work, we have developed a method for the isolation of pollen tube organelles that move along microtubules under an in vitro motility assay. Germinated pollen tubes were homogenized, and then low- and high-speed centrifugation was used to separate the cytosol from the organelle fraction. The pool of organelles was termed the postnuclear supernatant (PNS) because the generative cell and the vegetative nucleus were likely excluded after the low-speed centrifugation. The main fractions used in the motility assays were analyzed using 7.5% PAGE. Figure 1A shows the polypeptide composition of bovine brain tubulin (lane 2), of the organelle fraction before (lane 3) and after (lane 4) the second 20% pelleting step, and of cytosol (lane 5) from pollen tubes. The sample shown in lane 3 was used for the motility assay in the presence of cytosol. The second pelleting step through 20% Suc, which was introduced to clean the organelle surface of cytosolic proteins, yielded the material in lane 4, which was used for the assays in the absence of soluble proteins. Bovine brain tubulin, which was used for the in vitro polymerization of taxol-stabilized microtubules, was purchased from Cytoskeleton, Inc. (catalog number T238), and is 99% pure according to the technical information accompanying the product. The purity level of tubulin was confirmed by analysis with Fluor-S and Quantity One software. The tubulin sample did not contain contaminating motor proteins (which could invalidate our results), as demonstrated by the absence of motor and ATPase activity and by negative results with both MMR44 (Marks et al., 1994) and HYPIR pan-kinesin antibodies in immunoblotting assays (data not shown). When in vitro–polymerized microtubules were observed by Alien video-enhanced contrast–differential interference contrast microscopy (Figure 1B), no fields showed

![Figure 1. Components of the in Vitro Motility Assay.](image-url)

(A) Silver-stained SDS gel showing the components used for the in vitro motility assay. Lane 1, molecular mass standards (indicated in kD at left); lane 2, bovine brain tubulin (5 μg) used to prepare in vitro–polymerized microtubules; lane 3, PNS organelles from pollen tubes (5 μg); lane 4, PNS organelles after the second 20% pelleting step (5 μg); lane 5, cytosolic proteins from pollen tubes (5 μg). The front of migration of the electrophoresis is indicated at bottom left (df, dye front).

(B) Single video frame of in vitro–polymerized microtubules at a concentration (0.4 mg/mL) higher than that used for the motility assay. Bar = 2 μm.
organelles either on the microtubules or in solution, demonstrating that bovine brain tubulin was pure and not contaminated by animal organelles.

**Pollen Tube Organelles of Different Size Move along Microtubules**

Different conditions were used during the in vitro motility assays to study the molecular basis of the movement of pollen tube organelles along microtubules. Table 1 summarizes the components used in each motility assay. In vitro motility assays were performed by preattaching microtubules to the cover slips of perfusion chambers, which then were saturated with casein to prevent the aspecific adsorption of organelles. A mixture of organelles, cytosol, and ATP then was added to the microtubules (the so-called standard condition). Samples were observed by Allen video enhanced contrast–differential interference contrast microscopy, which allowed the observation of different types of dynamic interactions between microtubules and organelles. Some organelles showed Brownian motions in the medium and then suddenly attached to microtubules and, either immediately or soon after, started to move along. The distance covered by organelles along microtubules was variable but in the range of 5 to 20 μm. Some organelles that moved along microtubules suddenly dissociated, whereas others stopped moving and remained attached to microtubules. In some instances, binding and subsequent dissociation of organelles occurred without movement in between. Movements were smooth and continuous, even for the larger organelles, whereas recoils or saltatory movements were very infrequent.

When moving organelles reached the intersections of different microtubules, some of them switched to a different microtubule and resumed their movement. Moving organelles sometimes were observed to pass through unmoving organelles (Figure 2A, arrowhead) and smaller (Figure 2B, arrowhead) organelles showed Brownian motions in the medium and then crossed over them (Figure 2A, arrow). The binding of organelles to microtubules appeared to be specific, because the number of organelles out of microtubules was very low. The movie sequence of Figure 2A can be viewed in the supplemental data online.

Transport of organelles along in vitro–polymerized microtubules was inhibited by 5 mM AMPPNP (5'-adenyllylimidodiphosphate), a nonhydrolyzable ATP analog, which also caused more organelles to bind to microtubules. Every view field under these experimental conditions showed no organelles moving along microtubules. Some occasional Brownian motion of organelles occurred in solution, but we never observed organelles attaching/dissociating from microtubules or moving along. Furthermore, organelle movement was not observed when nucleotides were omitted from the microtubule-organelle mixture (data not shown).

**Cytochalasin D Does Not Affect Organelle Movement**

Short AFs could be present in the cytosol or in membrane fractions from pollen tubes. To establish that the in vitro movement of pollen tube organelles was dependent on microtubules but not on AFs, we added cytochalasin D (an actin-depolymerizing factor) to the organelle-cytosol-ATP mixture. The inhibitor was applied at concentrations (5 μM) that usually inhibit cytoplasmic streaming in the pollen tube. Under these experimental conditions, we still observed or-

<table>
<thead>
<tr>
<th>Condition</th>
<th>Cytosol</th>
<th>Nucleotide(s)</th>
<th>Other</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard condition</td>
<td>6.5 μg/μL</td>
<td>5 mM ATP</td>
<td></td>
</tr>
<tr>
<td>Without cytosol</td>
<td>–</td>
<td>5 mM ATP</td>
<td>Buffer&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cytochalasin plus cytosol</td>
<td>6.5 μg/μL</td>
<td>5 mM ATP</td>
<td>5 μM cytochalasin D</td>
</tr>
<tr>
<td>Cytochalasin without cytosol</td>
<td>–</td>
<td>5 mM ATP</td>
<td>5 μM cytochalasin D and buffer&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>AMPPNP</td>
<td>6.5 μg/μL</td>
<td>5 mM ATP and 5 mM AMPPNP</td>
<td>–</td>
</tr>
<tr>
<td>GTP</td>
<td>6.5 μL/μL</td>
<td>5 mM GTP</td>
<td>–</td>
</tr>
<tr>
<td>No nucleotide</td>
<td>6.5 μL/μL</td>
<td>–</td>
<td>Buffer&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Each test contained in vitro–polymerized microtubules (0.1 mg/mL), the PNS at 0.15 μg/mL, 20 μM taxol, and other components as indicated.

