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A Kinesin-like Protein, KatAp, in the Cells of Arabidopsis and Other Plants

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The kinesin-like proteins (KLPs) are a large family of plus- or minus-end-directed microtubule motors important in intracellular transport, mitosis, meiosis, and development. However, relatively little is known about plant KLPs. We prepared an antibody against two peptides in the microtubule binding domain of an Arabidopsis KLP (KatAp) encoded by the *KatA* gene, one of a family of genes encoding KLPs whose motor domain is located near the C terminus of the polypeptide. Such KLPs typically move materials toward the minus end of microtubules. An immunoreactive band (M_r of 140,000) corresponding to KatAp was demonstrated with this antibody on immunoblots of Arabidopsis seedling extracts. During immunofluorescence localizations, the antibody produced weak, variable staining in the cytoplasm and nucleus of interphase Arabidopsis suspension cells but much stronger staining of the mitotic apparatus during division. Staining was concentrated near the midzone during metaphase and was retained there during anaphase. The phragmoplast was also stained. Similar localization patterns were seen in tobacco BY-2 cells. The antibody produced a single band (M_r of 130,000) in murine brain fractions prepared according to procedures that enrich for KLPs (binding to microtubules in the presence of AMP-PNP but not ATP). A similar fraction from carrot suspension cells yielded a cross-reacting polypeptide of similar apparent molecular mass. When dividing BY-2 cells were lysed in the presence of taxol and ATP, antibody staining moved rapidly toward the poles, supporting the presence of a minus-end motor. Movement did not occur without ATP, with AMP-PNP, or with ATP plus antibody. Our results indicate that the protein encoded by *KatA*, KatAp, is expressed in Arabidopsis and is specifically localized to the midzone of the mitotic apparatus and phragmoplast. A similar protein is also present in other species.

INTRODUCTION

Microtubule-dependent motility relies primarily on mechanochemical proteins belonging to the dynein and kinesin superfamilies (Goldstein, 1993a; Goodson et al., 1994; Tanaka et al., 1995). By harnessing the chemical potential energy of ATP to do work, these motor proteins govern various motility phenomena associated with microtubules, including organelle/vesicle transport, nuclear migration and karyogamy, and chromosome motion (reviewed in Endow and Titus, 1992; Goldstein, 1993a; Walker and Sheetz, 1993; Bloom and Endow, 1994; Goodson et al., 1994; Schroer, 1994; Block, 1995; Brady, 1995; Cole and Lippincott-Schwartz, 1995). Several of these motors have been implicated in the assembly and alignment of the meiotic and mitotic apparatus or the generation of their characteristic morphology (Fuller and Wilson, 1992; Sawin and Endow, 1993; Bloom and Endow, 1994). Two recently described members of the kinesin superfamily, chromokinesin and Nod protein, contain DNA binding motifs as well as microtubule binding domains (Afshar et al., 1995; Wang and Adler, 1995). In addition, kinesin-like proteins (KLPs), such as CENP-E, can cross-link microtubules in a mitosis-regulated fashion (Liao et al., 1994). The data have produced a picture of complex, cooperative or antagonistic interactions between various motors in cell motility and division.

Diverse KLPs have been identified in various organisms such as mammals, yeasts, Aspergillus, Xenopus, and Drosophila, using analyses of mutants and polymerase chain reaction (PCR) procedures (Bloom and Endow, 1994). Multiple KLPs are present in each organism, and some may govern redundant functions (Goldstein, 1993a, 1993b). Kinesin and KLP heavy chains possess a number of general structural features in common, including a globular head or motor domain containing conserved microtubule and ATP binding regions and a nonconserved globular tail that determines the different cargoes with which each motor interacts. A linking α helical region is often present as well, which participates in oligomerization via coiled-coil interactions (Cole and Scholey, 1995). Kinesin and KLPs may also associate with additional light chain polypeptides.

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the movement of cross-reacting KLP in lysed tobacco cells. All of these observations support the conclusion that the antibody detected KLP.

The molecular mass of KatAp calculated from the cDNA open reading frame is 89,046 D (Mitsui et al., 1993); however, the M_r of the Arabidopsis polypeptide detected in our study was 140,000. Although this discrepancy appears to be rather large, significant differences in calculated and relative molecular masses have been reported for other KLPs. Indeed, Bernstein et al. (1994) proposed that migration slower than predicted during SDS-PAGE may be a general feature of KLPs. Bloom and Endow (1994) have also noted the complex electrophoretic mobility patterns of KLPs. It is noteworthy in this regard that anti-KatAp produced single immunoreactive bands of M_r closer to 100,000 in pollen tubes of tobacco and Tradescantia (Liu and Palevitz, 1996).

Although the anti-KatAp antibody appeared to be specific for KLP, we cannot rule out the possibility that it recognizes more than one homologous polypeptide with very similar molecular masses in Arabidopsis and other species. Such behavior would be consistent with the fact that several *Kat* genes are now known in Arabidopsis and that KLPs in general exhibit redundancy of function. Moreover, the superfamily of genes encoding KLPs in Arabidopsis undoubtedly encompasses genes in addition to the *Kat* sequences, because the *stalkless* (*stl*) mutation that produces aberrant trichomes in this species resides in a KLP gene different from the *Kat* genes (Oppenheimer and Marks, 1995). That the anti-KatAp antibody stained only a portion of the mitotic apparatus argues in favor of it binding to one KLP or to a limited subset of KLPs.

The data are consistent with the conclusion that our antibody detected a minus-end-directed motor. Unequivocal evidence of motor directionality would have been provided by an in vitro motility assay using intact microtubules and polypeptides identified and/or isolated by using the antibody. However, our attempts to use such an assay were unsuccessful. As an alternative, we assessed the redistribution of anti-KatAp binding protein in lysed BY-2 cells. Although it is less definitive than the in vitro motility assay, the use of lysed cells is based on available information on the polarity of microtubules in the mitotic apparatus and phragmoplast. Euteneuer et al. (1981) showed that the minus ends of microtubules are located at the poles in Haemanthus endosperm cells. More recent results from tobacco BY-2 are consistent with this conclusion (Asada et al., 1991; Asada and Shibaoka, 1994). Thus, the movement of antibody label toward the poles indicates a minus-end-directed motor. This conclusion is consistent with sequence analysis showing that the motor domain of KatAp is located at the C terminus of the KatAp polypeptide (Mitsui et al., 1993, 1994). So far, all known KLPs with C-terminal motor domains are minus-end-directed motors.

A polypeptide that cross-reacts with an antibody to kinesin heavy chain has been reported in the pollen tubes of tobacco and *Corylus avellana* (Cai et al., 1993; Liu et al., 1994b). However at first glance that protein may be different from KatAp and its homologs. The anti-KatAp antibody most prominently stains the mitotic apparatus of dividing somatic cells (data presented here) as well as tobacco generative cells (Liu and Palevitz, 1996), whereas the antibody to kinesin heavy chain reportedly does not stain generative cells.

We realize that the peptides used to prepare our antibody are partially conserved in kinesin heavy chain. However, that the antibody binds to multiple polypeptides is unlikely, based on the immunoblot and immunofluorescence data. It also seems unlikely that the antibody recognizes structural MAPs unrelated to KLPs, since a data base search of known structural MAPs did not reveal homologous antigenic peptides. Moreover, when the antibody was blotted against a different preparation of carrot MAPs isolated by tubulin affinity chromatography (Durso and Cyr, 1994), only a faint, nonspecific reaction was detected (data not shown). Thus, all of the data, including the enhanced binding to microtubules in the presence of AMP–PNP and movement of antigen in lysed cells in the presence of ATP, point to binding of KLP by our antibody.

