A Novel Plant Kinesin-Related Protein Specifically Associates with the Phragmoplast Organelles

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In higher plants, the formation of the cell plate during cytokinesis requires coordinated microtubule (MT) reorganization and vesicle transport in the phragmoplast. MT-based kinesin motors are important players in both processes. To understand the mechanisms underlying plant cytokinesis, we have identified AtPAKRP2 (for Arabidopsis thaliana phragmoplast-associated kinesin-related protein 2). AtPAKRP2 is an ungrouped N-terminal motor kinesin. It first appeared in a punctate pattern among interzonal MTs during late anaphase. When the phragmoplast MT array appeared in a mirror pair, AtPAKRP2 became more concentrated near the division site, and additional signal could be detected elsewhere in the phragmoplast. In contrast, the previously identified AtPAKRP1 protein is associated specifically with bundles of MTs in the phragmoplast at or near their plus ends. Localization of the tobacco homolog(s) of AtPAKRP2 was altered by treatment of brefeldin A in BY-2 cells. We discuss the possibility that AtPAKRP1 plays a role in establishing and/or maintaining the phragmoplast MT array, and AtPAKRP2 may contribute to the transport of Golgi-derived vesicles in the phragmoplast.

INTRODUCTION

In higher plants, cytokinesis involves the formation of the cell plate, which depends on the cytokinetic apparatus of the phragmoplast. The phragmoplast has a framework of microtubules (MTs) that are oriented perpendicular to the division plane by having their plus ends at or near the division site. Phragmoplast MTs are highly dynamic, as revealed by fluorescent analog histochemistry and the green fluorescent protein–tagging approach (Zhang et al., 1993; Granger and Cyr, 2000; Hasezawa et al., 2000). Derived from interzonal MTs of the anaphase spindle, phragmoplast MTs are arranged in the pattern of a solid cylinder before the cell plate is formed. Concomitant with the centrifugal buildup of the cell plate, phragmoplast MTs depolymerize in the region in which the cell plate has been formed. Remarkably, at the same time, new MTs form at the periphery of the phragmoplast. During the whole process of phragmoplast development, MT polymerization takes place at the MT plus ends, and new MT segments are constantly translocated away from the division site (Vantard et al., 1990; Asada et al., 1991). It has been shown that stabilization of MTs with taxol prevents the centrifugal growth of the phragmoplast MT array (Yasuura and Shibaoka, 2000).

During cell plate formation, Golgi-derived vesicles are transported rapidly along the MTs toward their plus ends. The vesicles contain xyloglucans and other components that contribute to the cell plate (Samuels et al., 1995; Otegui and Staehelin, 2000; Sonobe et al., 2000; Verma, 2001). Vesicle transport along MTs depends on MT-based motor proteins, the dyneins and the kinesins (Goldstein and Philp, 1999). Although a number of kinesin-related proteins (KRPs) have been reported in higher plants (Reddy, 2001), the motor(s) responsible for vesicle transport in the phragmoplast has not yet been identified.

Motor proteins play critical roles in multiple processes during cell division (Sharp et al., 2000; Wittmann et al., 2001). Kinesin and KRPs are members of the kinesin superfamily (Kim and Endow, 2000). In higher plants, several KRPs have been localized to the phragmoplast (Liu and Lee, 2001). Plus end–directed KRPs in the Block-In-Mitosis C protein (BIMC) subfamily are required for MT translocation in the phragmoplast, likely by a sliding filament mechanism (Asada et al., 1997; Sharp et al., 1999). One such KRP from carrot especially concentrates in the midline of the phragmoplast MTs (Barroso et al., 2000). Minus end–directed KRPs from the C-terminal motor KRP subfamily decorate phragmoplast MTs as well, but their roles have not been determined (Liu and Palevitz, 1996; Liu et al., 1996; Mitsui et al., 1996; Bowser and Reddy, 1997; Smirnova et al., 1998).
Among them, Kinesin-like Calmodulin-Binding Protein (KCBP) appears to be inactivated, possibly by Ca\(^{2+}\)/calmodulin binding in the phragmoplast, although it appears to be associated with the phragmoplast MTs (Vos et al., 2000).

In a previous study, we identified AtPAKRP1 (for Arabidopsis thaliana phragmoplast-associated kinesin-related protein 1), an N-terminal motor KRP that does not resemble KRPs from other organisms (Lee and Liu, 2000). It does not associate with the preprophase band or the mitotic spindle. During late anaphase, however, it appears to associate with interzonal MTs. Later, it decorates phragmoplast MTs at or near their plus ends. AtPAKRP1 likely plays a role in establishing and/or maintaining the phragmoplast MT array.

One of the central questions about phragmoplast operation is what the force generator(s) for unidirectional vesicle transport is during cell plate formation. Here, we report a phragmoplast-specific KRP, AtPAKRP2 (Arabidopsis phragmoplast-associated kinesin-related protein 2), which is named based on its intracellular localization pattern. AtPAKRP2 has distinct structure and localization patterns that differ from those of AtPAKRP1. On the basis of pharmacological data, we suggest that AtPAKRP2 is a candidate motor for transporting Golgi-derived vesicles toward the division site.

