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Dynamic, Rho1p-dependent localization of Pkc1p to sites of polarized growth

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SUMMARY

In eukaryotes, the Rho GTPases and their effectors are key regulators of the actin cytoskeleton, membrane trafficking and secretion, cell growth, cell cycle progression and cytokinesis. Budding yeast Pkc1p, a protein kinase C-like enzyme involved in cell wall biosynthesis and cytoskeletal polarity, is structurally and functionally related to the Rho-associated kinases (PRK/ROCK) of mammalian cells. In this study, localization of Pkc1p was monitored in live cells using a GFP fusion (Pkc1p-GFP). Pkc1p-GFP showed dynamic spatial and temporal localization at sites of polarized growth. Early in the cell cycle, Pkc1p-GFP was found at the pre-bud site and bud tips, becoming delocalized as the cell progressed further and finally relocalizing around the mother-daughter bud neck in an incomplete ring, which persisted until cell separation. Bud localization was actin-dependent but stability of Pkc1p-GFP at the neck was actin-independent, although localization at both sites required functional Rho1p. In addition, Pkc1p-GFP showed rapid relocalization after cell wall damage. These results suggest that the roles of Pkc1p in both polarized growth and the response to cell wall stress are mediated by dynamic changes in its localization, and suggest an additional potential role in cytokinesis.

Movies available on-line:
http://www.biologists.com/JCS/movies/jcs1621.html

Key words: Pkc1p, Actin, Cell wall, Rho1p, Yeast

INTRODUCTION

Polarized cell growth and division are critical cellular processes that play key roles in eukaryotic development (Drubin and Nelson, 1996). In the budding yeast *Saccharomyces cerevisiae*, polarized growth occurs towards an external cue during mating and to internal cues during the cell division cycle (Pruyne and Bretscher, 2000), involving the regulated and sequential assembly of proteins to the bud tip and mother-daughter bud neck during vegetative growth or to the shmoo tip during mating (Ayscough et al., 1997). Polarized cell growth therefore requires the coordinated function of the cytoskeleton, cell polarity proteins, the secretory machinery and signal transduction cascades.

The protein kinase C (PKC) superfamily is a group of phospholipid-dependent protein kinases which, in mammalian cells, display isotype-specific cellular localization and translocation in response to extracellular stimuli (reviewed by Mellor and Parker, 1998). Budding yeast Pkc1p should be considered an archetypal PKC (Mellor and Parker, 1998), since it possess all the domains found in the various PKC isotypes and in the PKC-related kinases (PRKs/PKN) and Rho-associated kinases (ROCK/ROK: reviewed in Aspenstrom, 1999), but like the latter group of enzymes, Pkc1p directly binds to and is regulated by a Rho guanosine triphosphatase (GTPase), Rho1p (Kamada et al., 1996). Pkc1p is an essential protein that is important both for maintaining cell wall integrity and for regulating actin cytoskeleton polarity by controlling signalling through the Bck1p-Mkk1,2p-Mpk1p MAP kinase module (Heinisch et al., 1999; Helliwell et al., 1998; Mazzoni et al., 1993). Rho1p acts upstream of Pkc1p in signalling to the actin cytoskeleton (Helliwell et al., 1998; Nonaka et al., 1995), responding via its exchange factor Rom2p to input from the phosphatidylinositol kinase homolog Tor2p (Schmidt et al., 1997) and to cell wall stresses (Bickle et al., 1998), which may be monitored by putative stress receptors such as Wsc1p and Mid2p (Delley and Hall, 1999; Ketela et al., 1999; Lodder et al., 1999). Rho1p and one of its other effectors, β-(1,3) glucan synthase (Fks1p), are both localized to sites of polarized growth (Delley and Hall, 1999; Qadota et al., 1996; Yamochi et al., 1994), that is to buds and to the mother-daughter bud neck, the site of cytokinesis. Like Rho1p and Pkc1p, Rho and its effectors PRK/PKN and the ROCK family in higher eukaryotes have been implicated in actin cytoskeletal dynamics (see Hall, 1998) and analysis of Pkc1p function and localization in yeast could therefore serve as a useful model for investigating the functions of its mammalian Rho-associated counterparts. Despite the evidence linking Pkc1p to Rho1p-mediated regulation of cell polarity and cell wall integrity, however, nothing is known about its spatial or temporal localization.

In this study, we have used a functional fusion of Pkc1p and green fluorescent protein (GFP) to examine the cellular localization of Pkc1p and to determine the principal factors which affect this. In particular, we sought to determine the role of Rho1p in Pkc1p localization, given its identification as a

critical activator of Pkc1p. We found that Pkc1p-GFP is localized dynamically in a cell cycle-specific manner to the pre-bud site, the tips of small- and medium-budded cells and to the mother-daughter bud neck, but is rapidly relocalized in response to cell wall damage. Pkc1p-GFP localization was partly coincident with actin and was Rho1p-dependent, but only pre-bud site and bud-tip localization required an intact actin cytoskeleton. Far from being a static component of the cell cortex, we were surprised to find that the distribution of Pkc1p-GFP is highly dynamic, both spatially and temporally, within the regions to which it is localized.

MATERIALS AND METHODS

Yeast growth conditions and general methods

Yeast strains were grown as described previously (Rose et al., 1990) in YPDA (YPD medium supplemented with 0.02% adenine). Cultures were grown at 24°C except where noted otherwise. Standard methods were used for general molecular genetic manipulations (Rose et al., 1990) and yeast transformation (Gietz et al., 1992).