Minus-end-directed KLPs, as well as cytoplasmic dynein, have been implicated in various aspects of cell division, including the organization of the mitotic apparatus in yeast, humans, and Drosophila. It has been proposed that minus-end KLPs are involved in setting up the architecture characteristic of mitotic and meiotic spindles (Fuller and Wilson, 1992; Sawin and Endow, 1993; Bloom and Endow, 1994; Fuller, 1995). Minus-end KLPs, working in conjunction with plus-end-directed microtubule motors, may be responsible for establishing a balance of forces necessary for spindle bipolarity (see above reviews). One or more such motors may also play a role in centrosome organization (Endow et al., 1994a). KLPs associated with the kinetochores may be responsible for elements of chromosome motion, including congression and anaphase A and B (Bloom and Endow, 1994; Wordeman and Mitchison, 1995; Hogan et al., 1992, 1993). Cytoplasmic dynein, a minus-end-directed motor, may cooperate with one or more KLPs to accomplish spindle elongation and may also govern or participate in nuclear migration (Eshel et al., 1993; Li et al., 1993; Plamann et al., 1994; Xiang et al., 1994; Saunders et al., 1995; Yeh et al., 1995; see also Schroer, 1994) and the initial organization or stabilization of the mitotic apparatus (Vaisberg et al., 1993). Recent evidence indicates that KLPs may affect tubulin flux in the spindle by enhancing depolymerization at the minus end (Endow et al., 1994b). Conversely, KLPs may participate in poleward chromosome motion specifically tied to microtubule depolymerization (Lombillo et al., 1995a). In the latter study, antibodies raised against the minus-end-directed motor CENP-E inhibited chromosome motion in an in vitro system under microtubule depolymerizing conditions, which indicates that a KLP can couple motor activity to microtubule depolymerization (Desai and Mitchison, 1995).

A minus-end-directed motor could serve a number of functions in the midzone and phragmoplast of plant cells. However, any function must account for the fact that KatAp remains in the midzone from metaphase onward. As proposed for motors in other dividing eukaryotes, KatAp could help establish a balance of forces necessary to maintain the pole-to-pole

Most KLPs identified so far appear to direct movement toward the plus end of microtubules, although a few KLPs, such as ncd in Drosophila, CHO2 in rodents, CENP-E in humans, and Kar3 in yeast, are minus-end-directed motors (Goldstein, 1993a; Bloom and Endow, 1994; Kuriyama et al., 1995). Most of the motor domains of these minus-end-directed KLPs are located at the C terminus, in contrast to the plus-end-directed proteins, in which the heads are situated primarily toward the N terminus of the molecule. In at least two cases, the motor domain is in the middle of the molecule (Noda et al., 1995; Wordeman and Mitchison, 1995). The position of the motor domain does not determine directionality, however. Instead, this property appears to be intrinsic to the domain itself (Stewart et al., 1993). Indeed, CENP-E was recently shown to direct minus-end motility in HeLa cells with a motor domain near the N terminus (Thrower et al., 1995).

Immunodetection of kinesin and KLPs is consistent with functions in microtubule-dependent motility. Immunolabeling is associated primarily with vesicles or organelles (Lippincott-Schwartz et al., 1995; see also Bloom and Endow, 1994) and at various positions in the mitotic apparatus, including the kinetochores, centrosome, spindle fibers, chromosome arms, poles, and interzone (Bloom and Endow, 1994). Thus, KLPs (but not kinesin) are strongly implicated in chromosome motion and spindle organization (Fuller and Wilson, 1992; Sawin and Endow, 1993; Bloom and Endow, 1994; Fuller, 1995). Mutants with defective KLPs show aberrant mitotic apparatuses, and injection of antibodies to KLPs has a similar effect on normal cells. Various KLPs appear to be important in spindle pole and centrosome organization and may drive the separation of the poles early in division and during anaphase B. The simultaneous action of different motors may govern an interplay or give-and-take between oppositely directed forces that is crucial for the establishment and/or stabilization of mitotic apparatus architecture and bipolarity (Fuller and Wilson, 1992; Sawin and Endow, 1993). Thus, KLPA and BIMC play opposing roles in spindle organization in Aspergillus (O'Connell et al., 1993), and Kar3p and Kip1p/Cin8p do the same in yeast (Hoyt et al., 1993). Minus-end-directed dynein also appears to be important in the organization and orientation of the mitotic apparatus and may generate forces that counterbalance those governed by KLPs (Vaisberg et al., 1993; Schroer, 1994).

Compared with animal cells and fungi, very little is known about KLPs in plants. However, there is ample reason to assume that KLPs are present in these organisms. For example, aside from the organization and operation of the mitotic apparatus, motor protein–related activities may govern the directed accumulation of secretory vesicles necessary for tip growth in root hairs and pollen tubes, the deposition of localized wall thickenings in a variety of cell types, and formation of the cell plate during cytokinesis. In addition, the switch from one microtubule array to another during the cell cycle may involve movement of intact microtubules (Palevitz, 1991), a process that would undoubtedly require the action of associated motor proteins. A cross-reacting homolog was recently detected in pollen tubes of two angiosperms by using an antibody directed against brain kinesin (Cai et al., 1993; Liu et al., 1994b). Polypeptides with plus-end-directed motor activity also have been reported in isolated phragmoplasts (Asada and Shibaoka, 1994). Plus-end-directed KLPs have been implicated in the operation of the central spindle in diatoms (Hogan et al., 1992, 1993) as well as in the function or assembly of Chlamydomonas flagella (see Bernstein and Rosenbaum, 1994; Walther et al., 1994). Recently, several genes (*Kat* genes) encoding KLPs were identified in Arabidopsis by using PCR (Mitsui et al., 1993, 1994). Sequence analysis of cDNAs indicates that the motor domain is located near the C terminus of the proteins, and the polypeptide belongs in the Kar3 subfamily, a feature consistent with minus-end-directed activity. A peptide fragment expressed in bacteria exhibits ATP-dependent microtubule binding (Mitsui et al., 1994). Recent evidence also supports the presence of cytoplasmic dynein in plants (Moscatelli et al., 1995).

Because very limited localization data have been obtained for any plant KLP, and because the KLPs encoded by the *Kat* genes are the first potential minus-end motors reported in plants, we wished to obtain information about the distribution of the *Kat* gene products in cells of this species. Therefore, we prepared a polyclonal antibody against peptides synthesized from the published sequence of one of the *Kat* genes, *KatA*, and used it for immunoblots and immunofluoresence imaging. The two peptides are in the predicted microtubule binding domain of the KatAp polypeptide (Mitsui et al., 1993, 1994), which is 74% homologous with the same region in Drosophila kinesin heavy chain. In addition to using the antibody in immunoblots of Arabidopsis polypeptides, we also ascertained whether cross-reacting polypeptides are present in tobacco and carrot. By taking advantage of attributes of different cell types (tobacco and carrot suspension cells), we were able to determine whether cross-reacting polypeptides exhibit properties expected of a KLP, including nucleotide-dependent association with and movement along microtubules in vitro and in lysed cells. A brief report on part of this work has appeared in abstract form (Liu and Palevitz, 1994).

RESULTS

Generation of a Polyclonal Antibody

Serum obtained after immunization reacts with several polypeptides in extracts of whole Arabidopsis shoots. Figure 1A, lanes 2 and 3, shows immunoblots using sera from the first and second bleeds. Among the observed immunoreactive bands was one with an M_r of 140,000, which was not evident in Arabidopsis shoot extracts blotted against preimmune serum (lane 1). When antiserum was affinity purified against immobilized antigenic peptides, a preparation was obtained that gave one immunoreactive signal with an M_r of 140,000

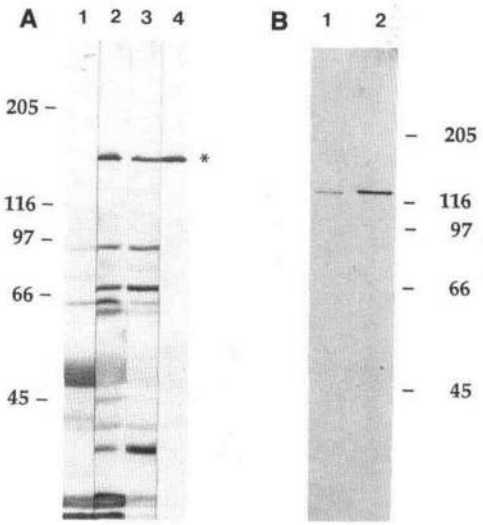

Figure 1. Immunoblots with Monospecific Anti-KatAp Antibody Detect Single Polypeptides from Plant and Animal Sources.