**RESULTS**

**Isolation of AtPAKRP2 cDNA**

To identify KRPs in Arabidopsis, the expressed sequence tag clone OAO358 from green shoots (GenBank accession number Z34049) was obtained because it overlapped with the AtFCA1.2 locus, which was predicted to encode a KRP (GenBank accession number Z97336). After being sequenced, however, the OAO358 clone did not show a full-length open reading frame. To determine the 5’ end of the coding sequence, 5’ rapid amplification of cDNA ends (RACE) experiments were performed. We predicted that the full-length open reading frame would encode a polypeptide of 869 amino acids, which has been named AtPAKRP2 (Figure 1). The deduced polypeptide has a calculated molecular mass of 97 kD and a pI of 5.8.

The deduced AtPAKRP2 sequence showed a number of differences from the amino acid sequence of the AtFCA1.2 locus annotated by the Arabidopsis Genome Initiative, especially toward the C terminus. This difference was caused by the discrepancy of intron/exon prediction by the Arabidopsis Genome Initiative and the cDNA sequence determined in the present study. The N-terminal part of AtPAKRP2 (amino acids 32 to 375) resembled the kinesin motor domain, although it had some unusual features (Figure 1). Typically, the motor domain of kinesin/KRPs contains an ATP binding site, which includes a highly conserved peptide of IF/VAYGQTGA/SGKS/T. The corresponding sequence in AtPAKRP2, however, was IMMYGPTGAGKS. Nevertheless, the AtPAKRP2 motor domain clearly had well-conserved motifs, such as SSRSH, LVDAG, and HVPFRDSLK, which have been found commonly in the MT binding site.

To date, identified KRPs are grouped into nine different subfamilies plus some ungrouped ones (Kim and Endow, 2000). To analyze the relationship between AtPAKRP2 and other KRPs in the kinesin superfamily, phylogenetic analysis was performed by aligning its motor sequence and the mo-
tor sequences of 17 representative kinesin/KRPs, including most if not all reported plant KRPs. First, AtPAKRP2 was very divergent from any of these plant KRPs (Figure 1B). Second, AtPAKRP2 did not fall into any of the existing subfamilies. Therefore, we considered AtPAKRP2 an ungrouped or orphan KRP, like many others such as the Saccharomyces cerevisiae Smy1p protein (http://www.blocks.fhcrc.org/~kinesin/BE6c_ungrouped.html).

Many kinesin/KRPs bear coiled-coil domains required for oligomerization. To further analyze whether AtPAKRP2 might oligomerize, its polypeptide sequence was analyzed for the probability of the formation of coiled coils. Coiled coils could be formed near the central region of the polypeptide (amino acids 390 to 517; P > 0.5) (Figure 2A). The C-terminal region after the coiled coils does not have significant similarity to any available sequence in GenBank, nor is any structural motif found in this so-called tail domain. A typical tripartite structure was predicted for AtPAKRP2 (Figure 2B). It would not be surprising if AtPAKRP2 formed a dimer in its native form, given such a tripartite structure.

AtPAKRP2 Binds to MTs in an ATP-Dependent Manner

Because AtPAKRP2 is very divergent from other KRPs, and the ATP binding site of AtPAKRP2 had three amino acid substitutions compared with the majority of kinesin superfamily members, we wondered whether it could still behave like a motor protein. Most members of the kinesin superfamily bind to MTs in an ATP-dependent manner. A glutathione S-transferase (GST) fusion protein (GST-AtPAKRP2-M) containing the AtPAKRP2 motor domain (amino acids 1 to 435) was expressed in Escherichia coli and affinity purified (Figure 2B). This fusion protein was subjected to MT cosedimentation experiments as described previously (Lee and Liu, 2000). In the absence of exogenous nucleotide, GST-AtPAKRP2-M largely cosedimented with the MT pellet (Figure 2C). The presence of ATP rendered at least 50% of the fusion protein in the supernatant instead of in the MT pellet (Figure 2C). When ATP was replaced with the nonhydrolyzable ATP analog adenylylimidodiphosphate (AMPPNP), the fusion protein appeared almost exclusively in the MT pellet (Figure 2C). Apparently, the fusion protein itself did not sediment (Figure 2C). In a control experiment, the fusion tag GST alone did not show any sign of cosedimentation with MTs (data not shown). Therefore, we concluded that AtPAKRP2 behaved like a typical kinesin/KRP in vitro.

Generation of Anti-AtPAKRP2 Antibodies

Antibodies against two independent regions of AtPAKRP2 were generated. Affinity-purified anti-PAKRP2-N’ and anti-PAKRP2-T antibodies from four different animals gave identical intracellular localization patterns in dividing Arabidopsis cells (see below). Therefore, we concluded that both

**Figure 2.** AtPAKRP2 Structure and Its ATP-Dependent MT Binding Activity.

(A) Coiled-coil domains of AtPAKRP2 were predicted by the Lupus algorithm. The x axis represents the amino acid residue number, and the y axis represents the probability of the formation of coiled coils. A P value >0.5 indicated that the region likely forms a coiled coil.