Construction of strains carrying a PKC1-GFP fusion

A strain expressing Pkc1p with GFP fused in-frame at its carboxy terminus was constructed by tagging the chromosomal *PKC1* locus. The wild-type W303 haploid strain AY925 (*MAT***a** *ade2-1 his3-11 leu2-3,112 trp1-1 ura3-1 can1-100 ssd1-d2* Gal^+) was transformed with a KanMX-marked cassette, generated by PCR using pFA6a-GFP(S65T)-kanMX6 as template and employing primers specific for the regions flanking the *PKC1* stop codon, as described by Longtine et al. (1998). The primers were: PKC-TAG-5′(GTTTTGACAACG-AGCCAGCAAGAAGAGTTTAGAGGATTTTCCTTTATGCCAGAT-GATTTGGATTTA*CGGATCCCCGGGTTAATTAA*) and PKC-TAG-3′(CAACTTTTCCGCTTAGATGTTTTATATAAAATTAAATAAAT-CATGGCATGACCTTTTCT*GAATTCGAGCTCGTTTAAAC*), where identity to the *PKC1* locus and to the plasmid template are shown underlined and in italics, respectively). Correct integration at the *PKC1* locus was confirmed by PCR amplification of genomic DNA from individual transformants, using standard conditions and suitable combinations of primers specific for the KanMX cassette (K2: CGCGGCCTCGAAACGTGA and K3: GATGACGAGCGTAATGG-CT) and *PKC1* (PKC1-DIAG5: GGTTGGGTGCTGGTCCCAGG-GAC and PKC1-DIAG3: CGCGGATTTGATAGCATTTGACAC). Fusion of GFP at the C terminus of Pkc1p in a haploid wild-type strain generated derivatives that grew at essentially wild-type rates, were not temperature-sensitive, did not show morphological or cell integrity defects and did not require osmotic support for growth. Since mutation or deletion of *PKC1* results in temperature-sensitivity, severe cell lysis and a requirement for osmotic support for wild-type growth rates, the Pkc1p-GFP fusion is likely to be fully functional. Strains YOC784 (*RHO1*), YOC772 (*rho1-2*), YOC774 (*rho1-4*) and YOC776 (*rho1-5*) (Helliwell et al., 1998) were similarly used for direct GFP-tagging of Pkc1p.

Live cell imaging of Pkc1p-GFP

Pkc1p-GFP cells were grown to mid-log phase in YPDA and were harvested, resuspended in synthetic complete medium, placed under a coverslip on a microscope slide and sealed. Cells were able to grow for several hours under these conditions. When monitoring the response to cell wall damage, resuspended cells were supplemented with Zymolyase 100T (ICN, UK) at a final concentration of 12 μg/ml. In some experiments, exponentially growing or G_0 cells were prepared and treated with Latrunculin A (LAT-A) at a final concentration of 200 μM, as described by Ayscough et al. (1997), except that YPDA medium was used with G_0 cells instead of YPD-Sorbitol. An equal amount of the LAT-A solvent (DMSO) was added to the control cultures. Actin was stained with Rhodamine-Phalloidin at selected time points to verify disruption of the actin cytoskeleton. Images were acquired using a DeltaVision Restoration Microscope (Applied Precision Inc., USA) fitted with a Nikon PlanApo 100× (1.4 NA) objective and a Photometrics series 350 cooled CCD camera, taking a *Z*-series to encompass the entire cell. Each exposure was restricted to less than 0.2 seconds to reduce phototoxicity/bleaching and images were collected every minute. Images were deconvolved and processed using the Deltavision SoftWorx application on a Silicon Graphics Octane Workstation (Silicon Graphics Inc., USA). Figures were assembled using Showcase (Silicon Graphics Inc., USA) and Adobe Photoshop (Adobe Systems Inc., USA), in each case generating 2-D projections of the 3-D images that had been acquired.

Visualization of GFP, actin and cell wall chitin

Exponentially growing cells were prepared for simultaneous visualization of actin and GFP as described in Bi et al. (1998), with the addition of 10 μg/ml Calcofluor White to stain cell wall chitin concurrently with Rhodamine-Phalloidin. Images were acquired and processed as above.

RESULTS

Pkc1p is localized to sites of polarized growth

To examine Pkc1p localization in budding yeast, GFP^{S65T} was fused to the Pkc1p carboxy terminus by modifying the 3′ end of the chromosomal *PKC1* locus, allowing expression of Pkc1p-GFP from the endogenous *PKC1* promoter. Cells carrying the *PKC1-GFP*^{S65T} locus behaved like *PKC1* wild-type cells with respect to growth rate and other phenotypic criteria, indicating that the fusion protein was functional. Pkc1p-GFP was visualized using high-resolution deconvolution fluorescence microscopy (Fig. 1) and found to be present in small- and medium-sized buds and at the mother-daughter bud neck in large-budded cells. We simultaneously labelled actin and cell wall chitin and found that a proportion of Pkc1p-GFP colocalized with the cortical actin cytoskeleton at the tips of growing buds and with cortical actin patches in large-budded cells (Fig. 1). In fact, a small amount of Pkc1p-GFP colocalized with 60-70% of actin patches in large-budded cells (Fig. 1C,D). Strikingly, Pkc1p-GFP was also seen at the mother-daughter bud neck, the site of cytokinesis in budding yeast (Fig. 1C-E). Again Pkc1p was partially coincident with actin, but much more extensive in its distribution and not present in patches. Pkc1p-GFP was also found at the shmoo tips of α mating pheromone-treated cells (data not shown). When the neck region of live cells expressing Pkc1p-GFP was examined in more detail in three dimensions, we noted that Pkc1p-GFP was present as an incomplete ring or a ‘horse-shoe’ in large-budded cells (Fig. 2A-D). In cells later in the cell cycle, which possessed a narrower neck when viewed laterally, Pkc1p-GFP was localized in a slightly smaller lobed-ring structure. Pkc1p-GFP was consistently observed in the middle of this neck region either partially, or occasionally completely, filling it (Fig. 2E,F). This pattern of localization is suggestive of a direct role for Pkc1p in the development of the septum.