The positions of molecular mass markers (in kilodaltons) are indicated at left in **(A)** and at right in **(B)**.

(A) Proteins from Arabidopsis shoot apices were separated on a 7.5% SDS-PAGE gel (80 μg per lane). Total proteins were stained with Ponceau S (not shown) and probed with preimmune serum (lane 1), serum from the first bleed (lane 2), serum from the second bleed (lane 3), and affinity-purified antibody (lane 4). A polypeptide with an M_r of 140,000 (asterisk) was recognized by the antibody.

(B) The affinity-purifed anti-KatAp antibody was used to probe fractions prepared from carrot suspension cells (lane 1) and mouse brain (lane 2), according to procedures that select for kinesin and KLP. Polypeptides with similar M_r values (~130,000) were detected in the carrot and brain preparations.

(lane 4). Very faint staining of lower molecular mass material was also detectable, but this probably represented degradation products.

Nature of the Polypeptides Recognized by the Anti-KatAp Antibody

To verify that the material recognized by the antibody was KatAp, we performed additional experiments. First, we isolated a kinesin/KLP–enriched fraction from murine brain according to published procedures employing nucleotide-sensitive binding to microtubules. When subjected to immunoblotting, this preparation produced a single immunoreactive band with an M_r of 130,000 (Figure 1B, lane 2), a value within the range of that reported elsewhere for kinesin heavy chain and KLPs (Bloom and Endow, 1994; Brady, 1995). Second, we subjected carrot suspension cells to a similar procedure, but instead of relying on native carrot microtubules, we used taxol-stabilized brain microtubules as an affinity matrix to pull out KLPs. A similar strategy was used successfully in a study of other plant microtubule-associated proteins (MAPs; Cyr and Palevitz, 1989). Carrot cells instead of Arabidopsis were used for this purpose because they are small and densely cytoplasmic, contain few vacuoles, and therefore are more amenable to protein isolations (Cyr and Palevitz, 1989). We obtained an immunoreactive signal from carrot extracts with an M_r of ~125,000 (Figure 1B, lane 1) that copelleted with microtubules.

Figure 2 shows that binding to microtubules was nucleotide modulated; the polypeptide weakly associated with microtubules in the presence of ATP but was more strongly bound when supernatants were reexposed to microtubules in the presence of AMP–PNP, a criterion that has been widely used to enrich for kinesin and KLPs compared with other (e.g., structural) MAPs. Despite the presence of the protease inhibitors, a few polypeptides of lower relative molecular mass were produced during these procedures, two of which reacted strongly with microtubules and the antibody (Figure 2). We believe that these are proteolytic degradation products, based on several

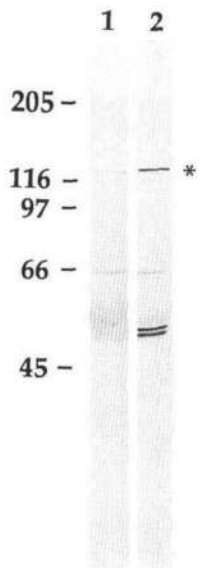

Figure 2. The Carrot KatAp Homolog Binds to Microtubules in a Nucleotide-Modulated Manner.

The anti-KatAp antibody was used to probe for carrot KLP in microtubule pellets. Total carrot supernatant was incubated with bovine microtubules in the presence of ATP. The microtubules were then pelleted (lane 1). The supernatant from this first centrifugation was incubated with bovine microtubules in the presence of AMP–PNP, and a second pellet was obtained (lane 2). The KLP (asterisk) preferentially associated with microtubules in the presence of AMP–PNP. Lower molecular mass degradation products were also produced during these procedures, two of which appear to bind specifically to microtubules. The positions of molecular mass markers (in kilodaltons) are indicated at left.

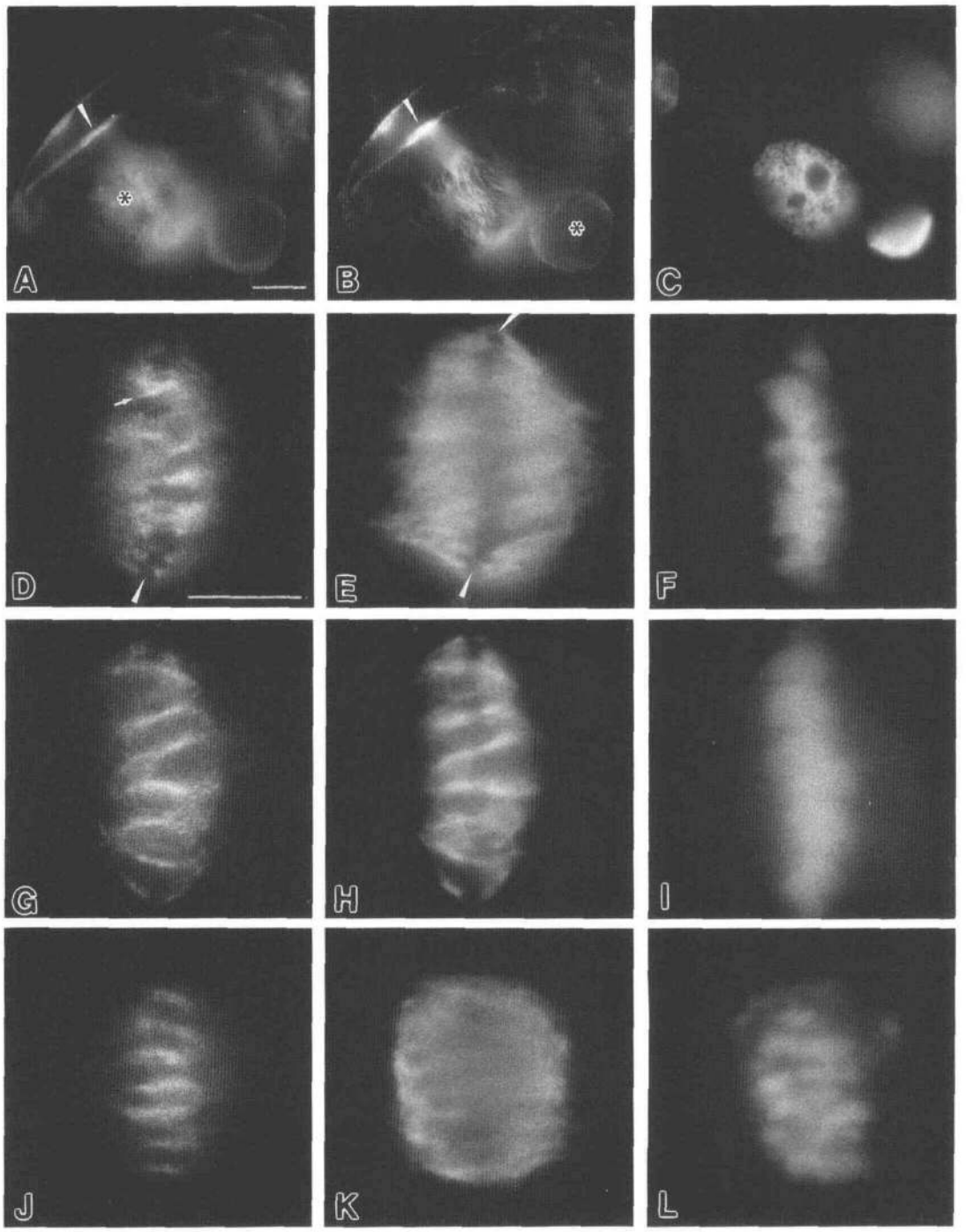

Figure 3. Triple Localization of KatAp, Microtubules, and Nucleus/Chromosomes in Suspension Cells of Arabidopsis.

