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Roles for kinesin and myosin during cytokinesis

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Cytokinesis in higher plants involves the phragmoplast, a complex cytoplasmic structure that consists of microtubules (MTs), microfilaments (MFs) and membrane elements. Both MTs and MFs are essential for cell plate formation, although it is not clear which motor proteins are involved. Some candidate processes for motor proteins include transport of Golgi vesicles to the plane of the cell plate and the spatiotemporal organization of the cytoskeletal elements in order to achieve proper deposition and alignment of the cell plate. We have focused on the kinesin-like calmodulin binding protein (KCBP) and, more broadly, on myosins. Using an antibody that constitutively activates KCBP, we find that this MT motor, which is minus-end directed, contributes to the organization of the spindle and phragmoplast MTs. It does not participate in vesicle transport; rather, because of the orientation of the phragmoplast MTs, it is supposed that plus-end kinesins fill this role. Myosins, on the other hand, based on their inhibition with 2,3-butanedione monoxime and 1-(5-iodonaphthalene-1-sulphonyl)-1H-hexahydro-1,4-diazepine (ML-7), are associated with the process of post-mitotic spindle/phragmoplast alignment and with late lateral expansion of the cell plate. They are also not the principal motors involved in vesicle transport.

Keywords: actin microfilaments; microtubules; phragmoplast; cytokinesis; myosin; kinesin

1. INTRODUCTION

MTs and actin MFs are major components of the phragmoplast and contribute to the process of cell plate formation during cytokinesis in plant cells (Staehelin & Hepler 1996). They are organized parallel to one another, but perpendicular to the division plane, where they are arranged in two oppositely polarized sets. MTs and MFs show a similar dynamic rearrangement during cytokinesis, in which they first cluster to form a dense cylinder of cytoskeletal elements in the centre of the mitotic apparatus during late anaphase and early telophase (Zhang *et al.* 1993). Subsequently, as a cell plate initial is being deposited within the cylinder, both MTs and MFs shorten in length while the array as a whole expands centrifugally. Because elements break down in the centre of the cylinder, the expanding phragmoplast becomes a torus that eventually fuses with the parental cell walls (Valster & Hepler 1997). Although most studies have focused on the structure and role of phragmoplast MTs, it must be recognized that MFs are also major contributors to the process of cell plate formation since their disruption with drugs, e.g. cytochalasin (Palevitz & Hepler 1974), or agents that affect actin polymerization, such as the actin monomer binding protein profilin (Valster *et al.* 1997), markedly affect normal progression of cytokinesis or inhibit the process altogether.

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The oppositely polarized orientation of both sets of MTs (Euteneuer & McIntosh 1980) and MFs (Kakimoto & Shibaoka 1988), with plus ends proximal to the plane of the cell plate, invites speculation about the possible participation of associated motor proteins. The conventional kinesins, for example, are plus-end directed MT motors (Goldstein & Philp 1999; Reddy 2001), while most myosins are plus-end directed MF motors (Berg *et al.* 2001; Shimmen *et al.* 2000; Reddy 2001). It would therefore seem highly plausible that these motors could transport the Golgi-derived cell plate vesicles inward to the point of aggregation and fusion, which is the region defined by the position of the MT and MF plus ends. There are also major issues concerning the initial organization and dynamic rearrangement of the phragmoplast cytoskeleton. For example, how is it that these elements are orientated normal to the plane of the cell plate and how is their precise polar organization achieved and maintained? Increasingly, in other systems it is recognized that motor proteins play a major role in cytoskeletal organization. Among the kinesins, both plus- and minus-end directed motors can contribute to the self-assembly of radial arrays of MTs *in vitro* (Surrey *et al.* 2001). Myosins are also implicated in the organization of MFs through the formation of complexes (e.g. Arp2/3) that participate in the localization and stabilization of F-actin (Berg *et al.* 2001; Lee *et al.* 2000).