Localization of Pkc1p-GFP to sites of polarized growth depends on functional Rho1p

Since Pkc1p binds to and is regulated by Rho1p and Rho1p is localized to sites of polarized growth, we next asked whether the polarized localization of Pkc1p was dependent on functional

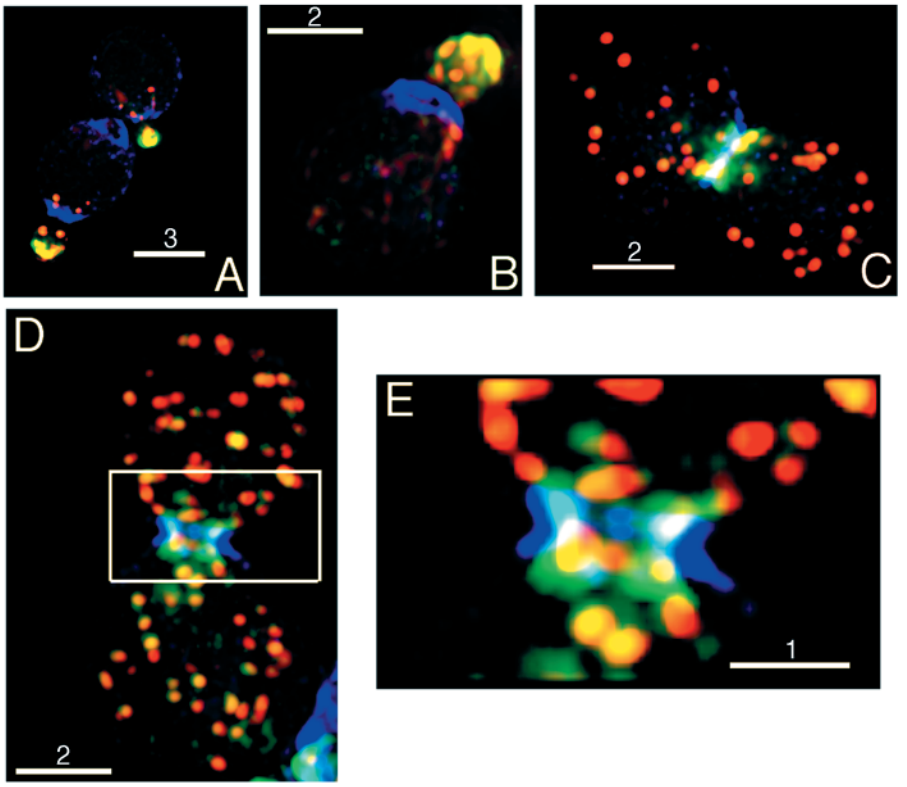

Fig. 1. Pkc1p-GFP localizes to sites of polarized growth. Pkc1p fused at its carboxy terminus to GFP was localized using DeltaVision restoration microscopy. Ethanol-fixed yeast cells were prepared for simultaneous visualization of actin, GFP and cell wall chitin and images were coloured green for Pkc1p-GFP, red for actin and blue for chitin. Overlapping actin and Pkc1p-GFP appears yellow. Pkc1p-GFP was localized to small (A) and medium buds (B) and to the mother-daughter bud neck during cytokinesis (C-E). E shows detail from within the box drawn on D. Scale bars are in μm. Movies showing rotations of three-dimensional images of the cells depicted in A and C can be viewed at http://www.biologists.com/JCS/movies/jcs1621.html.

Rho1p. We GFP-tagged Pkc1p in a panel of *rho1* mutant strains (Helliwell et al., 1998) and simultaneously visualized Pkc1p-GFP and the actin cytoskeleton (Fig. 3). In a wild-type *RHO1* strain, Pkc1p-GFP was found to be polarized at 26°C and remained polarized after a 15-minute period at 37°C. In contrast, while Pkc1p-GFP displayed polarized localization at 26˚C in three different *rho1* mutant strains (*rho1-2*, *rho1-4* and *rho1-5*), it rapidly delocalized after shifting the *rho1* cells to 37°C for only 15 minutes. In all three mutants, this short temperature shift was insufficient to cause obvious changes in actin cortical patch polarity but Pkc1p-GFP became rapidly delocalized either across the cell cortex or to diffuse patches (Fig. 3). In *rho1-4* cells, Pkc1p-GFP additionally delocalized to the cell interior into bar-shaped areas or patches in some cells, and was no longer visible in small buds despite clear retention of a polarised actin cytoskeleton (Fig. 3). It was difficult to determine unambiguously whether bud neck localisation of Pkc1p in large-budded cells was also lost in any of the mutants due to the general cortical delocalization of Pkc1p. These results suggest an important role for Rho1p, directly or indirectly, in tethering Pkc1p to sites of active secretion and cell wall deposition.

Pkc1p-GFP localization changes spatially and temporally across the cell cycle

We next examined the dynamic nature of Pkc1p-GFP localization through the cell cycle by time-lapse microscopy of live cells. Pkc1p-GFP was localized at the pre-bud site in unbudded cells (Fig. 4A), in small buds and at the tip of some medium-budded cells (Fig. 4A,B). Within the bud we observed

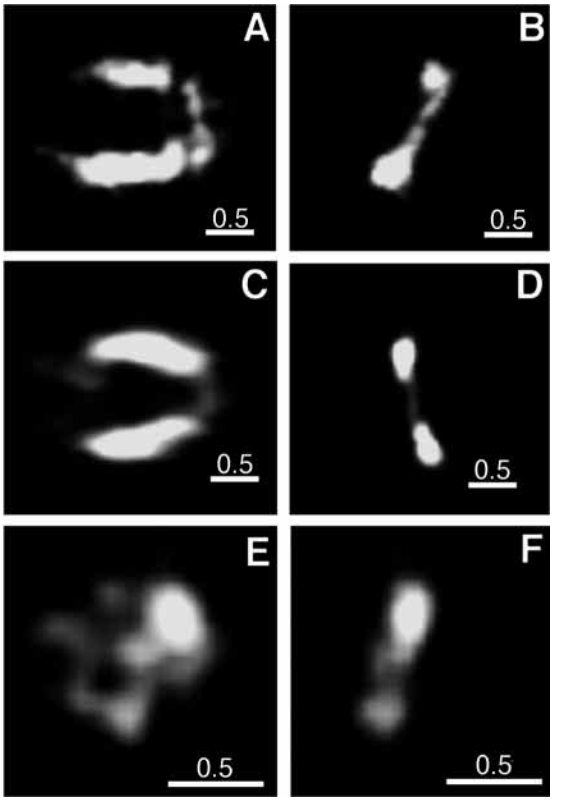

Fig. 2. Pkc1p-GFP localized in a ‘horse-shoe’ configuration at the neck of live, medium- to large-budded yeast cells (A-D). In cells at a later stage of cytokinesis Pkc1p-GFP was observed in a partially filled, lobed ring (E,F). Projections of the neck face-on shown in the left-hand panels have been rotated 90° around the *Y*-axis in the right-hand panels. Scale bars are in μm.