(B) Schemes of AtPAKRP2 and its protein constructs used for MT binding and antibody preparation. The relative positions of the motor, coiled coil, and tail are indicated.

(C) The MT binding property of the GST-AtPAKRP2-M fusion protein was demonstrated by cosedimentation with MTs. The resulting supernatant (S) and pellet (P) were analyzed by SDS-PAGE and visualized by Coomassie blue staining. (−), the GST-AtPAKRP2-M fusion protein without addition of MTs; MTs, the fusion protein incubated with MTs alone; MTs + ATP, the fusion protein incubated with MTs and 2 μM ATP; MTs + AMPPNP, the fusion protein incubated with MTs and 2 μM AMPPNP.
anti-PAKRP2-N’ and anti-PAKRP2-T antibodies recognized the native AtPAKRP2 protein.

Immunoblotting experiments were performed to detect the AtPAKRP2 polypeptide from protein extracts of etiolated Arabidopsis seedlings and young inflorescence buds. We could not detect the protein in extracts from etiolated seedlings. But in protein extracts of young inflorescence buds, a single 88-kD band was detected with anti-PAKRP2-N’ antibodies (Figure 3A). Unfortunately, the anti-PAKRP2-T antibodies did not work in immunoblotting, possibly because of the availability of the corresponding epitope(s) in our protein preparations. Because of the difference in the apparent molecular mass and the calculated 97 kD, we wanted to verify whether this 88-kD band was in fact the AtPAKRP2 protein. We preincubated the antibodies with the AtPAKRP2-N’ fusion polypeptide before they were applied to the protein blots. The preincubation prevented the 88-kD band from being revealed (Figure 3A). These results led us to believe that our antibodies had recognized the native AtPAKRP2 protein.

AtPAKRP2 Is Concentrated in the Phragmoplast

To gain insights into the function of AtPAKRP2 in plant cells, we used purified anti-AtPAKRP2-N’ and anti-AtPAKRP2-T antibodies to perform immunolocalization experiments in cells from three different species. Although both antibodies gave identical localization signals in Arabidopsis and Brassica oleracea cells, only anti-AtPAKRP2-N’ antibodies worked in tobacco BY-2 cells.

When the purified antibodies were used for immunofluorescence in Arabidopsis root tip cells, they labeled the central region of telophase cells (Figure 3B). To verify the specificity of the purified antibodies for immunolocalization, the anti-AtPAKRP2-N’ antibodies were subjected to preincubation with the AtPAKRP2-N’ polypeptide before they were applied to the cells. The preincubation completely abolished the localization signal (Figure 3C). Identical results were obtained when the anti-AtPAKRP2-T antibodies were used (data not shown).

Because the AtPAKRP2 protein obviously was present in the dividing cells, we wondered whether its localization changed dynamically during the cell division cycle by using Arabidopsis root tip cells that were dividing actively (Figures 4 and 5). Dual localizations of AtPAKRP2 and tubulin were performed. During interphase, AtPAKRP2 was present throughout the cytoplasm and the nucleus, with a concentration of signals at or near the periphery of the nucleus (Figures 4A to 4D). During the progression of mitosis, no signal was detected in the MT preprophase band and in MTs on the nuclear envelope (data not shown). In metaphase cells, judged by their spindle MTs, very little if any anti-AtPAKRP2 signal could be detected with MT bundles by fluorescence microscopy (Figures 4E to 4H). The same was true for early anaphase cells (data not shown). When sister chromatids were separated completely during anaphase, AtPAKRP2 appeared among the interzonal MTs between segregated chromatids (Figures 4I to 4L). Unlike clear bundles of MTs in the spindle midzone, AtPAKRP2 appeared in a punctate pattern among these MT bundles.

After the completion of anaphase, MTs coalesced and were organized into an early phragmoplast array with a dark
midline between the two mirror halves, as judged by anti-tubulin staining (Figure 5B). At this stage, AtPAKRP2 appeared very concentrated at the midline of the phragmoplast, and more punctate AtPAKRP2 signal could be detected weakening away from this midline (Figures 5A to 5D). Although the phragmoplast MT array expanded toward the cell periphery, AtPAKRP2 remained in a punctate pattern among phragmoplast MTs, with a peak of fluorescence intensity near the midline (Figures 5E to 5H). The expansion of AtPAKRP2 signal was concomitant with the expansion of the MT array. MTs depolymerized from the central region of the phragmoplast, as indicated by the anti-tubulin fluorescence signal (Figure 5J) upon the formation of daughter nuclei. At this stage, AtPAKRP2 remained distributed among phragmoplast MTs by being pronounced toward the midline, and the anti-AtPAKRP2 signal was more obvious where more MTs were present toward the periphery of the phragmoplast (Figures 5I and 5L). In the area in which MTs had been completely depolymerized, there was little if any AtPAKRP2 signal (Figure 5I). Therefore, the results suggested that AtPAKRP2 localization correlated with phragmoplast MT organization.

