Identification of a phragmoplast-associated kinesin-related protein in higher plants

Y-R. Julie Lee and Bo Liu

The phragmoplast executes cytokinesis in higher plants. The major components of the phragmoplast are microtubules, which are arranged in two mirror-image arrays perpendicular to the division plane [1]. The plus ends of these microtubules are located near the site of the future cell plate. Golgi-derived vesicles are transported along microtubules towards the plus ends to deliver materials bound for the cell plate [2,3]. During cell division, rapid microtubule reorganization in the phragmoplast requires the orchestrated activities of microtubule motor proteins such as kinesins. We isolated an *Arabidopsis* cDNA clone of a gene encoding an amino-terminal motor kinesin, AtPAKRP1, and have determined the partial sequence of its rice homolog. Immunofluorescence experiments with two sets of specific antibodies revealed consistent localization of AtPAKRP1 and its homolog in *Arabidopsis* and rice cells undergoing anaphase, telophase and cytokinesis. AtPAKRP1 started to accumulate along microtubules towards the spindle midzone during late anaphase. Once the phragmoplast microtubule array was established, AtPAKRP1 conspicuously localized to microtubules near the future cell plate. Our results provide evidence that AtPAKRP1 is a hitherto unknown motor that may take part in the establishment and/or maintenance of the phragmoplast microtubule array.

Address: Section of Plant Biology, University of California Davis, Davis, California 95616, USA.

Correspondence: Bo Liu
E-mail: bliu@ucdavis.edu

Received: **25 February 2000**
Revised: **15 May 2000**
Accepted: **15 May 2000**