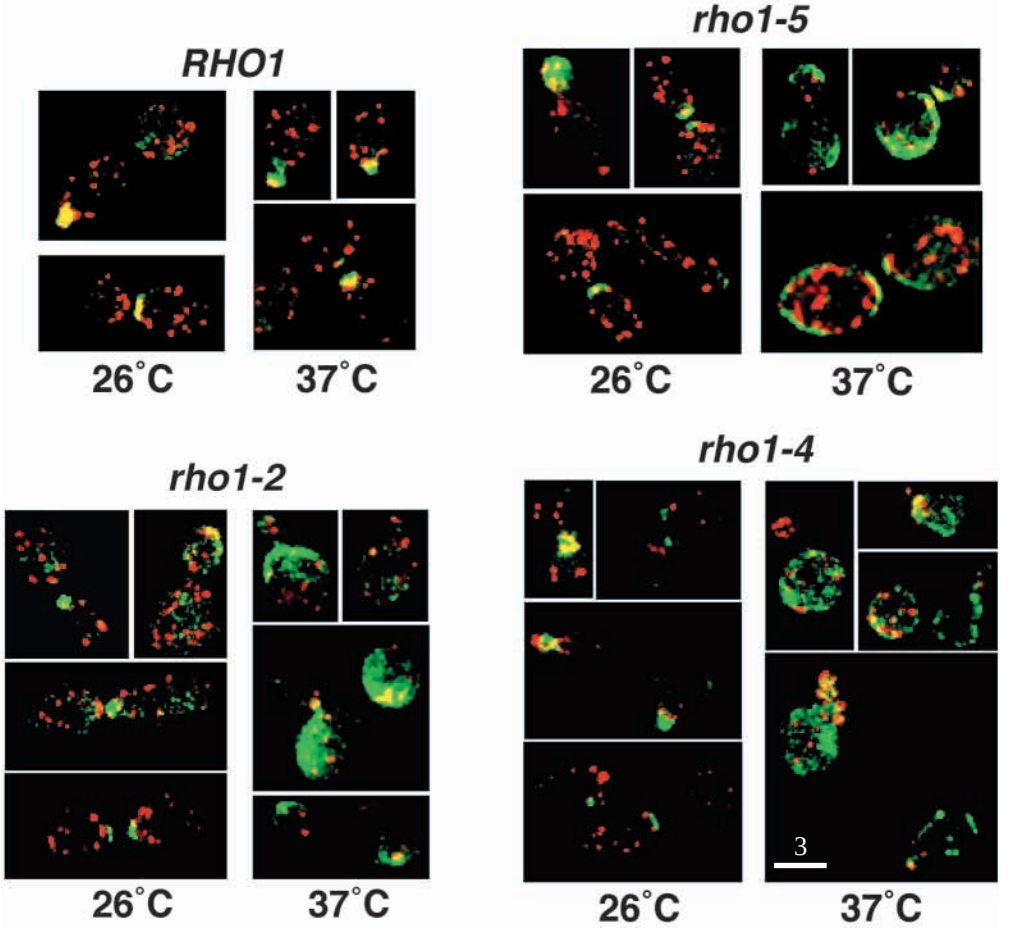

Fig. 3. Polarized localization of Pkc1p-GFP depends on functional Rho1p. Pkc1p-GFP was visualized in *RHO1* mutant and wild-type mid-log phase cells growing at either 24°C or after shifting to 37°C for 15 minutes. Samples of cells were processed for simultaneous visualization of actin and GFP. All three of the *rho1* mutants examined delocalized Pkc1p-GFP after incubation at the high temperature for 15 minutes, yet actin remained polarized in *rho1-4* as expected (Helliwell et al., 1998). Scale bars are in μm.

oscillations in overall fluorescence intensity (Fig. 4B) and mobility of Pkc1p-GFP localization within the plane of the cell surface. Quantification of Pkc1p-GFP fluorescence in the bud or neck region demonstrated that Pkc1p-GFP regularly fluctuated in intensity with a periodicity of approx. 8-10 minutes (Fig. 4C). Pkc1p-GFP fluorescence diminished as bud growth continued beyond the medium-budded stage but at later times, when large-budded cells would have been completing mitosis and beginning cytokinesis, relocalization of Pkc1p-GFP to the mother-daughter bud neck was observed. Neck localization persisted until after cell separation was complete (Fig. 4A,B). We measured the diameter of the neck localization over time in this and other cells, and found an approx. 60% reduction in the diameter of the Pkc1p-GFP 'ring' over 9 minutes. This may indicate that Pkc1p-GFP, at least in part, follows the constriction of the actomyosin ring that occurs during cytokinesis. As cell separation occurred, Pkc1p-GFP neck localization became more diffuse before relocalizing to the incipient bud site (Fig. 4A).

Cell wall damage causes dynamic relocalization of Pkc1p-GFP

Cell wall damage or stress in budding yeast has been shown to cause a Rom2p- and Wsc1p-dependent transient depolarization of the actin cytoskeleton, Rho1p GTPase, Fks1p and Wsc1p itself (Delley and Hall, 1999). To test whether Pkc1p also responds to cell wall stress, we damaged yeast cell walls with Zymolyase and followed Pkc1p-GFP localization in real-time. Surprisingly, instead of delocalizing generally across the mother cell cortex as seen for Pkc1p-GFP in *rho1* mutants shifted to 37°C, Pkc1p-GFP showed highly dynamic relocalization to specific regions underlying the cell surface within approx. 10 minutes of Zymolyase addition (Fig. 5A), remaining in the same plane but changing in intensity and position on a sub-minute time-scale (Fig. 5B,C). Thus rapid relocalization of Pkc1p in response to cell wall damage may coordinate events required to bolster the strength of the cell wall at weak points anywhere on the cell surface.

