Cytokinesis mediated through the recruitment of cortexillins into the cleavage furrow

Igor Weber, Günther Gerisch¹,
Christina Heizer, John Murphy, Kim Badelt,
Alexander Stock, Jean-Marc Schwartz and
Jan Faix

Max-Planck-Institut für Biochemie, Am Klopferspitz 18a,
D-82152 Martinsried, Germany

¹Corresponding author
e-mail: gerisch@biochem.mpg.de

The fact that substrate-anchored *Dictyostelium* cells undergo cytokinesis in the absence of myosin II underscores the importance of other proteins in enabling the cleavage furrow to constrict. Cortexillins, a pair of actin-bundling proteins, are required for normal cleavage. They are targeted to the incipient furrow in wild-type and, more prominently, in myosin II-null cells. No other F-actin bundling or cross-linking protein tested is co-localized. Green fluorescent protein fusions show that the N-terminal actin-binding domain of cortexillin I is dispensable and the C-terminal region is sufficient for translocation to the furrow and the rescue of cytokinesis. Cortexillins are suggested to have a targeting signal for coupling to a myosin II-independent system that directs transport of membrane proteins to the cleavage furrow.

Keywords: cleavage furrow/cortexillin/cytokinesis/
Dictyostelium/protein targeting

Introduction

Attempts to attribute specific physical properties to the cleavage furrow of a dividing cell trace back to early measurements on sea urchin blastomeres, where Wolpert (1966) found an increased stiffness of the cell cortex in the furrow relative to the polar regions. In recent experiments, the forces associated with constriction of the furrow have been analysed by determining the traction that an adherent fibroblast applies on a compliant silicon rubber surface (Burton and Taylor, 1997). Atomic force microscopy has been used to track local changes in the viscoelastic properties of bovine embryo skin and muscle cells in mitosis (Dvorak and Nagao, 1998). A decrease in stiffness in the midzone of these cells during anaphase is followed by an increase in telophase, coincident with cleavage. Although a marked difference in physical properties between the cleavage furrow and polar regions has become a tenet in theories of cytokinesis (White and Borisy, 1983; He and Dembo, 1997), molecules that form a pattern in the cell cortex responsible for this difference remain to be specified (for a review on cytokinesis, see Glotzer, 1997).

Cytokinesis is under intricate control by small GTPases

and is mediated by a variety of effector proteins. In HeLa cells, citron kinase and its substrate rho are localized to the cleavage furrow (Madaule *et al.*, 1998). In *Dictyostelium*, deficiencies in cytokinesis have been observed in mutants that lack racE (Laroche *et al.*, 1996; Gerald *et al.*, 1998) or rasG (Tuxworth *et al.*, 1997), or overexpress an IQGAP homologue specifically interacting with the GTP-bound form of rac1 (Faix and Dittrich, 1996; Faix *et al.*, 1998). As to the cytoskeletal target proteins, previous findings indicated a primary role for myosin II in interacting with membrane-associated actin filaments to form a ring that separates the daughter cells by contraction (White, 1990; Satterwhite and Pollard, 1992; Fishkind and Wang, 1995). Although myosin II undoubtedly plays an important role in mitotic cleavage, its singular function has been questioned by the finding that cells of *Dictyostelium discoideum* undergo cytokinesis in the absence of myosin II when they are attached to an appropriate substrate (Neujahr *et al.*, 1997a; Zang *et al.*, 1997). This finding has provoked a search for proteins responsible for the formation of a cleavage furrow in the absence of myosin II. Here we explore the role of cortexillins, a pair of actin-bundling proteins that accumulate in the cortex of *Dictyostelium* cells (Faix *et al.*, 1996).

The N-terminal halves of the cortexillin polypeptides comprise a conserved actin-binding domain that identifies cortexillins as members of the α -actinin/spectrin superfamily. The cortexillins are distinguished from other members of this superfamily by a coiled-coil domain responsible for parallel dimer formation (Steinmetz *et al.*, 1998). Cortexillin isoforms I and II differ in their C-terminal domains, which contain putative phosphatidylinositol bisphosphate (PIP₂)-binding motifs and other regulatory sites. An important role for cortexillins in cytokinesis has been indicated by mutants that lack both isoforms of cortexillins. Cells of these mutants tend to greatly increase in size and to become multinucleate when cultivated in nutrient medium (Faix *et al.*, 1996). The elimination of cortexillin I alone causes a less dramatic increase in the average number of nuclei per cell, and the elimination of cortexillin II an even lower increase, indicating that the two isoforms can largely replace each other (Faix *et al.*, 1996). Here we show that formation of a cleavage furrow is tightly linked to the targeting of cortexillins into the furrow region, and that the C-terminal portion plays a primary role in the targeting and function of cortexillins.

Results

Precise cleavage furrow formation in *Dictyostelium* requires cortexillins

Wild-type cells of *D. discoideum* undergo a characteristic sequence of shape changes during cytokinesis: they round

Fig. 1. Wild-type and cortaxillin-deficient cells of *D. discoideum* in cytokinesis. (A–D) Stages of cytokinesis in wild-type (A), cortaxillin I-null (B) and cortaxillin I/II double-null cells (C and D). (A) In normal cytokinesis, the cleavage furrow is located in the equatorial region of the cell and demarcated by its smooth surface from the ruffling polar regions. (B) In this cell, which is representative of the majority of cortaxillin I-null mutants, the cleavage furrow is less sharply defined than in wild-type, but division proceeds to completion. (C and D) In cortaxillin I/II double-mutant cells, cytokinesis is impaired to various degrees. The cell in (C) finally succeeded to divide, whereas the cell in (D) failed. Time is indicated in seconds after the first frame of each series. Bars, 10 μ m. (E) Lengths of time required for cytokinesis in wild-type and cortaxillin mutants; time required from elongation of the cells up to almost complete separation of the daughter cells. Differences between wild-type cells ($n = 17$), cortaxillin I-null cells ($n = 12$) and cortaxillin double-null cells ($n = 23$) were significant ($p < 10^{-5}$, Mann–Whitney U test). In the double-null mutant, 10 out of 33 cells did not complete cytokinesis, and are therefore not represented in the histogram.

up, elongate in the direction of the spindle, and are cleaved in a symmetrical fashion by ingression of a furrow at the equatorial region of the cell (Figure 1A). The cleavage furrow with its smooth surface is sharply demarcated from the ruffling polar regions.

