Different mechanisms of cell polarisation in vegetative and shmooing growth in fission yeast

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Summary

Schizosaccharomyces pombe **cells have two polarised growth modes: an intrinsic vegetative growth mode, determined by an internal positioning mechanism and an extrinsic shmooing growth mode, activated by external pheromone. We have analysed the role of the cell end marker Tea1p, the CLIP170 like protein Tip1p, the kinesin like protein Tea2p and the Dyrk-like kinase Pom1p, during the switch between the two growth patterns, with the intention of studying the switch away from the vegetative growth mode. In vegetative growth these morphological factors are concentrated at cell ends, whereas during shmooing growth they are delocalised from the cell ends. In the absence of Tea1p, Tip1p and Tea2p, vegetative cells display microtubule and cell polarisation defects, but shmooing cells are indistinguishable from wild-type and shmoo more readily. These results suggest that Tea1p, Tip1p and Tea2p are not required for polarised growth during shmooing, but form part of the intrinsic vegetative growth mode that needs to be dismantled before cells can generate an extrinsic growth pattern. In contrast, Pom1p appears to have a role in the initial stages of the switch to the shmooing growth mode.**

Key words: Fission yeast, Shmooing, Morphogenesis, Tea1, Tea2, Tip1, Pom1

Introduction

Polarisation can be defined as the generation and maintenance of directional cellular organisation. Eukaryotic cells can become polarised in response to signals of two types: (1) intrinsic signals generated within a particular cell, for example nuclear migration in the *Drosophila* oocyte (van Eeden and St Johnston, 1999), or bud emergence in budding yeast (Hartwell, 1971); and (2) extrinsic signals provided by sources external to the cell such as diffusible hormones or growth factors, cell to cell contacts, or signals from the extracellular matrix. In both types of polarisation, cells have to be able to select growth sites and reorganise their cytoskeleton and growth machinery accordingly (Drubin and Nelson, 1996). Cells responding to an internal program may have their growth sites marked by an internal memory system, as in the case of budding yeast, where the site of bud emergence is determined by an earlier bud position (Cabib et al., 1998; Chant and Herskowitz, 1991; Taheri et al., 2000). When polarisation is externally directed, the site and direction of cell growth are dictated by the position of the external signal (Chenevert, 1994). In this case, a cell needs to be able to detect the signal's location and directionality, relay this information to its interior, and then redirect its cytoskeleton to generate polarised growth or directionalised movement. Individual cells can switch between these intrinsic and extrinsic growth modes, but how this transition occurs is not well understood.

Some of the major players involved in the selection and maintenance of sites for polarised growth, such as Cdc42p (Miller and Johnson, 1994; Johnson, 1999), Rho proteins (Arellano et al., 1999), PAKS (Ottilie et al., 1995) and G-protein-coupled receptors (Kitamura and Shimoda, 1991), are conserved from yeast to mammals, suggesting that the basic mechanisms involved may have been conserved throughout evolution. Studies in higher eukaryotes have shown that some signals inducing differentiation also alter microtubular dynamics, suggesting that this might be an essential step in the regulation of cell shape during the process of differentiation (Chausovsky et al., 2000; Spencer et al., 2000), although it is not known how the switch in microtubular dynamics takes place and how this leads to an alteration in cell shape.

The genetically amenable fission yeast (*Schizosaccharomyces pombe*) is a useful model system to study this problem. It displays both modes of polarised cell growth: one intrinsically established during vegetative growth and one extrinsically determined during mating. The intrinsic growth mode directs polarisation to produce cylindrical rods extending mostly in a bipolar fashion from the ends of the cell. When such a cell undergoes medial fission it generates two daughter cells, each with a new end at the site of cell division and an old end inherited from the mother cell (Mitchison and Nurse, 1985). After cytokinesis cells begin to grow monopolarly from the old end and at a specified point during the cell cycle they undergo NETO (new end take off), when growth at the new end is activated to generate a bipolarly growing cell. Actin is located at actively growing ends during interphase and is relocated to form a medial ring at the site of cell division at cytokinesis (Marks et al., 1986). Cytoplasmic microtubules nucleate from sites adjacent to the nuclear membrane and span the length of the cell terminating at the cell ends (Drummond and Cross, 2000; Hagan, 1998; Hagan and Hyams, 1988). This mode of growth allows cells to extend in a straight line (Brunner and Nurse, 2000b). Fission yeast cells have two mating types, h^+ and h^-, and can conjugate only with a cell of an opposite mating type

(Egel, 1971; Egel, 1989; Gutz and Doe, 1975). During mating, fission yeast cells activate a new pattern of cell growth that allows them to bend towards a mating partner. This extrinsic growth mode is induced by a pheromone secreted from cells of opposite mating type and is, therefore, extrinsically determined (Fukui et al., 1986; Leupold, 1987). Cells are capable of detecting the directionality of the pheromone gradient, and orient their mating projection towards the pheromone source (Fukui et al., 1986; Leupold, 1987). Cells then touch and undergo cellular and nuclear fusion, which is followed by meiosis and the formation of four haploid spores (Nielsen and Davey, 1995). Shmooing cells are characteristically bent, with one pointed and one rounded end, and extend in a monopolar fashion from the pointed end, where actin is localised (Petersen et al., 1998b). Microtubules curve round the non-growing end and terminate at the shmooing end (Petersen et al., 1998a). Thus the vegetative and the shmooing growth modes are characterised by different cell shapes and these may be the result of differences in the regulation of microtubular dynamics and the cell polarisation machinery.

Various morphological factors may have roles in regulating the intrinsic and extrinsic growth modes. Tea1p is a cell end marker involved in regulating microtubular dynamics and the selection of growth sites during vegetative polarised growth. *Tea1*Δ cells have some long microtubules that bend round the cell ends (Mata and Nurse, 1997) and are often bent with occasional branching. Cells that have been starved and then returned to growth exhibit a dramatic increase in the number of branches formed (M. Arellano, unpublished). This suggests that the internal memory, marking growth sites at the ends of the cell, is lost during starvation. Tea1p is normally located at the cell ends and at microtubular tips and may trigger microtubule depolymerisation once the microtubules have reached the cell ends, thus maintaining growth along a single axis (Mata and Nurse, 1997). In the presence of pheromone, Tea1p was found to delocalise from the cell ends, consistent with it being part of a vegetative specific machinery that is shut down in shmooing cells (Mata and Nurse, 1997), allowing them to grow away from the long axis of the cell.

Two further factors, Tea2p and Tip1p, which act upstream in the Tea1 pathway also play a role in microtubular dynamics and cell polarity (Browning et al., 2000; Brunner and Nurse, 2000a). In the absence of either of these factors, microtubules are short and rarely reach the cell ends. Tip1p, a CLIP 170-like protein, has been shown to stabilise microtubules when they reach the cell periphery, allowing them to grow just beneath the cell cortex until they have reached the cell ends. This enables microtubules to align along the long axis of the cell (Brunner and Nurse, 2000a). Tip1p forms a complex with Tea2p (D. Brunner and P.N., unpublished), a kinesin-like protein, and both are found at the tips of microtubules and at the ends of cells (Browning et al., 2000; Brunner and Nurse, 2000a). Like Tea1p, these factors play a role in growth site selection. *Tip1*Δ and *tea2*Δ cells are bent and branch at a low frequency during exponential growth. On re-growth from starvation, like *tea1*Δ cells, the number of branched cells increases dramatically (Browning et al., 2000; Brunner and Nurse, 2000a). The short microtubules of *tip1*Δ and *tea2*Δ cells could be partly or wholly responsible for the defects in cell polarity, given that short microtubules are known to lead to cell branching (Sawin and Nurse, 1998). It is possible that Tea1p, Tea2p and Tip1p are part of the machinery regulating microtubular dynamics, which ensures that cells grow from opposite poles and extend along the long axis of the cell (Browning et al., 2000; Brunner and Nurse, 2000a; Mata and Nurse, 1997; Sawin and Nurse, 1998). Markers of cell ends also play an essential role in maintaining cell shape. Pom1p, a protein kinase found at cell ends, is involved in NETO, septation and marking cell ends (Bahler and Pringle, 1998). In the absence of Pom1p, cells are bent or T-shaped, monopolar, and have misplaced septa (Bahler and Pringle, 1998) and slightly longer microtubules (Bahler and Nurse, 2001).