LAT-A treatment disrupts localization of Pkc1p-GFP to the bud but not the neck

Since Latrunculin-A (LAT-A), an inhibitor of actin polymerization, disrupts the localization of a number of polarity-associated proteins including Rho1p (Ayscough et al., 1999, 1997), we next examined the effect of LAT-A on the localization of Pkc1p-GFP to sites of polarized growth. We found that LAT-A rapidly abolished Pkc1p-GFP bud localization (Fig. 6A). In contrast, Pkc1p-GFP localization at the mother-daughter bud neck was largely unaffected by disrupting actin over a 3-hour time-course (Fig. 6A,B). In addition, these experiments allowed a detailed examination of Pkc1p-GFP localization at the neck region in three-dimensions over time. Both in control and LAT-A-treated cells, localization

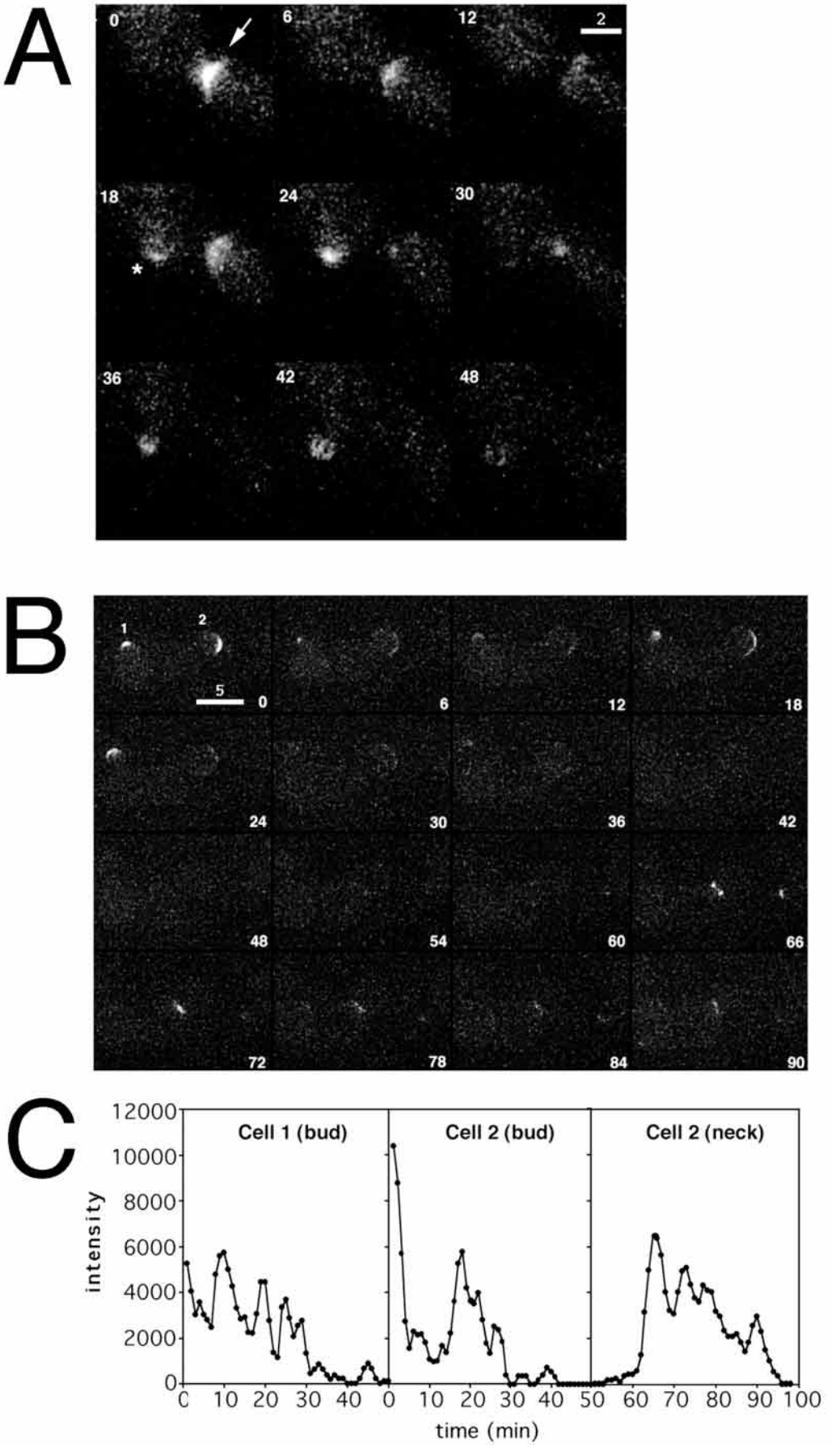

Fig. 4. Localization of Pkc1p-GFP during the cell cycle in exponentially growing cells in real time. Images were acquired at 1-minute intervals and each montage shows images separated by 6-minute intervals taken from such series, arranged chronologically from top left to bottom right. (A) Pkc1p-GFP is present at the neck until after cell separation, when it redistributes to the new bud site of the mother cell and then becomes localized to the tips of small buds; arrow, mother-daughter bud neck; *, mother cell pre-bud site. (B) Pkc1p-GFP is present at the tips of buds, fluctuating in position and intensity. As cells progress through the cell cycle Pkc1p-GFP is no longer observed in a localized pattern, but later still reappears at the bud neck. (C) Quantification of Pkc1p-GFP fluorescence in buds and at the neck across the cell cycle. The oscillation in Pkc1p-GFP fluorescence intensity was quantified using the DeltaVision SoftWorx application and plotted against time for the bud of cell 1, the bud of cell 2 and the neck of cell 2 shown in B. Pkc1p-GFP fluorescence oscillated with a periodicity of between 8 and 10 minutes. Scale bars are in μm. Movies showing the full time-course of the experiments presented in A and B can be viewed at http://www.biologists.com/JCS/movies/jcs1621.html.