Published: **16 June 2000**

Current Biology 2000, 10:797–800

0960-9822/00/$ – see front matter

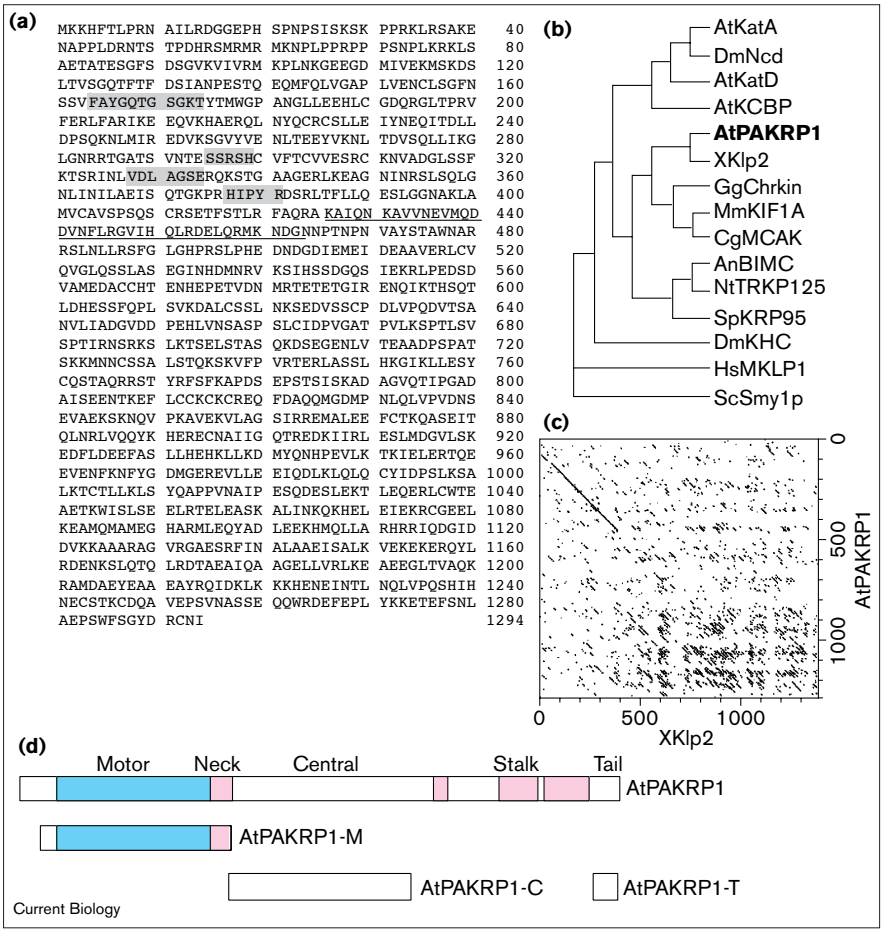
Results and discussion

To identify plant kinesin-related proteins (KRPs) with a role in cell division, we identified a rice expressed sequence tag (EST) clone, RICS4872A, which potentially encoded a KRP. Because anti-RICS4872A antibodies gave an identical localization pattern in *Arabidopsis* cells and in rice cells (see below), we postulated the existence of an *Arabidopsis* RICS4872A homolog, and used the RICS4872A cDNA as a probe to screen an *A. thaliana* cDNA library. A cDNA clone of 3923 bp was isolated. When compared with the corresponding genomic sequence a start codon was identified upstream of the 5′ end of the cDNA clone. A deduced open reading frame encoded a polypeptide of 1294 amino acids (Figure 1a) with a predicted molecular mass of 145 kDa and predicted isoelectric point of 5.78. The amino-terminal region (amino acids 93–424) of the deduced polypeptide sequence resembled a typical kinesin motor domain. Highly conserved peptide sequences in the ATP-binding site (FAYGQTGSGKT; single-letter amino-acid notation) and the microtubule-binding site (SSRSH, VDLAGSE and HIPYR) were found in this region. The region downstream of the motor domain (residues 426–463) matched a consensus neck sequence among kinesin/KRPs that move towards microtubule plus ends [4]. Because of the presence of a conserved kinesin amino-terminal motor domain, as well as its cellular localization in the phragmoplast (see below), we named this polypeptide AtPAKRP1, for *A. thaliana* phragmoplast-associated KRP1.

To study the relationship between AtPAKRP1 and other kinesin/KRPs, a phylogenetic analysis based on the alignment of motor domain sequences was carried out. AtPAKRP1 was divergent from *Arabidopsis* AtKatA, AtKatD and AtKCBP proteins, and tobacco NtTRKP125 protein. AtPAKRP1 was also divergent from major kinesin/KRP subfamilies (Figure 1b), but was most closely related to ungrouped XKlp2 from *Xenopus laevis* [5]. AtPAKRP1 and XKlp2 shared 58% amino-acid similarity within the motor and neck domains, but no significant sequence similarity was found outside these domains (Figure 1c). AtPAKRP1 and RICS4872A are likely to belong to a new KRP subfamily as they shared 66% overall similarity in amino-acid sequence, and 85% in part of their motor and neck domains; the RICS4872A clone lacked the first ~200 amino acids in its motor domain.

The majority of kinesin/KRPs dimerize through coiled-coil domains. The Lupus algorithm was used to predict potential α-helical coiled-coil structure in AtPAKRP1 [6]. Amino acids 433–465, 867–894, 1044–1129 and 1139–1236 tended to form coiled-coil structures ($p > 0.5$) in this test, implying that AtPAKRP1 might dimerize. Upon dimerization, the coiled-coil domain downstream of the neck domain is often referred to as the stalk domain. The relative positions of the motor, neck and stalk domains are shown in Figure 1d. To our knowledge, a KRP bearing a long non-coiled-coil region between coiled coils has not been reported previously. The significance of this unique structural pattern is not clear.