Here we investigate the role of Tea1p, Tea2p and Tip1p in response to an external pheromone signal, with the intention of studying what parts of the vegetative growth mode machinery need to be dismantled to allow the switch to a new extrinsic mode of growth. We show that these factors no longer play a role in regulating microtubular dynamics in the presence of pheromone. In contrast, Pom1p, a factor involved in the identification of cell ends during vegetative growth, may play a role in the switch to the extrinsic growth mode.

Materials and Methods

Schizosaccharomyces pombe strains and methods

All strains used are listed in Table 1. Standard protocols were used to construct and culture strains (for manual see http://www.bio.uva.nl/pombe/handbook/). All cells were grown at 25°C (if not stated otherwise) in minimal EMM2 medium supplemented with leucine. Pheromone experiments were carried out as described (Stern and Nurse, 1997), using 3 μg/ml of P factor in liquid culture and plates.

Construction of Tip1YFP strain

The *tip1* gene was PCR amplified using the primers: CGCG-TCGACCTAAATGTTTCCTCTTGGG (3′) and CGGGATCCCC-AGCTTCGTCTGTGCTGCC (5′), and the resulting product was cloned as a *SalI/Bam*HI-digested fragment into pREP5X-YFP (Decottignie et al., 2001) to create a *tip1* gene tagged at the C-terminus with YFP.

The pREP5 *tip1YFP* plasmid was integrated into a wild-type *ade 6-704* strain placing the endogenous *tip1* gene under the control of the *nmt* promoter and *tip1 YFP* under the control of the endogenous promoter. The *nmt tip1 sup3-5* genomic fragment was then replaced with a *KanR* cassette by a further integration of the pFA-kanMX6 (Bähler et al., 1998). The resulting strain was kanamycin resistant, adenine deficient and had *tip1YFP* driven by the *tip1* promoter. This strain was then crossed into the *cyr1*Δ *sxa2*Δ background. Tip1YFP (A. Decottignie, personal communication) is almost completely functional; microtubules look normal and the shmooing rate is similar to wild-type but there is a slight NETO defect.

Immunofluorescence microscopy

To detect tubulin and Tea1p, cells were fixed in –70°C methanol and processed as previously described (Mata and Nurse, 1997). Tubulin was visualised using TAT1 monoclonal antibody [a gift from K. Gull, University of Manchester, UK (Woods et al., 1989)] at 1:50, Tea1p with anti-Tea1 at 1:1000 (Mata and Nurse, 1997) and Tip1p with anti-Tip1 at 1:200 (Brunner and Nurse, 2000a). The secondary antibodies were goat anti-mouse Alexa 546 and goat anti-rabbit Alexa 488 at 1:1000. Images were taken with a Zeiss LSM 510 laser scanning confocal microscope. To visualise F-actin, cells were fixed in 4% formaldehyde at 25°C and stained with rhodamine phalloidin (Sawin and Nurse, 1998). Cells were visualised using a Zeiss Axioplan microscope mounted with a mercury lamp.

Table 1. *Schizosaccharomyces pombe* strains

cyr1Δ::LEU2 sxa2Δ::ura4 leu1-32 ura4D18 h-	Kawamukai et al., 1991
cyr1::LEU2 sxa2::ura4 tea1Δ::ura4 leu1-32 ura4D18 h-	Mata and Nurse, 1997
cyr1Δ::LEU2 sxa2Δ::ura4 pom1-3HA::ura4 ura4-D18 leu1-32 h-	Bahler and Pringle, 1998
cyr1Δ::LEU2 sxa2Δ::ura4 pom1Δ::ura4 leu1-32 ura4D18 h-	This study
cyr1Δ::LEU2 sxa2Δ::ura4 tip1Δ::kanR leu1-32 ura4-D18 h-	This study
cyr1Δ::LEU2 sxa2Δ::ura4 tea2-1 leu1-32 ura4-D18 h-	This study
cyr1Δ::LEU2 sxa2Δ::ura4 pom1::GFP(kanR) leu1-32 ura4-D18 h-	This study
cyr1Δ::LEU2 sxa2Δ::ura4 tipYFP (kanR) tea1GFP(kanR) leu1-32 ura4D18 h-	This study
cyr1Δ::LEU2 sxa2Δ::ura4 tipYFP (kanR) tea2GFP(kanR) leu1-32 ura4D18 h-	This study
cyr1Δ::LEU2 sxa2Δ::ura4 tea2GFP(kanR) leu1-32 ura4D18 h-	This study
cyr1Δ::LEU2 sxa2Δ::ura4 tipYFP (kanR) leu1-32 ura4D18 h-	This study
cyr1Δ::LEU2 sxa2Δ::ura4 tea1GFP(kanR) leu1-32 ura4D18 h-	This study
cdc25-22cyr1Δ::LEU2 sxa2Δ::ura4+ ura4-D18 leu1-32 tea1GFP (kanR) h-	Stern and Nurse, 1997
wt h90	Cell cycle laboratory
tea1Δ::ura4 ura4D18 h90	Cell cycle laboratory
tea2.1 h90	This study
tip1Δ::kanR h90	This study
tea2GFP(kanR) h90	This study

Immunoblot analysis

Preparation of total boiled protein extracts, western blot analysis and detection of proteins were carried out as previously described (Yamaguchi et al., 2000). The result for Tea1p differs from previously published data (Mata and Nurse, 1997) and is caused by a different extraction procedure. Mata collected the cells, boiled them and broke the cells using glass beads, he then spun the samples and only took the supernatant for further analysis; we never spun the samples and loaded the whole extract onto the gel.

SDS-polyacrylamide gels for Tea1p and Tip1p were prepared using a 203.25/1, mono/bisacrylamide mix and samples were run on long gels. The antibodies used were polyclonal anti-Tea1 at 1:1000 (Mata and Nurse, 1997), anti-Tip1 at 1:2000 (Brunner and Nurse, 2000a) and polyclonal anti-GFP at 1:1000 (a gift from Ken Sawin, Wellcome Trust Centre for Cell Biology, Institute of Cell and Molecular Biology, University of Edinburgh, UK). Pom1HA was detected with monoclonal 16B12 anti HA at 1:2000 (BABCO).

Phosphatase assay

Native cell extracts were made as described (Yamaguchi et al., 2000) in HB buffer (25 mM MOPS pH 7.2, 15 mM $MgCl_2$, 15 mM EGTA, 1 mM DTT, 1% Triton X-100) with the Protease Inhibitor Set (Roche). Cell extracts were then incubated with Lambda Phosphatase (New England Biolabs) in its buffer supplemented with MnCl for 25 minutes at 30°C in the presence or absence of phosphatase inhibitors (60 mM β-glycerophosphate, 12 mM p-nitrophenylphosphate, 0.1 mM sodium vanadate). The reactions were stopped by the addition of 2× sample buffer and boiling for 3 minutes.

Assay of Tea1GFP binding to microtubules

A *cdc25-22 cyr1Δsxa2Δ* culture was grown overnight at 25°C to 4×10^6 cells/ml, shifted to 36°C for 90 minutes to arrest cells in G2 and then 3 μg/ml of P factor was added. After a further 2 hours, the cells were shifted to 25°C for 2 hours and 20 minutes to allow progression into G1. Samples were taken for microtubular repolymerisation at 90 minutes, just before the shift-down and at the end of the time course.

25 μg/ml of MBC (Carbendazim) (added from a 5 mg/ml freshly made stock in DMSO) was added to a cell culture for 10 minutes at 36°C or 15 minutes at 25°C to totally depolymerise microtubules. To visualise tubulin, cells were collected onto Millipore filters (0.45 μm pore size) and washed for 50 seconds with minimal medium without MBC to allow partial repolymerisation of the microtubules. The filters were then dropped into 20 ml of –70°C methanol to fix the cells, which were then processed as described for tubulin immunofluorescence. For visualisation of Tea1GFP in live cells, 50 μl of cells with MBC were placed on 35 mm glass bottom dishes (MatTek Corporation) coated with 20 μg/ml Soybean Lectin (Biochem), which allows the cells to stick without moving while the media is changed. The dish with the cells was placed on an inverted LSM 510 confocal microscope and 1 ml of preconditioned media without MBC was added to the dish. This diluted the MBC to a concentration that allowed microtubules to repolymerise, and the cells were imaged 50 seconds later. For experiments carried out at 36°C, the dishes were pre-heated to 36°C before adding the cells, all media and dishes were kept at 36°C in a heating block and the microscope and stage used were inside a chamber heated to 36°C.