KatAp is shown in **(A)**, **(D)**, **(G)**, and **(J)**; microtubules are shown in **(B)**, **(E)**, **(H)**, and **(K)**; and nucleus/chromosomes are shown in **(C)**, **(F)**, **(I)**, and **(L)**.
(A) to **(C)** A preprophase cell showed some localization in the nucleus, indicated by the asterisk in **(A)**, as well as relatively faint staining elsewhere in the cytoplasm and preprophase band (arrowheads in **[A]** and **[B]**). An extruded interphase nucleus with a thin shell of surrounding cytoplasm (see asterisk in **[B]**) was not labeled with the anti-KatAp antibody.
(D) to **(I)** Two metaphase cells stained with antibodies raised against KatAp and β-tubulin and with Hoechst 33258 (for chromosomes). KatAp was localized around the metaphase plate (**[D]** and **[G]**). Signal was detected at the ends of kinetochore fibers and on branches of kinetochore fibers that cross the midzone (e.g., arrow in **[D]**). The latter are particularly evident in **(G)**. There is a paucity of staining at the poles in **(D)**. The kinetochores are indicated by the dark, unstained blocks (e.g., arrowheads) in **(D)**, **(E)**, and **(H)**.
(J) to **(L)** A triple-stained anaphase cell. KatAp remains in the midzone.
Bars = 10 μm. The bar in **(A)** is for **(A)** to **(C)**. The bar in **(D)** is for **(D)** to **(L)**.

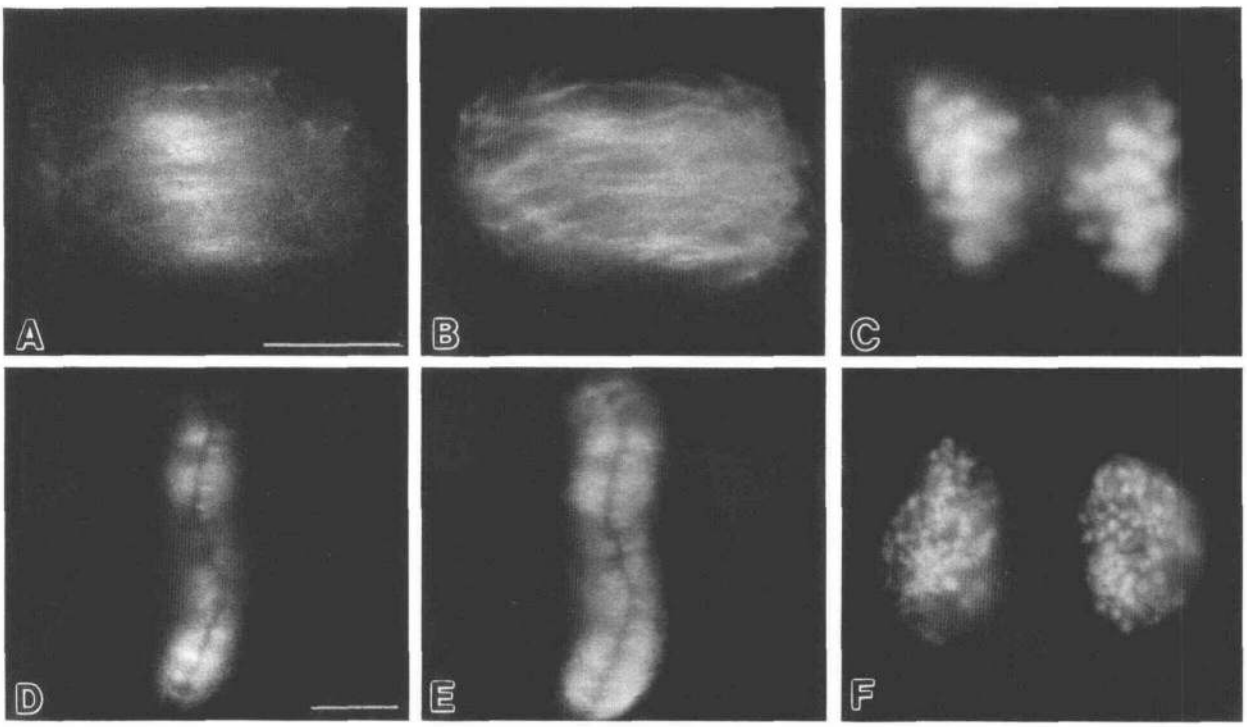

Figure 4. Triple Localizations in Telophase and Cytokinetic Cells.

(A) to **(C)** A telophase cell that contained an elaborate array of interzonal microtubules **(B)** between two masses of daughter chromosomes **(C)**. KatAp was concentrated in the middle of the interzone in **(A)**.

(D) to **(F)** This cytokinetic cell contained a mature phragmoplast **(E)** bisected by a cell plate (dark lines in **[D]** and **[E]**). KatAp in **(D)** was located in the phragmoplast. Bars = 10 μm. The bar in **(A)** is for **(A)** to **(C)**; the bar in **(D)** is for **(D)** to **(F)**.

criteria: they increased not only when purification increased but also when samples were stored; lower molecular mass bands appeared in the ATP supernatant, not just the AMP–PNP microtubule pellet; and apparent proteolysis problems also had an impact on attempts to induce microtubule motility in vitro in the presence of purified proteins (data not shown).

KLP Is Localized in the Mitotic Apparatus and Phragmoplast

Using the antibody purified against the antigenic peptides, we localized KatAp in intact Arabidopsis and tobacco BY-2 suspension cells. Because the data were identical for both, we used only those obtained from Arabidopsis. However, images of lysed BY-2 cells confirmed that the anti-KatAp antibody reacts with cells of this species. No specific staining was seen in control preparations in which cells were exposed to a purified antibody preparation preadsorbed to the antigenic peptides. We were not able to obtain adequate immunoblots of BY-2 cells for technical reasons.

Staining with the anti-KatAp antibody was variable and generally weak in interphase cells. As illustrated in Figures 3A to 3C, nuclear staining was evident in some cells but not others. Likewise, localization along interphase and preprophase band microtubules, if present, was also weak. Bright anti-KatAp fluorescence appeared in the mitotic apparatus during mitosis (Figures 3D to 3L). Fluorescence was not uniform within the mitotic apparatus; it was present mainly in the midzone at metaphase. Comparisons with anti–β-tubulin antibody staining showed that KatAp was associated with the ends of the kinetochore fibers near the kinetochores (Figures 3D and 3E) and with microtubules that branch from the fibers and cross the midzone (Figures 3G and 3H). Fluorescence was much weaker in the vicinity of the poles. No enhanced signal was evident at the kinetochores proper (Figure 3D). The kinetochores appear as dark blocks in Figures 3D, 3E (arrowheads), and 3H.

At the onset of anaphase, fluorescence remained in the interzone between separating chromosomes but was lost from the ends of the kinetochore fibers (Figures 3J to 3L). At no time during anaphase did the poles become stained.