In cortaxillin I-null cells, and more dramatically in double-null cells, this series of shape changes was disarranged. The majority of cortaxillin I-null cells did not fully round up during mitosis; they formed numerous protrusions at their surface, including the furrow region. The appearance of cleavage furrows was therefore indistinct and their progression often asymmetrical (Figure 1B, 180 s frame). In double-mutant cells, the mildest defects observed resembled those in cortaxillin I-null cells. In exceptional cases, cleavage furrows were still initiated but tended to regress afterwards. The majority of double-mutant cells, lacking both cortaxillins I and II, exhibited severe irregularities. The examples shown in Figure 1C and D represent cortaxillin double-mutant cells that divided late or not at all. The cell in Figure 1C eventually succeeded by stretching out the connecting strand between a larger and smaller portion of the cell body until this cytoplasmic bridge broke. In the cell of Figure 1D, a primordial cleavage furrow is seen at the 240 s stage, which subsequently became indistinct when the cell was subdivided into three interconnected portions (590 s). Finally, these segments re-united to form one compact, binucleate body (740 s and later stages not shown).

For the experiments illustrated in Figure 1, we cultivated the mutant cells with bacteria as nutrients, i.e. under conditions where mitotic division was less inhibited than in cultures with liquid nutrient medium. Under these optimal conditions, all cortaxillin I-null cells and ~70% of the cortaxillin I/II double-null cells tested finally completed cytokinesis. However, the cortaxillin I-deficient cells needed, on average, twice as long as wild-type cells. The cortaxillin I/II double-mutant cells that succeeded to divide completed cytokinesis after much more variable periods of time (Figure 1E).

Different patterns of cortaxillins, F-actin and the actin-bundling protein p34 in cytokinesis

The irregularities in cleavage of cortaxillin I/II double-null mutant cells suggest a direct involvement of cortaxillins in shaping the furrow. To determine whether localization of cortaxillins distinguishes the furrow from the polar regions of a dividing cell, mitotic cells were immunolabelled and the three-dimensional distribution of proteins reconstructed from confocal image series. Since cortaxillins initially have been characterized as actin filament-bundling proteins (Faix *et al.*, 1996), their distribution was compared with the distributions of F-actin and p34, a potent actin-bundling protein that shows no sequence relationship to cortaxillins or any other member of the α -actinin/spectrin superfamily (Fechheimer *et al.*, 1991).

In interphase and mitotic cells, cortaxillins were distributed in the cytoplasm and enriched in a cortical layer, similarly to F-actin and p34. In mitotic cells, however, the patterns were different. In accord with previous findings (Neujahr *et al.*, 1997b), F-actin was much more enriched in the protrusions emanating from the poles than it was in the cleavage furrow (Figure 2A and B). p34 showed a similar localization, suggesting that the distribution of this protein in the cell cortex is determined by its affinity for F-actin (Figure 2C and D). In sharp contrast, cortaxillins were more strongly enriched in the cleavage furrow than in the polar regions (Figure 2E and F). Equatorial cross-sections through the furrow revealed a closed cortical ring

Fig. 2. Localization of F-actin, the actin-bundling protein p34 and cortexillin I in dividing wild-type and myosin II-null cells. Three-dimensional images were constructed from fluorescence intensities recorded by confocal laser-scanning microscopy. Sagittal sections through the three-dimensional reconstructions are shown in the left panels, equatorial cross-sections in the right panels. (A) F-actin labelled with fluorescent phalloidin in a wild-type cell and (B) the same in a myosin II-null cell. (C) Actin-bundling protein p34 in a wild-type cell and (D) in a myosin II-null cell. (E) Cortexillin I in a wild-type cell and (F) in a myosin II-null cell. The colour scales represent, from blue to red, linear increases in fluorescence intensity within the limits indicated. The mAb 241-36-2 used is specific for cortexillin I, but similar results were obtained with mAb 241-71-3 that cross-reacts with cortexillin II. This coincidence in the labelling pattern is in accord with the formation of heterodimers *in vivo*.

of accumulated cortexillins. To explore the possibility that transport to the cleavage furrow is common to proteins that harbour an α -actinin/spectrin type of actin-binding domains, we have immunolocalized in mitotic cells three other actin-bundling or cross-linking proteins that share this type of domain with cortexillins. These proteins, *Dictyostelium* fimbrin/plastin, α -actinin and 120 kDa gelation factor (ABP120), were not distinctly enriched in the equatorial zone of dividing cells (data not shown), arguing that it is not the common type of actin-binding domain which directs cortexillins towards the cleavage furrow.

Cortexillin accumulation is related to furrow formation in uni- and multinucleate myosin II-null cells

As judged from the comparison of Figure 2E and F, the enrichment of cortexillins in the furrow region was even more pronounced in myosin II-null cells than in wild-type cells. Quantitative comparison of uninucleate myosin II-null and wild-type cells confirmed this effect (Figure 3), indicating a role for myosin II in modulating the redistribution of cortexillins in dividing cells.