<sup>a</sup>–, no data.

<sup>b</sup>Buffer (BRB25 buffer containing 20 μM taxol and 1 mM DTT) was added to adjust the assay volume to 200 μL.
The percentage of moving organelles was relatively the same compared with that in the standard conditions. Figure 3 shows images from two different time-lapse video sequences of organelles moving along microtubules in the presence of cytochalasin D. In Figure 3A, two to three organelles were observed to move simultaneously in the same direction along a single microtubule. Two organelles (arrowheads outlined in black and white) covered a longer distance without interruption at velocities of ~285 nm/s. The organelle indicated by the white arrowhead without outline detached from the microtubule or disappeared from the focal plane when it collided with the unmoving organelle marked by the arrow (the movie sequence of Figure 3A can be viewed in the supplemental data online). Figure 3B shows images from a time-lapse video sequence of a pollen tube organelle (arrowhead) that switched between three different microtubules. This specific assay was performed in the absence of cytosol.

Cytosol Is Not Critical for Organelle Movement

To evaluate the importance of cytosolic proteins in promoting the transport of pollen tube organelles along microtubules, the organelle fraction was assayed for motility in the absence of cytosol. The high-speed pellet was centrifuged again on a
20% Suc cushion to further separate the organelle fraction from cytosolic proteins. A mixture of organelles and ATP (but not cytosol) was added to microtubules in the perfusion chamber. Figure 4 shows two examples of pollen tube organelles that moved along microtubules in the absence of cytosol with velocities of 208 and 156 nm/s (A and B, respectively; the movie sequence of Figure 4B can be viewed in the supplemental data online). Dynamic interactions between organelles and microtubules under these experimental conditions were the same as those described for the standard conditions. Organelles were observed to move continuously along microtubules even when they approached motionless organelles and crossed over them. These results indicate that cytosolic factors are not necessary for the movement of pollen tube organelles along microtubules.

**Organelle Proteins Move Latex Beads**

As further evidence for the presence of microtubule-based motors on the surface of pollen tube organelles, peripheral...
proteins were extracted from organelles and assayed for their ability to move latex beads by in vitro motility assay. Peripheral membrane proteins were removed from pollen tube organelles by treatment with KI. The sample was centrifuged to separate the KI-washed organelles (Figure 5A, lane 2) from extracted proteins. The supernatant (Figure 5A, lane 3) was desalted to remove KI from extracted proteins and then used to coat latex beads. As a control, we performed in vitro motility assays with a commercial kinesin from Cytoskeleton, Inc. (Figure 5A, lane 4) that was attached to beads under exactly the same experimental conditions as were used for pollen proteins. In this case, many beads moved at high speed (~400 nm/s) in the same direction, as shown by the two video frames in Figure 5B. When organelle protein-coated beads were introduced into the perfusion chambers, they stuck to the microtubule surface just as pollen tube organelles did. In most cases, the beads moved along microtubules (Figures 5C and 5D). The velocity of beads was equivalent to that of unextracted organelles (183 ± 44 nm/s for beads versus 171 ± 58 nm/s for organelles). Beads showed unidirectional and continuous movement. As an additional control, KI-washed organelles were centrifuged, resuspended in KI-free buffer, and assayed for the ability to translocate along microtubules by in vitro motility assay.

Figure 4. Motility Assay of Pollen Tube Organelles in the Absence of Cytosol.

Two time-lapse video sequences of pollen tube organelles (arrowheads) that move along a microtubule in the absence of cytosol. Velocities of the organelles were ~208 and 156 nm/s. Numbers in each frame indicate the time in seconds. The movie sequence of (B) can be viewed in the supplemental data online. Bars = 2 μm.
vitro motility assay. In this case, we observed no organelles showing active movement along microtubules (data not shown). Uncoated beads never moved along microtubules (data not shown).

Organelle Velocities under Different Conditions

Organelle velocity was calculated with the Speed command of the Argus 20 image processor, taking into account all moving organelles regardless of their size. Figure 6 shows the average velocities of pollen tube organelles in different experimental conditions. Under standard conditions (organelles, ATP, and cytosol), we determined an average speed of 171 ± 58 nm/s. Both the average speed and the standard deviation increased in the absence of cytosol (309 ± 205 nm/s) compared with the presence of soluble factors. The addition of cytochalasin D to the standard conditions increased the average speed to 360 ± 67 nm/s. The effect of cytochalasin D was analyzed in the absence of cytosol as well. In this case, the average velocity was comparable to that observed in the presence of cytosol and cytochalasin D, and the standard deviation was higher. Organelle protein–coated beads moved along microtubules with an average speed of 183 ± 44 nm/s.

Organelles of Different Fractions Move along Microtubules

To demonstrate that microtubule-based motility is not restricted to a particular class of pollen tube organelles, we re-
quired fractionation of the initial PNS. A discontinuous Suc gradient was used to separate pollen tube organelles into five distinct fractions, whose polypeptide profiles are shown in Figure 7A (lanes 4 to 8). When tested by in vitro motility assay, the organelles of each fraction were observed to move along microtubules in both the presence and the absence of cytosol (data not shown). This finding suggested that most pollen tube organelles are able to move along microtubules. We found that organelles of the 50/57 fraction (lane 7) showed a higher level of motor activity than the other organelle fractions. In this context, higher motor activity means that moving organelles in the 50/57 fraction were greater in number and covered longer distances. Figure 7B shows one organelle from the 50/57 fraction that moved along a microtubule. The movement of 50/57 organelles was smooth and continuous without recoils or saltatory activities and was similar to that of PNS organelles under the same experimental conditions.

The organelle fractions were assayed for the presence of specific organelle marker proteins to provide indications of the organelle composition of each fraction. We selected four organelle markers: cytochrome c oxidase activity for mitochondria, inosine 5'-diphosphatase activity for the Golgi apparatus and secretory vesicles, P-ATPase activity for the plasma membrane, and cytochrome c reductase activity for rough and smooth endoplasmic reticulum. The results are expressed in Figure 7C as relative percentages of enzymatic activity. We found that the 50/57 organelle fraction did not contain peaks of relative enzymatic activity. However, this fraction was quite enriched in cytochrome c oxidase activity; therefore, it could be ascribed as a mitochondrial membrane fraction. More specifically, the S33 fraction contained the highest level of inosine 5'-diphosphatase activity (it was enriched in the Golgi apparatus and/or secretory vesicles). The 33/44 fraction had the highest intensity of both cytochrome c reductase and P-ATPase activity (which suggested enrichment in endoplasmic reticulum and plasma membrane, respectively). The highest level of cytochrome c oxidase activity was found in the 57/65 fraction, which likely contains mitochondria.