Because AtPAKRP2 behaved like a typical kinesin motor localized among phragmoplast MTs, we tested whether the localization was dependent on MTs. The MT depolymerization agent amiprophos methyl (APM) was used to depolymerize MTs in root cells. After Arabidopsis seedlings were treated with 100 μM APM for 2 hr, no filamentous structure

Figure 4. AtPAKRP2 Was Localized among Interzonal MTs.

Triple localizations of AtPAKRP2 with anti-AtPAKRP2-N' ([A], [E], and [I]), of MTs with anti-α-tubulin ([B], [F], and [J]), and of DNA with DAPI ([C], [G], and [K]) are shown in Arabidopsis root tip cells at interphase ([A] to [D]), metaphase ([E] to [H]), and late anaphase ([I] to [L]). The pseudocolored composite images ([D], [H], and [L]) show AtPAKRP2 in green, α-tubulin in red, and DNA in blue. Bar in (L) = 5 μm for (A) to (L).
was detected in the root cells by anti-tubulin immunofluorescence, indicating that MTs had been depolymerized completely (Figure 6B). In this APM-treated cell with reforming daughter nuclei indicated by 4,6-diamidino-2-phenylindole (DAPI) staining (Figure 6C), AtPAKRP2 no longer had distinct localization between the nuclei; rather, it became distributed diffusely across the cell (Figure 6A). In the control cells treated with the identical amount of isopropanol used to dissolve APM, intact MTs and phragmoplast-localized AtPAKRP2 were observed (Figures 6D to 6F). Therefore, we concluded that the localization of the AtPAKRP2 protein in these root cells was dependent on the integrity of MTs.

Distinct Localization Patterns of AtPAKRP2 and AtPAKRP1

In a previous study, another Arabidopsis motor, AtPAKRP1, was shown to localize in the midline of the phragmoplast MTs (Lee and Liu, 2000). We wanted to determine whether AtPAKRP2 colocalized with AtPAKRP1 in the phragmoplast. Dual localizations of AtPAKRP2 and AtPAKRP1 were performed in Arabidopsis cells at different stages of cell division (Figure 7). At an early stage of cytokinesis, as judged by the appearance of the reforming daughter nuclei (Figure 7C), AtPAKRP2 was detected as particles with different sizes between two daughter nuclei (Figure 7A). Compared with the punctate localization of AtPAKRP2 in the phragmoplast, AtPAKRP1 was much more discrete and concentrated in the midline (Figures 7A and 7B). At a later stage of cytokinesis, the punctate localization pattern of AtPAKRP2 was still clear in the phragmoplast (Figure 7D). AtPAKRP1, however, remained exclusively at the equatorial plane (Figure 7E). We realized the limitation of the resolution by immunofluorescence and did not intend to exclude the possibility of partial colocalization of these two proteins. It was concluded that the two proteins might have distinct functions in the phragmoplast because of the difference in localizations.

AtPAKRP2 Is Enriched in the Insoluble Cytosolic Fraction

The punctate localization pattern of AtPAKRP2 suggested that this motor might be associated with membranous compartment(s) such as Golgi-derived vesicles in the phragm-
A Novel Phragmoplast-Associated Kinesin

To determine whether AtPAKRP2 was in fact associated with the insoluble cytosolic fraction, protein extracts of Arabidopsis inflorescence buds were subjected to fractionation (see Methods); the resulting supernatant was designated the soluble cytosolic fraction, and the pellet was designated the fraction of endomembranes and other insoluble cytosolic compartments. When equal amounts of protein from the two fractions were loaded on an SDS-PAGE gel followed by immunoblotting with anti-AtPAKRP2-N, it appeared that AtPAKRP2 was enriched greatly in the insoluble fraction (Figure 8A). Our results suggest that AtPAKRP2 could be a motor protein that associates with vesicles that are enriched in such preparations. When anti-AtPAKRP1 antibodies were applied, AtPAKRP1 protein was found mainly in the supernatant fraction, suggesting that AtPAKRP1 was unlikely to be associated with the endomembranes (data not shown).

AtPAKRP2 Localization Is Altered by Brefeldin A

We further tested whether AtPAKRP2 associated with the Golgi-derived vesicles because such vesicles were commonly found in the phragmoplast. To disrupt the distribution of these vesicles, we used brefeldin A (BFA), which disrupts the Golgi apparatus and inhibits cell plate formation in BY-2 cells (Yasuhara and Shibaoka, 2000). Although BFA could prevent MT depolymerization in the phragmoplast, it did not change the overall organization of phragmoplast MTs (Figure 8C) (Yasuhara and Shibaoka, 2000). Because BFA was dissolved in methanol, control cells were treated with an amount of methanol equal to that used to dissolve BFA. In the phragmoplasts of control cells, the AtPAKRP2 homolog was concentrated near the equatorial plane (Figure 8E), which was similar to what was observed in cells not treated with methanol (Figure 8H). In BFA-treated cells, however, much reduced anti-AtPAKRP2 signal could be detected in the same area (Figure 8B). Such a result was unlikely to be caused by an overall reduction of fluorescence intensity because diffuse signal was present in the cytoplasm (Figure 8B). To quantify the distortion of AtPAKRP2 localization caused by BFA treatment, we measured the relative fluorescence intensities in both the BFA-treated cell and control cells (Figures 8D, 8G, and 8J). Although a clear peak of AtPAKRP2 signal could be detected near the equatorial plane in the control cells, such a peak was much less obvious in the same area of the BFA-treated cell (Figures 8D, 8G, and 8J). Moreover, the overall level of fluorescence intensity of