Because of the strong enrichment of cortexillins in the cleavage furrow of myosin II-null cells, green fluorescent protein (GFP)- α -tubulin was expressed in these cells in order to relate cortexillin redistribution to stages of mitosis and cytokinesis. After fixation, the cells were labelled with 4',6-diamidino-2-phenylindole (DAPI) for DNA and with an antibody specific for cortexillin I. The mitotic stages of Figure 4A–G show that from interphase to metaphase, the cortexillin remains almost uniformly accumulated in the cell cortex (Figure 4A and B). Before cleavage commences, cortexillin I becomes accumulated in the furrow region (Figure 4C) and remains enriched there until cleavage is nearly completed (Figure 4D and E). At the final stage of cytokinesis, cortexillin returns to

Fig. 3. Quantification of the increased accumulation of cortexillin I in the cleavage furrow of myosin II-null cells as compared with wild-type cells. (A) As illustrated on top of the curves, fluorescence intensity profiles were determined in sagittal sections through the cleavage furrow (upper panels) and through polar regions of the cells (lower panels). These panels represent examples of single scans; the fluorescence intensities are in arbitrary units. (B) Ratios of cortical fluorescence intensities in the furrow over intensities in polar regions of the cells. The data represent measurements on 13 myosin II-null cells and the same number of wild-type cells labelled with mAb 241-36-2. In the absence of myosin II, the distribution is broader but non-overlapping with wild-type values. The vertical bars indicate the means of the two data sets.

Fig. 4. Translocation of cortexillin I linked to mitosis in uni- and multinucleate myosin II-null cells. Uninnucleate (A–G) and multinucleate (H–K) myosin II-null cells producing GFP- α -tubulin (green) were immunolabelled for cortexillin I (red) and stained for DNA with DAPI (blue). The cortexillin I distribution is shown in confocal sections. (A–G) In interphase (A) and metaphase (B), the cortexillin is distributed uniformly in the cell cortex. In late telophase, immediately before the onset of cleavage, cortexillin I becomes accumulated distinctly in the cleavage furrow (C), and this accumulation is retained up to late stages of cytokinesis (D and E). Before separation of the daughter cells is completed, the interphase state of cortexillin distribution is reached, whereby the two daughter cells are equally (F) or unequally (G) supplied with cortexillin. (H–K) Multinucleate myosin II-null cells grown up in suspension and then transferred to a glass surface. The stages of cytokinesis correspond to the stage of the uninnucleate cell shown in (D). In multinucleate cells, cortexillin I accumulates between the microtubule asters wherever bilateral (H), tripartite (I) or multifold (K) cleavage furrows are formed. Bar, 10 μ m.

the interphase state of a more or less uniform distribution in both daughter cells (Figure 4F). Exceptionally, stages have been observed in which cortexillin is strongly enriched in a zone derived from the cleavage furrow of one cell and depleted from the other cell (Figure 4G). This asymmetric distribution is probably related to the unequal cleavage previously found in a fraction of myosin II-null cells (Neujahr *et al.*, 1997a). Together, these data indicate that a myosin II-independent translocation system, which targets cortexillins from the cell poles to the incipient furrow, comes into play between the metaphase and telophase stages.

To establish that the direction of cortexillin gradients is determined by the sites of docking of microtubule asters to the cell cortex, we have examined the distribution of cortexillin I in cleavage stages of multinucleate myosin II-null cells. These cells are produced abundantly in suspension culture (DeLozanne and Spudich, 1987; Knecht and Loomis, 1987). When transferred to a glass surface, synchronous division of the nuclei is accompanied by differentiation of the cell cortex into large cleavage furrows and into ruffling areas, which correspond to the poles of a normal cell. The ruffling areas are formed on top of the microtubule asters which emanate from the centrosomes. Furrows are induced at spaces that separate the docking sites of the asters (Neujahr *et al.*, 1998). Figure 4H–K shows that the pattern of cortexillin I accumulation in multinucleate cells coincides with the pattern of cleavage

furrows that are formed between the docking sites of the asters at the cell cortex.

Cross-linkage with concanavalin A reveals myosin II-independent translocation of membrane proteins to the cleavage furrow

One way of directing cortexillins to the cleavage furrow would be to recruit these proteins from the cytoplasmic pool. Alternatively, cortexillins might be translocated from the poles to the midzone along the plasma membrane or within the actin-rich cortical layer of a dividing cell. To provide support for a system that shuffles membrane-associated proteins from the poles to the equatorial region, we have treated dividing wild-type or myosin II-null cells with concanavalin A (ConA). Cross-linkage by ConA is known to couple cell surface proteins to a system that transports them to the tail of a cell in interphase, where they form a cap. In *Dictyostelium* cells, this system has a myosin II-independent component (Aguado-Velasco and Bretscher, 1997).