![Figure 6. Velocities of Pollen Tube Organelles and Beads under Different Experimental Conditions.](image)

Velocities of organelles (expressed as nm/s) under the standard conditions increased when the cytosol was not added to the mixture. Higher velocities also were obtained in the presence of cytochalasin D (in both the presence and the absence of cytosol). The velocity of beads coated with proteins from pollen tube organelles was comparable to the velocity of organelles under standard conditions. Approximately 20 organelles (or beads) were tested to calculate the speed and standard deviation for each experimental condition.

**Proteins from the 50/57 Organelle Fraction Bind to and Glide Microtubules in Vitro**

Because the 50/57 organelles exhibited higher motor activity, this organelle fraction was investigated for the presence of microtubule-based motors. Proteins from the 50/57 organelle fraction (Figure 8A, lane 3) were extracted with KI. After desalting, KI-extracted proteins were assayed for the ability to bind to microtubules in an ATP-sensitive manner. The pool of KI-extracted proteins (Figure 8A, lane 4) was incubated with microtubules in the presence of AMPPNP. Microtubules were pelleted by centrifugation. The AMPPNP-microtubule pellet (lane 6) was washed with ATP and KCl at a low concentration (200 mM). Centrifugation was used to separate the ATP/KCl-released proteins (S1-ATP; lane 7) from microtubule-bound proteins. The S1-ATP contained essentially polypeptides at 105 kD (arrowhead) and 74 kD (dot). A 53-kD band represented tubulin, as indicated by positive blots with a monoclonal antibody to tubulin (data not shown). The microtubule pellet was washed further with ATP and KCl at a higher concentration (500 mM) and then centrifuged to yield a second ATP/KCl-released sample (S2-ATP; lane 8) and the final microtubule pellet (lane 10). The second ATP supernatant contained predominantly two bands at 183 kD (lane 8, arrow) and 53 kD. The presence of polypeptides at 105 kD was not detected, showing that they had been released quantitatively in the first ATP/KCl wash. The ATP-dependent microtubule binding assay also was performed on proteins extracted from the PNS by KI treatment. In this case, the final ATP supernatant contained additional bands but still included polypeptides at 183 and 105 kD (lane 9, arrow and arrowhead, respectively).

A key feature of motor proteins is the ability to glide microtubules in an in vitro motility assay. The motor activity of the S1-ATP fraction was determined by gliding assay of microtubules on S1-ATP proteins bound to cover slips. Single microtubules bound to cover slips coated with S1-ATP proteins and moved in the presence of ATP with a velocity of
Immunoblot Analysis of the 105-kD Polypeptide

To determine if any S1-ATP proteins belonged to the kinesin superfamily, we probed different anti-kinesin antibodies.

Specifically, we used the polyclonal HYPIR antibody, which was raised against highly conserved amino acid sequences of the kinesin superfamily (Sawin et al., 1992); the polyclonal AKIN01, raised against bovine brain kinesin; the monoclonal AKIN02, which corresponds to SUK4 (Ingold et al., 1988) and was raised originally against the head of sea urchin kinesin; and the polyclonal K1005, which corresponds to the HD antibody (Rodionov et al., 1993) and was raised originally against the motor domain of Drosophila kinesin. Al-

133 ± 47 nm/s. Figure 8B shows an example of three to four microtubules that moved on S1-ATP proteins. The motor activity of S2-ATP was not investigated.

Figure 7. Motility Assay of the 50/57 Organelle Fraction.

(A) Silver-stained SDS gel showing the organelle fractions obtained after separation of PNS on a discontinuous Suc gradient. Lane 1, molecular mass standards (indicated in KD at left); lane 2, cytosolic proteins (5 μg); lane 3, PNS organelles; lane 4, the S33 fraction (the organelle fraction just above the 33% Suc layer); lane 5, the 33/44 fraction; lane 6, the 44/50 fraction; lane 7, the 50/57 fraction; lane 8, the 57/65 fraction. Lanes 3 to 8 contained 10 μg of protein. The front of migration of the electrophoresis is indicated at bottom left (df, dye front).

(B) In vitro motility assay of the 50/57 organelle fraction. The arrowhead indicates one organelle that moved along in vitro–polymerized microtubules. Numbers in each frame indicate the time in seconds. Bar = 2 μm.

(C) Detection of organelle marker proteins in the discontinuous Suc fractions. The specific activity of each marker is expressed as the relative percentage of enzymatic activity. The results are averages of three different experiments. Cytochrome c reductase, P-ATPase, inosine 5'-diphosphatase, and cytochrome C oxidase activities are markers of endoplasmic reticulum, plasma membrane, the Golgi apparatus, and mitochondria, respectively.
though the first three antibodies failed to give a positive reaction, the polyclonal K1005 cross-reacted with the 105-kD polypeptide (Figure 9A). The K1005 anti-kinesin antibody was probed on the protein fractions shown in Figure 8A to analyze the distribution of the 105-kD polypeptide during the isolation of microtubule binding proteins. As shown in Figure 9A, the 105-kD polypeptide was detected throughout the purification steps (arrowhead). In the PNS (lane 2), the 105-kD polypeptide was not the only band detected by the K1005 antibody. The protein was detected in the 50/57 organelle fraction (lane 3) and in the KI-extracted protein sample (lane 4). At this stage, most of the contaminating bands were lost. The 105-kD polypeptide bound preferentially to microtubules in the presence of AMPPNP (lane 6) and was released after washing with ATP/KCl (lane 7). The band was almost undetectable in the S2-ATP sample (lane 8), and only a small amount was found in the microtubule pellet (lane 10).