From studies of dividing plant cells, there is evidence supporting a role for kinesins during cytokinesis. Several different kinesins, including both plus-end directed examples—TKRP125 (Asada *et al.* 1997), AtPAKRP1 and 2 (Lee & Liu 2000)—and minus-end directed examples—AtKatA (Liu *et al.* 1996), KCBP (Smirnova *et*

al. 1998)—associate with the phragmoplast. Recent findings reveal that AtKRP1 and 2 are confined to the plane of the cell plate or to the plus-end overlap zone of the phragmoplast MTs (Liu *et al.* 1996). By contrast, both KCBP and AtKatA, minus-end motors, distribute along the length of the phragmoplast MTs and are not localized to the region of overlap. Functional studies suggest that TKRP125 participates in MT sliding in the phragmoplast. The importance of MT sliding in the phragmoplast can be appreciated if we assume that the inter-zonal MT array, which arises during anaphase, contains elements of mixed polarity. Therefore, a plus-end kinesin, which generates inter-MT sliding, would sort out the array and establish the two oppositely polarized sets of MTs, with a central overlap zone, as is seen in the phragmoplast (Asada *et al.* 1997). Curiously, no direct evidence has thus far shown a link between any of the plus-end kinesins with vesicle transport.

Our understanding of myosins during cytokinesis in plants is less well developed. Earlier studies, using a monoclonal antibody to a non-muscle myosin heavy chain, indicated the presence of cross-reacting antigens in the phragmoplast of onion root tip cells (Parke *et al.* 1986). However, more recent studies with antibodies against maize myosin XI (Liu *et al.* 2001) and *Arabidopsis* myosin VIII (Reichelt *et al.* 1999), while showing reactivity within cells, do not reveal an accumulation in the phragmoplast. Myosin VIII does stain the post-cytokinetic cell wall region, perhaps indicating an association with the newly formed plasmodesmata (Reichelt *et al.* 1999). Maize myosin XI antibodies instead stain small organelles or vesicles, but there is no accumulation in the phragmoplast or enhanced association with the cell plate (Liu *et al.* 2001). As with kinesins, there are no data demonstrating a role for myosin in cell plate vesicle transport, despite the presence of a favourable polarity of actin MFs.

To expand our understanding of motor proteins in cell plate formation we have taken advantage of some recently developed probes. In studies on kinesin, we have focused on KCBP, using an antibody that constitutively activates the motor. For myosin, we have used two inhibitors, 2,3-BDM and ML-7. Briefly, our results show that KCBP participates in the organization of MTs in the phragmoplast, while the myosin inhibitors provide evidence that this motor contributes importantly to the rotation and alignment of the phragmoplast to ensure proper alignment of the cell plate. More detailed accounts of these results have been published (Vos *et al.* 2000) or are currently in press (Molchan *et al.* 2002).

2. MATERIAL AND METHODS

The procedures used in this study have been previously published in detail (Vos *et al.* 1999, 2000). Briefly, dividing stamen hair cells from young buds of *Tradescantia virginiana* were isolated and mounted in low-temperature gelling agarose in microscope chambers for analysis with an inverted light microscope or by confocal microscopy. For studies on KCBP, cells at the appropriate stage of division were microinjected with affinity-purified KCBP antibody (KCBP-Ab) (needle concentration of 0.2–1.7 mg ml^{-1}). The effects were recorded using time-lapse imaging and compared with those injected with control buffer, which was prepared from pre-immune serum (Vos *et al.* 2000). The two myosin inhibitors, 2,3-BDM and ML-7, are permeant and thus were simply added to the culture medium at concentrations of 30 mM and 100 μM, respectively. Cells were examined by time-lapse cinemicroscopy. Progress of treated cells through division, in particular during cytokinesis, was compared with untreated controls.