No gross redistribution of cell surface glycoproteins was observed at metaphase after the incubation of wild-type or myosin II-null cells for 10 min with ConA. Since wild-type and myosin II-null cells behaved in a similar way, we show only the latter in Figure 5. Beginning with anaphase, the cross-linked proteins were translocated within a few minutes to the midzone of the cell, leaving sharply demarcated areas around the microtubule asters

Fig. 5. Myosin II-independent translocation to the cleavage furrow of membrane proteins cross-linked by ConA. (A–C) Myosin II-null cells that express GFP- α -tubulin were incubated with ConA for 10 min to show that translocation of cross-linked proteins depends on the mitotic stage. The fluorescence of TRITC-labelled ConA is shown in red in one confocal section per cell, and is superimposed to fluorescence images of GFP- α -tubulin in green. The GFP fusion protein visualizes the mitotic apparatus (A) at metaphase, (B) at telophase and (C) at a post-telophase stage after dispersal of the spindle. Only at the stages shown in (B) and (C) did the cell poles become depleted of ConA-labelled proteins. (D–F) Advanced stages of cytokinesis in cells incubated with ConA for 1 min (D), 5 min (E) or 10 min (F) show the time dependence of the translocation of membrane proteins. Two confocal sections through each cell showing ConA distribution are superimposed on each other. A section close to the glass surface, to which the cell was attached, is pseudo-coloured in blue, a section through a plane at 1–2 μ m above the substrate in red. ConA glued the cells to the substrate and left the blue-coloured remnants there while the cleavage furrow contracted. Comparison of the blue and red images in (F) reveals that the furrow in this myosin II-null cell contracted during the 10 min of incubation with ConA without significant changes in the pole positions. Bar, 10 μ m.

at each pole of the cell that were depleted of ConA-bound proteins (Figure 5A–C). When wild-type or myosin II-null cells were incubated with ConA, translocation to the furrow and clearance of the polar regions took no longer than 5 min (Figure 5D–F). The fact that cells succeeded in forming a cleavage furrow in the presence of ConA without the help of myosin II demonstrates the existence of a myosin II-independent system that is capable of translocating membrane proteins from the cell poles to the midzone before and during the formation of a cleavage furrow. We propose that one of the intrinsic functions of this process is to move, at the beginning of cytokinesis, cortexillins and probably other proteins bound to the cytoplasmic phase of the plasma membrane towards the cleavage furrow.

The C-terminal region of cortexillin I is sufficient for targeting to the furrow and for the rescue of cytokinesis

In order to identify the region responsible for the targeting of cortexillin I, N- or C-terminal fragments of this protein were fused to GFP. These fragments did not overlap with the long coiled-coil region in the centre of the molecule, which is responsible for dimerization (Steinmetz *et al.*, 1998). Cortexillin I-null cells producing the GFP fusion proteins were subjected to confocal fluorescence imaging in parallel with cells producing the full-length cortexillin I fusion protein, which served as a reference (Figure 6). The full-length fusion protein was translocated to the cleavage furrow as the endogenous cortexillin I in wild-type cells (Figure 6B). The N-terminal actin-binding domain was located predominantly in the cytoplasm, with a weak enrichment in the polar protrusions known to be enriched in F-actin (Figure 6C). Cells producing the N-terminal fragment exhibited the same type of impaired cytokinesis as cortexillin I-null cells in the absence of this fragment: the cleavage furrow was indistinct due to the formation of pseudopods over the entire cell surface.

In contrast to the N-terminal domain, the C-terminal region associated most strongly with the cell cortex and localized to the cleavage furrow. Less fluorescence was observed in the cytoplasm than with the full-length construct (Figure 6D). No association with the membranes of intracellular vesicles was observed, indicating that the C-terminal region contains recognition sites to couple cortexillin I specifically to the plasma membrane or the cell cortex.

The C-terminal fragment that accumulated in the cleavage furrow also rescued the capability of the cells to undergo cytokinesis up to a level indistinguishable from wild-type (Figure 7A). No rescue was observed with the N-terminal domain (Figure 7B), although this domain was expressed more strongly than the C-terminal one (Figure 7C). These results indicate that the sequence motifs crucial for cortexillin localization and activity reside in the C-terminal region rather than in the N-terminal actin-binding domain.

Discussion

The role of cortexillins in cytokinesis is consistent with their activities in vivo and in vitro

In wild-type cells of *D.discoideum*, the cleavage furrow is sharply separated by its smooth, concave surface area from the ruffling polar regions. The elimination of both cortexillin isoforms causes highly variable irregularities. The furrow is covered with protrusions and is undefined in shape, and often the cells fail to divide after no recognizable furrow has been made. The importance of cortexillins for cytokinesis is corroborated by the accumulation of these proteins in the incipient cleavage furrow.

The influence of cortexillins on the physical properties of the cell cortex has been determined in interphase cells. Cortexillin I/II-null mutants show a marked decrease in cortical tension (Simson *et al.*, 1998). According to these

Fig. 6. Targeting of cortexillin I fragments to the furrow. (A) GFP-tagged full-length cortexillin-I, N-terminal actin-binding domain (N1–233) and C-terminal region (C352–444) were expressed in cortexillin I-null cells. (B) The full-length construct localized to the furrow in accord with the antibody-labelling pattern in the wild-type cells shown in Figure 2E. (C) The N-terminal domain showed no accumulation in the cleavage furrow; it was dispersed within the cytoplasm and slightly enriched in the polar protrusions that are also rich in F-actin (Figure 2A). (D) The C-terminal region was bound excessively to the plasma membrane and targeted efficiently to the furrow. Bar, 10 μ m.

results, the equatorial accumulation of cortexillins is supposed to increase cortical stiffness in the furrow region relative to the poles of a dividing cell, in accord with theoretical considerations (Harris, 1990; He and Dembo, 1997) and experimental data on other cells (Hiramoto, 1990; Rappaport, 1996; Dvorak and Nagao, 1998). Diminished cortical tension in cortexillin I/II-null cells might explain why during mitosis of these cells the furrow region can be stretched extensively in an axial direction by traction produced at the poles of the cells (Figure 1C and D).