Figures 4A to 4C show that fluorescence remained associated with the interzone in early telophase. Interestingly, its distribution was much more narrow than that of the interzonal microtubules revealed by staining with the anti-tubulin

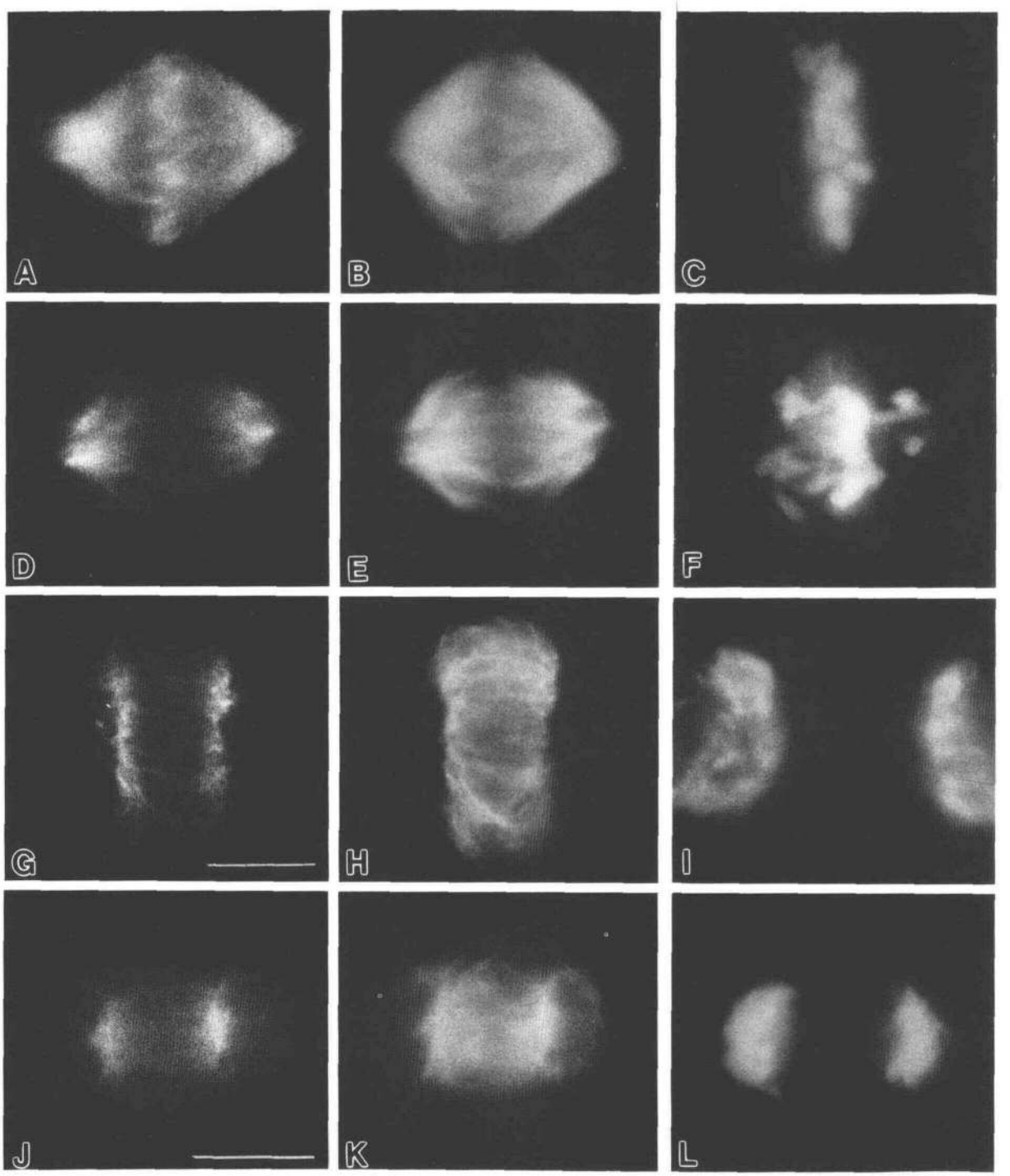

Figure 5. Movement of the KatAp Homolog in Lysed Tobacco BY-2 Suspension Cells.

The tobacco protein moved toward the minus ends of microtubules at the poles and daughter nuclei in lysed mitotic and cytokinetic cells in the presence of ATP. Cells were fixed 5 min after exposure to ATP solution and labeled for KLP (with anti-KatAp in **[A]**, **[D]**, **[G]**, and **[J]**), microtubules (with anti–β-tubulin in **[B]**, **[E]**, **[H]**, and **[K]**), and nuclei/chromosomes (with Hoechst 33258 in **[C]**, **[F]**, **[I]**, and **[L]**).
(A) to **(C)** Staining with the anti-KatAp antibody was present at the poles as well as the midzone in the metaphase cell shown in **(A)**. Moreover, the poles of the mitotic apparatus are now very focused **(B)**.

antibody. Anti-KatAp fluorescence gradually assumed a distribution similar to that of phragmoplast microtubules by late cytokinesis, including the presence of an unstained layer that corresponded to the new cell plate (Figures 4D to 4F).

Anti-KatAp Reactive Polypeptide Moves toward the Mitotic Poles in Lysed Cells

To assess the potential motor activity of the anti-KatAp cross-reactive protein, we tried to ascertain whether it promotes the movement of microtubules on the surface of glass. However, those experiments were unsuccessful due to the rapid proteolysis of the polypeptide in vitro. As an alternative approach, we conducted an in situ assay using lysed tobacco BY-2 cells in which endogenous microtubules were stabilized by the addition of taxol to the lysis buffer. Thus, loss of microtubules should not have been a complicating factor. This assay was also based on the known polarity of microtubules in the mitotic apparatus and phragmoplast, in which minus ends are located at the poles (Euteneuer et al., 1981). First, we established that the anti-KatAp antibody bound to fixed BY-2 cells in a manner similar to that seen in Arabidopsis (data not shown). Next, we assessed the distribution of the cross-reactive KLP in cells that were lysed before fixation. As shown in Figures 5A to 5F, when metaphase BY-2 cells were lysed in the presence of taxol and ATP, the anti-KatAp signal moved to the poles. Changes in the anti-KLP localization pattern were noticeable in as little as 1 min. In some cells, such as that shown in Figures 5A to 5C, signal was evident both at the midzone and the poles, perhaps representing an intermediate pattern (Figure 5A). Eventually, all the fluorescence reached the poles (Figure 5D).

A change in anti-KatAp fluorescence was also seen in the phragmoplast of lysed cytokinetic cells: cross-reacting polypeptide moved toward the daughter nuclei in the presence of ATP (Figures 5G and 5J).

Interestingly, other changes also accompanied the redistribution of anti-KatAp fluorescence. First, some of the chromosomes in metaphase cells moved toward the poles, although we could not determine whether they were unseparated homologs or separated daughter chromosomes (Figure 5F). Second, the microtubules became more focused at the poles (compare Figures 5B and 5E with Figures 6B and 6H). Third, the microtubule signal became relatively stronger near the daughter nuclei in cytokinetic cells and less intense near the midzone (Figures 5H and 5K). Fourth, daughter nuclei appeared to move farther apart during treatment (compare Figure 5I with Figures 6F and 6L), although we have not quantitated this effect. In some cases, daughter nuclei and associated microtubules seemed to separate into two distinct complexes (Figures 5J to 5L).

Figure 6 shows that redistribution of the cross-reacting KLP was blocked by various treatments. No redistribution of polypeptide occurred in the absence of ATP (Figures 6A to 6F) or in the presence of the nonhydrolyzable analog AMP-PNP (Figures 6G to 6I), indicating that such distribution requires the activity of a nucleotide-dependent motor. To test further whether the movement was due to KLP, we lysed cells in the presence of ATP plus the anti-KatAp antibody. No redistribution was seen (Figures 6J to 6L). Interestingly, redistribution was evident in the presence of GTP as well as ATP (data not shown).

DISCUSSION

Our results show that a kinesin-like protein, KatAp, is expressed in suspension cells and seedlings of Arabidopsis, as ascertained with an antibody raised against peptides derived from the published sequence of the *KatA* gene. Cross-reacting polypeptides are also present in tobacco and carrot cells. KatAp was seen primarily in dividing cells, where it was restricted to the mitotic interzone and cytokinetic phragmoplast. Observations made with lysed cells indicate that KatAp contains a minus-end-directed motor.