To visualize the effect of KCBP-Abs on the MT arrays during mitosis, cells were first microinjected with rhodamine-labelled bovine tubulin (needle concentration of 0.25 mg ml^{-1}), followed by antibody injection and time-lapse observation by confocal microscopy (Vos *et al.* 2000). Effects of BDM and ML-7 on the MFs were observed by microinjection of fluorescein isothiocyanate (FITC)-phalloidin at selected moments during BDM or ML-7 treatment. The approach here uses the 'snap shot' strategy, which employs a reasonably high level of phalloidin to ensure that all the MFs are labelled (Valster & Hepler 1997). The injected cells were then immediately observed with the confocal microscope.

3. RESULTS AND DISCUSSION

(a) *KCBP-Ab activates KCBP*

KCBP is a 140 kDa, minus-end directed motor whose concentration increases in dividing cells (Bowser & Reddy 1997). Immunolocalization reveals that KCBP specifically associates with MTs of the spindle and phragmoplast (Bowser & Reddy 1997; Smirnova *et al.* 1998), further suggesting that it may function in the MT-dependent events of division. Somewhat unique among kinesins, KCBP is regulated by Ca^{2+} and calmodulin (Reddy *et al.* 1999). When Ca^{2+} is elevated and binds calmodulin, the latter binds to a unique 23 amino-acid domain on KCBP and acts as a negative regulator by inhibiting KCBP motor activity and its ability to bind MTs (Narasimhulu *et al.* 1997; Narasimhulu & Reddy 1998). However, an antibody against this same 23 amino-acid region blocks the ability of Ca^{2+}–calmodulin to regulate KCBP negatively. When treated with KCBP-Ab, KCBP retains its ability to bind and sediment MTs, and thus the antibody is thought to activate the motor protein constitutively (Narasimhulu & Reddy 1998). It is important to emphasize that KCBP-Ab is selective for KCBP and does not appear to alter the activity of other calmodulin-binding proteins. To support this contention, we note that this antibody was produced against the unique 23 amino-acid region and not against the whole protein. In addition, KCBP-Ab was affinity purified against the chemically synthesized peptide so that only those molecules that react with the peptide were used (Narasimhulu *et al.* 1997; Narasimhulu & Reddy 1998). Finally, KCBP-Ab only reacted with one band in Western blots, indicating a single target protein (Vos *et al.* 2000).

In the studies below, we injected KCBP-Ab at selected times during the cell cycle, thereby activating KCBP at these times (Vos *et al.* 2000). Through time-lapse cinemicroscopy, we followed the effect of constitutively active KCBP on the ensuing progress of division. As with inhibitors, the extent to which the events of division are modulated provides information about the processes that are regulated.

(b) *KCBP activation accelerates prometaphase onset but arrests metaphase*

While the primary focus of this report is on cytokinesis, it is important for comparative purposes to note how KCBP-Ab affects other stages of cell division. Quite surprisingly, the results show that cells injected in late prophase undergo dissolution of their nuclear envelope and enter into prometaphase at a rate accelerated by nearly a factor of three (Vos *et al.* 2000). Although late prophase cells could be hastened into prometaphase by activation of KCBP, these same cells became arrested in metaphase and many failed to enter anaphase. Similarly, cells injected in early prometaphase also became arrested or retarded, when compared with those injected with the control buffer (Vos *et al.* 2000). However, while prophase can be accelerated and metaphase retarded, if cells are injected during late metaphase or early anaphase there is no effect on the onset of anaphase or on anaphase chromosome motion (Vos *et al.* 2000).

(c) *KCBP activation blocks or retards cytokinesis*

The above observations make it clear that the process of spindle formation and the events of mitosis are exquisitely tuned to the level of activity of KCBP. How then do the processes of phragmoplast formation and cell plate initiation respond to injection of KCBP-Ab? Again, the results are quite dramatic. Injection of KCBP-Ab during mid-anaphase, under the most severe instances, completely abolishes the formation of the phragmoplast (figure 1). More commonly, cytokinesis is delayed and those cell plates that do arise are often wavy or misaligned (Vos *et al.* 2000). Cells injected at similar times in mid-anaphase with control buffer showed no effects on the normal control processes or transitional times.