In vitro, recombinant cortexillin I bundles actin filaments preferentially in an anti-parallel manner (Faix

Fig. 7. Expression of GFP constructs in cortexillin I-null cells and rescue of cytokinesis. (A and B) Histograms illustrating the proportion of uni- and multinucleate cells in wild-type AX2, cortexillin I-null mutant, and in this mutant transfected with the GFP–cortexillin I fusions outlined in Figure 6. A decrease in the portion of multinucleate cells indicates rescue of cytokinesis by the C-terminal region (A) and inefficiency of the N-terminal domain (B). (C) Blots of total cellular proteins of wild-type and cortexillin I mutant strains, labelled with mAb 241-36-2, which recognizes specifically the N-terminal domain of cortexillin I (left panel), or with anti-GFP mAb 264-449-2 (right panel). Lysates of 2×10^5 cells were loaded per lane, and the primary antibodies recognized by [125 I]sheep anti-mouse IgG. Numbers below the blots indicate radioactivity in the antibody-labelled bands relative to endogenous cortexillin I in wild-type cells, which was set to 100%. The GFP fusion of the full-length cortexillin I was, on average, slightly less abundant than the endogenous cortexillin I in wild-type cells, the GFP fusion of the N-terminal domain was more abundant and that of the C-terminal domain less abundant. These data show that the rescue of cytokinesis by the C-terminal domain is not a matter of overexpression.

et al., 1996), in contrast to a parallel bundling protein such as fimbrin that associates with the core of actin filaments in microvilli (Glenney *et al.*, 1981). Anti-parallel connection of actin filaments in the cleavage furrow has been attributed to bipolar filaments of myosin II (Satterwhite and Pollard, 1992). The peculiar role of cortexillins in cytokinesis may be related to the fact that they arrange the actin filaments in a similar way to myosin II.

Myosin II modifies the translocation of cortexillins in cytokinesis

In wild-type cells, a smooth bilateral cortexillin gradient develops, with the highest cortexillin concentration in the mid-region of the cell and a continuous decline towards each pole (Figure 2E). The machinery for transport of cortexillins to the cleavage furrow is established at anaphase and acts independently of myosin II, indicating that the force for translocation is provided by molecular interactions other than those exerted by the filamentous myosin on actin.

However, in cells lacking myosin II, cortexillins accumulate in the form of a more compact cylinder (Figure 2F), indicating that the motor protein has a modulating effect on the accumulation of cortexillins within the furrow (Figure 3). This accumulation is not related to the variability of cell size and of the time required for cytokinesis previously observed in myosin II-null cells (Neujahr *et al.*, 1997a). As shown in Figure 4C, cortexillin accumulation in the midzone of the cells precedes the formation of a cleavage furrow. The translocation of cortexillins might be slowed down by virtue of their N-terminal actin-binding domains. Cross-linkages of actin filaments produced by the binding of bipolar myosin filaments could act as barriers against the movement of actin-bound cortexillin molecules, thus giving rise to a more graded increase in cortexillin concentration from the poles to the midline of a dividing cell in the presence of myosin II (Figures 2E–F and 3).

Translocation to the cleavage furrow is not common to actin-binding proteins

Redistribution of cortexillins during cytokinesis does not mirror the distribution of their binding partner F-actin, nor is the localization of cortexillins typical of actin-bundling proteins. Under our conditions, F-actin forms a shallow layer beneath the surface of the furrow region, which contrasts markedly with its strong enrichment in protrusions at the poles of a dividing cell (Neujahr *et al.*, 1997a,b). From the fact that p34, a potent actin-bundling protein, largely matches the distribution of F-actin (Figure 2C and D), we conclude that the actin-bundling activity, as such, does not drive a protein towards the cleavage furrow. It is consistent with this view that the bundling activity of p34 does not imply a role for the protein in cytokinesis (Rivero *et al.*, 1996a).

In line with these data, the actin-binding domain of the α -actinin/spectrin type, which is located in the N-terminal half of cortexillins, does not specify the particular function of cortexillins in cytokinesis. Three *Dictyostelium* proteins carrying similar actin-binding domains, fimbrin/plastin, α -actinin and ABP120, do not co-localize with cortexillins in the furrow. Moreover, no substantial impairment of

cytokinesis has been reported upon elimination of these proteins by gene disruption (Witke *et al.*, 1992; Prassler, 1995; Rivero *et al.*, 1996b).

Targeting to the furrow involves recognition sites in the C-terminal region of cortexillin I

The translocation of cortexillins seems to be governed by signals emanating from the mitotic apparatus at the poles of the cells, since it is specifically the area around the microtubule asters which is mostly cleared of cortexillins (Figure 4C and D). This notion is in line with data indicating that in multinucleate myosin II-null cells, the positions of cleavage furrows are negatively controlled by the interaction of aster microtubules with the cell cortex (Neujahr *et al.*, 1998). The pattern of cortexillin translocation parallels the transport of glycoproteins cross-linked by ConA, suggesting that proteins on the cell surface are coupled to the same system as cortexillins at the inside of the plasma membrane.

As in other cells, cross-linkage by ConA induces membrane glycoproteins in *Dictyostelium* to couple to the underlying cytoskeleton, causing these proteins to be transported to the tail of the cells, where they are deposited as a cap. Two pieces of evidence suggest that the capping in interphase cells is related to the specification of a cleavage furrow in mitotic cells. First, during mitosis in macrophages, ConA-bound glycoproteins are translocated to the furrow in a way that indicates a general contraction during cleavage (Koppel *et al.*, 1982). Secondly, in *Dictyostelium* cells, ConA induces rearward transport of attached particles (Jay and Elson, 1992) and cell surface capping (Aguado-Velasco and Bretscher, 1997) in the presence and, less efficiently, in the absence of myosin II. The myosin II-independent translocation of cell surface proteins and cortexillins to the cleavage furrow has, therefore, a counterpart in interphase cells.