Live GFP microscopy

Cells were mounted on a glass slide in a volume of growth medium sufficient to trap but not to squeeze the cells. Cells were visualised with a Zeiss LSM 510 laser scanning confocal microscope. A GFP specific filter was used to visualise GFP alone. For colocalisation, a single section through the middle of the cell was taken using a YFP optimised filter set to visualise Tip1YFP and a CFP optimised filter set for Tea2GFP and Tea1GFP. YFP was excited at 514 nm and detected with a LP530 filter; GFP was excited at 458 nm and detected with a LP475 filter. The images were collected by line scanning and the two channels were excited sequentially at very high speed. Because of laser variability the amplitude gain and the offset were adjusted every time to ensure that there was no significant crossover between channels and all imaging was then carried out with the same settings. Pom1GFP and phase images were taken using a Zeiss Axioplan microscope mounted with a Hamamatsu camera.

Results

Tea1p, Tea2p and Tip1p are present during shmooing growth

We investigated the differences between the vegetative and shmooing growth modes by looking at the behaviour of the morphological factors Tea1p, Tea2p and Tip1p after pheromone addition. All our experiments were carried out in a *cyr1Δsxa2Δ* strain (Imai and Yamamoto, 1994), which responds to exogenous pheromone in rich media. This allows us to separate the pheromone response from the effects of nitrogen starvation, which is required to induce shmooing in wild-type cells, and therefore permits us to analyse the effects of a single external signal (pheromone) on cell shape. In our time courses, pheromone was added to exponentially growing

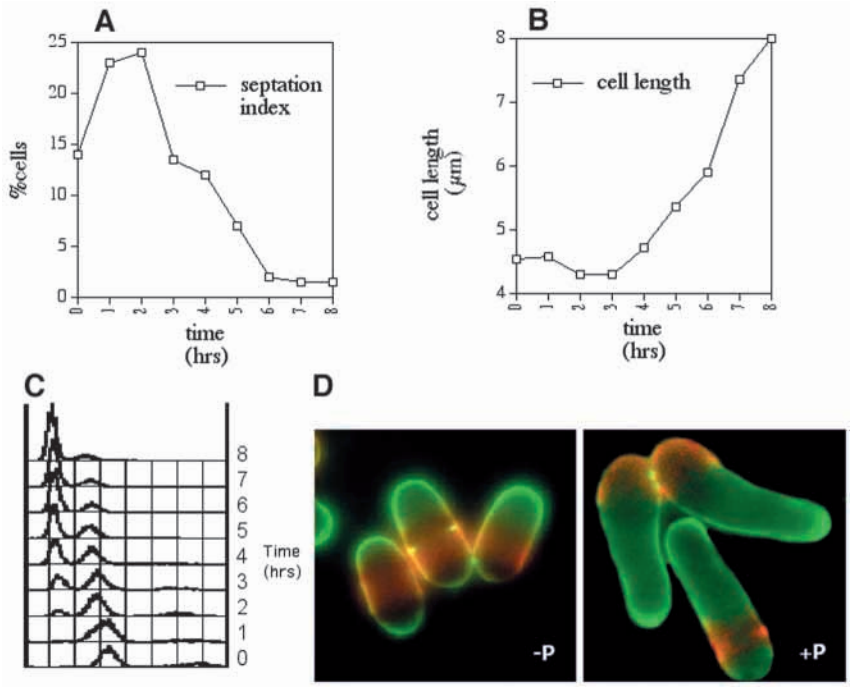

Fig. 1. *Cyr1*Δ*sxa2*Δ strain in the presence of pheromone. 3 μg/ml of P factor was added at time 0 and samples were taken every hour. (A) Cells were stained with calcofluor and the sepation index was monitored. (B) The cells were imaged with a phase microscope mounted with a Hamamatsu camera and cell length was measured using NIH Image software. (C) Cells were fixed in ethanol and processed for FACS analysis. (D) The cell wall was stained with 1:1000 (from a 5 mg/ml stock in water) FITC-lectin (red), for 10 minutes; the lectin was then washed out and the cells were allowed to grow for 8 hours in the presence or absence of pheromone. Cells were then stained with calcofluor (green), which stains growing ends. The areas in red have not grown since the lectin pulse, whereas the areas in green have.

cells and these cells were then followed for up to 8 hours. During this time the cells arrested in G1, with the septation index dropping to 3% after 6 hours and remaining low for up to 9 hours (Fig. 1A,C), but continued to grow actively from one end, as shown by lectin staining and cell length (Fig. 1B,D).

Initially we examined the levels of Tea1p, Tea2p and Tip1p by western blotting at different time points after pheromone addition and found that they remain essentially constant during the pheromone time course (Fig. 2A). The only change we detected was a slightly different band pattern for Tip1p and Tea1p after 2.5 hours in pheromone (marked by arrows in Fig. 2A,B). Phosphatase treatment suggests that the new faster migrating form for Tea1p may be due to dephosphorylation (Fig. 2C). We conclude that all three proteins are present in the cell during shmooing growth, although they might be differentially phosphorylated compared with vegetatively growing cells. The result for Tea1p differs from previously published data (Mata and Nurse, 1997), and is due to a more complete extraction procedure during the sample preparation in the present study (see Materials and Methods for details)

Localisation of Tea1p, Tea2p and Tip1p

If Tea1p, Tea2p and Tip1p exert a similar role in cell morphogenesis during shmooing growth to that in vegetative growth, they might be expected to localise to the same place within the cell. To investigate this we analysed the localisation of GFP tagged versions of the three proteins in live cells in the presence or absence of pheromone. Tea1GFP (R. Bahrens, personal communication), which replaces the endogenous Tea1p, fully rescues the mutant phenotype of *tea1*Δ cells. Cells

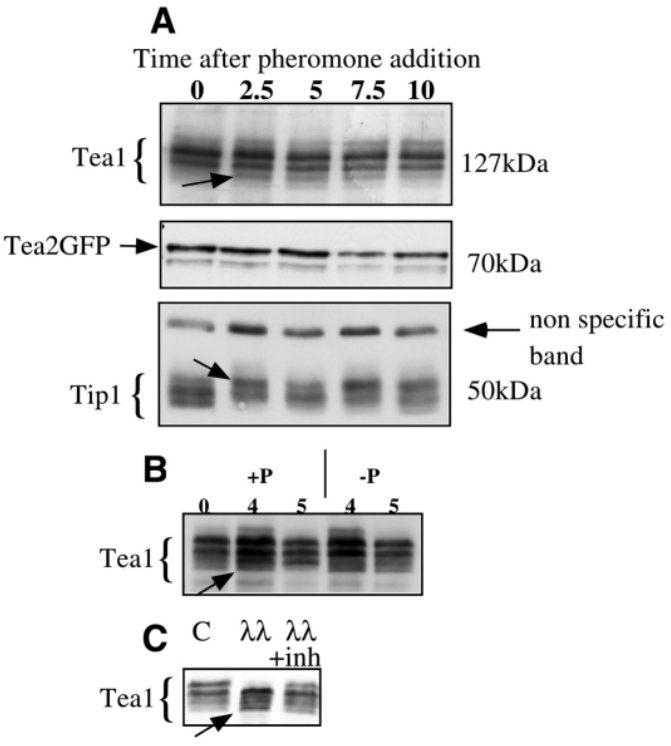

Fig. 2. Protein levels during a pheromone time course. (A) Western blot of total cell extracts from a pheromone time course probed with anti-Tea1, anti-GFP (for Tea2GFP) and anti-Tip1 antibodies. New forms of protein appearing after pheromone addition are marked by arrows. (B) Cells were shifted to 36°C in the presence or in the absence of pheromone for 4 hours. Cells at 36°C in the presence of pheromone arrest in G1 but do not activate the shmooing growth mode (Fig. 7B,C). Cells were then released at 25°C to allow synchronous progression into shmooing growth. Samples were taken at time 0, after 4 hours at 36°C and after 1 hour release and western blots were carried out on total cell extracts for Tea1p. (C) Native cell extracts were made from vegetatively growing cells and treated with λ-phosphatase in the absence (λλ) or in the presence (λλ+inh) of phosphatase inhibitors. An untreated sample is run as a control (C). Western blots were probed for Tea1p. The lower dephosphorylated form, which increases after phosphatase treatment, is marked by an arrow.