We took extensive precautions to ensure the specificity of our results. First, antibody was raised against synthetic peptides rather than impure proteins or cell extracts. Two peptides were used that bracket much of the microtubule binding domain of KatAp. Immunoblots of Arabidopsis seedling extracts showed that the antibody produces a single immunoreactive band. Moreover, single bands were also detected in carrot and brain extracts prepared according to procedures used to enrich for kinesin and KLPs. These procedures employed nucleotide-dependent binding to microtubules. The anti-KatAp antibody stained microtubules in situ in a cell-cycle–dependent manner, and binding was inhibited by pre-exposure of antibody to the antigenic peptides. Similar localization patterns were seen in two different species. The antibody also prevented

Figure 5. (continued).

(D) to **(F)** Most of the anti-KatAp signal in **(D)** is located at the poles in the metaphase mitotic apparatus shown in **(E)**. Some chromosome movement toward the poles has occurred in **(F)**.

(G) to **(I)** Staining with the anti-KatAp antibody redistributed toward the daughter nuclei in the lysed cytokinetic cell in **(G)**, whereas phragmoplast microtubules now show an uneven distribution **(H)** with some concentration near the daughter nuclei **(I)**. The distance between the two daughter nuclei appears to be greater than normal (see Figures 6F and 6L).

(J) to **(L)** In this cytokinetic cell, staining with the anti-KatAp antibody (shown in **[J]**) was concentrated at the faces of the re-forming daughter nuclei. Moreover, the phragmoplast seemed to consist of two separate arrays of microtubules, shown in **(K)**, associated with the daughter nuclei **(L)**. Bars = 5 μm. The bar in **(G)** is for **(A)** to **(I)**; the bar in **(J)** is for **(J)** to **(L)**.

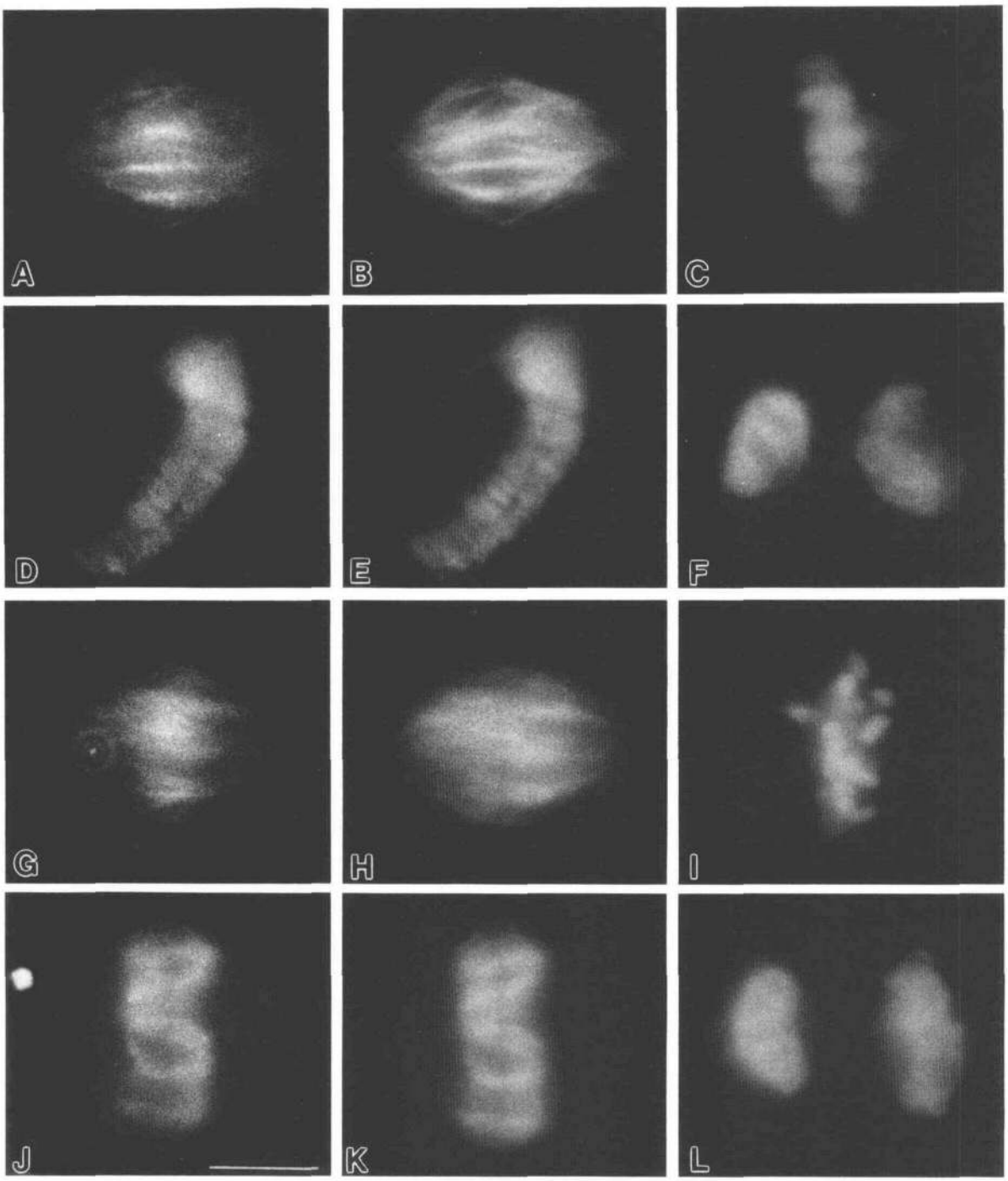

Figure 6. Lysed Tobacco Cell Controls.

Cells were labeled for KLP (with anti-KatAp antibody in **[A]**, **[D]**, **[G]**, and **[J]**), microtubules (with anti–β-tubulin antibody in **[B]**, **[E]**, **[H]**, and **[K]**), and nuclei/chromosomes (with Hoechst 33258 in **[C]**, **[F]**, **[I]**, and **[L]**).

(A) to **(F)** In the absence of ATP, KLP signal did not redistribute and remained in the midzone and phragmoplast in metaphase (**[A]** to **[C]**) and cytokinetic (**[D]** to **[F]**) cells, respectively. In the latter, daughter nuclei retained a normal distance between each other, as shown in **(F)**, and no redistribution of phragmoplast microtubules occurred, as shown in **(E)**.

(G) to **(I)** Shown is a lysed metaphase cell that was incubated with AMP–PNP instead of ATP. The KLP signal remained in the midzone, as shown in **(G)**.

(J) to **(L)** No movement of KLP (shown in **[J]**) along phragmoplast microtubules **(K)** occurred with ATP in the presence of the anti-KatAp antibody. In addition, there was no redistribution of microtubules. The distance between daughter nuclei shown in **(L)** appeared normal. The cell appears to be at approximately the same stage of cytokinesis as that shown in Figures 5G to 5I.

Bar in **(J)** = 5 μm for **(A)** to **(L)**.

distance and bipolar architecture of the plant mitotic apparatus. Thus, movement of intact microtubules toward the minus ends of other microtubules, perhaps in a manner similar to that proposed by Endow et al. (1994a, 1994b), could counterbalance forces generated in the opposite direction. Movement of microtubules could be coupled to depolymerization at the minus ends. This hypothesis is supported by our in vitro studies on lysed cells, in which microtubules moved toward the poles along with KatAp in the presence of ATP. That this redistribution was not seen in intact cells indicates that under lysed-cell conditions a delicate counterbalance is disturbed, perhaps due to the loss of one or more critical components such as another motor. That the poles became more focused in the lysed cells indicates that the counterbalancing forces may be important in maintaining the more open arrangement of the poles in plant cells. Indeed, Bloom and Endow (1994) have proposed that, in the absence of the focusing action of a discrete centrosome, one or more minus-end-directed motors, such as KatAp, play an important role in organizing the plant spindle poles. It is worth reiterating, however, that KatAp is not localized at the poles in intact cells.