**Figure 6.** Requirement of MTs for AtPAKRP2 Localization.

(A) After APM treatment, AtPAKRP2 appeared in a diffuse pattern in the cytoplasm in an Arabidopsis root tip cell after the completion of mitosis.
(B) MTs were depolymerized completely in this cell, as indicated by anti-α-tubulin.
(C) Daughter nuclei stained with DAPI.
(D) to (F) A control root tip cell at a similar stage as in the cell (A) to (C), (shown by DAPI staining of the nuclei in [F]) was treated with isopropanol alone. AtPAKRP2 localization was not disturbed (D), nor were phragmoplast MTs (E). Bar in (F) = 5 μm for (A) to (F).
AtPAKRP2 across the cell, or the basal level, increased significantly in the BFA-treated cell (Figure 8D). We concluded that as a result of the reduced amount of these vesicles among phragmoplast MTs after BFA treatment, AtPAKRP2 was not able to localize among the phragmoplast MTs properly and was distributed randomly in the cytoplasm.

AtPAKRP2 and KNOLLE Show Distinct Localization Patterns

The KNOLLE protein is a cytokinesis-specific syntaxin that localizes to the developing cell plate and functions in vesicle targeting during cell plate formation (Lauber et al., 1997). Because no vesicle motor had been found in the phragmoplast, we tested whether AtPAKRP2 was involved in the transport of KNOLLE-bearing vesicle(s) to the cell plate. Dual localizations of AtPAKRP2 and KNOLLE were performed in B. oleracea because of its large cells compared with those of Arabidopsis and because of its evolutionary relationship with Arabidopsis. Our antibodies cross-reacted with the B. oleracea homolog of AtPAKRP2 and gave similar localization in B. oleracea cells and Arabidopsis cells (Figure 9A). In a cell undergoing cytokinesis, as judged by the appearance of the daughter nuclei (Figure 9C), the B. oleracea AtPAKRP2 homolog was in a punctate pattern in the phragmoplast, being more pronounced toward the division site (Figure 9A). In this cell, KNOLLE was in a sharp line clearly marking the cell plate (Figure 9B). We concluded, therefore, that the KNOLLE protein was not present in the punctate structure(s) bearing the AtPAKRP2 homolog.

DISCUSSION

We have identified AtPAKRP2 cDNA encoding a novel N-terminal motor KRP in Arabidopsis and determined the intracellular localization of this AtPAKRP2 protein. AtPAKRP2 and its homologs in tobacco and B. oleracea localize specifically to the phragmoplast during cell division. In addition to AtPAKRP1 (Lee and Liu, 2000), AtPAKRP2 is the second plant KRP that associates only with the phragmoplast MT array. Unlike AtPAKRP1, which is localized exclusively at or near the plus end of phragmoplast MT bundles, however, AtPAKRP2 appears in a punctate pattern with more pronounced distribution toward the division site. On the basis of biochemical and pharmacological data, AtPAKRP2 likely associates with possibly Golgi-derived vesicles in the phragmoplast. Thus, this protein is a potential motor for vesicle transport during cell plate formation.

Although AtPAKRP2 has a tripartite structure with a coiled-coil domain flanked by the motor and tail domains, it does not fall into any of the established kinesin/KRP subfamilies. Phylogenetic analysis based on the alignment of the motor domains of AtPAKRP2 and kinesin/KRPs from dif-
different subfamilies suggested that AtPAKRP2 is very divergent from various KRPs among eukaryotes. Therefore, it should be considered an outgroup KRP. Such a divergence, however, did not affect its ATP-dependent MT binding activity. There is little doubt that it behaves as a motor protein in plant cells. AtPAKRP2 does not contain a clear consensus sequence in the neck domain that is present among plus end–directed kinesin/KRP motors (Vale and Fletterick, 1997), nor does it have a C-terminal motor KRP-specific neck region. Because the neck sequence of C-terminal motor KRPs has been suggested to direct KRPs to move toward the MT minus ends (Endow, 1999), we believe that AtPAKRP2 cannot be a minus end–directed motor. Because of the nature of the intrinsic plus end–directed activity of the motor domain, AtPAKRP2 most likely is a plus end–directed motor. Unfortunately, under our experimental conditions, GST-AtPAKRP2-M failed to demonstrate motility in vitro. This might be because the GST fusion protein was not robust enough or the conditions we tested were not favorable for the motility assays.