We also checked the distribution of the 105-kD polypeptide in the organelle fractions obtained after discontinuous Suc gradients (shown in Figure 7A). The K1005 antibody cross-reacted with all of the organelle fractions, but the 105-kD polypeptide was found mostly in the 50/57 and 57/65 fractions (Figure 9B). Densitometric analysis was performed in all lanes with Quantity One software to calculate the relative intensity of the blots. Assuming that the signal in the 50/57 fraction is 100% and that equal protein amounts were loaded in each lane, the 105-kD polypeptide was detected with a relative percentage of 13.4% in S33, 39.5% in 33/44, 32.8% in 44/50, and 86.1% in 57/65.

**ATPase Activity on S1-ATP Proteins**

Almost all microtubule-based motor proteins have microtubule-enhanced ATPase activity. To establish whether the S1-ATP proteins share this feature, we separated the S1-ATP polypeptides by Suc gradient centrifugation. Figure 10A shows a typical SDS gel representing the separation of S1-ATP polypeptides. The main proteins were eluted in distinct fractions: the 74-kD polypeptide at ~4 Svedberg units and the 105-kD protein at ~9 S. The ATPase activity of each fraction was determined in both the presence and the absence of microtubules. We found evidence for microtubule-enhanced ATPase activity (Figure 10B) that peaked in fraction 11 (from 0.08 to 3.7 nmol Pi/mL) and that corresponded exactly with the elution pattern of the 105-kD polypeptide. Analysis of the other fractions showed lower ATPase activity for the 74-kD polypeptide, which did not require the presence of microtubules.

**DISCUSSION**

Here, we present evidence that membrane-bound organelles of pollen tubes move along microtubules in in vitro motility assays. In vitro organelle movement occurs in the absence of cytosol, indicating that motor proteins are attached firmly to the organelle surface and that no cytosolic factors are required. The motility of organelles along microtubules is continuous, not saltatory, and is dependent on ATP. Motor protein(s), which can be extracted from the organelle surface, are able to move polystyrene beads along microtubules. The ATP-dependent microtubule binding of peripheral organelle proteins allowed the isolation of a protein fraction that glides microtubules and that contains a 105-kD protein showing microtubule-enhanced ATPase activity and cross-reactivity with anti-kinesin antibodies. These data constitute evidence that pollen tube organelles move along microtubules under in vitro conditions and that microtubule-dependent organelle movements are driven by motor proteins. A rational extension of these findings is that pollen tube organelles can move along microtubules in vivo conditions as well.

We used a reconstituted system to examine the movement of pollen tube organelles along microtubules. The in vitro motility assay using video-enhanced techniques is a powerful tool with which to study the movement of membrane-bound organelles under defined experimental conditions. This technique was used to study the motility of organelles from a wide variety of different cells and to characterize the proteins involved in the transport of organelles along microtubules (Schnapp et al., 1985). We used bovine brain tubulin in our motility assays mainly because of the poor quantity of tubulin that can be obtained from tobacco pollen tubes. Although it would be preferable to use the native track (i.e., pollen tube microtubules), extensive evidence indicates that animal tubulin can efficiently replace plant tubulin: the binding of plant MAPs to neuronal or plant tubulin is the same (Schellenbaum et al., 1993), microinjected brain tubulin incorporates into plant microtubules (Wasteneys et al., 1993), and sea urchin dynein translocates efficiently along plant microtubules (Yokota et al., 1995). Under standard conditions (i.e., in the presence of cytosol), we found that pollen tube organelles of different size move along microtubules. We have not yet investigated the polarity of organelle movement along microtubules.

Organelles can switch from different microtubules and can pass each other without colliding, as described in previous studies (Schnapp et al., 1985). Organelles move only after the addition of ATP, which implies the presence of ATP-driven motor proteins on the organelle surface. The mean velocity of such organelle movement (171 ± 58 nm/s) is very low compared with the velocity of pollen tube organelles involved in cytoplasmic streaming (~1 μm/s). Other authors have reported that isolated vesicles can translocate along microtubules in vitro motility assays with velocities close to the speed that vesicles attain within cells (Schnapp et al., 1985). This equivalence was not found when isolated pollen tube organelles were assayed for the ability to slide along actin bundles in characean cell models (Kohno and Shimmen, 1988). In that case, the velocity of organelle movement was
greater than that of the cytoplasmic streaming in pollen tubes, indicating that the potential velocity of pollen tube organelles is moderated within the cell. Nevertheless, these examples suggest that organelle speed in an in vitro motility assay is comparable to or greater than the in vivo organelle velocity. Consequently, evidence that the in vitro velocity of pollen tube organelles along microtubules is considerably less than that in vivo suggests that either (1) we have been unable to reproduce the in vivo conditions (which may be more stringent for pollen tubes), or (2) microtubules are not involved in the primary route of cytoplasmic streaming in the pollen tube. Organelle movement along microtubules does not occur in the presence of nucleotides other than ATP and is sensitive to AMPPNP, as shown for animal kinesins (Urrutia et al., 1991) and for pollen tube myosin (Kohno et al., 1990).

Whether organelle speed is dependent on size is not known; it appeared that larger organelles exhibited a lower velocity, but statistical analysis is needed to confirm this observation. Movement of pollen tube organelles was not salatory, as was observed in other cases (Vale et al., 1985a). Organelle movement in our assay was not actin dependent. The addition of cytochalasin D did not prevent organelle movement but, surprisingly, increased organelle speed to 360 ± 67 nm/s (possibly by removing networks of AFs that could prevent the free movement of organelles along microtubules). In addition, no AFs were visualized in our assay samples after staining with rhodamine-phalloidin.

The in vitro movement of pollen tube organelles occurred in both the presence and the absence of cytosol. This result indicated that organelles do not require the presence of soluble motor proteins to move along microtubules and, consequently, that motor proteins are present on the surface of isolated organelles. The association of microtubule motor proteins with membrane-bound organelles has been shown for different cells (Allan and Schroer, 1999), leading to the conclusion that functional motor proteins are attached stably to the organelle surface and that the cytosol contains a nonfunctional pool of motor proteins (Reilein et al., 2001). The requirement of soluble components other than motor proteins to activate or regulate organelle-associated motor proteins in eukaryotic cells still is under debate. For example, the first evidence that soluble factors other than kinesin are essential for organelle motility (Schroer et al., 1988) was not confirmed by subsequent studies, in which purified kinesin was shown to promote vesicle motility (Urrutia et al.,

in S1-ATP. The arrows indicate the 183-kD polypeptide released in S2-ATP. The front of migration of the electrophoresis is indicated at bottom left (df, dye front).