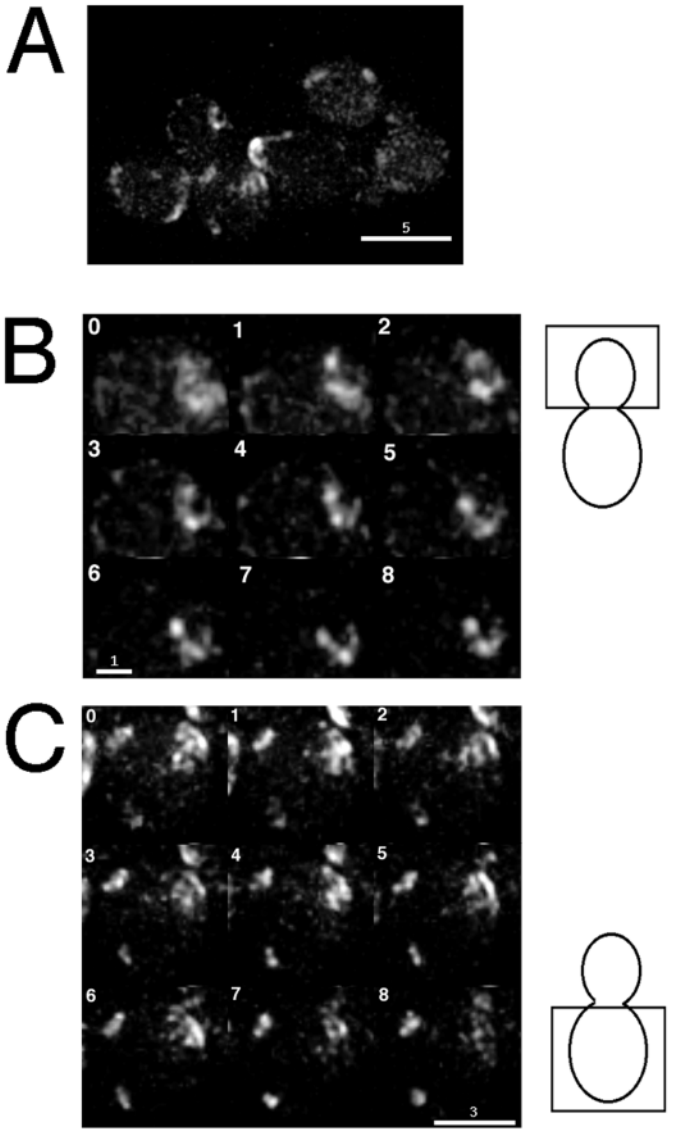

Fig. 5. Pkc1p-GFP relocalizes in response to cell wall damage. Cells were immediately mounted after addition of Zymolyase to damage their cell walls and time-lapse images were acquired. (A) Numerous regions of non-polarized Pkc1p-GFP localization were observed within minutes of initiating cell wall damage. (B) Dynamic movement within a localized region at the side of a bud, and (C) in various parts of a mother cell. The unpolarized localization after cell wall damage is in direct contrast to untreated cells, which show distinct bud-tip and neck localization (see Fig. 4). Scale bars are in μm. Movies showing the full time-course of the experiments presented in A-C, can be viewed at http://www.biologists.com/JCS/movies/jcs1621.html.

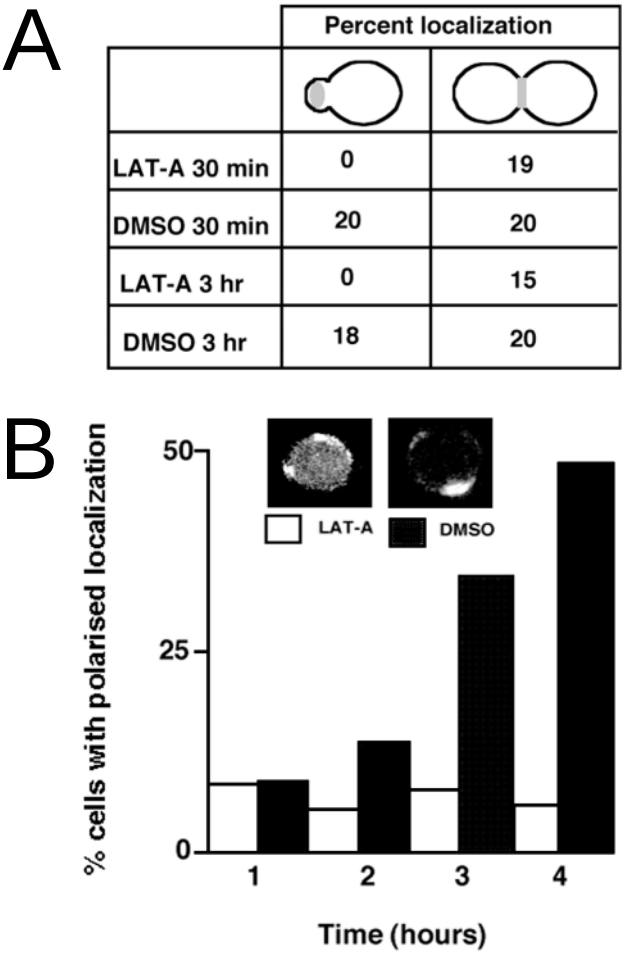

Fig. 6. Effect of LAT-A on Pkc1p-localization and dynamics. Exponentially growing Pkc1p-GFP-expressing yeast cells were treated with 200 μM LAT-A to disrupt actin polymerization and Pkc1p localization monitored after 30 minutes or 3 hours. (A) Quantitation of Pkc1p-GFP localization patterns after growth in LAT-A (or DMSO as a control), showing that bud localization of Pkc1p-GFP was rapidly abolished by LAT-A. (B) Unpolarized G_0 cells were released from stationary phase in the presence of LAT-A or DMSO and Pkc1p-GFP localization monitored, showing that Pkc1p-GFP failed to localize to the usual single, polarized site in the presence of LAT-A.

of Pkc1p at the neck was highly dynamic, suggestive of a high degree of local mobility (Fig. 7A,B). However, in LAT-A-treated cells the diameter of the Pkc1p-GFP fluorescence in the neck region only decreased by around 16% over the course of the experiment, compared to approx. 50% in control cells, a figure in agreement with results obtained for Myo1p-ring contraction in the presence of LAT-A (Bi et al., 1998). Additionally, when unpolarized G_0 cells were released from stationary phase, Pkc1p failed to localize to a polar prebud site in the presence of LAT-A (Fig. 6B). These results indicate that polarized localization of Pkc1p-GFP to the bud was dependent on the actin cytoskeleton but that in contrast, the stability of Pkc1p-GFP's localization in the neck region may be largely independent of actin.