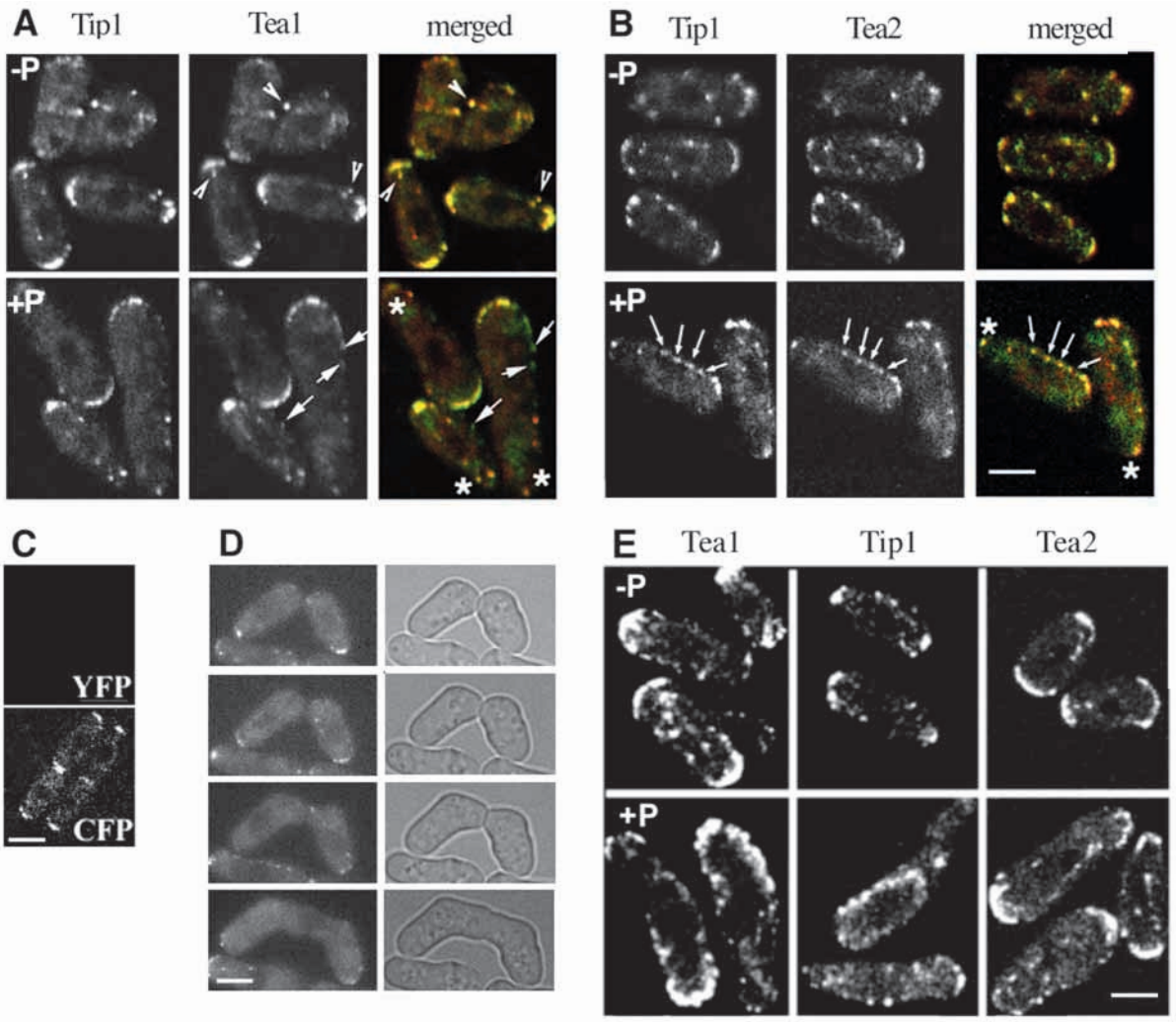

Fig. 3. Tea1p, Tip1p and Tea2p partially delocalise in the presence of pheromone. (A) *tip1YFP-tea1GFP* (B) and *tip1YFP-tea2GFP* strains were used to determine colocalisation in vivo. Cells were grown in minimal medium without or with pheromone for 5 (A) or 7 (B) hours. Pictures were taken with a confocal microscope using YFP and CFP filters, which allow excitation and detection of each fluorophore separately. The more narrow ends, marked by an asterisk, are known to be growing from previous calcofluor and actin stainings. The arrows in A indicate the Tea1GFP dots co-localising with Tip1YFP in the absence of pheromone but not in the presence of pheromone. In B the arrows indicate the Tea2GFP dots co-localising with Tip1YFP both in the presence and absence of pheromone. Scale bar, 3μm. (C) Images of a *tea2GFP* strain in the YFP and CFP channels, showing there is no bleed through in the YFP channel. Bar, 5 μm. (D) To confirm the localisation in a wild-type background, we mated an h90 Tea2GFP overnight on glutamate plates and photographed mating cells. Bar, 3 μm. (E) Images of Tea1GFP, Tip1YFP and Tea2GFP in the absence or after 6 hours in the presence of pheromone. Bar, 3 μm.

with Tea2GFP appear to be wild-type during vegetative (Browning et al., 2000) and shmooing growth.

During vegetative growth, Tea1GFP, Tip1YFP and Tea2GFP localise to cell ends and to a few dots on microtubules (R. Bahrens, personal communication; A. Decottignie, personal communication) (Browning et al., 2000).

After pheromone addition, Tea1GFP became mostly lost from the growing end and was redistributed along the cell periphery at the nongrowing larger end (Fig. 3E). After pheromone addition, Tea2GFP and Tip1YFP were also reduced at the growing end, accumulating at the non-growing end and in the cytoplasm, often as dots in a row (Fig. 3E). The same relocalisation was observed for Tea2GFP during an h90 mating. In conjugating cells, Tea2GFP was found to localise to the nongrowing ends with some dots in the cytoplasm (Fig. 3D).This demonstrates that the delocalisation of these factors is also observed in cells undergoing normal conjugation. We conclude that Tea1p, Tea2p and Tip1p are no longer specifically located at the growing ends of shmooing cells, and therefore their role in polarising cellular growth in these cells may not be the same as that in vegetatively growing cells.

Tea2p and Tip1p are known to be associated during the vegetative cell cycle (D. Brunner, personal communication) and Tea2p may be the motor protein that transports Tea1p to the ends of the cell (Browning et al., 2000). These factors might therefore colocalise during vegetative growth and, if they no longer have a role in shmooing growth, their association might fall apart. Visualisation of Tea2GFP in combination with Tip1YFP revealed that Tea2p always co-

Table 2. Quantitation of the colocalisation of Tea1GFP with Tip1YFP

	–pheromone	+pheromone
Free Tea1p	1.5% (*n*=193)	17% (*n*=95)
Free Tip1p	16% (*n*=227)	23% (*n*=102)

The colocalisation of Tip1YFP and Tea1GFP was quantitated for the strain *tea1GFP tip1YFP cyr1*Δ*sxa2*Δ in the absence and in the presence of pheromone. The number of Tip1YFP dots without any Tea1GFP and the number of Tea1GFP dots without any Tip1YFP was scored and each expressed as a percentage of the total number of dots scored (*n*).

localises with Tip1p in the presence and absence of pheromone (Fig. 3B). In contrast, analysis of Tea1GFP in combination with Tip1YFP revealed that there is a tenfold increase in the number of Tea1GFP dots that do not co-localise with Tip1YFP in the presence of pheromone (Fig. 3A, Table 2). Most of the free Tea1p dots localise to the peripheral region near the non-growing end (Fig. 3A). The Tea1p in the cytoplasm may still be on microtubules where it is co-localised with Tip1p, but once it reaches the cell end it diffuses along the periphery of the cell while Tip1p remains more concentrated at the cell tip. Therefore, during shmooing growth the colocalisation of Tip1p and Tea2p is maintained, but the colocalisation of Tea1p and Tip1p, and by inference Tea1p and Tea2p, is reduced.