(B) Microtubules gliding on cover slips coated with S1-ATP. Microtubules (arrows and arrowheads) were observed to move in the focus plane with velocities of 133 ± 47 nm/s. Numbers in each frame indicate the time in seconds. Bar = 2 μm.
Because the addition of cytochalasin D increased counteract the activity of the organelle-associated motor proteins, microtubule binding proteins that hinder the movement of organelles along microtubules, or other motors that regulate organelle velocity. These factors could be regulatory proteins on the cargo.

The ATP-dependent binding of KI-extracted proteins to microtubules could highlight most of the microtubule-based motor proteins by KI treatment or the requirement of receptor proteins. However, extracted proteins succeeded in moving polystyrene beads, which suggested that functionally active motors are detached from the organelle surface by KI treatment. The presence of microtubule-based motors on the surface of pollen tube organelles is not unexpected. Microtubule-based motors other than the 105-kD polypeptide are present in the pollen tube and are found in association with membrane-bound organelles. The pollen kinesin homolog was identified initially as a soluble protein that interacted with microtubules in an ATP-dependent manner (Tiezzi et al., 1992); however, the protein was immunolocalized at the tip and on the flanks of pollen tubes with a spot-like pattern resembling the distribution of organelles or vesicles (Cai et al., 1993). Furthermore, an immunorelated pollen kinesin homolog was detected in association with the surface of isolated 80-nm membranous structures from hazel pollen (Liu et al., 1994). Dynein-related polypeptides were discovered as soluble proteins as well (Moscatelli et al., 1995); however, they also were found in association with membranous structures (Moscatelli et al., 1998). The 90-kD ATP-MAP was isolated as a soluble protein; however, the MMR44 antibody detected the 90-kD ATP-MAP in association with organelles isolated from pollen tubes (Cai et al., 2000). At present, pollen kinesin homolog, dynein-related polypeptides, and 90-kD ATP-MAP have not been shown to translocate organelles along microtubules, even though the 90-kD ATP-MAP has motor activity and interacts with organelles. Nevertheless, these data suggest that the pollen tube contains two distinct populations of microtubule-based motors—soluble and membrane associated—whose relationships are not known.

The present evidence on the movement of isolated pollen tube organelles along microtubules in the absence of soluble proteins confirms previous findings and suggests that microtubule-based motors are bound to the surface of pollen tube organelles as in other cells.

Motor proteins have been extracted from different organelles by treatment with KI (Schoer et al., 1988) or NaOH (Lacey and Haimo, 1992), but kinesin was shown to bind to membranes after strong treatment conditions, such as 1 M KI or 1 M NaCl (Schnapp et al., 1992). By contrast, pollen tube myosin was shown to be released from isolated organelles after treatment with 0.5 M KCl (Kohno et al., 1990). In our hands, 0.6 M KI was sufficient to extract motor proteins from the surface of pollen tube organelles, because KI-washed organelles did not show motility along microtubules. Presumably, most but not all microtubule-based motors were extracted from the surface of pollen tube organelles after KI treatment. However, extracted proteins succeeded in moving polystyrene beads, which suggested that functionally active motors are detached from the organelle surface by KI treatment. The average velocity of beads is lower compared with that of organelles in the absence of cytosol, which suggests a partial denaturation of motor proteins by KI treatment or the requirement of receptor proteins on the cargo.

The ATP-dependent binding of KI-extracted proteins to microtubules could highlight most of the microtubule-based proteins involved in this mechanism of control.

Movement of Pollen Tube Organelles along Microtubules 263

The ATP-dependent binding of KI-extracted proteins to microtubules could highlight most of the microtubule-based proteins involved in this mechanism of control.

Figure 9. Immunoblot Analysis of the 105-kD Polypeptide.

(A) Immunoblot analysis with the polyclonal K1005 antibody on fractions obtained during the preparation of S1-ATP (lanes 2 to 10 are the same as in Figure 8A). The antibody cross-reacted with the 105-kD polypeptide in all lanes (arrowhead). Other contaminating cross-reacting bands were lost during the procedure; as a result, the 105-kD polypeptide is the only band that binds to and is released from the microtubules. Lane 1, Coomassie blue–prestained molecular mass markers. The first three bands correspond to molecular masses of 205, 115, and 93 kD, respectively.

(B) Cross-reactivity of the K1005 antibody with the organelle fractions obtained by discontinuous Suc gradients. Lane 1 to 6, PNS, S33, 33/44, 44/50, 50/57, and 57/65 fractions, respectively. The 105-kD polypeptide was detected in all fractions, but more strongly in the 50/57 and 57/65 fractions (lanes 5 and 6, respectively). Equal protein amounts were loaded in each lane.
motors associated with pollen tube organelles. When the assay was performed using proteins extracted from the PNS, the final ATP supernatant revealed many polypeptides, which likely correspond to putative microtubule-based motors. However, we concentrated our efforts on the 50/57 organelle fraction because it showed a higher motility rate. The 183-, 105-, and 74-kD polypeptides are likely candidates for motor proteins. We did not investigate the 183-kD polypeptide, which is eluted only when KCl concentration is >500 mM. In addition, the in vitro microtubule gliding assay on S2-ATP-coated cover slips was not performed. The S1-ATP sample, which contained the 105- and 74-kD polypeptides in addition to tubulin, was able to glide microtubules at a speed of 133 ± 47 nm/s. This value is comparable to the velocity of kinesin (Saxton et al., 1988) but is lower than the velocity of microtubules gliding on dynein-coated slides (Sakakibara and Nakayama, 1998).