Figure 1



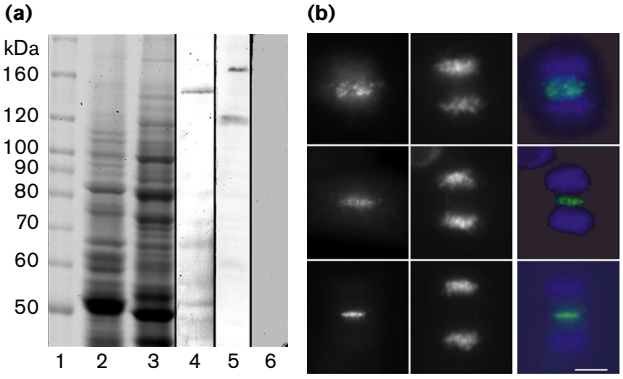
The amino-terminal region of AtPAKRP1 has sequence homology to KRP motor domains. **(a)** The amino-acid sequence was deduced from *AtPAKRP1* cDNA and genomic sequences (GenBank accession numbers AF193767 and AF193768). The highly conserved peptides in the motor domain are shaded, and the conserved neck sequence commonly found in plus end-directed KRPs is underlined. **(b)** A phylogenetic tree was built from sequence alignment of KRP motor domains of AtPAKRP1; *A. thaliana* AtKatA (D11371), *Drosophila melanogaster* DmNcd (X52814), *A. thaliana* AtKatD (AF080249), *A. thaliana* AtKCBP (L40358), *X. laevis* XKlp2 (X94082), *Gallus gallus* GgChrkin (U18309), *Mus musculus* MmKIF1A (D29951), *Cricetulus griseus* CgMCAK (U11790), *Aspergillus nidulans* AnBIMC (M32075), *Nicotiana tabacum* NtTPKP125 (U52078), *Strongylocentrotus purpuratus* SpKRP95 (U00996), *D. melanogaster* DmKHC (M24441), *Homo sapiens* HsMKLP1 (X67155) and *Saccharomyces cerevisiae* ScSmy1 (M69021). **(c)** Comparison of amino-acid sequences of AtPAKRP1 and XKlp2 in a dot plot. XKlp2 sequence is on the *x*-axis, and AtPAKRP1 on the *y*-axis. **(d)** Schematic structure of AtPAKRP1. Coiled coils are shown in pink. The relative positions of motor, neck and stalk are shown. The region between neck and stalk and the region downstream of stalk are referred to as the central and tail regions, respectively. The positions of three recombinant polypeptides, AtPAKRP1-M, -C and -T, are also shown.

To understand the function of AtPAKRP1, we wanted to determine its intracellular localization. To obtain specific antibodies against AtPAKRP1 and/or its rice homolog, we prepared two antigens. One was derived from RICS4872A (residues 364–767). In immunoblots of a protein extract from rice root tips, affinity-purified anti-RICS4872A antibodies stained a 145 kDa band (Figure 2a). The RICS4872A antigen corresponded to AtPAKRP1 residues 828–1246, and the sequences had 57% identity (67% similarity). The second antigen was AtPAKRP1 residues 466–848 (AtPAKRP1-C in Figure 1d). Anti-AtPAKRP1-C antibodies recognized a 160 kDa band in an immunoblot of a protein extract of *Arabidopsis* 2-day-old etiolated seedlings (Figure 2a). Anti-AtPAKRP1-C also cross-reacted with a 120 kDa band on the blot. The intensity of the band decreased, however, when protease inhibitors were added during protein extraction. Preincubation of antibodies with fusion protein antigen before immunoblotting abolished the detection of both bands. The 120 kDa band is therefore likely to be a degradation product of AtPAKRP1. The AtPAKRP1-C sequence was unique to AtPAKRP1 and is not found in RICS4872A.

We used the purified antibodies to determine the intracellular localization of the corresponding proteins by immunofluorescence. Anti-RICS4872A antibodies on fixed rice root tip cells gave no obvious staining pattern before anaphase (data not shown). A relatively broad localization was detected in the midzone in late anaphase cells (Figure 3b). During telophase and cytokinesis, the staining gradually narrowed down in the middle of the cell (Figure 3b). Anti-RICS4872A antibodies gave a similar localization in *Arabidopsis* cells and in rice cells (data not shown). To confirm that the localization of anti-RICS4872A in *Arabidopsis* represented that of AtPAKRP1, *Arabidopsis* cells were treated with purified anti-AtPAKRP1-C antibodies. Anti-AtPAKRP1-C staining was identical to that of anti-RICS4872A (see below). Addition of AtPAKRP1-C abolished the staining pattern.