Tea1p association with microtubules

It is thought that cells predominantly respond to pheromone in the G1 phase of the cell cycle (Stern and Nurse, 1998). Therefore, when cells enter G1 in the presence of pheromone, the properties of microtubules or Tea1p may be altered, reducing the association between them. To test this possibility, we compared the efficiency of Tea1p binding to re-polymerising microtubules in G2 arrested cells that do not respond to pheromone, and in G1 cells that do respond to pheromone. Live Tea1GFP images (Fig. 4Ea,b) during a G2 block, in the absence and presence of pheromone, show short linear arrays of dots, which are similar to the re-polymerising microtubule arrays seen by tubulin immunostaining (Fig. 4Da,b) of the same population. This suggests that Tea1GFP may co-localise with re-polymerising microtubules. On the contrary, when cells enter G1 and become responsive to pheromone, Tea1GFP is no longer found on re-polymerising microtubules (Fig. 4Dc, Ec).

We confirmed that the Tea1GFP dots corresponded with microtubules in G2 arrested cells by repeating the experiment using an untagged *tea1* strain, fixing the cells in methanol and co-staining for tubulin and Tea1p in the same cells. Cells arrested in G2 without pheromone (Fig. 4A) and G2 cells with pheromone (Fig. 4B) show Tea1p on microtubules. As before, after entry into G1 in the presence of pheromone (Fig. 4C), Tea1p is not found on microtubules.

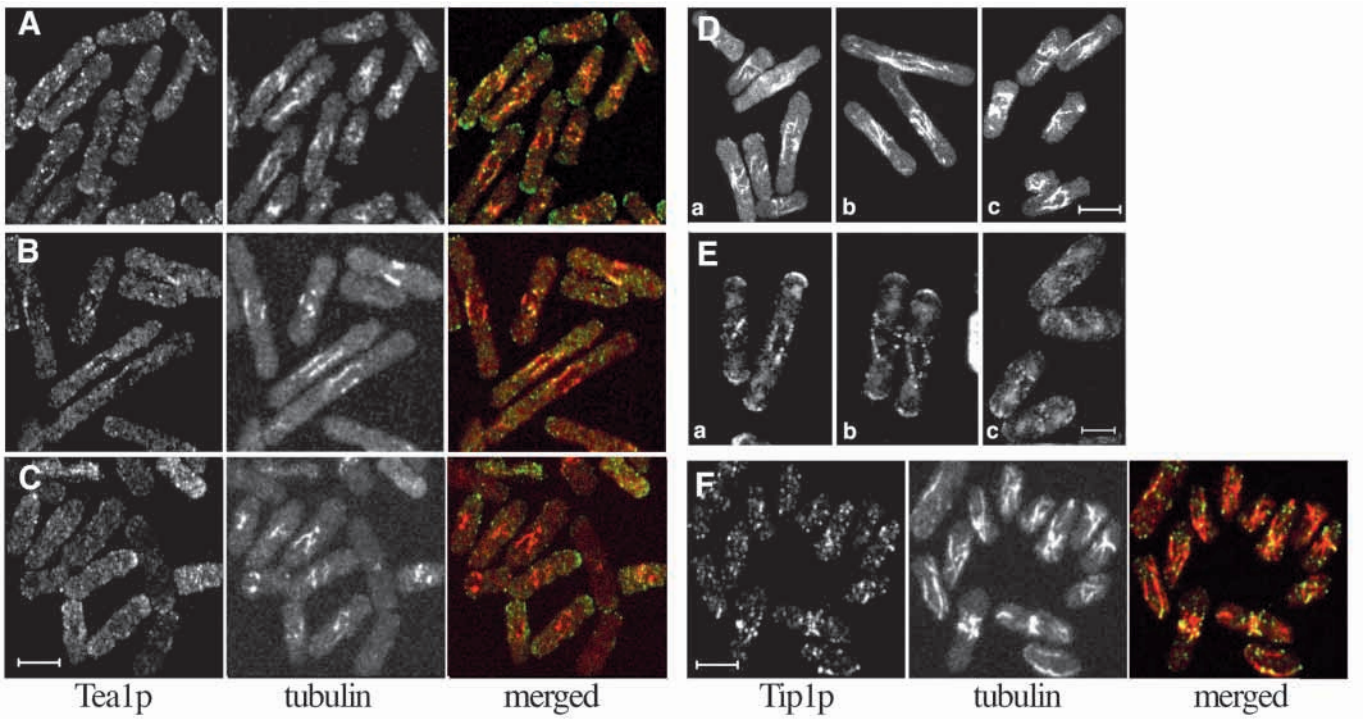

Fig. 4. Tea1 localisation in the presence of pheromone in G2 and G1. A *cdc25-22cyr1*Δ*sxa2*Δ strain was arrested in G2 at 36°C for 90 minutes; pheromone was added and cells were incubated for a further 2 hours; cells were then released into cell cycle progression at 25°C in the continued presence of pheromone. Samples were taken at three different time points: (A) after cells had been arrested in G2 at 36°C in the absence of pheromone; (B) after having been incubated with pheromone for 2 hours at 36°C; and (C) after cells had been allowed to enter G1 at 25°C in the continued presence of pheromone. Cells from each time point were treated with MBC, a microtubule depolymerising drug, to totally depolymerise microtubules. The drug was then washed out and microtubules were allowed to repolymerise for 50 seconds. Cells were fixed in methanol and processed for tubulin and Tea1p immunofluorescence. (D,E) The same experiment was repeated with a *cdc25-22cyr1*Δ*sxa2*Δ*tea1GFP* strain, timepoints a, b and c correspond to points A, B and C. (D) Cells were fixed in methanol and processed for tubulin while Tea1GFP was imaged live on a confocal microscope (E; see Materials and Methods for details). (F) Cells from C (after the release into G1 in the presence of pheromone) were also immunostained for Tip1p and tubulin. Bars, 5 μm.

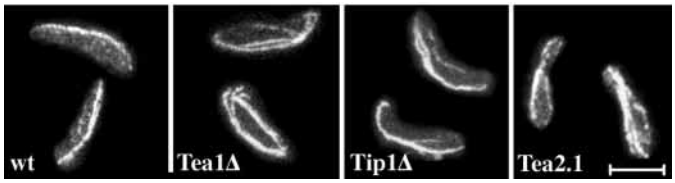

Fig. 5. Microtubules in morphological mutants. Cells were treated with 3 μg/ml P factor for 9 hours, fixed in methanol and then stained with anti-tubulin antibody. All strains are *cyr1*Δ*sxa2*Δ. Bar, 5 μm.

The lack of association of Tea1p with microtubules in cells responsive to pheromone could mean that Tea1p is no longer efficiently transported along microtubules and so does not accumulate at the growing end of the cell. In contrast, Tip1p can still associate with growing microtubules in G1 cells treated with pheromone (Fig. 4F), and therefore a lack of microtubular association is unlikely to be the reason for Tip1p not being found at growing cell ends.

Phenotype of cells lacking Tea1p, Tea2p and Tip1p

Since Tea1p, Tea2p and Tip1p are delocalised in response to pheromone, they might no longer play a role during shmooing. To investigate this we examined the microtubular phenotype in *tea1*Δ, *tea2.1* and *tip1*Δ cells treated with pheromone. Microtubules of vegetatively growing *tea1*Δ cells are slightly longer than wild-type and some can curl around their cell ends (Mata and Nurse, 1997) whereas, in *tea2.1* (a null mutant) and *tip1*Δ cells, microtubules are shorter and rarely reach the cell ends (Verde et al., 1995; Browning et al., 2000; Brunner and Nurse, 2000a). If these factors no longer have a role during shmooing growth, then microtubules should look like wild-type after pheromone treatment. This was indeed the case. All three mutants were able to detect and respond to pheromone by arresting in G1, as shown by FACS analysis (Fig. 6B), and displayed wild-type microtubules (Fig. 5; Tables 3, 4). Control cells arrested in HU still showed the mutant phenotype, which demonstrated that the effect was not related to cell elongation.