The results reported here indicate that the intracellular localization of cortexillin I is determined by a balance between the actin-binding activity of the N-terminal domain and a membrane-binding capacity of the C-terminal region. Whereas the N-terminal domain might play a modulating role in translocation, the C-terminal region harbours, within a sequence of 93 amino acid residues, all sites that are crucial for the function of cortexillin I in mitotic cleavage. Two of the activities have been specified herein: (i) the capability of coupling to a system that in mitotic cells translocates the protein to the midregion of the cell; and (ii) the capacity to alter locally the physical properties of the cell cortex in a way that supports cleavage. Based on these results, a combined approach will be pursued to characterize, *in vitro* and *in vivo*, the motifs in the C-terminal region that are responsible for the various activities of cortexillins in cytokinesis.

Materials and methods

Vector construction

The pDGFP-MCS vector was constructed to express, under control of the actin 15 promoter, fusion proteins in *D. discoideum* that contain GFP at the N-terminal side and cortexillin I sequences at the C-terminal side. This vector was derived from plasmid pDBsr kindly supplied by A. Erdmann. A 650 bp sequence encoding red-shifted GFP S65T (Heim and Tsien, 1996) was amplified from plasmid p133, kindly provided by

M. Westphal, and modified by PCR in order to add in-frame a sequence encoding the linker (GGG)₂ and a multiple cloning site that contains *Sall*, *HincII*, *XhoI* and *EcoRI* restriction sites behind the GFP sequence. Primers carrying *HindIII* sites for subsequent cloning used for PCR were 5'-GCCAAGCTTAAATGAGTAAAGGAGAGAACTTTTC-3' and 5'-CGCAAGCTTGAATTCTCGAGTCGACTTCCACCTGAACCACCTTGTATAGTTCATCCATGCC-3'. The amplified fragment was digested with *HindIII* and ligated in sense orientation into the *HindIII* site of pDBsr, giving rise to pDGFP-MCS. The full-length protein and two fragments, one encoding the N-terminal residues 1–233, the other the C-terminal residues 352–444 of cortexillin I, were amplified from cortexillin I cDNA with sequence-specific primers. All three fragments carried a stop codon and a polyadenylation signal and were flanked by *Sall* sites. The fragments were digested with *Sall* and cloned in sense orientation into the *Sall* site of pDGFP-MCS. The sequences of all fragments generated by PCR were verified by DNA sequencing.

Cell culture and transformation

Cells of the AX2 wild-type strain, of the myosin II-null strain HS2205, of cortexillin-null mutants and transformants producing GFP fusion proteins were cultivated at 23°C axenically in nutrient medium (Watts and Ashworth, 1970), either in shaken suspension at 150 r.p.m. or on polystyrene Petri dishes. *Dictyostelium discoideum* cells were transformed by electroporation. Transformants were selected on plates in nutrient medium containing 10 µg/ml blasticidin S (ICN) and cloned on agar plates with *Klebsiella aerogenes*. For transfected cells, blasticidin S was added to a final concentration of 10 µg/ml.

Immunoblotting and fluorescent labelling of cells

Proteins were resolved by SDS-PAGE in 10% gels, and immunoblotted using hybridoma supernatant containing either anti-GFP antibody mAb 264-449-2, kindly provided by M. Maniak, mAb 241-36-2 specific for cortexillin I (Faix *et al.*, 1996) or mAb 241-71-3 reacting with both cortexillin I and II, and [¹²⁵I]sheep anti-mouse IgG (Amersham) as second antibody. Bound antibody was quantified by analysing the [¹²⁵I]-labelled bands with a Fuji Phosphorimager using the PCBAS program (Raytest Isotopenmessgeräte, Straubenhardt, Germany).

For immunofluorescence labelling, growth phase cells were washed twice with 17 mM Na/K-phosphate buffer, pH 6.0, and allowed to adhere on glass coverslips for 20 min. The cells were then fixed with picric acid/paraformaldehyde (Humbel and Biegelmann, 1992), and labelled for cortexillin with mAb 241-36-2 and for the 34 kDa bundling protein with mAb 159-291-1, followed by incubation with tetramethylrhodamine isothiocyanate (TRITC)-conjugated goat anti-mouse IgG (Dianova, Hamburg, Germany). F-actin was visualized with TRITC-conjugated phalloidin (Sigma) and nuclei with DAPI (Sigma).

To record the redistribution of cell surface proteins cross-linked with ConA, myosin II-null cells that express GFP- α -tubulin (Neujahr *et al.*, 1998) were incubated for various times at 23°C with 30 µg/ml of TRITC-conjugated ConA type IV (Sigma) in 20 mM MES buffer, pH 6.8, supplemented with 2 mM MgCl₂ and 0.2 mM CaCl₂, immediately fixed as described for immunolabelling, and scanned for ConA redistribution.

Confocal microscopy and three-dimensional image reconstruction

Confocal images were taken with an LSM 410 microscope using a 40×/1.3 Neofluar objective (Zeiss). For the GFP fluorescence, the 488 nm argon-ion laser band was used for excitation together with a 510–525 nm band-pass emission filter; for TRITC, the 543 nm helium-neon-ion band and a 570 nm high-pass filter; and for DAPI, the 364 nm argon-ion band and a 400–435 nm band-pass filter. For the triple-labelled images in Figure 4, the same optical section of a cell was scanned successively in the GFP, TRITC and DAPI channels.

For three-dimensional reconstructions, cells were scanned in the TRITC channel at 0.2 µm distance intervals in the z-axis along their entire height. Three-dimensional images were constructed using the AVS software package (Advanced Visual Systems, Waltham, MA) as described by Neujahr *et al.* (1997b).