To see whether there was a direct effect of KCBP activation on MTs, cells in anaphase were first injected with rhodamine-conjugated bovine tubulin. When this fluorescent analogue had incorporated into the endogenous MT arrays, the cell was then injected with KCBP-Ab. No particular effect on the structure of spindle MTs was observed, although misaligned phragmoplasts and lagging, incomplete cell plates were evident (Vos *et al.* 2000).

When taken together, these results indicate that KCBP is possibly downregulated during cytokinesis, since its activation with KCBP-Ab causes marked abnormalities and deviations from the control process. Since KCBP is negatively regulated by Ca^{2+} and calmodulin, it is attractive to suggest that during the normal course of cytokinesis the Ca^{2+} concentration elevates, thereby activating calmodulin, which then binds to and inactivates KCBP. It is possible that the Ca^{2+} elevation would be temporally controlled at the anaphase/telophase transition and spatially restricted through release from the endoplasmic reticulum system near the developing cell plate. The ability of 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetra-acetic acid (BAPTA) buffers, which dampen elevations in Ca^{2+}, to delay and/or inhibit cell plate formation (Jürgens *et al.* 1994) in much the same way as does the activation of KCBP, provides indirect support for this scheme.

(d) *Myosin inhibitors block streaming but not chromosome motion*

BDM and ML-7 are inhibitors of myosin; BDM is a general uncompetitive inhibitor of myosin ATPase (Herrmann *et al.* 1992), while ML-7 blocks activity of myosin light chain kinase, and thus is more specific for non-muscle myosin II-like motors (Saitoh *et al.* 1987). While BDM has been used more extensively in animal systems, there is evidence that it specifically blocks myosin activity in plants. For example, BDM inhibits cytoplasmic streaming *in vivo* and specifically blocks the sliding of actin MFs by plant myosin *in vitro* (Tominaga *et al.* 2000). It also inhibits the movement of plant Golgi stacks (Nebenführ *et al.* 1999), the constriction of the neck region of plasmodesmata (Radford & White 1998) and alters the distribution of actin MFs and myosins in corn root tips (Šamaj *et al.* 2000). The lack of a myosin II, the normal target for ML-7, in the *Arabidopsis* genome database raises questions about which molecules are being blocked by this reagent. However, we note that the plant myosin class VIII bears considerable similarity to myosin II and thus could be the target for ML-7. Moreover, myosin VIII is localized to the newly formed cross-walls in maize root cells (Reichelt *et al.* 1999; Reichelt & Kendrick-Jones 2000), suggesting that it might participate in some aspect of cell division.

Regardless of the specific molecular mechanism and of the target molecules, it is important to emphasize that, under the experimental conditions used, both BDM and ML-7 blocked cytoplasmic streaming but not chromosome motion. The former, as noted above, is a well-known actomyosin-dependent process, while the latter is widely thought to be independent of actomyosin. These inhibitors are thus not simply blocking cell viability. It is additionally pertinent with BDM that the architecture of MFs, as revealed by injection with FITC-phalloidin, appeared normal. By contrast, ML-7 did cause a fragmentation of the MFs normally present in the transvacuolar strands (Molchan *et al.* 2002).

(e) *Myosin inhibition blocks late lateral expansion of the cell plate*

To appreciate the effect of BDM and ML-7 on cell plate formation it is first important to note that the normal events during cytokinesis can be experimentally divided into two distinct phases (Valster & Hepler 1997). During the first phase, the inter-zone MTs and MFs coalesce into a dense cylinder, the phragmoplast initial, which then expands laterally across *ca.* 70% of the cell width. During the second phase, the central phragmoplast MTs and MFs degrade and presumably new ones polymerize at the edge of the plate creating a torus that expands and makes contact with the parental cell wall (Valster & Hepler 1997). The breakpoint between the first and second phases seems to be related to the diameter of the reforming daughter nuclei. Thus, during the first phase the plate progresses to a width that corresponds to the diameter of the nuclei, while during the second phase the plate expands by a distance that corresponds to the interval between the daughter nuclei and the width of the cell. From the standpoint of the phragmoplast structure, the first phase appears to correlate with a time when the cytoskeletal elements are under the influence of the perinuclear region, while the second phase occurs when these same elements detach from the perinuclear region and establish contact with the cell cortex.