The previously identified AtPAKRP1 protein also is associated exclusively with the phragmoplast (Lee and Liu, 2000). Although both AtPAKRP1 and AtPAKRP2 are N-terminal motor KRPs, they have very different structural features. AtPAKRP1 has a motor domain that is much less...
divergent from other known kinesins/KRPs than is AtPAKRP2. AtPAKRP1 also has an extremely long nonmotor domain and does not have a typical tripartite structure, as does AtPAKRP2 (Lee and Liu, 2000). AtPAKRP1 has coiled coils more scattered in the nonmotor domain. AtPAKRP2, however, has coiled coils immediately after the motor domain. The significance of the nonmotor domains of both motors has yet to be characterized.

Our results suggest that AtPAKRP1 and AtPAKRP2 do not colocalize in the phragmoplast. Their distinct localizations could be distinguished from the very beginning stage when they started to appear in the spindle midzone at late anaphase. AtPAKRP1 clearly associated with MT bundles, whereas AtPAKRP2 was not restricted to the MT bundles. In later stages of phragmoplast development, AtPAKRP1 is conspicuous in the midline of the phragmoplast, coinciding with the plus ends of MT bundles in a narrow area, as revealed by immunofluorescence. As a result of the limitations of our fixation and staining procedures, it is possible that we missed some AtPAKRP1 proteins in the phragmoplast that were not concentrated along MT bundles. But identical procedures allowed us to clearly detect AtPAKRP2 in the phragmoplast, which was not restricted to MT bundles. It is conceivable that phragmoplast operation requires both motors that are responsible for MT reorganization and motors that are responsible for vesicle transport. Obviously, motors bearing two distinct roles will have different tail domains. Elucidation of the functions of the nonmotor domains of both motors will shed light on the mechanisms of plant cytokinesis. For example, it would be interesting to determine whether either tail domain could bind MTs in the absence of the motor domain in vivo or in vitro.

Treatment with BFA suggested that AtPAKRP2 associated with Golgi vesicles that were destined to the phragmoplast, although careful examination with immunogold labeling is needed to reveal the AtPAKRP2 localization at the ultrastructural level. When the formation of these vesicles was hampered by BFA as it disrupted the Golgi apparatus, fewer vesicles were expected in the phragmoplast. We suggest that binding to the vesicle might be necessary for AtPAKRP2 to associate with the phragmoplast MTs. Therefore, when fewer vesicles were produced, AtPAKRP2 was mislocalized. Conversely, AtPAKRP2 was required for Golgi vesicles to travel along phragmoplast MTs because AtPAKRP2 is a MT-based motor. It was not surprising that AtPAKRP2 localization to the phragmoplast was dependent on the integrity of phragmoplast MTs.

Because KNOLLE is a syntaxin-like protein that plays an essential role in vesicle docking during cytokinesis in plant cells (Lauber et al., 1997), it is reasonable to wonder whether AtPAKRP2 is the motor protein that transports KNOLLE-bearing vesicles. However, our results from double localizations of homologs of AtPAKRP2 and KNOLLE in B. oleracea cells suggested that they had different localization patterns. Thus, it is possible that other motor protein(s) might transport KNOLLE-bearing vesicles.

It is worth noting that dynamic activities of vesicles are required in animal cytokinesis as well (Straight and Field, 2000). The ras-like GTPase Rabs play critical roles in vesicle targeting (Deacon and Gelfand, 2001). In a search for interactors with Rab6, the kinesin-like Rab6-KIFL has been identified (Echard et al., 1998). Rab6-KIFL localizes to the spindle midzone and the midbody in mammalian cells (Hill et al., 2000). Introduction of an–Rab6-KIFL antibodies into dividing cells blocks cytokinesis (Hill et al., 2000). Rab6-KIFL probably is involved in transporting Golgi-derived vesicles during cytokinesis in animal cells. Such speculation is fostered by the evidence that Rab6-KIFL also associates with the Golgi apparatus (Echard et al., 1998). Thus, cytokinesis in animals requires the active transport of Golgi vesicles by MT-based motor(s), as in plant cells.

A number of KRPs have been implicated in cytokinesis in higher plants (Liu and Lee, 2001). The most studied ones are members of the BIMC subfamily, which form homotetramers in their native forms (Kashina et al., 1996; Asada et al., 1997; Barroso et al., 2000). Among them, the tobacco TKRP125 protein and the carrot DcKRP120-2 protein are activated to render the sliding activity of antiparallel MTs to establish the phragmoplast MT array (Asada et al., 1997; Barroso et al., 2000). The activity of BIMC-like KRPs likely is balanced by C-terminal motor KRPs, most likely KatA/B/C, which also localize to phragmoplast MTs (Liu and Palevitz, 1996; Liu et al., 1996; Mitsu et al., 1996). Moreover, downregulation of KCBP is required for the organization of phragmoplast MTs (Vos et al., 2000). In addition to these motors, AtPAKRP1 is believed to play a role in the maintenance of the mirror-shaped phragmoplast MT array after the sliding activity is complete (Liu and Lee, 2001). It is conceivable that stabilization of the phragmoplast MT array allows vesicles to be transported to the right place at the division site, possibly by AtPAKRP2. In a simplified view of cytokinesis, we believe there are collaborative efforts of different motors that result in the smooth progression of cell plate formation.