In vivo microscopy

For observing the morphology of dividing cells, a double-view microscope was used, which combines phase-contrast and RCM imaging (Weber *et al.*, 1995). To provide optimal conditions for cytokinesis, the cells were cultivated on SM agar plates with *K.aerogenes* (Sussman, 1951), washed in 17 mM Na/K-phosphate buffer, pH 6.0, and transferred

together with bacteria onto a glass surface coated with bovine serum albumin.

For studying the localization of different GFP-containing fusion proteins during cytokinesis, a laser-scanning confocal microscope was used (LSM 410, Zeiss), equipped with a 40×/1.3 NEOFLUAR objective. The 488 nm band of an argon-ion laser was used for excitation and a 515–565 nm band-pass filter for emission. The medial sagittal plane through a cell was scanned at time intervals of 20 s. Cells were cultivated in nutrient medium on the plastic culture dishes, washed and transferred to a glass surface, where they were incubated with a suspension of *K.aerogenes* in the phosphate buffer for at least 2 h.

Quantification of cortical cortexillin I accumulation

Fixed cells were immunolabelled using mAb 241-36-2 against cortexillin I as first antibody, and TRITC-conjugated second antibody. The middle horizontal plane through a cell was scanned using the confocal microscope. Fluorescence intensity profiles along lines drawn perpendicular to the long axis of a cell were then determined at two locations: in the cleavage furrow and at the distal portion of the cell, using the Optimas image-processing program (Optimas Corporation, Bothell, WA). Ratios between the peak intensity values in the cleavage furrow and in the distal cell cortex were determined for 13 wild-type and myosin II-null cells.

Acknowledgements

We thank Chris Clougherty and Ralph Neujahr for comments on the manuscript, Markus Maniak for anti-GFP antibody, and Francisco Rivero for confirming specificity of the anti-p34 antibody using a p34-null mutant.

References

- Aguado-Velasco, C. and Bretscher, M.S. (1997) *Dictyostelium* myosin II null mutant can still cap ConA receptors. *Proc. Natl Acad. Sci. USA*, **94**, 9684–9686.
- Burton, K. and Taylor, D.L. (1997) Traction forces of cytokinesis measured with optically modified elastic substrata. *Nature*, **385**, 450–454.
- DeLozanne, A. and Spudich, J.A. (1987) Disruption of the *Dictyostelium* myosin heavy chain by homologous recombination. *Science*, **236**, 1086–1091.
- Dvorak, J.A. and Nagao, E. (1998) Kinetic analysis of the mitotic cycle of living vertebrate cells by atomic force microscopy. *Exp. Cell Res.*, **242**, 69–74.
- Faix, J. and Dittrich, W. (1996) DGAP1, a homologue of rasGTPase activating proteins that controls growth, cytokinesis, and development in *Dictyostelium discoideum*. *FEBS Lett.*, **394**, 251–257.
- Faix, J. *et al.* (1996) Cortexillins, major determinants of cell shape and size, are actin-bundling proteins with a parallel coiled-coil tail. *Cell*, **86**, 631–642.
- Faix, J., Clougherty, C., Konzok, A., Mintert, U., Murphy, J., Albrecht, R., Mühlbauer, B. and Kuhlmann, J. (1998) The IQGAP-related protein DGAP1 interacts with Rac and is involved in the modulation of the F-actin cytoskeleton and control of cell motility. *J. Cell Sci.*, **111**, 3059–3071.
- Fechheimer, M., Murdock, D., Carney, M. and Glover, C.V.C. (1991) Isolation and sequencing of complementary DNA clones encoding the *Dictyostelium discoideum* 30 000-Dalton actin-bundling protein. *J. Biol. Chem.*, **266**, 2883–2889.
- Fishkind, D.J. and Wang, Y.-L. (1995) New horizons for cytokinesis. *Curr. Biol.*, **7**, 23–31.
- Gerald, N., Dai, J., Ting-Beall, H.P. and DeLozanne, A. (1998) A role for *Dictyostelium* RacE in cortical tension and cleavage furrow progression. *J. Cell Biol.*, **141**, 483–492.
- Glenney, J.R., Jr, Kaulfus, P., Matsudaira, P. and Weber, K. (1981) F-actin binding and bundling properties of fimbrin, a major cytoskeletal protein of microvillus core filaments. *J. Biol. Chem.*, **256**, 9283–9288.
- Glotzer, M. (1997) The mechanism and control of cytokinesis. *Curr. Opin. Cell Biol.*, **9**, 815–823.
- Harris, A.K. (1990) Testing cleavage mechanisms by comparing computer simulations to actual experimental results. *Ann. NY Acad. Sci.*, **582**, 60–77.
- He, X. and Dembo, M. (1997) On the mechanics of the first cleavage division of the sea urchin egg. *Exp. Cell Res.*, **233**, 252–273.