During vegetative growth, Tea1p, Tip1p and Tea2p also affect the ability of a cell to position a growth zone correctly (Browning et al., 2000; Brunner and Nurse, 2000a; Mata and Nurse, 1997). Therefore we investigated the ability of the three null mutants to correctly reorganise a single growth zone during the switch from vegetative to shmooing growth. Vegetatively growing cells mostly extend in a bipolar fashion (Mitchison and Nurse, 1985), whereas shmooing cells set up a single growth zone (Petersen et al., 1998b). Actin mostly localises to sites of active growth and is usually localised to both ends of vegetative cells (Marks and Hyams, 1985), but only to one end during shmooing growth (Petersen et al., 1998b). After pheromone addition cells switch from a vegetative bipolar actin localisation to a monopolar localisation. The relocalisation of actin can therefore be taken as an indicator of the onset of shmooing. This assay allows the switch between the bipolar actin localisation seen during vegetative growth and the tight monopolar actin localisation seen during shmooing to be monitored. Cells were scored as being monopolar only if they showed no actin or only one actin dot at the other end. Most monopolar mutants, *tea1*Δ included, are monopolar for growth and show actin mostly localised at the growing end but often have a few dots of actin at the non-growing end and therefore would have been scored as bipolar. The assay gives an accurate indication of the switch from vegetative growth to a shmooing growth pattern, which shows no actin at all at the non-growing end.

In vegetative wild-type cells actin was initially bipolar, and after 2.5 hours in pheromone it became monopolar in 50% of the cells (Fig. 6A), indicating that half of the cells have activated shmooing growth by 2.5 hours. In contrast, all three mutants responded more rapidly, relocalising actin in 50% of the cells within 1.5 hours (Fig. 6A). These results show that Tea1p, Tea2p and Tip1p are not required to reorganise a shmooing tip and, in fact, their presence can cause some delay in the switch to the new growth mode. To analyse the effect of faster shmooing rates on mating we scored the number of fused cells in a time course for mating in h90 wild-type and mutant strains. *Tea2.1* and *tip1*Δ strains mated faster than wild-type, suggesting that the faster shmooing rates may lead to faster conjugation (Fig. 6C). In contrast, *tea1*Δ cells mated at the same rate as wild-type. It is possible that Tea1p could be required for a subsequent step in the mating pathway or for efficient arrest during nitrogen starvation. In support of this we observed more septating cells in *tea1*Δ than in wild-type after 6 hours without nitrogen (data not shown).

Table 3. Microtubule length in the presence and absence of pheromone

Mutant	Mean cell length	Mean MT length	TTEST
wt+HU	7.35±1.60	7.06±1.61	0.13
wt+P	7.00±2.26	6.80±2.17	0.59
Tea2.1+HU	6.02±1.82	4.34±1.26	1.09×10^{-5}
Tea2.1+P	6.44±1.40	6.44±1.40	0.933
*Tip1*Δ+HU	6.86±1.68	4.90±1.82	1.83×10^{-12}
*Tip1*Δ+P	6.86±1.96	6.58±2.10	0.20

Cells were treated with P factor or HU for 8 hours. The length of microtubules and the length of the cells was measured in μm by using NIH Image software in three independent experiments (n=100×3). The length of the microtubules was then compared with the length of the cells by using a TTEST, which gave an indication of how similar the microtubule length was to the cell length. A TTEST value of 1 means that the length of microtubules is the same as the length of the cell.

Table 4. Microtubules bending round the end of the cell

Strain	+HU	+P
wt	3%	20%
*tea1*Δ	17%	23%
*tip1*Δ	0%	25%
tea2.1	0%	25%

The percentage of cells with microtubules curving round the cell ends was scored for wild-type and mutant cells treated with P factor or HU, as a control, for 8 hours. At least 100 cells were scored in three independent experiments.

Roles of microtubules and actin during shmooing

Next we decided to analyse the role of the cytoskeleton itself in the shmooing process. First we tested whether properly organised actin was required for shmooing. We depolymerised actin using the drug latrunculin A (LatA), which inhibits actin

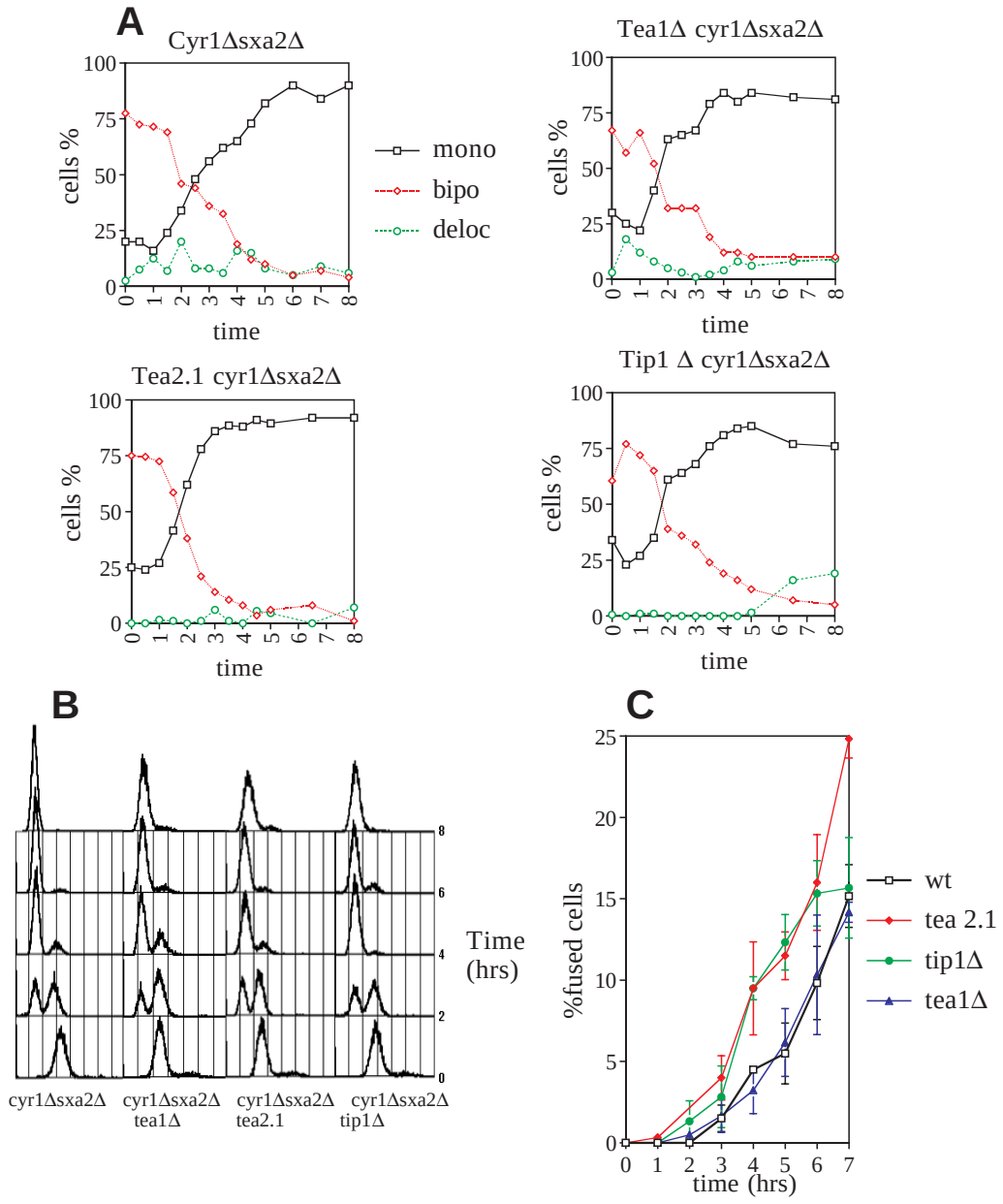

Fig. 6. Actin relocalisation in the mutants strains. 3 μg/ml of P factor was added at time 0. (A) Samples were taken every 30 minutes, fixed in formaldehyde for 40 minutes and stained for actin with rhodamine phalloidin. Actin localisation was scored for each time point. (B) Samples were taken every 2 hours, fixed in ethanol and processed for FACS analysis. (C) h90 wild-type and mutant strains were grown overnight in full nitrogen and low glucose medium (10 g/l) to 2×10^7 cells/ml. Cells were then spun and re-suspended in low glucose, nitrogen-free medium at 1×10^6 cells/ml to induce mating and fused cells were scored every hour. Experiments were repeated at least three times and at least 200 cells were scored for each time point. Standard deviation was calculated and error bars are shown.

polymerisation, and looked at the ability of the cells to shmoo. Not surprisingly, since polymerised actin is known to be essential for growth, cells were unable to shmoo in the presence of LatA (Fig. 7A).