Recently, the tobacco mitogen-activated protein kinase kinase kinase NPK1 has been implicated in a signaling pathway that regulates cytokinesis in tobacco cells (Machida et al., 1998). Upon overexpression of a dominant negative construct or a kinase-negative mutant, cytokinesis can be blocked and multinucleate cells can be formed (Nishihama et al., 2001). It appears that this kinase is required for the expansion of the phragmoplast MT array toward the cell periphery during cell plate formation (Nishihama et al., 2001). NPK1 localizes with spindle MTs at metaphase, at the equatorial plane during late anaphase, and in the midzone of the phragmoplast (Nishihama et al., 2001). More interestingly, it has been mentioned that a novel tobacco KRP, NACK1, acts as an activator of NPK1 and localizes to the division site as well (Machida et al., 1998; Nishihama et al., 2001). It will be interesting to compare NACK1 and AtPAKRP1/2 once the Nack1 sequence is revealed.
METHODS

Isolation and Characterization of AtPAKRP2 cDNAs

The Arabidopsis thaliana expressed sequence tag clone OA0358 was provided by Dr. Alain Lecharny (Laboratoire de Biologie du Développement des Plantes, Universite de Paris Sud, France). 5’ RACE (rapid amplification of cDNA ends; Clontech, Palo Alto, CA) was applied to obtain the 5’ sequence of AtPAKRP2 cDNA. Trizol (Life Technologies, Rockville, MD) was used to isolate total RNA from inflorescence buds of Arabidopsis Columbia according to the manufacturer’s protocol. Two AtPAKRP2-specific primers were designed for 5’ RACE: one primer (5’-GTTGTAAGAGGATCTTAAGCAGC-3’) was used for reverse transcription, and the other nested primer (5’-ATCTTCCCCACAGTTGCGACATC-3’) was used for subsequent polymerase chain reaction (PCR). DNA sequences were sampled in a commercial laboratory (Davis Sequencing, Davis, CA). The GCG program (Genetics Computer Group, Madison, WI) was used to analyze DNA sequences. Phylogenetic analysis of kinesin/KRP motor domain was performed with the heuristic search method of PAUP included in GCG. A maximum parsimony with random stepwise addition was used. In addition to the AtPAKRP2 reported here, the phylogenetic analysis included Arabidopsis AtKatA (GenBank accession number D11371), AtKatB (D21137), AtKatC (D21138), AtPAKRP1 (AF193767); Aspergillus nidulans AnBIMC (M32075); Cricetulus griseus CgMCACK (U11790); Daucus carota DcKRP120-2 (AF283505); Drosophila melanogaster DmNcd (X52814) and DmKHC (M24441); Gallus gallus GgChrkin (U18309); Homo sapiens HsMLP1 (X67156); Mus musculus MmKIF1A (D29951); Nicotiana tabacum NtKRP17 (L40358); Apis mellifera A1 (AF285127); and Homo sapiens HsKRP (X67009). The phylogenetic tree shown in Figure 1B was one of two optimal trees and was rooted arbitrarily using ScSmyp1 as an outgroup KRP. The CoiScan program of GCG was used to predict the coiled-coil domains of a protein.

Production of Recombinant Fusion Proteins

To construct plasmids encoding fusion proteins of glutathione S-transferase (GST) and various regions of AtPAKRP2, new restriction sites were introduced into AtPAKRP2 cDNA by PCR. Four plasmids were made for this study: (1) pGEX-AtPAKRP2-M contained the DNA fragment encoding amino acid residues 1 to 435, which was amplified by PCR with the primers 5’-TCTTGGAATTCGACGACACTACATC-3’ and 5’-GTTGTCGAGAGATCTTAAAGCAGC-3’; (2) pGEX-AtPAKRP2-N contained the DNA fragment encoding amino acid residues 1 to 202, which was amplified with the primers 5’-GACGATCCGACACCATTTCTTCTC-3’ and 5’-AGTCTGAGAGATCTTAAAGCAGC-3’; (3) pGEX-AtPAKRP2-T contained the DNA fragment encoding amino acid residues 1 to 27, which was amplified with the primers 5’-GACGAGACATCCGACACCATTTCTTCTC-3’ and 5’-GCTGAGACATCATACTGACGCA-3’; and (4) pGEX-AtPAKRP2-T contained the DNA fragment encoding amino acid residues 516 to 869, which was amplified with the primers 5’-CCGCTCCATGGAGGAAAGTGGTATG-3’ and 5’-AATCTGAGGACAGCATGGCGAC-3’. The PCR fragments for the first three plasmids were digested with EcoRI and XhoI (sites underlined in the primers) and ligated to pGEX-KG EcoRI-XhoI (Guan and Dixon, 1991). The PCR fragment for the last plasmid was digested with NcoI and XhoI and ligated to pGEX-KG NcoI-XhoI.