Motor proteins are machines that convert the energy of ATP hydrolysis in mechanical work; therefore, microtubule gliding correlates well with the enzymatic ability to hydrolyze ATP (Oosawa, 1995). In our case, the motor activity of S1-ATP was related to the 105-kD polypeptide, because it exhibited exclusively microtubule-dependent ATPase activity. Therefore, the 105-kD polypeptide is a potential candidate for moving pollen tube organelles along microtubules. This protein is extracted from pollen tube organelles, is able to bind to microtubules in an ATP-sensitive manner, is the main band of a protein fraction (S1-ATP) that glides microtubules, and is the only protein that shows microtubule-dependent ATPase activity. In addition, the protein cross-reacts with the anti-kinesin antibody K1005, formerly known as HD, which was raised against the motor domain of Drosophila kinesin (Rodionov et al., 1993). These findings indicate that the 105-kD polypeptide is related functionally, biochemically, and

Figure 10. Suc Gradient Centrifugation of S1-ATP and Quantitation of Mg-ATPase Activity.
(A) The S1-ATP fraction was fractionated on a 5 to 25% Suc gradient, and fractions of 0.25 mL were collected. Black arrowheads indicate standard proteins in Svedberg units: thyroglobulin (19.1 S), catalase (11.3 S), and BSA (4.4 S). The positions of both the 105- and 74-kD polypeptides are indicated by white arrowheads. Lane M, molecular mass standards; lane L, protein sample loaded onto the Suc gradient (S1-ATP); lanes 2 to 19, fractions from the Suc gradient. The front of migration of the electrophoresis is indicated at bottom left (df, dye front).
(B) Mg-ATPase activity with and without microtubules (MT) was analyzed in the Suc gradient fractions shown in (A). Activity is expressed as nmol/mL inorganic phosphate.
immunologically to kinesin. The 105-kD polypeptide was not the only band that cross-reacted with the K1005 antibody; however, it was the only polypeptide that bound to and was released from microtubules. Therefore, the other cross-reacting bands are likely to be nonmotor proteins that share common epitopes. Because the K1005 antibody stained bands other than the 105-kD polypeptide and cross-reacted with all organelle fractions, it could not be used for immunocytochemistry analysis of pollen tubes.

Studies with cytoskeletal inhibitors have indicated that cytoplasmic streaming of organelles in the pollen tube depends essentially on AFs (Heslop-Harrison et al., 1988). By contrast, our data from motility assays indicate that pollen tube organelles are competent to move along microtubules. Furthermore, we found that all organelle fractions obtained by Suc gradient centrifugation showed microtubule-dependent motility and that the 50/57 organelle fraction had a higher motility rate. These findings suggest two possible hypotheses. The first hypothesis suggests that microtubule-based transport is a common feature of pollen tube organelles. This hypothesis, which is confirmed indirectly by the presence of the 105-kD motor protein in all organelle fractions, proposes that microtubules play a general role in the positioning or movement of pollen tube organelles. Because the long-range transport of organelles occurs along AFs, microtubules could be important for the localization of organelles at specific sites (perhaps temporarily) or for the short-range movement of organelles and vesicles in the pollen tube. These assumptions are supported by data showing that the positioning and transport of membranous structures in eukaryotic cells depend on the coordinated activity of AFs, microtubules, and related motors (Goode et al., 2000). In plant cells, organelle transport relies mainly on AFs and myosin, as shown for the Golgi apparatus (Boevink et al., 1998) and peroxisomes (Mathur et al., 2002). However, recent evidence has shown that microtubules also could play a role in the relocation and positioning of other plant organelles, such as chloroplasts (Sato et al., 2001) and mitochondria (Van Gestel et al., 2002).

The second hypothesis suggests that a few classes of pollen tube organelles move more actively along microtubules. Specific enrichment of the 105-kD polypeptide in the most active organelle fraction indicates that this protein is one of the microtubule-based motors that drives organelles or vesicles of the 50/57 fraction. The 50/57 fraction is more enriched in cytochrome c oxidase activity than in other markers, suggesting that mitochondria may be the relevant organelle class moving along microtubules in the 50/57 fraction. Furthermore, the appearance of membranes is very similar to that of mitochondria moving in vitro along microtubules in other cell systems (Nangaku et al., 1994). To reinforce these data, we found that the 57/65 organelle fraction (which contained the peak mitochondrial marker) has motor activity lower but comparable to that of the 50/57 fraction and is correspondingly enriched in the 105-kD motor polypeptide. Although we cannot exclude the presence of other mobile membranes in the 50/57 fraction, these findings suggest that moving organelles in the 50/57 fraction are enriched in mitochondria and that the 105-kD polypeptide participates in their positioning or transport along microtubules, as shown for both animal (Nangaku et al., 1994) and plant (Van Gestel et al., 2002) cells.

Evidence has been reported that the microtubule cytoskeleton is involved in the transport of both the generative cell and the vegetative nucleus in the pollen tube (Astrom et al., 1995). The precise role of microtubules during generative cell and vegetative nucleus movement is unknown, because the drug-dependent disorganization of microtubules did not prevent but markedly reduced the transport efficiency and speed of these larger particles. No microtubule-based motors have been localized on the surface of or close to the generative cell and the vegetative nucleus. Furthermore, no in vitro motility assays with isolated generative cells have been reported. Therefore, the involvement of microtubule motors in the transport of the generative cell and the vegetative nucleus is far from established. We hypothesize that the role of microtubules is to organize the pollen tube cytoplasm (including the actin cytoskeleton) during the movement of the generative cell and the vegetative nucleus. The motor protein and the motility events we have demonstrated here could take part in this process.

METHODS

Chemicals and Antibodies

Chemical reagents for electrophoresis, including molecular mass standards (unstained or prestained with Coomassie Brilliant Blue R250), were purchased from Bio-Rad (Hercules, CA). Blotting membranes, secondary antibodies, and enhanced chemiluminescence reagents were from Amersham Pharmacia Biotech (Uppsala, Sweden). General reagents used in the preparation of buffers (including nucleotides) were purchased from Sigma-Aldrich (St. Louis, MO). Chromatographic columns were from Amersham Pharmacia Biotech. Polystyrene beads for motility assays were obtained from Sigma-Aldrich. Purified tubulin and recombinant kinesin were purchased from Cytoskeleton, Inc. (Denver, CO). Anti-kinesin antibodies were obtained as follows: the polyclonal HYPIR antibody was a generous gift from K.E. Sawin (Institute of Cell and Molecular Biology, University of Edinburgh, UK) (Sawin et al., 1992); polyclonal AKIN01 and monoclonal AKIN02 (SUK4) were purchased from Cytoskeleton, Inc.; and polyclonal K1005 was obtained from Sigma-Aldrich.