Next we examined the requirement for an intact microtubular cytoskeleton, which is not essential for cell end extension during vegetative growth (Sawin and Nurse, 1998).

Since cells treated with pheromone at 36°C arrest in G1 with bipolar actin and do not activate monopolar shmooing growth (Fig. 7B,C), we could arrest cells at 36°C in the presence of pheromone, depolymerise microtubules with the drug MBC, and then release the cells at 25°C to allow them to activate shmooing growth in the absence of microtubules (Fig. 7D). We then monitored the rate of shmooing by looking at the relocalisation of actin from bipolar to monopolar. The switch from bipolar to monopolar actin localisation was unaffected by the absence of microtubules (Fig. 7D). This demonstrates that microtubules play no role in the establishment of a single polarised growth zone during shmooing. However, some T-shaped cells were observed (7%), similar to the proportion seen in vegetative growth in the presence of MBC (K. Sawin, personal communication), suggesting that microtubules may play some role in the correct positioning of a growth zone within the cell.

Pom1p does play a partial role in the switch to shmooing growth

We also examined the role of Pom1p in the pheromone response. Pom1p plays a major role in the establishment of cell polarity (Bahler and Pringle, 1998); acting downstream of Tea1p (Bahler and Pringle, 1998). The Pom1HA protein was still present during shmooing growth (Fig. 8A), and Pom1GFP,

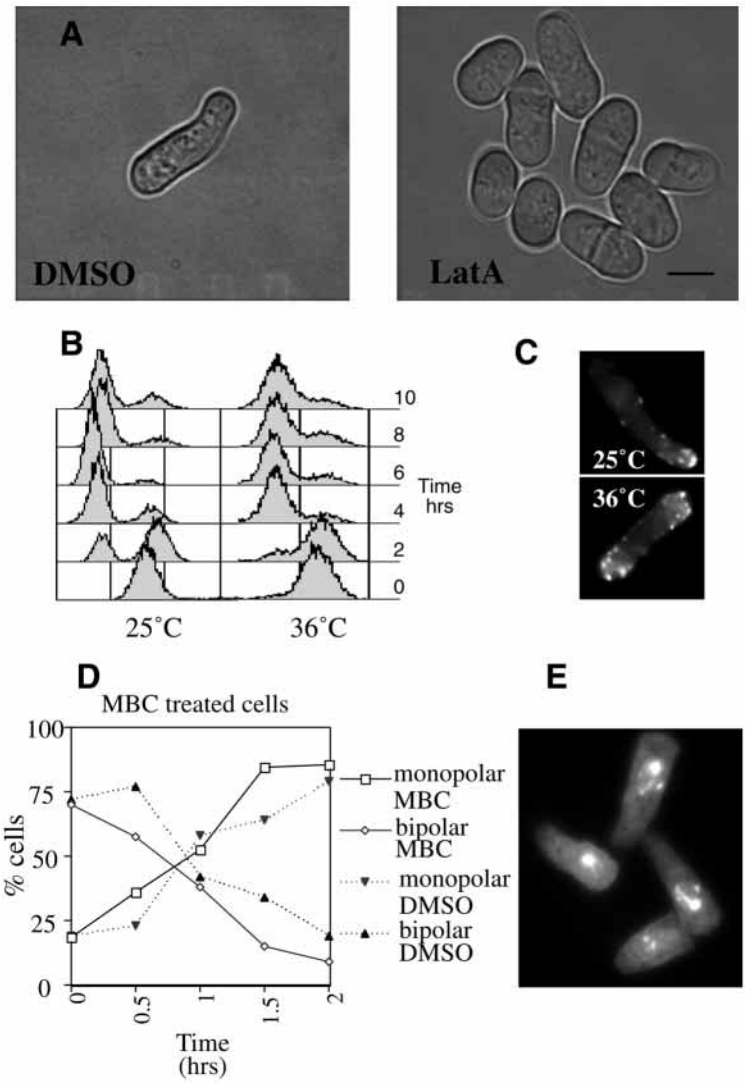

Fig. 7. Ability of the cells to shmoo in the presence of drugs. (A) Cells were treated with 3 μg/ml of P factor for 4 hours at 36°C. Cells were then shifted to 25°C in the presence of 200 μg/ml of LatA or DMSO, as a control, and visualised under the light microscope. 3 μg/ml of P factor was added to cells at 25°C and at 36°C. (B) Samples were taken every 2 hours, fixed in ethanol and processed for FACS. (C) After 8 hours, cells were fixed in formaldehyde and stained for actin. (D) Cells were arrested at 36°C in the presence of P factor for 4 hours; 25 μg/ml of MBC or DMSO, as a control, were added and the cells were shifted to 25°C to induce shmooing. Samples were taken every 30 minutes, fixed in formaldehyde, stained with rhodamine phalloidin to visualise actin and scored for actin localisation. Times shown are calculated from the shift to 25°C. (E) Cells treated with MBC were fixed in methanol at the end of the time course and processed for tubulin immunofluorescence to verify that no microtubules were present. Bar, 5 μm.

which is located at the cell ends during vegetative growth, became distributed more generally throughout the cell after addition of pheromone (Fig. 8B).

Next, we analysed the role of Pom1p by looking at the ability of *pom1*Δ cells to shmoo. FACS analysis showed that the cells responded to pheromone, arresting with similar kinetics to wild-type (Fig. 8C). Actin was mostly monopolar during vegetative growth but, as soon as pheromone was added, the proportion of cells with monopolar actin dropped and that of cells with delocalised actin increased (Fig. 8D). Close comparison of actin localisation for the first 2 hours in a *pom1*Δ strain and wild-type (Fig. 8E) showed a marked difference between the two. In wild-type cells actin remained bipolar for the first 1.5 hours whereas, in *pom1*Δ cells, delocalised actin increased within 0.5 hours.

In wild-type monopolar cells actin becomes bipolar upon commitment to mating, and then relocalises to the shmooing end (Petersen et al., 1998b). Pom1p therefore may be required to maintain the cell end localisation of actin during the early stages of the switch to shmooing growth. The subsequent establishment of a monopolar localisation for actin was also slower and less efficient in *pom1*Δ cells. Even after 6.5 hours, only 65% of the cells were monopolar for actin and 27% of cells still had delocalised actin whereas, by this time point, over 90% of wild-type cells were monopolar for actin (Fig. 6).

Discussion

In this paper we have shown that the three morphological factors, Tea1p, Tea2p and Tip1p, required for cell polarisation in vegetative growth do not play a role in polarising cell growth in pheromone. In the presence of pheromone these three factors are all present in the cell but are no longer localised to the growing end. The cell appears to shut down the vegetative mechanism of polarisation, which maintains the cell growing in a straight line, by triggering the delocalisation of these key morphological factors. This would allow the new, shmooing growth mode to be established. Tea1p and Tip1p display a different banding pattern, which might be due to differences in phosphorylation (D. Brunner, personal communication; this study) and these modifications might contribute to their delocalisation.

In vegetative growth, Tea1p is thought to be transported along microtubules in association with Tea2p and Tip1p. Live imaging data suggest that Tea1p and Tip1p mostly co-localise during vegetative growth, but in the presence of pheromone there is an increase in free Tea1p. Most free Tea1p is localised to sites near the cell cortex close to the non-growing end, suggesting that the complex is less strongly held together and falls apart when it reaches the cell ends. By contrast, Tip1p and Tea2p, which also co-localise during vegetative growth (D. Brunner, personal communication; this study), still co-localise in the presence of pheromone, suggesting that their association remains intact. These results support the idea that the association of Tea1p with Tip1p-Tea2p is not as strong during shmooing growth as it is during vegetative growth.