All pGEX constructs were expressed in the E. coli strain BL21(DE3)pLysS (Novagen, Madison, WI). GST fusion proteins were affinity purified with glutathione–Sepharose as described by the manufacturer (Pierce Chemical Co., Rockford, IL), except for the protein expressed by pGEX-AtPAKRP2-N. This protein was not soluble, so it was purified from inclusion bodies with B-PER bacterial protein extraction reagent as described by the manufacturer (Pierce).

Microtubule Sedimentation Assay

This assay was performed essentially as described previously (Lee and Liu, 2000). In brief, purified GST-AtPAKRP2-M fusion protein (5 μg) was incubated with 25 μg of microtubules (MTs), 10 μM taxol, and 2 μM ATP (or adenylylimidodiphosphate, AMPPNP) in BRB80 buffer (80 mM Pipes, 1 mM MgCl2, and 1 mM EGTA, pH 6.8). After incubation at room temperature, the reaction was loaded on top of a sucrose cushion (15% sucrose and 10 μM taxol in BRB80) and then subjected to centrifugation at 25,000g. The supernatant above the sucrose cushion and the MT pellet were analyzed by SDS-PAGE. Proteins of the supernatant and pellet were visualized by staining the gels with Coomassie Brilliant Blue R250 (Sigma, St. Louis, MO).

Antibody Preparation and Immunoblotting

The GST fusion proteins GST-AtPAKRP2-N and GST-AtPAKRP2-T were used as antigens to raise polyclonal antibodies in rabbits and rats, respectively, at commercial facilities (Strategic BioSolutions, Romona, CA; and Antibodies, Inc., Davis, CA). Sera were passed through a column that had the GST protein covalently linked to the matrix (GST Orientation Kit; Pierce) to deplete antibodies against GST. Depletion of anti-GST antibodies was confirmed by immunoblotting against the GST protein. Because the PAKRP2-N polypeptide covered approximately half of the motor domain, the anti-PAKRP2-N sera might contain antibodies against conserved kinesin/EBP epitopes. To eliminate such antibodies, PAKRP2-N polypeptide was used for the purpose of antibody purification because it was upstream of the ATP binding site and few conserved residues were found in this region. Antibodies specific to AtPAKRP2 were affinity purified with GST-AtPAKRP2-N or GST-AtPAKRP2-T by a blot purification method (Olmsted, 1981). To test the specificity of antibodies, control experiments were performed by incubating antibodies with blots containing their corresponding antigens before immunostaining.

Total protein extraction from plant tissues and immunoblotting was performed as described previously (Liu et al., 1998). To prepare subcellular fractions, Arabidopsis inflorescence buds were ground in the extraction buffer containing 30 mM Pipes, 10 mM EGTA, 6 mM MgCl2, 1 mM phenylmethylsulfonyl fluoride, 2 mM DTT, and 250 mM sucrose, pH 7.0, with a mortar and pestle. The homogenate was centrifuged at 10,000g for 10 min at 4°C to remove large cellular debris. This low-speed supernatant was centrifuged at 100,000g for 30 min at 4°C. The resulting supernatant (the cytosolic fraction) and the pellet (the insoluble cytosolic fraction) then were used for immunoblotting analysis.

Fluorescence Microscopy and Image Processing

Slides containing Arabidopsis root tip cells of 2-day-old etiolated seedlings or tobacco BY-2 suspension cells were prepared and processed for immunofluorescence as described previously (Lee and...
Liu, 2000). In addition to the rabbit anti-AtPAKRP2-N’ and rat anti-AtPAKRP2-T antibodies reported here, antibodies used in this study included rabbit anti-AtPAKRP1-C (Lee and Liu, 2000), mouse anti-α-tubulin (DM1A; Sigma), fluorescein isothiocyanate–conjugated goat anti-rabbit IgG (Sigma), Texas Red X–conjugated goat anti-rabbit IgG (Molecular Probes, Eugene, OR), Texas Red X–conjugated goat antimouse IgG (Molecular Probes), fluorescein isothiocyanate–conjugated goat anti-rat IgG (Sigma), and rabbit anti-KNOLLE (Rose Biotech, Winchendon, MA). Images were acquired by conventional epifluorescence microscopy as described previously (Lee and Liu, 2000).

Pharmacological Studies

To depolymerize MTs, Arabidopsis 2-day-old etiolated seedlings were incubated in 5 mL of germination medium (half-strength Murashige and Skoog [1962] salts [Life Technologies] and 5 mM Mes, pH 5.7) containing 100 μM APM (a gift from Dr. C. Gregg of Bayer Corp., Kansas City, MO) for 2 hr. The same amount of isoamyl alcohol, used to dissolve APM, was added in the control sample. BFA treatment was performed in tobacco BY-2 suspension cells 4 days after subculture. Ten-milliliter cells were treated with 20 μM BFA for 2 hr. The solvent methanol was added in the control sample. The measurement of fluorescence intensity was performed using the Line Profile measurement function in the ImagePro software package (Media Cybernetics, Silver Spring, MD). Data then were exported to Microsoft Excel software to generate Figures 8D, 8G, and 8J.

Accession Number

The GenBank accession number for AtPAKRP2 is AF320248.

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