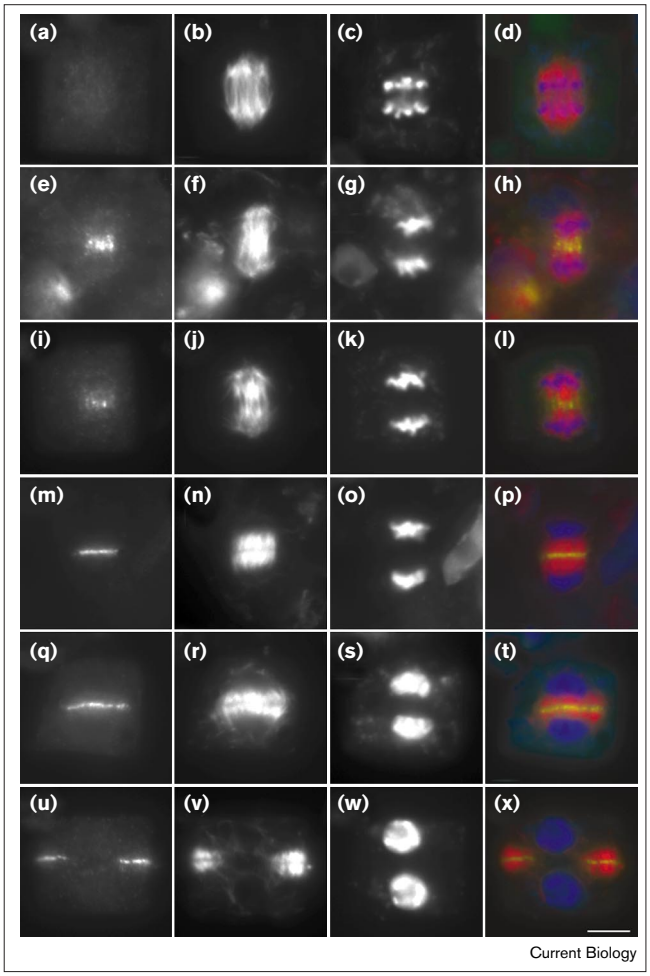
Because of the conspicuous AtPAKRP1 localization pattern, we wished to reveal the relationship between AtPAKRP1 and microtubules, especially in the phragmoplast. A double localization experiment was carried out in *Arabidopsis* cells using anti-AtPAKRP1 and anti-α-tubulin

Figure 2



(a) Immunoblots of cell extracts with affinity-purified anti-RICS4872A and anti-AtPAKRP1 antibodies. Total protein was extracted from rice root tips (lanes 2,4) or *A. thaliana* seedlings (lanes 3,5,6). After resolution by SDS–PAGE, proteins were stained with Coomassie blue (lanes 2,3) or transferred to a nitrocellulose membrane and blotted with anti-RICS4872A (lane 4) or anti-AtPAKRP1-C (lane 5). Preincubation of antibodies with AtPAKRP1-C abolished both bands (lane 6). Molecular weight markers are shown in lane 1.
(b) Localization of RICS4872A in dividing rice root cells by immunofluorescence. Localization of RICS4872A (first column) and DNA (second column) in one late anaphase cell (first row) and two telophase cells (second and third rows) are shown. The third column shows composite images in which RICS4872A is pseudocolored green and DNA blue. The scale bar represents 5 μm.

Figure 3

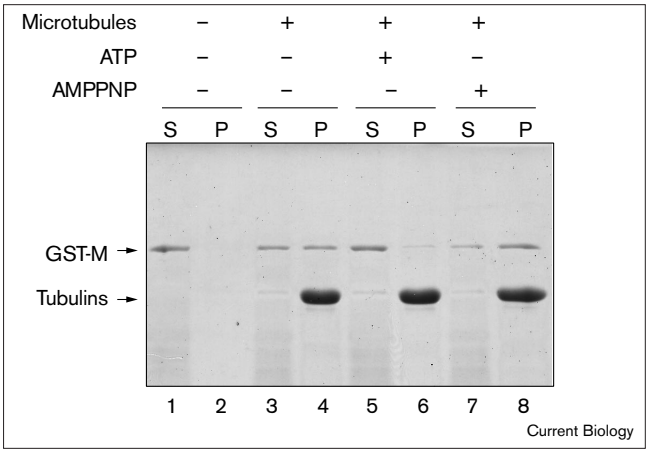


Localization of AtPAKRP1 in *Arabidopsis* cells established by immunofluorescence. Cells are in **(a–h)** anaphase, **(i–p)** telophase and **(q–x)** cytokinesis. The separate localization of (a,e,i,m,q,u) AtPAKRP1, (b,f,j,n,r,v) α-tubulin and (c,g,k,o,s,w) DNA are shown. (d,h,l,p,t,x) Composite images pseudocolored for AtPAKRP1 (green), α-tubulin (red) and DNA (blue). The scale bar represents 5 μm.