When cells are treated with BDM under conditions that

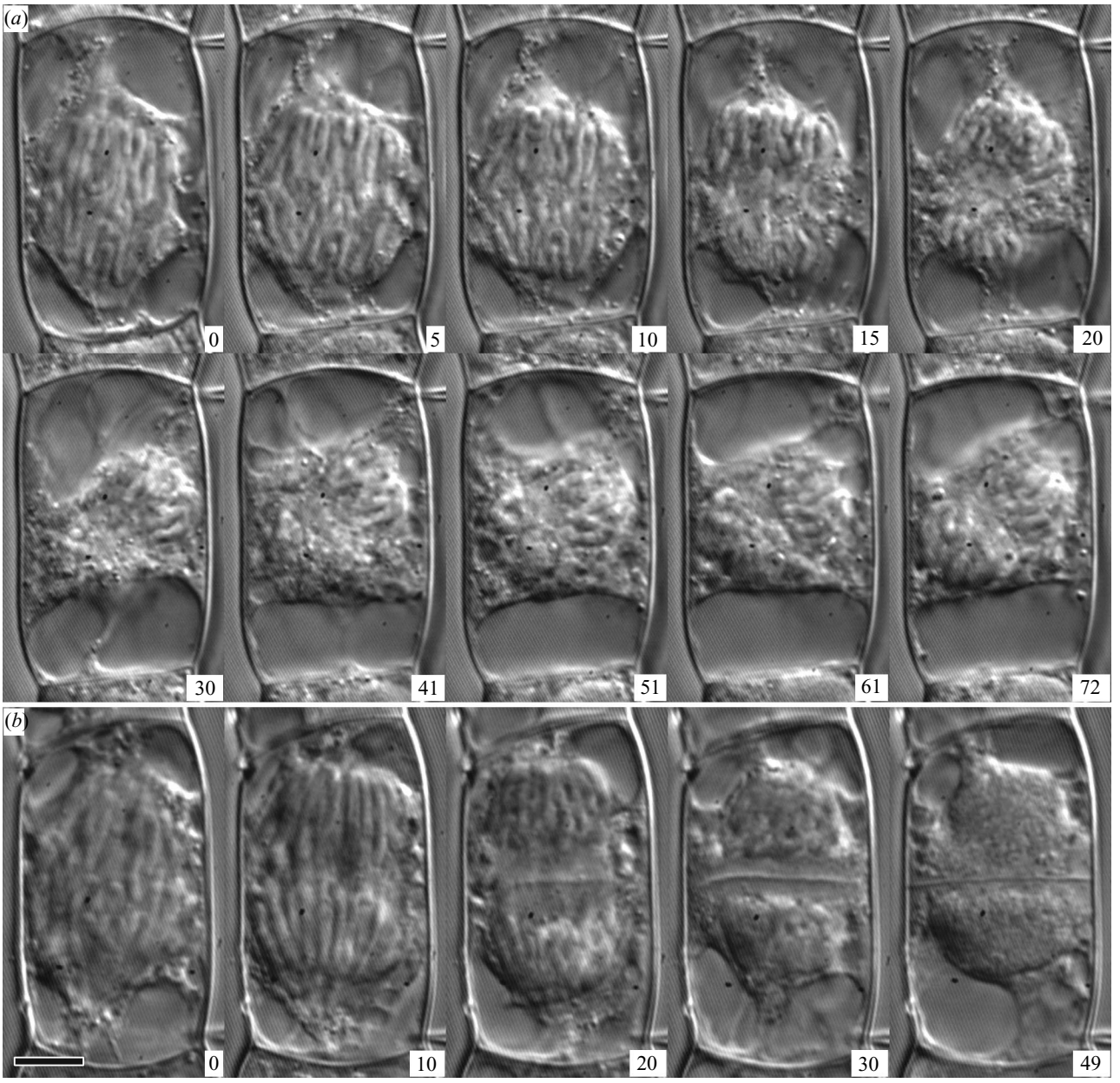

Figure 1. (*a*) Injection of KCBP-Ab during anaphase. Activation of KCBP with the antibody markedly disturbs phragmoplast and cell plate formation. Notice the lack of a clear zone between the separating sets of chromosomes at late anaphase and early telophase (15 and 20 min after injection). As a consequence, the reforming nuclei move closely together (51–72 min after injection) and no cell plate is formed. (*b*) Injection of an anaphase cell with control buffer. This cell progressed normally through cytokinesis; it formed a cell plate initial at 20 min after injection and completed the cell plate at 49 min after injection. Bar, 10 μm. (From Vos *et al.* 2000.)

block streaming, those in division complete the first phase of cell plate formation. However, the second phase is markedly affected, leading to a delay or even inhibition in cell plate fusion with the parental cell wall. Also evident are several morphological abnormalities, in which the plates appear tilted, wavy or fragmented. Many affected cell plates show two or three of these abnormalities, with none being normal. Examination of the phragmoplast cytoskeleton using FITC-phalloidin for MFs and FITC-tubulin for MTs, reveals that in the presence of BDM the expanding phragmoplast fails to break its contact with the daughter nuclei. Thus, both MTs and MFs become orientated at angles of up to 45° to the plane of the cell plate rather than being perpendicular, as they are in controls (Molchan *et al.* 2002).

ML-7 also delays late lateral expansion of the cell plate and can lead to situations where fusion with the parental wall will occur on one side but not the other. In this way, incomplete cell plates arise, as with BDM. However, in contrast to BDM, ML-7 does not appear to cause the formation of tilted and wavy plates. Also, despite the delay in cell plate completion, the structure and organization of the MFs in the phragmoplast do not deviate appreciably from that seen in controls.

The ability of BDM and ML-7 to perturb various aspects of cell plate formation further supports the contention that the actomyosin system is an important component of the cytokinetic apparatus in plants. Although organized rather differently than it is in animal cells, it nevertheless plays an important and, to a large extent,