Preparation of Taxol-Stabilized Microtubules

Microtubules were polymerized from monomeric tubulin (10 mg/mL in 80 mM PIPES, pH 6.8, 1 mM MgCl2, and 1 mM EGTA) in the presence of 1 mM GTP and 10% glycerol at 35°C for 20 min. The microtubule sample was diluted 1:25 in microtubule resuspension buffer (80 mM PIPES, pH 6.8, 1 mM MgCl2, 1 mM EGTA, 1 mM DTT, and 20 μM taxol), placed at room temperature, and used for several motility assays.
Pollen Culture

Anthers of tobacco (*Nicotiana tabacum*) were collected from plants grown in the Botanical Garden of Siena University. After dehiscence, pollen was collected, dried on silica gels, and stored at -20°C. Before use, the pollen was acclimatized progressively at room temperature and then hydrated in a moist chamber. The pollen was germinated in BK medium [1.62 mM H$_3$BO$_3$, 1.25 mM Ca(NO$_3$)$_2$·4H$_2$O, 2.97 mM KNO$_3$, and 1.65 mM MgSO$_4$·7H$_2$O] containing 15% (w/v) Suc (Brewbaker and Kwack, 1963). Germination was performed for 2 to 3 h in a rotary incubator set at 24°C. Germinated pollen then was used for protein preparation.

Preparation of Organelle and Cytosol Fractions from Tobacco Pollen Tubes

After germination in BK medium containing 15% (w/v) Suc, the pollen was washed twice with 25 mL of BRB25 buffer (25 mM Hepes, pH 7.5, 2 mM EGTA, and 2 mM MgCl$_2$) plus 15% Suc. The pollen was resuspended in 1 volume of lysis buffer (BRB25 buffer containing 2 mM DTT, 1 mM phenylmethylsulfonyl fluoride [PMSF], 1 μM protease inhibitor cocktail [Sigma-Aldrich], 1 mM NaN$_3$, and 10% mannitol). After lysis on ice with a motor-driven Potter-Elvehjem homogenizer (Millville, NJ), the sample was centrifuged at 10,000 g for 10 min at 4°C to remove large cellular debris. The low-speed supernatant was centrifuged again at 100,000 g to remove large cellular debris. The low-speed supernatant was resuspended with 200 μL of resuspension buffer (BRB25 buffer, 10% mannitol, 1 mM DTT, and 1 mM PMSF) for 50 mg of germinated pollen. The sample of tobacco pollen tube organelles was named the postnuclear supernatant.

Organelle Motility Assay

The movement of organelles along microtubules was analyzed by in vitro motility assay (Waterman-Storer, 2001). Perfusion chambers were assembled by attaching two pieces of double-sided scotch tape on clean microscope slides, and then clean cover slips were placed on top of them to form a perfusion chamber of 10 to 15 μL. The movement of organelles along microtubules was analyzed by in vitro motility assay (Waterman-Storer, 2001). After the addition of 20 μM taxol and 5 mM ATP, the mixture was used for the in vitro motility assay, which was performed like the organelle motility assay.

Fractionation of Pollen Tube Organelles by Suc Gradient Centrifugation

Pollen tube organelles were separated into five different classes by a discontinuous Suc gradient (Robinson and Hinz, 2001). Suc solutions at different concentrations (33, 44, 50, 57, and 65% [w/v]) were layered into a centrifuge tube (0.8 mL of each solution). Postnuclear supernatant organelles (~500 μL) were loaded directly onto 4.0 mL of the discontinuous Suc gradient and centrifuged at 200,000g for 60 min at 4°C. The five organelle fractions obtained were named S33 (for the organelle layer just above the 33% Suc layer), 33/44, 44/50, 50/57 and 57/65 (for the interfaces between each Suc concentration). Fractionation of pollen tube organelles by suc gradient centrifugation.

Extraction of Peripheral Proteins from Pollen Tube Organelles

Peripheral proteins from pollen tube organelles were extracted by incubating organelles for 30 min on ice with 0.6 M KI (Schoer et al., 1988). KI-washed organelles were centrifuged at 100,000g for 90 min at 4°C. The pellet was resuspended with an identical volume of BRB25 buffer containing 1% mannitol, 1 mM DTT, and 1 mM PMSF. Stripped organelles were used for in vitro motility assays. The supernatant obtained after centrifugation was desalted using the Hi-Trap desalting column (Amersham Pharmacia Biotech) in BRB25 buffer containing 1 mM DTT and 1 mM PMSF. Desalted KI-extracted proteins were used for in vitro motility assay with carboxylated latex beads.

Motility Assay with Carboxylated Latex Beads

For the in vitro motility assay of carboxylated latex beads, KI-extracted proteins after desalting were mixed with carboxylated latex beads (1:1500 final dilution) and incubated for 5 min on ice (Vale et al., 1985b). After the addition of 20 μM taxol and 5 mM ATP, the mixture was used for the in vitro motility assay, which was performed like the organelle motility assay.

Argus 20 (Hamamatsu) was used to visualize microtubules and organelles. The Background Subtraction and Average commands of Argus 20 were used to enhance the image quality. Video sequences were recorded onto U-matic tapes (Sony, Tokyo, Japan). For printing of single video frames, individual frames were captured using ATI TV-Player software (ATI Technologies, Markham, Canada) running on a personal computer equipped with the ATI Radeon All-In-Wonder video capture card connected to the video recorder. To evaluate organelle velocity, single organelles were tracked with the mouse cursor and their velocity was calculated with the Speed command of Argus 20. To visualize the presence of actin filaments, samples were counterstained with 1 μM rhodamine-phalloidin, which was added directly to the organelle-cytosol mixture.
ATP-Dependent Microtubule Binding Assay