antibodies. The AtPAKRP1 signal was hardly detectable at early anaphase (Figure 4a–d). At late anaphase, when the kinetochore microtubule fibers were almost completely shortened, AtPAKRP1 was clearly localized to the central region of interzonal microtubules, but was not associated with kinetochore microtubules (Figure 4e–h). When the phragmoplast microtubule array started to appear, evident as a dark equatorial zone in cells treated with anti-tubulin antibody, AtPAKRP1 concentrated in this dark zone where the plus end of phragmoplast microtubules were located (Figure 4i–l). While phragmoplast microtubules shortened at the distal ends and expanded in girth, AtPAKRP1 clearly localized at or near the microtubule plus ends (Figure 4m–t). When the cell plate is laid down during cytokinesis, phragmoplast microtubules depolymerize and expand centrifugally towards the parental plasma membrane. AtPAKRP1 was always localized at or near the plus ends of phragmoplast microtubules during centrifugal expansion (Figure 4u–x). To visualize the relation of AtPAKRP1, microtubules and the cell plate in *Arabidopsis* cells undergoing cytokinesis, we used calcofluor to stain the cell plate, in addition to immunofluorescence for AtPAKRP1 and microtubules. We found that AtPAKRP1 localization did not overlap with the already formed cell plate (data not shown). We investigated if AtPAKRP1 localization depended on microtubule integrity. Microtubule depolymerization with colchicine totally abolished the AtPAKRP1 staining pattern (data not shown). Thus, AtPAKRP1 localization is microtubule dependent.

In higher plants, several amino- and carboxy-terminal motor KRPs localize along microtubules in the spindle and the phragmoplast [7–9]. Unlike other plant KRPs, however, AtPAKRP1 has a unique localization pattern. It is unclear how such stage-dependent localization is established. A post-translational modification of AtPAKRP1, for example phosphorylation, may contribute to such localization. We speculate that AtPAKRP1 only associates with microtubules at or near their plus ends. Microtubule plus ends are distributed widely among anaphase interzonal

Figure 4



Assays for the ability of AtPAKRP1 to bind microtubules. The purified GST–AtPAKRP1 motor domain fusion protein (GST-M) was used. Microtubules and their associated proteins were collected by centrifugation. Proteins of the supernatant (S) and pellet (P) were analyzed by SDS–PAGE and visualized by Coomassie blue staining. ATP or AMPPNP (2 μM) was included to test for ATP-dependent binding of GST-M to microtubules (lanes 5–8). The presence (+) or absence (–) of microtubules, ATP or AMPPNP are shown at the top.

microtubules as these microtubules have mixed polarities. When the antiparallel microtubule pattern is established, the plus ends are restricted to the region near the future cell plate [10]. AtPAKRP1 is therefore localized widely in the anaphase spindle and then becomes restricted to a narrow area of the phragmoplast microtubules. By associating with microtubules at or near their plus ends in the phragmoplast, AtPAKRP1 may keep antiparallel microtubule bundles from becoming distanced from each other. Our preliminary results with inhibitors of antibodies and truncated fusion proteins support this hypothesis (see Supplementary material). Interestingly, two KRPs, which remain to be revealed, have been proposed to be the activators of MAP kinase kinase kinase in the phragmoplast [11].