- Heim, R. and Tsien, R.Y. (1996) Engineering green fluorescent protein for improved brightness, longer wavelength and fluorescence resonance energy transfer. *Curr. Biol.*, **6**, 178–182.
- Hiramoto, Y. (1990) Mechanical properties of the cortex before and during cleavage. *Ann. NY Acad. Sci.*, **582**, 22–30.
- Humbel, B.M. and Biegelmann, E. (1992) A preparation protocol for postembedding immunoelectron microscopy of *Dictyostelium discoideum* with monoclonal antibodies. *Scanning Microsc.*, **6**, 817–825.
- Jay, P.Y. and Elson, E.L. (1992) Surface particle transport mechanism independent of myosin II in *Dictyostelium*. *Nature*, **356**, 438–440.
- Knecht, D.A. and Loomis, W.F. (1987) Antisense RNA inactivation of myosin heavy chain gene expression in *Dictyostelium discoideum*. *Science*, **236**, 1081–1086.
- Koppel, D.E., Oliver, J.M. and Berlin, R.D. (1982) Surface functions during mitosis. III. Quantitative analysis of ligand–receptor movement into the cleavage furrow: diffusion vs. flow. *J. Cell Biol.*, **93**, 950–960.
- Larochelle, D.A., Vithalani, K.K. and DeLozanne, A. (1996) A novel member of the *rho* family of small GTP binding proteins is specifically required for cytokinesis. *J. Cell Biol.*, **133**, 1321–1329.
- Madaule, P., Eda, M., Watanabe, N., Fujisawa, K., Matsuoka, T., Bito, H., Ishizaki, T. and Narumiya, S. (1998) Role of citron kinase as a target of the small GTPase Rho in cytokinesis. *Nature*, **394**, 491–494.
- Neujahr, R., Heizer, C. and Gerisch, G. (1997a) Myosin II-independent processes in mitotic cells of *Dictyostelium discoideum*: redistribution of the nuclei, re-arrangement of the actin system and formation of the cleavage furrow. *J. Cell Sci.*, **110**, 123–137.
- Neujahr, R., Heizer, C., Albrecht, R., Ecke, M., Schwartz, J.-M., Weber, I. and Gerisch, G. (1997b) Three-dimensional patterns and redistribution of myosin II and actin in mitotic *Dictyostelium* cells. *J. Cell Biol.*, **139**, 1793–1804.
- Neujahr, R., Albrecht, R., Köhler, J., Matzner, M., Schwartz, J.-M., Westphal, M. and Gerisch, G. (1998) Microtubule-mediated centrosome motility and the positioning of cleavage furrows in multinucleate myosin II-null cells. *J. Cell Sci.*, **111**, 1227–1240.
- Prassler, J. (1995) Platin aus *Dictyostelium discoideum*: Isolierung, biochemische Charakterisierung und Ausschaltung dieses aktinbindenden Proteins durch Geninaktivierung. PhD Thesis, Technical University München, Germany.
- Rappaport, R. (1996) *Cytokinesis in Animal Cells*. Cambridge University Press, Cambridge, New York.
- Rivero, F., Furukawa, R., Noegel, A.A. and Fechheimer, M. (1996a) *Dictyostelium discoideum* cells lacking the 34,000-dalton actin-binding protein can grow, locomote, and develop, but exhibit defects in regulation of cell structure and movement: a case of partial redundancy. *J. Cell Biol.*, **135**, 965–980.
- Rivero, F., Koppel, B., Peracino, B., Bozzaro, S., Siegert, F., Weijer, C.J., Schleicher, M., Albrecht, R. and Noegel, A.A. (1996b) The role of the cortical cytoskeleton: F-actin crosslinking proteins protect against osmotic stress, ensure cell size, cell shape and motility, and contribute to phagocytosis and development. *J. Cell Sci.*, **109**, 2679–2691.
- Satterwhite, L.L. and Pollard, T.D. (1992) Cytokinesis. *Curr. Opin. Cell Biol.*, **4**, 43–52.
- Simson, R., Wallraff, E., Faix, J., Niewöhner, J., Gerisch, G. and Sackmann, E. (1998) Membrane bending modulus and adhesion energy of wild-type and mutant cells of *Dictyostelium* lacking talin and cortexillins. *Biophys. J.*, **74**, 514–522.
- Steinmetz, M.O., Stock, A., Schulthess, T., Landwehr, R., Lustig, A., Faix, J., Gerisch, G., Aebi, U. and Kammerer, R.A. (1998) A distinct 14 residue site triggers coiled-coil formation in cortexillin I. *EMBO J.*, **17**, 1883–1891.
- Sussman, M. (1951) Origin of cellular heterogeneity in the slime molds. *J. Exp. Zool.*, **118**, 407–418.
- Tuxworth, R.I., Cheetham, J.L., Machesky, L.M., Spiegelmann, G.B., Weeks, G. and Insall, R.H. (1997) *Dictyostelium* RasG is required for normal motility and cytokinesis, but not growth. *J. Cell Biol.*, **138**, 605–614.
- Watts, D.J. and Ashworth, J.M. (1970) Growth of myxamoebae of the cellular slime mould *Dictyostelium discoideum* in axenic culture. *Biochem. J.*, **119**, 171–174.
- Weber, I., Wallraff, E., Albrecht, R. and Gerisch, G. (1995) Motility and substratum adhesion of *Dictyostelium* wild-type and cytoskeletal mutant cells: a study by RICM/bright-field double-view image analysis. *J. Cell Sci.*, **108**, 1519–1530.
- White, J.G. (1990) Laterally mobile, cortical tension elements can self-assemble into a contractile ring. *Ann. NY Acad. Sci.*, **582**, 50–59.
- White, J.G. and Borisy, G.G. (1983) On the mechanism of cytokinesis in animal cells. *J. Theor. Biol.*, **101**, 289–316.
- Witke, W., Schleicher, M. and Noegel, A. (1992) Redundancy in the microfilament system: abnormal development of *Dictyostelium* cells lacking two F-actin cross-linking proteins. *Cell*, **68**, 1–10.
- Wolpert, L. (1966) The mechanical properties of the membrane of the sea urchin egg during cleavage. *Exp. Cell Res.*, **41**, 385–396.
- Zang, J.H., Cavet, G., Sabry, J.H., Wagner, P., Moores, S.L. and Spudich, J.A. (1997) On the role of myosin-II in cytokinesis—division of *Dictyostelium* cells under adhesive and nonadhesive conditions. *Mol. Biol. Cell*, **8**, 2617–2629.

Received September 24, 1998; revised November 24, 1998;
accepted November 27, 1998