The same motor could also function during cytokinesis, maintaining the distance between daughter nuclei by counterbalancing plus-end-directed microtubule motors that would tend to push the nuclei apart through microtubule–microtubule sliding of the kind reported to occur in the central spindle of diatoms (Hogan et al., 1992, 1993). Kakimoto and Shibaoka (1989) reported that the distance between daughter nuclei is maintained in isolated phragmoplasts in which microtubules are eliminated during cell lysis. However, this result is seemingly at odds with other work showing that anti-microtubule drugs used in vivo cause daughter nuclei to approach each other as phragmoplast microtubules depolymerize (Palevitz and Hepler, 1974). Our results with lysed cells indicated that phragmoplast microtubules moved toward the daughter nuclei along with KatAp in the presence of ATP (Figures 5H and 5K). In addition, the nuclei tended to move apart under these circumstances. Thus, our data support a role for microtubules, KatAp, and perhaps other motor proteins in maintaining internuclear distance during cytokinesis. The activity of KatAp could be linked to the shortening of phragmoplast microtubules (again, presumably at the minus ends, as discussed above) typically seen during the course of cytokinesis.

If this process occurred, it would increase our appreciation of the complexity of microtubule dynamics and motor activity in the phragmoplast. Net microtubule assembly is known to occur at the plus ends of phragmoplast microtubules, based on studies of lysed tobacco cells (Asada et al., 1991). Because the width of the microtubule overlap zone remains relatively uniform over time, a plus-end-directed motor is thought to govern sliding of antiparallel cross-linked microtubules. A candidate for such a motor was later identified (Asada and Shibaoka, 1994). Thus, phragmoplast organization could be a function of the activity of at least two microtubule motors working in opposite directions. Because actin microfilaments are important components of the phragmoplast (Kakimoto and Shibaoka, 1987; Palevitz, 1987; Traas et al., 1987; Schmit and Lambert, 1990; Schopfer and Hepler, 1991), myosin motors may provide additional sources of complexity (Parke et al., 1986). Finally, KatAp could function in some aspect of vesicle transport in the phragmoplast, consistent with the role of KLPs in vesicle/organelle movement in other systems (see Bloom and Endow, 1994; Sekine et al., 1994; Cole and Lippincott-Schwartz, 1995; Lippincott-Schwartz et al., 1995).

Unfortunately, our understanding of KatAp is clouded by general uncertainties about the functions of the various microtubule motors in dividing cells. Indeed, the situation at this juncture might generously be described as confusing. This is due to a number of factors, including concurrent localizations of both plus-end- and minus-end-directed microtubule motors at the same site in cells. Both plus-end- and minus-end-directed motors have been localized to the midzone, midbody, or phragmoplast, including KLP3A (Williams et al., 1995), Xklp1 (Vernos et al., 1995), CHO1 (Nislow et al., 1992; Kuriyama et al., 1994), cut7 protein (Hagan and Yanagida, 1992), chromokinesin (Wang and Adler, 1995), CENP-E (Yen et al., 1991), and KatAp (our results). The location of KLPs can also change during the cell cycle. Thus, CENP-E is found at the kinetochores until anaphase, when it redistributes to the midzone and midbody (Yen et al., 1991). The CHO1 motor is located in the nucleus and centrosome but redistributes to the midzone late in division (Nislow et al., 1992). Although different motors may have some localization sites in common, they may differ in their presence elsewhere in the cell. For example, CENP-E is located at the kinetochores, midzone, and midbody but is not associated with spindle fibers. On the other hand, antibodies raised against the ncd protein bind to the centrosome and meiotic and mitotic spindles, as well as to the midbody, but do not stain kinetochores (Hatsumi and Endow, 1992; Endow et al., 1994a).

The KatAp motor described here is found in the midzone of the mitotic apparatus from metaphase through anaphase and remains in that position in association with the phragmoplast during cytokinesis. Thus, its distribution is somewhat similar to that of other midzone-localized motors such as CENP-E and CHO1. Unlike CENP-E, however, it is not associated with the kinetochores. Unlike CHO1, anti-KatAp fluorescence is not unequivocally localized in the nucleus prior to mitosis but displays a variable, dim pattern in the nucleus and cytoplasm. It is notable in this regard that the anti-KatAp antibody produces distinct, albeit transient, labeling of the nucleus just before division in tobacco generative cells (Liu and Palevitz, 1996). The multiplicity of KLPs (as well as cytoplasmic dynein) in the same organism and cell indicates that they govern a variety of functions, including several in the mitotic apparatus alone.

Still other complexities cloud our understanding of the nature and scope of microtubule motor protein function, including the fact that members of the kinesin and dynein superfamilies are active in the same direction along microtubules and

govern potentially redundant functions. Thus, dynein (Vaisberg et al., 1993) as well as certain KLPs direct movement toward the minus end. In addition, the directionality of a given motor may vary, depending on microtubule dynamics (Lombillo et al., 1995b). Lastly, KLPs may interact not only with other microtubule motors but also with elements of the actin cytoskeleton, such as members of the myosin superfamily (Lillie and Brown, 1994; Langford, 1995).

In all likelihood, the KLP superfamily in Arabidopsis is substantial and complex, with new members yet to be discovered, and it governs important cellular and developmental processes. Additional data must be secured before the functions of KatAp and the other proteins encoded by the superfamily are fully understood.

METHODS

Plant Material

Seedlings (*Arabidopsis thaliana* cv Norway) were grown as described previously by Liu et al. (1994a). Suspension cells of Arabidopsis, tobacco BY-2, and carrot were maintained according to Liu et al. (1994a), Hasezawa et al. (1991), and Cyr and Palevitz (1989), respectively.

Production and Purification of the Anti-KatAp Antibody

Two peptides were synthesized based on the published Arabidopsis *KatA* sequence (Mitsui et al., 1993). Peptides 637-652 (RSVGKTQMNEQSSRSH) and 750-765 (SPDPTSAGESLCSRF) were synthesized using the multiple-antigen peptide system (Tam, 1988) by the Molecular Genetics Instrumentation Facility at the University of Georgia. Peptide 637-652 is more conserved among the KatA, B, and C proteins, Kar3, ncd, and kinesin heavy chain than is peptide 750-765. Approximately 1 mg of each peptide complex was dissolved in 100 μL of PBS and then mixed in 1 mL of Freund's complete adjuvant. A female New Zealand White rabbit was injected intradermally at multiple spots. Booster injections with 500 μg of each peptide in incomplete Freund's adjuvant were performed 20, 48, and 181 days after the initial injection. Two bleeds were done, with the first at 41 days and the second (final) at 191 days. Serum was obtained from both bleeds.

In some cases, serum was diluted and used directly. However, specific anti-KatAp antibody was also obtained from serum by affinity purification against the synthetic peptides. The peptides were covalently attached to Immobilon-AV membrane (Millipore Corp., Bedford, MA) as described by Canas et al. (1993). Briefly, 1 mg of each peptide was dissolved in 1 mL of 0.5 M Na_2HPO_4, pH 7.5, containing 0.1% SDS. This solution (200 μL) was applied to a 0.5 × 10–cm strip of membrane and air dried. The strip was then incubated in 10 mM Tris, pH 7.5, containing 0.15 M NaCl and 0.1% Tween 20, for 2 hr. Freshly prepared 10% ethanolamine in 1 M $NaHCO_3$ was applied as a blocking agent for 3 hr, followed by an additional blocking step of 5% nonfat milk in PBS for 30 min. Serum diluted 10-fold in PBS containing 1% BSA and 0.05% Tween 20 was applied to the membrane for 1 hr at room temperature. The membrane was then rinsed five times with PBS containing 0.05% Tween 20, after which bound antibodies were eluted with 100 mM glycine, pH 2.5. The eluate was neutralized with one-tenth volume of 1 M Tris, pH 8.0. The antibody was either first concentrated with a Centricon-30 filter (Amicon, Inc., Beverly, MA) or immediately diluted with PBS for immunoblotting and immunolocalizations.