DISCUSSION

The spatial and temporal localization of signal transduction proteins plays an important role in the way cells both respond to extracellular stimuli and control their growth and division. This notion is exemplified by the discovery of subcellular targeting by A-kinase anchoring proteins, MAP kinase scaffolds, protein kinase recruitment to the plasma membrane, and the role of nuclear import/export and nucleolar

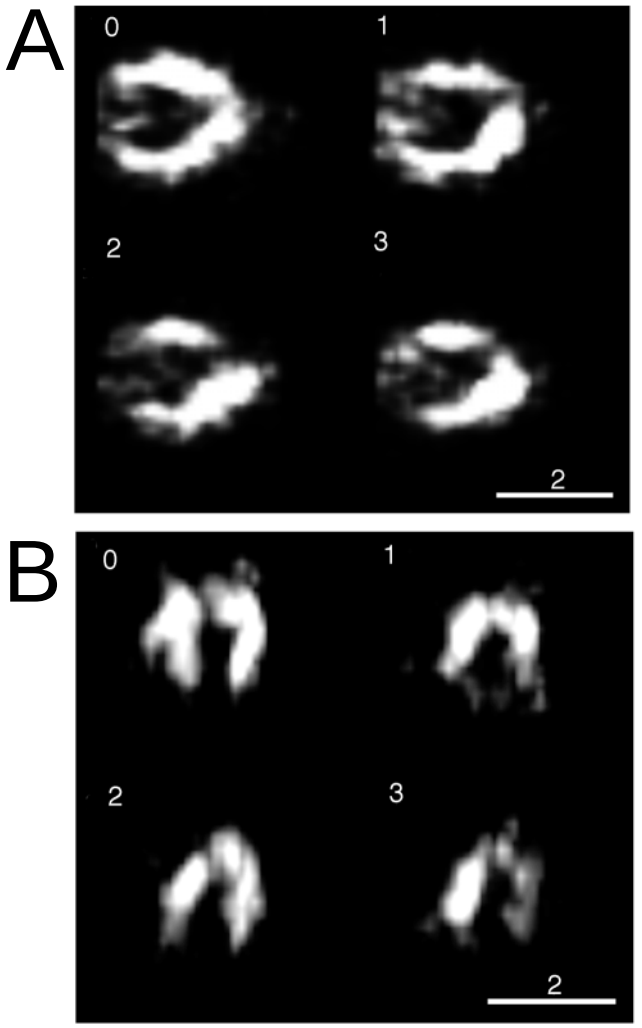

Fig. 7. Effect of LAT-A on Pkc1p bud neck localization and dynamics. Exponentially growing Pkc1p-GFP-expressing yeast cells were treated with 200 μM LAT-A to disrupt actin polymerization and Pkc1p localization in the neck region was examined in detail, capturing images at 1 minute time intervals. Pkc1p-GFP localization and mobility in the neck region of LAT-A-treated cells (A) and DMSO-treated control cells (B) was essentially identical. Scale bars are in μm. Movies showing the full time-course of the experiments shown in A and B can be viewed at http://www.biologists.com/JCS/movies/jcs1621.html.

sequestration in cell cycle regulation (Pawson and Scott, 1997; Pines, 1999). We have described for the first time the spatial and temporal localization of Pkc1p in live yeast cells using high-resolution digital restoration microscopy. Pkc1p was localized to the cell cortex at sites of polarized secretion and was partially coincident with the actin cytoskeleton, appearing first at the pre-bud site prior to bud emergence and then being restricted to a localized region at the tip of the bud until buds were of medium size. After disappearing altogether as cell progressed further through the cell cycle, Pkc1p then reappeared at the mother-daughter bud neck. Despite the established role of Pkc1p as a positive regulator of genes involved in cell wall biosynthesis (Igual et al., 1996), we found no evidence for any nuclear localisation of Pkc1p, suggesting that it is the downstream signalling components rather than Pkc1p itself which act within the nucleus.

The pattern of Pkc1p localization is therefore dissimilar to that of the septins, Myo1p and Nim-kinases (which persist at the neck throughout the cell cycle in a well-defined ring: Barral et al., 1999; Bi et al., 1998; Longtine et al., 1996), to the PAK-kinase Cla4p (which is present only in small buds; Holly and Blumer, 1999) and to the actin patch-associated kinases Ark1p and Prk1p (Cope et al., 1999), but is very similar to that shown by Rho1p (Yamochi et al., 1994). Given that we have shown Pkc1p localization to be Rho1p-dependent, at least in small buds, that Rho1p shows a very similar pattern of localization to Pkc1p both in buds and at bud necks and that Rho1p binds directly to and activates Pkc1p in vitro (Kamada et al., 1996), it is likely that Pkc1p localization is determined by its physical association with the Rho1p GTPase. Thus loss of Rho1p polarity would be predicted to cause depolarisation of Pkc1p as well. In support of this contention, localization of Pkc1p-GFP to the pre-bud site and bud-tip was abolished by LAT-A, which also causes depolarization of Rho1p across the cell cortex (Ayscough et al., 1999). Thus we favour a model in which the requirement for an intact actin cytoskeleton for bud localization of Pkc1p is indirect through its effects on Rho1p. Furthermore, the finding that Pkc1p-GFP was delocalized away from the buds of the *rho1-4* mutant in conditions under which actin remained polarized at 37°C (Fig. 3) further indicates that Pkc1p localization is primarily Rho1p- rather than actin-dependent. Like Rho1p and the putative cell wall stress receptor Wsc1p, Pkc1p therefore both mediates changes in the organization of the actin cytoskeleton and yet in turn is affected by such changes (Delley and Hall, 1999; Helliwell et al., 1998). Localization of Pkc1p at the bud neck of large-budded cells was insensitive to prolonged treatment with LAT-A. It is not known whether Rho1p localisation in this region is also LAT-A- resistant, but our model predicts that this would be the case. Alternatively, it is possible that Pkc1p localization to the bud neck is dependent on components other than Rho1p, for example on the septins. In any event, our data show that an intact actin cytoskeleton is dispensable for the maintenance of neck-localized Pkc1p.