To test whether AtPAKRP1 could bind to microtubules in an ATP-dependent manner, we carried out co-sedimentation of AtPAKRP1 and microtubules *in vitro*. A glutathione-*S*-transferase (GST) fusion protein containing the AtPAKRP1 motor domain (AtPAKRP1-M, Figure 1d) was expressed and purified. When incubated with polymerized microtubules in the absence of ATP, nearly 50% of the GST–AtPAKRP1-M co-sedimented with microtubules (Figure 4, lanes 3,4). The addition of ATP significantly reduced the amount of the fusion protein co-sedimenting (Figure 4, lanes 5,6). When the non-hydrolyzable ATP analog AMPPNP was used in place of ATP, most of the fusion protein remained associated with microtubules in the pellet (Figure 4, lanes 7,8). GST alone did not noticeably co-sediment with microtubules (data not shown). The results threfore support the notion that AtPAKRP1 can bind to microtubules in an ATP-sensitive manner like typical kinesin/KRPs.

Our localization results in late anaphase cells suggested that AtPAKRP1 might associate with midzone microtubules via a microtubule-binding site outside the motor domain. We then tested the fusion proteins GST–AtPAKRP1-C and GST–AtPAKRP1-T in the microtubule sedimentation assay, but neither sedimented with microtubules (data not shown). Despite this negative result, an *in vivo* microtubule-binding activity might be established via a post-translational modification of the protein in plant cells. Another possibility is that a microtubule-binding domain was not present in the region we tested. The interaction with microtubules could also be mediated through a microtubule-associated protein. Our results have revealed a new KRP in higher plants. AtPAKRP1 is an amino-terminal motor KRP that associates with microtubule arrays in a cell-cycle-dependent manner. Its association with phragmoplast microtubules indicates that it might have a role in cytokinesis.

Supplementary material

Supplementary material including the results of functional inhibition of AtPAKRP1 and detailed methodology is available at http://current-biology.com/supmat/supmatin.htm.

Acknowledgements

This work was supported by the USDA CSREES/NRICGP grant 99-35304-8142. We are grateful to the National Institute of Agrobiological Resources in Japan for plasmids and the *Arabidopsis* Biological Resource Center at the Ohio State University for cDNA libraries. B.L. thanks Barry Palevitz for ordering the rice clone and for encouragement. We also thank G. Rogers and J. Scholey for their comments on the manuscript.

References

1. Wick SM: **Spatial aspects of cytokinesis in plant cells.** *Curr Opin Cell Biol* 1991, **3:**253-260.
2. Staehelin LA, Hepler PK: **Cytokinesis in higher plants.** *Cell* 1996, **84:**821-824.
3. Sylvester AW. **Division decisions and the spatial regulation of cytokinesis.** *Curr Opin Plant Biol* 2000, **3:**58-66.
4. Vale RD, Fletterick RJ: **The design plan of kinesin motors.** *Annu Rev Cell Dev Biol* 1997, **13:**745-777.
5. Boleti H, Karsenti E, Vernos I: **Xklp2, a novel *Xenopus* centrosomal kinesin-like protein required for centrosome separation during mitosis.** *Cell* 1996, **84:**49-59.
6. Lupas A, Van Dyke M, Stock J: **Predicting coiled coils from protein sequences.** *Science* 1991, **252:**1162-1164.
7. Asada T, Kuriyama R, Shibaoka H: **TKRP125, a kinesin-related protein involved in the centrosome-independent organization of the cytokinetic apparatus in tobacco BY-2 cells.** *J Cell Sci* 1997, **110:**179-189.
8. Bowser J, Reddy ASN: **Localization of a kinesin-like calmodulin-binding protein in dividing cells of *Arabidopsis* and tobacco.** *Plant J* 1997, **12:**1429-1437.
9. Liu B, Cyr RJ, Palevitz BA: **A kinesin-like protein, KatAp, in the cells of *Arabidopsis* and other plants.** *Plant Cell* 1996, **8:**119-132.
10. Euteneuer U, Jackson WT, McIntosh JR: **Polarity of spindle microtubules in *Haemanthus* endosperm.** *J Cell Biol* 1982, **94:**644-653.
11. Machida Y, Nakashima M, Morikiyo K, Banno H, Ishikawa M, Soyano T, Nishihama R: **MAPKKK-related protein kinase NPK1: regulation of the M phase of plant cell cycle.** *J Plant Res* 1998, **111:**243-246.