Preparation of Proteins

Proteins were prepared from murine brain according to procedures that enrich for kinesin and kinesin-like protein (KLP), and a similar procedure was used to partially purify cross-reacting KLP from carrot suspension cells. Suspension cells of carrot were used for this purpose because they are small and densely cytoplasmic and therefore very amenable to protein purifications.

A murine neuronal kinesin/KLP fraction was prepared using a method slightly modified from Vale et al. (1985). Briefly, one mouse brain was homogenized with a Dounce homogenizer (Kontes, Vineland, NJ), using 1:1 (w/v) PM buffer (50 mM Pipes, 1 mM $MgSO_4$, pH 6.9) containing 2 mM DTT, a cocktail of protease inhibitors (1 mM phenylmethylsulfonyl fluoride [PMSF], 10 μg/mL each of leupeptin, pepstatin, and Nα-*p*-tosyl-L-arginine methyl ester [TAME]), 0.2 mM GTP, 0.2 mM ATP, and 1 M glycerol. The homogenate was left on ice for 30 min and then spun at 27,000*g* for 20 min at 4°C. The supernatant was collected, taxol (Sigma) was added to 20 μM, GTP and ATP were both added to 1 mM, and the endogenous tubulin was allowed to assemble at room temperature for 30 min, after which the microtubules were pelleted by centrifugation at 48,000*g* for 30 min at 25°C. The supernatant was collected and dialyzed against PM buffer, after which ATP was depleted by adding hexokinase to 10 units per mL and glucose to 20 mM. After the supernatant was incubated for 15 min at room temperature, it was centrifuged at 48,000*g* for 20 min, AMP–PNP was added to the supernatant to 10 mM and to taxol-assembled bovine microtubules to 200 μg/mL, and the kinesin/KLP was allowed to bind for 20 min at room temperature. The microtubules with associated kinesin/KLP were collected by centrifugation at 48,000*g* for 20 min, washed with PM buffer containing 1 mM AMP–PNP and 10 μM taxol, and then recentrifuged as before. The kinesin/KLP was released from the sedimented microtubules by resuspension in PM buffer containing 75 mM KCl and 1 mM ATP. The suspension was centrifuged at 48,000*g* for 20 min, and the supernatant was collected and frozen. This fraction was capable of supporting the gliding of microtubules on a glass surface in an ATP-dependent fashion.

Soluble proteins from 4- to 5-day-old carrot suspension cells were prepared as described by Cyr and Palevitz (1989). After washing in PM buffer, grind buffer (5 mM EGTA, 5 mM $MgCl_2$, 100 mM Pipes, pH 6.9, 10 mM DTT, 2 M glycerol, 0.05% Triton X-100, 0.2 mM ATP, 0.2 mM GTP, 1 mM PMSF, and 10 μg/mL each of leupeptin, pepstatin, and TAME) was added to the cells (1:1 [w/v]) on ice until the solution reached 4°C. The cells were then burst with a French pressure cell. After debris was removed by centrifugation at 100,000*g* at 4°C for 20 min, the supernatant was collected and used as starting material. Taxol was added to 20 μM, ATP and GTP to 1 mM, and taxol-stabilized bovine brain microtubules to 2 mg/mL. After sitting on ice for 30 min, the solution was centrifuged at 100,000*g* for 20 min. The supernatant was desalted on a P6DG column (Bio-Rad, Hercules, CA) previously equilibrated with PM buffer containing 2 M glycerol and protease inhibitors. Taxol was then added to 10 μM, AMP–PNP to 10 mM, and brain microtubules to 200 μg/mL, and the solution was allowed to sit on ice for 30 min. An AMP–PNP pellet was then collected by centrifugation at 100,000*g* for 20 min, washed with PM buffer containing 10 μM taxol and 1 mM AMP–PNP, and pelleted again.

Total protein was extracted from Arabidopsis seedlings using the trichloroacetic acid precipitation method described by Yokota and Shimmen (1994). The proteins were then subjected to immunoblot analysis.

Immunoblot Analysis of Proteins

Proteins from various samples were separated by SDS-PAGE (Laemmli, 1970) and transferred to Immobilon-P membranes (Millipore Corp.). After blocking with 3% gelatin and 5% lamb serum in PBS for 5 min, the membranes were incubated with the anti-KatAp antibody (2000-fold dilution for serum, 40-fold dilution for purified antibody) for 1 hr. The membrane was then washed with PBS containing 0.05% Tween 20, followed by incubation with alkaline phosphatase–conjugated goat anti–rabbit IgG (Sigma) diluted 8000-fold. The membrane was developed with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (both from Sigma).

Immunofluorescence Localizations

Arabidopsis suspension cells were processed for immunofluorescence localizations as described by Liu et al. (1994a). Intact tobacco BY-2 cells were processed according to Liu et al. (1993, 1994a). Lysed BY-2 cells (see below) were prepared for immunofluorescence staining by fixing in 4% formaldehyde plus 0.1% glutaraldehyde in PME buffer (PM buffer plus 5 mM EGTA) and exposed to antibodies. The anti-KatAp antibody was applied alone at a fourfold dilution in PBS or in combination with a 400-fold dilution of anti–β-tubulin antibody (Sigma) for 1 hr at room temperature. Secondary antibodies were fluorescein isothiocyanate–conjugated goat anti–rabbit IgG (Sigma) and Texas Red–conjugated goat anti–mouse IgG (Amersham), both diluted 100-fold. As a control, cells were treated with a combination of anti–β-tubulin and anti-KatAp antibodies that had been pretreated with antigenic peptides linked to Immobilon-AV membrane. The rinse and secondary antibody protocols were the same as those already described. Cells were visualized on an Axioskop microscope (Carl Zeiss, Inc., Thornwood, NY) equipped with epifluorescence optics.

Analysis of Movement of KLP in Lysed Cells

A tobacco BY-2 suspension culture was used to prepare lysed cells with which to study movement of anti-KLP cross-reacting proteins. Tobacco cells were chosen rather than Arabidopsis or carrot cells because the number of dividing cells is too low at any given time with the former, and the small size of the latter makes definitive imaging difficult. Tobacco BY-2 cultures have been valuable in other studies on cell division and the cell cycle in plants (e.g., Hasezawa et al., 1991; Fisher and Cyr, 1993; Liu et al., 1993; Asada and Shibaoka, 1994; Nagata et al., 1994). Our localizations with anti-KatAp showed that BY-2 has a cross-reacting polypeptide.

Protoplasts were isolated from 4-day-old cells using 1% cellulase Onozuka RS (Yakult Honsha Ltd., Tokyo) and 0.1% pectolyase (Sigma) in 0.3 M mannitol, pH 5.3. The protoplasts were collected by centrifugation and rinsed in PM buffer containing 10 μM taxol and 0.3 M mannitol. The resuspended protoplasts were then placed on polylysine-coated multiwell slides and allowed to adhere for 2 min before application of lysis buffer. The lysis buffer consisted of 5 mM DTT, 10 μM taxol, and a cocktail of protease inhibitors (10 μg/mL each of antipain, aprotinin, chymostatin, leupeptin, pepstatin; 1 mM PMSF; 50 μg/mL each of TAME and Nα-benzyl-L-arginine methyl ester) in PM, with the addition of no nucleotide, 1 mM ATP, GTP, or AMP-PNP, or 1 mM ATP plus fourfold-diluted anti-KatAp antibody. The protoplasts were incubated in the lysis media for 1, 5, or 15 min and then fixed as described in the previous section. After rinsing in PME and PBS, the lysed cells were processed for immunofluorescence localizations, as described previously.

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