The localization of Pkc1p to polarized growth sites mirrors that of the secretory landmark Sec3p (Finger et al., 1998) and also Sec1p, a protein involved in a late step of exocytosis (Carr et al., 1999). At each of its locations in the cell, Pkc1p might control the addition of new membrane or cell wall deposition either by regulating the polarity and function of the actin cytoskeleton to target secretory vesicles or by acting directly on components of the secretion machinery. Mammalian cells provide possible parallels for both of these mechanisms, since Rho and its effectors PRK/PKN and the ROCK family are involved in actin cytoskeletal dynamics (reviewed in Hall, 1998), while mammalian PKC has also been implicated in the regulation of the exocytic machinery on the plasma membrane (Chen et al., 1999) and fusion pore expansion (Scepek et al., 1998). The idea that changes in Pkc1p localization may serve to target secretion to places where it is most needed is strengthened by the very rapid relocalization of Pkc1p away from the normal sites of polarized growth in unstressed cells which we have observed in response to damage by Zymolyase treatment.

Previous work has suggested that Pkc1p functions

downstream of the Cdc28p protein kinase in bud emergence (Gray et al., 1997; Marini et al., 1996; Mazzoni et al., 1993; Zarzov et al., 1996) and Cdc28p activation at START parallels the in vivo production of the PKC activator diacylglycerol (Marini et al., 1996). It is therefore possible that Cdc28p may help to bring about localization of Pkc1p to its required site of action in bud tips early in the cell division cycle. Interestingly, bud tip localization of the secretory landmark protein Sec3p is clearly dependent on Cdc28p function, although unlike Pkc1p it is localized independently of actin (Finger et al., 1998).

Time-lapse microscopy of live Pkc1p-GFP-expressing cells revealed that Pkc1p localization is highly dynamic within all the regions to which it is localized, both spatially and temporally. Strikingly, fluorescence of Pkc1p-GFP in the bud consistently oscillated in intensity, peaking every 8-10 minutes. The observed ultradian oscillation of Pkc1p is of considerable interest since yeast and other unicellular eukaryotes have been shown to possess internal 'clock' mechanisms (Haase and Reed, 1999; Kippert and Hunt, 2000), although little is understood of their molecular nature. The localized, dynamic changes in the spatial distribution of Pkc1p suggest that far from being rigidly anchored, it responds rapidly to cues from the cell cortex, which direct it to very defined regions of cell surface growth. Such cues might be provided by candidate cell wall stress receptors such as Wsc1p and Mid2p (Ketela et al., 1999; Lodder et al., 1999), and our data suggest that this aspect of Pkc1p function is important both in normal cell growth as well as after cell wall damage. It will be interesting to determine whether Wsc1p, Mid2p and the Rho1p GTPase also show the same spatial and temporal dynamics that this model predicts. It remains to be discovered whether Pkc1p itself or one of its upstream regulators is the primary determinant of its dynamic localization patterns. It will also be interesting to determine what drives the localized motility within the areas where Pkc1p is found. Since Pkc1p at the bud neck continued to demonstrate such motility even in the presence of LAT-A it is unlikely that the localized mobility is an actin-based phenomenon. In fact cortical actin patches themselves retain motility either when actin filaments are stabilised (in *act1*^{V159N} or *cof1-22* cells) or destabilised in the presence of LAT-A (Belmont and Drubin, 1998; Lappalainen and Drubin, 1997) and it will be interesting to determine whether Pkc1p and cortical patch motility share a common mechanism.

Pkc1p at the neck region formed an incomplete ring or horseshoe that consistently appeared to fill in as cells completed the cell cycle. This suggests a hitherto unappreciated role for Pkc1p in cytokinesis and is reminiscent of the phragmoplast, the microtubule and actin-containing equivalent of the septum in plants, which plays an important role in cytokinesis. Since Pkc1p is known to regulate at least one MAP kinase module in budding yeast, it is interesting that certain plant MAP kinases required for cytokinesis are localized to the phragmoplast (Bogre et al., 1999; Calderini et al., 1998). In addition, mammalian Rho and its effectors PRK/PKN and ROCK have been implicated as regulators of cytokinesis and are localized to the midzone/cleavage furrow (Kosako et al., 1999; Lu and Settleman, 1999; Madaule et al., 1998; O'Connell et al., 1999), providing another possible parallel with yeast Pkc1p. By analogy with higher eukaryotes, Pkc1p's function in yeast cytokinesis could be to regulate myosin II function. In mammalian cells, Rho-kinase phosphorylates the myosin-binding regulatory subunit (MBS/M-subunit) of the type-1 protein phosphatase PP1, inactivating the dephosphorylation of myosin light chains and thereby reducing contractility and causing disassembly of stress fibres (Kimura et al., 1996). However, yeast lack a convincing homologue of the MBS/M-subunit while genetic studies fail to support the notion that Pkc1p inhibits PP1 function (Andrews and Stark, 2000), hence it is unlikely that this model is applicable.

In conclusion, our data show that Pkc1p is localized in a Rho1p-dependent manner to sites of polarized growth through the cell cycle and also to regions of cell wall damage. Localized Pkc1p-GFP fluorescence also displays ultradian periodicity and spatial mobility, showing minute-to-minute changes in its precise location. A detailed dissection of the mechanisms driving these dynamic changes should lead to a much-improved understanding of Pkc1p function.

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