Peripheral organelle proteins were extracted with KI and desalted with the HiTrap desalting column into BRB25 buffer containing 1 mM DTT and 1 mM PMSF. Microtubules used in the binding assay were prepared as described for the motility assay. Microtubules (0.66 mL for 1 mL of protein solution), 20 μM taxol, and 10 mM AMPPNP were added to the protein solution. The mixture was incubated for 30 min at room temperature, and the sample was centrifuged at 40,000 g for 30 min at 25°C. The pellet was washed for 10 min at room temperature with 1 mL of EDTA buffer (25 mM Hepes, pH 7.5, 3 mM EGTA, 1 mM AMPPNP, 20 μM taxol, 1 mM DTT, and 10 mM EDTA). The sample was centrifuged at 40,000 g for 30 min at 25°C. The pellet was incubated overnight with 0.5 mL of release buffer 1 (25 mM Hepes, pH 7.5, 3 mM EGTA, 2 mM MgCl₂, 1 mM DTT, 200 mM KCl, 20 μM taxol, and 10 mM ATP). After centrifugation at 40,000 g for 30 min at 25°C, the supernatant was named S1-ATP. The pellet was washed for 60 min with 0.5 mL of release buffer 2 (25 mM Hepes, pH 7.5, 3 mM EGTA, 2 mM MgCl₂, 1 mM DTT, 500 mM KCl, 20 μM taxol, and 10 mM ATP). The sample was centrifuged at 40,000 g for 30 min at 25°C, and the resulting supernatant was named S2-ATP.

Microtubule Motility Assays

The in vitro motility assay of microtubules was performed exactly as described previously (Cai et al., 2000).

Velocity Sedimentation

Centrifugation through continuous Suc gradients was used to separate the proteins extracted from the organelle fractions and that bound to microtubules in an ATP-sensitive manner. Samples of S1-ATP (∼300 μL) were loaded onto 4.5 mL of 5 to 25% Suc gradients prepared using a gradient former from Amersham Pharmacia Biotech and a peristaltic pump from Bio-Rad. Suc was dissolved in BRB25 buffer containing 1 mM PMSF and 2 mM DTT. Thyroglobulin (19.1 Svedberg units), catalase (11.3 Svedberg units), and BSA (4.4 Svedberg units) were used as standards to calculate the sedimentation coefficient (Martin and Ames, 1961). Samples were centrifuged at 80,000 g for 15 h at 4°C. Fractions of ∼250 μL each were collected from the top of the centrifuge tubes using a peristaltic pump.

Electrophoresis of Proteins and Immunoblot Analysis

SDS-PAGE analysis was performed using 7.5% linear acrylamide concentration (Laemmli, 1970) on a mini-gel apparatus (Bio-Rad). Gels were stained with silver according to the protocol and kit provided by Amersham Pharmacia Biotech. Images of gels were captured using Quantity One software and the Fluor-S Multimager apparatus from Bio-Rad. Immunoblot analysis (Towbin et al., 1979) was performed on a TE22 Transfer Unit (Amersham Pharmacia Biotech). Primary antibodies were used for 1 h diluted as follows: the HYPIR antibody at 25 μg/mL, AKIN01 and AKIN02 (SUK4) at 0.25 and 1 μg/mL, respectively, and K1005 at 1:500. Secondary antibodies were horse-radish peroxidase–conjugated goat anti-mouse and goat anti-rabbit antibodies from Amersham Pharmacia Biotech and were used at 1:3000 for 1 h. Blots were developed with the enhanced chemiluminescence kit from Amersham Pharmacia Biotech and captured with the Fluor-S apparatus. Images of molecular mass standards, which were prewashed with Coomassie Brilliant Blue R250, were captured with the Fluor-S apparatus and superimposed to the blot results.

Protein Concentration

The protein concentration was determined by the colorimetric method (Bradford, 1976) using BSA as a standard.

ATPase Activity

The ATPase activity of protein fractions was measured as follows. A total of 80 μL of protein samples was supplemented with 10 μL of ATP solution (20 mM Hepes, pH 7.5, 20 mM ATP, 20 mM MgCl₂, and 10 mM DTT). For analysis in the presence of microtubules, 10 μL of microtubule solution was added to the mixture. In the control sample, 10 μL of BRB25 buffer was added to the mixture in place of microtubules. Samples were incubated at 25°C for 45 min. The release of Pi was measured according to the PhosFree phosphate assay kit purchased from Cytoskeleton, Inc. A linear working curve was made from 4.5 to 90 μM K-phosphate and used to calculate the nanomoles of phosphate produced by the protein fractions.

ACKNOWLEDGMENTS

We thank Kenneth E. Sawin (Institute of Cell and Molecular Biology, University of Edinburgh, UK) for kindly donating the HYPIR antibody. We also thank Ronald D. Vale (Department of Cellular and Molecular Pharmacology, University of California, San Francisco), Clive Lloyd (Department of Cell Biology, The John Innes Centre, Colney, Norwich, UK), and Antonio Tiezzi (Dipartimento Scienze Ambientali, Università della Tuscia, Viterbo, Italy) for helpful and critical discussions.

Received June 25, 2002; accepted October 8, 2002.

REFERENCES


Move along Microtubules

In Vitro Assays Demonstrate That Pollen Tube Organelles Use Kinesin-Related Motor Proteins to Move along Microtubules
Silvia Romagnoli, Giampiero Cai and Mauro Cresti

*Plant Cell* 2003;15;251-269; originally published online December 13, 2002;
DOI 10.1105/tpc.005645

This information is current as of September 30, 2017

<table>
<thead>
<tr>
<th>Supplemental Data</th>
<th>/content/suppl/2002/12/31/15.1.251.DC1.html</th>
</tr>
</thead>
<tbody>
<tr>
<td>References</td>
<td>This article cites 72 articles, 31 of which can be accessed free at: /content/15/1/251.full.html#ref-list-1</td>
</tr>
<tr>
<td>eTOCs</td>
<td>Sign up for eTOCs at: <a href="http://www.plantcell.org/cgi/alerts/ctmain">http://www.plantcell.org/cgi/alerts/ctmain</a></td>
</tr>
<tr>
<td>CiteTrack Alerts</td>
<td>Sign up for CiteTrack Alerts at: <a href="http://www.plantcell.org/cgi/alerts/ctmain">http://www.plantcell.org/cgi/alerts/ctmain</a></td>
</tr>
<tr>
<td>Subscription Information</td>
<td>Subscription Information for <em>The Plant Cell</em> and <em>Plant Physiology</em> is available at: <a href="http://www.aspb.org/publications/subscriptions.cfm">http://www.aspb.org/publications/subscriptions.cfm</a></td>
</tr>
</tbody>
</table>