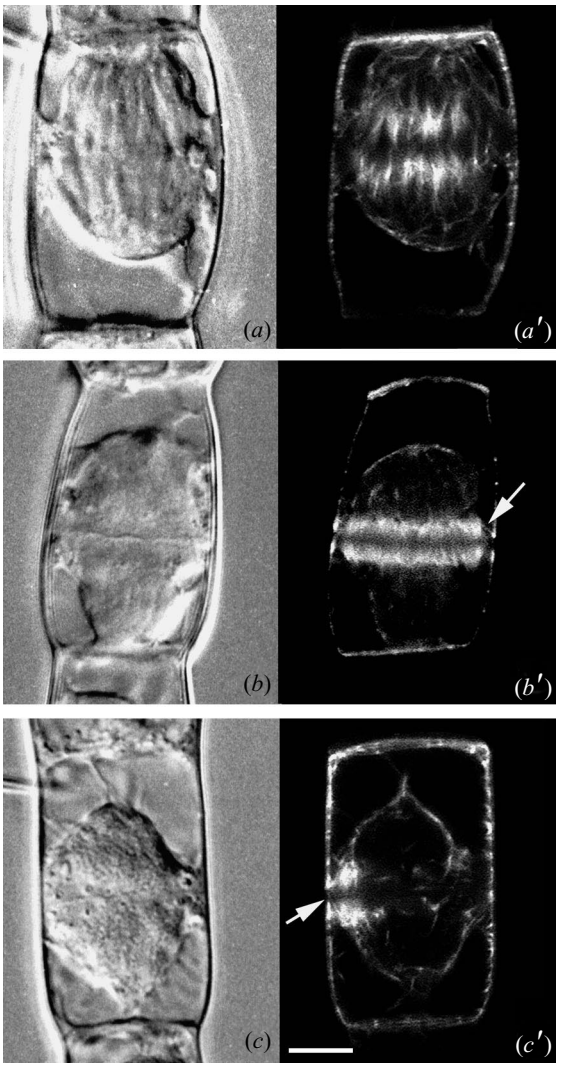

Figure 2. Distribution of actin microfilaments in living cells during normal cell plate formation. The actin is visualized by confocal microscopy of cells that have been microinjected with FITC-phalloidin. A Nomarski differential interference contrast image of each cell provides a comparative image of its general morphology. Three different cells were injected in progressive phases of cytokinesis. (*a*,*a*′) Early initiation phase. Actin microfilaments are abundant in the phragmoplast initial. (*b*,*b*′) Transition between early initiation and late lateral expansion of the cell plate. Actin microfilaments can be observed in the phragmoplast and they also connect the phragmoplast with the cell cortex (see arrow). (*c*,*c*′) Late lateral expansion. One side of the cell plate has fused with the parental cell wall, while the other side is somewhat delayed. Actin microfilaments are associated with the side that has not yet fused, where connections between the phragmoplast and the cortex can be observed (see arrow). Bar, 10 μm. (From Valster & Hepler 1997.)

unappreciated role. Since cell plates do arise, myosin does not appear to be the motor that transports vesicles along phragmoplast MFs inward to the cell plate despite the favourable polarity and the realization that one of the common plant myosins, namely XI, is a strong candidate for the movement of small organelles or vesicles (Liu *et al.* 2001). However, myosins do appear to play a key role in generating the forces responsible for late lateral expansion of the phragmoplast. Initially, the phragmoplast MTs and MFs arise in the spindle mid-zone and establish an association with the proximal perinuclear zone. Since this early phase is not affected by BDM or ML-7, the cell plate initiates under circumstances that may not require the activity of myosin. Nevertheless, to complete cell plate formation there must be a transition in which the phragmoplast cytoskeleton changes its primary association from the perinuclear region to the cell cortex. It is in this step that myosin appears to play a central role. Possibly under the control of myosin, the MF bridges, observed between the edge of the phragmoplast and the cell cortex (figure 2), are able to form and exert tension, which overcomes the earlier connection between the MFs and the perinuclear region.

Given the unique organization of the cortical actin cytoskeleton, namely the presence of numerous elements on either side of an actin-depleted zone that marks the fusion site of the cell plate, together with the bipolar organization of the phragmoplast MFs, conditions may be established that cause the phragmoplast to rotate and align, with a null point in force generation centring around the actin-depleted zone. Actomyosin thus may be the element most responsible for the precise alignment of the cell plate, an idea that was suggested years ago when it became apparent that cytochalasin blocked proper positioning of the cell plate in guard mother cells of onion (Palevitz & Hepler 1974).

In conclusion, these studies, aimed at deciphering the activity of one kinesin (KCBP) and myosins in general, provide positive support for the role of these cytoskeletal motors during cytokinesis. A low activity of KCBP may be important to allow for MT unbundling and turnover during phragmoplast expansion. Actomyosin, on the other hand, appears to have a direct role in controlling the late lateral expansion of the plate and its fusion with the parental cell wall, perhaps by cross-linking the MFs in the edge of the phragmoplast with those in the cell cortex. In this regard, MFs appear to be more intimately involved with the positioning of the cell plate than MTs. While the motor for cell plate vesicle transport has not been identified thus far, it seems most likely that one of the several plus-end directed kinesins (Reddy 2001) will support this activity.

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