In response to pheromone, Tea1p also no longer appears to be associated efficiently with microtubules and this could explain why Tea1p does not accumulate at the cell ends. Preventing a cell end marker for vegetative growth, such as Tea1p, from reaching cell ends may be an important step in the initial dismantling of the intrinsic growth mechanism, allowing the assembly of a new polarised projection directed by an extrinsic signal. Since Tea1p may act as an anchoring factor

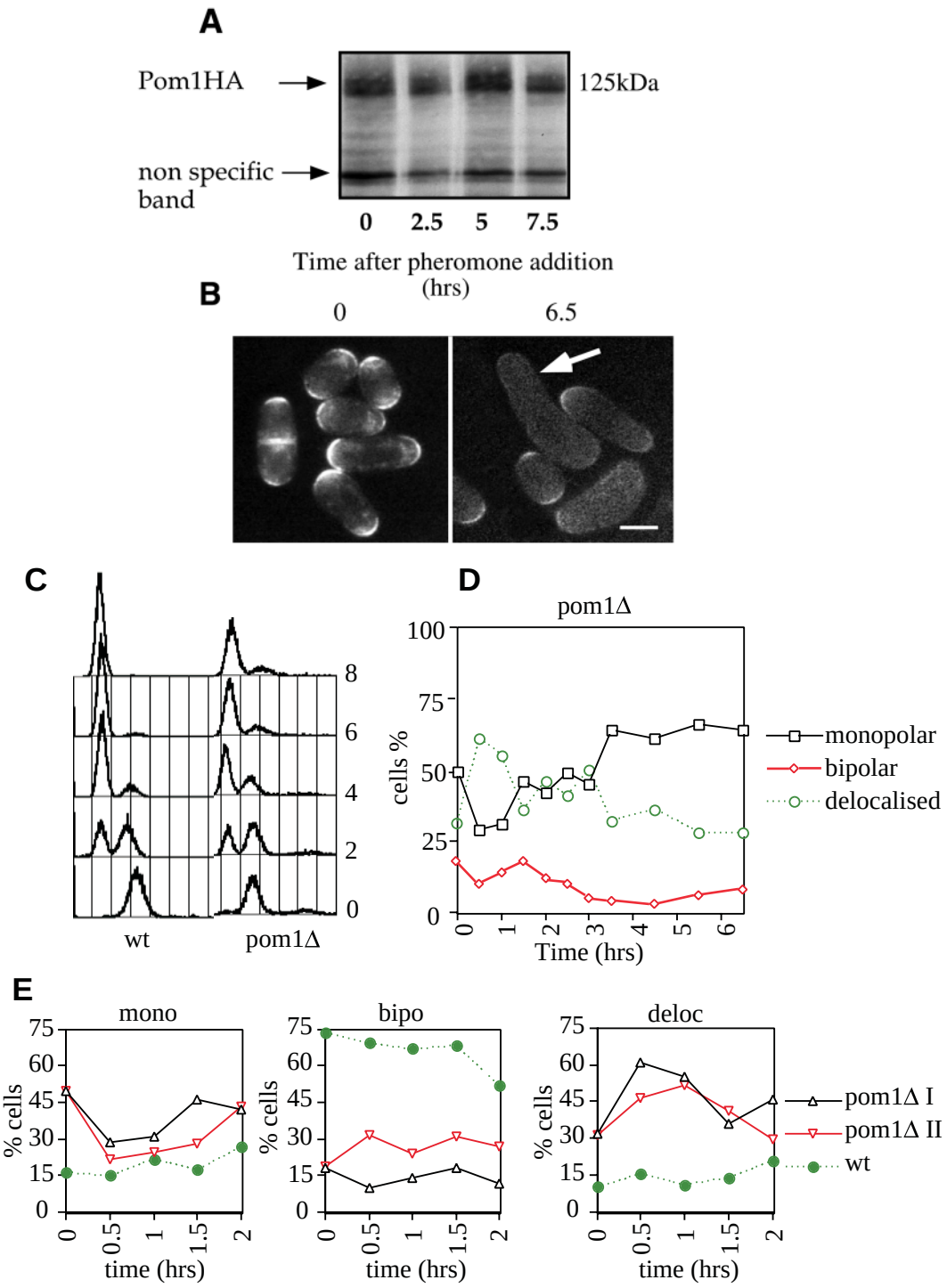

Fig. 8. Pom1 behaviour in the presence of pheromone. 3 μg/ml of P factor was added at time 0 to a *cyr1*Δ*sxa2*Δ*pom1HA* strain (A) or a *cyr1*Δ*sxa2*Δ*pom1GFP* strain (B). (A) Western blot of total cell extracts probed with anti-HA antibody to visualise Pom1p. (B) Pom1GFP in live cells at time zero and after 6.5 hours in pheromone. The arrow indicates the longest shmoo, which shows Pom1GFP diffused throughout the cell. 3 μg/ml of P factor was added at time 0 to a *pom1*Δ*cyr1*Δ*sxa2*Δ cell. (C) Samples were taken every 2 hours, fixed in ethanol and processed for FACS analysis. (D) Samples were taken every 30 minutes, fixed in formaldehyde, stained with rhodamine phalloidin to visualise actin and scored for actin localisation. (E) The first 2 hours of the time course for two independent experiments (I and II) are plotted together with a wild-type control to show the rise in delocalised actin and the reduction in monopolar actin for *pom1*Δ.

for Tea2p and Tip1p at the cells ends (Browning et al., 2000; Brunner and Nurse, 2000a), the loss of Tea1p from the growing end may also stop the cell end accumulation of Tea2p and Tip1p. The microtubular dynamics might also be different during shmooing, since their appearance is different, and this could contribute to the loss of morphological factors from one

end. The loss of association between Tea1p and microtubules was only observed in G1 and not G2 cells with pheromone, suggesting that the decision to set up a new, shmooing polarised projection or to maintain the old vegetative growth pattern is made during G1.

In the presence of pheromone, *tea1*Δ, *tip1*Δ *and tea2.1*, all of which have microtubule defects during vegetative growth, exhibit wild-type microtubules, suggesting that Tea1p, Tea2p and Tip1p do not play a major role in regulating microtubular dynamics during shmooing. In fact, the presence of these factors seems to be inhibitory for shmooing growth as, in response to pheromone, all the mutants set up the new shmooing pattern of growth faster than the wild-type. FACS analysis showed that the mutants arrested with similar kinetics to wild-type cells, indicating that the response to pheromone occurs normally, and that it is only the switch to shmooing growth that is accelerated in these mutants. In the absence of any of these factors the vegetative system is probably partially defective and can be dismantled more easily, allowing the new mode of growth to be activated faster.

We found that microtubules themselves are not essential for the organisation of a single polarised projection. However, the generation of some T-shaped cells in the absence of microtubules, suggests that microtubules might play some role in the correct positioning of the growth zone during shmooing. Microtubules could also play a role in directing the polarised projection towards the pheromone source, but this possibility requires further investigation. Disrupting microtubules with TBZ concomitant with nitrogen starvation totally inhibits projection tip formation (Petersen et al., 1998a). This may be due to the fact that actin, as well as microtubules, is disrupted in the presence of TBZ (K. Sawin, personal communication), and lower amounts of TBZ were found to lead to the formation of H-shaped zygotes (Petersen et al., 1998a). Using LatA we found that actin was completely essential for a polarised projection to be formed, in agreement with previously published data (Petersen et al., 1998b).

After addition of pheromone to cells, actin first becomes bipolar and then relocalises to the shmooing end (Petersen et al., 1998b). This may be an initial re-setting step, which allows the cell to pick either end for shmooing growth, depending on which one is experiencing the higher pheromone concentration. Pom1p may be important for marking the cell ends for this initial relocalisation step since actin does not become bipolar in *pom1*Δ cells after pheromone addition. The subsequent relocalisation to one end is not as efficient as in wild-type cells, but this could be a consequence of the initial actin delocalisation.

We have shown that the morphological factors, Tea1p, Tea2p, Tip1p and Pom1p, involved in setting up and maintaining an internally established axis for vegetative cell growth are not required for shmooing growth. This probably reflects a fundamental difference in the way cell polarity is established during the two growth modes. In vegetative growth the landmarks for cell polarisation are set up internally and once the cell shape has been established the cell must be able to read that shape and maintain it. The microtubular dynamics and the ability of the cell to remember its previous ends appear to be suited for this purpose. During shmooing, the landmark for growth is positioned by an external signal so the cell needs to recognise that signal and position its growth site accordingly, thus altering its current cell shape. The externally directed machinery will have to alter rather than maintain cell shape. It will be interesting to establish whether, like *S. cerevisiae* (Nern and Arkowitz, 2000; O'Shea and Herskowitz, 2000; Shimada et al., 2000), there is a core polarisation machinery, which is directed to the internal landmarks during vegetative growth and to the externally marked site during shmooing growth.

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