A role for the yeast actin cytoskeleton in pheromone receptor clustering and signalling

Kathryn R. Ayscough* and David G. Drubin†

The development of cell polarity in response to external stimuli is a feature of most eukaryotic cell types. Haploid cells of the budding yeast *Saccharomyces cerevisiae* secrete peptide pheromones to induce conjugation with cells of the opposite mating type. Pheromone binding triggers transcription of mating-specific genes, cell cycle arrest in the G1 phase and the formation of a mating projection oriented toward the source of pheromone [1,2]. Based on a multitude of studies in diverse eukaryotic cells, it has been hypothesized that hierarchies of proteins function to govern the generation of cell polarity [3,4]. Numerous proteins have been identified in yeast that accumulate both at a position on the cell cortex that will develop into a mating projection in response to pheromone binding and at the site of bud formation in response to an intrinsic cue during mitotic growth. When the actin cytoskeleton is disrupted before bud formation by the addition of latrunculin-A (LAT-A), several proteins involved in budding, including the GTPase Cdc42p, are still able to achieve their appropriate polarized localization [5]. In contrast, we show here that following pheromone addition, an intact actin cytoskeleton is required for localization of several proteins to a discrete position on the cell cortex. We also demonstrate a role for actin in pheromone-induced receptor clustering and signalling. We propose that actin-mediated pheromone receptor clustering might consolidate signalling from Cdc42p to one region of the cell cortex so that small differences in receptor occupancy across the cell surface can be amplified into dramatic cellular polarity.

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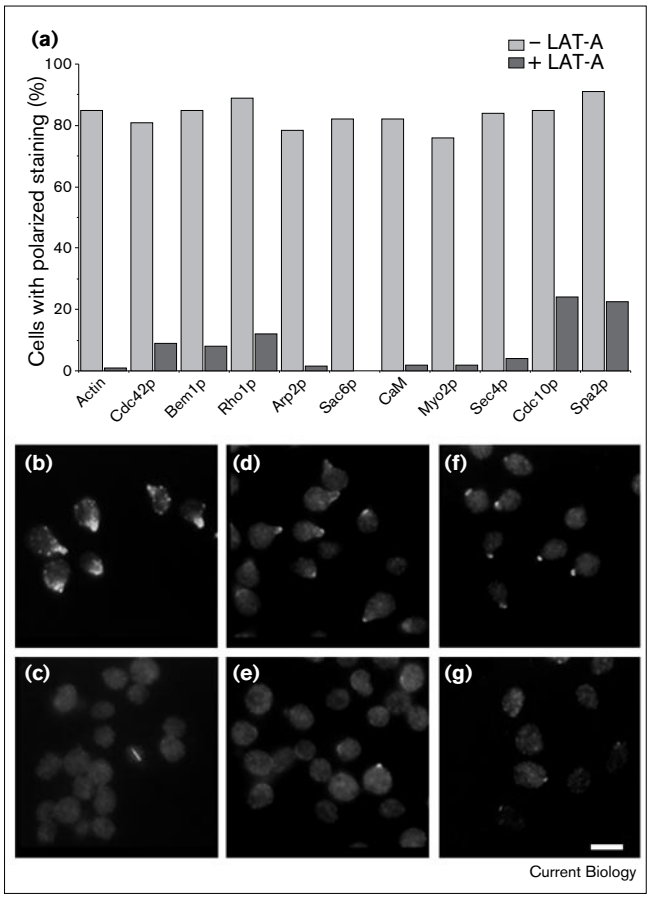
Results and discussion

Haploid cells of the MAT**a** mating type were released from stationary phase (G0) in the presence of the α-factor pheromone. The culture was divided, and LAT-A was added to one of the cultures to disrupt the actin cytoskeleton. Samples taken at different time points over a 3 hour period were fixed and processed for immunofluorescence microscopy. To establish a baseline for our comparisons, we first looked at the organization of actin itself. In cells released from stationary phase in the presence of α factor, cortical actin patches initially showed no polarized localization; 90 minutes after release, before projections were formed, actin patches were seen to be clustered on the cell

Figure 1



The effect of LAT-A on the localization of polarized marker proteins following release from G0 in the presence of α factor. **(a)** Samples were fixed and the indicated proteins were detected by immunofluorescence 3 h after cells were released from G0 in the presence of α factor and in the presence or absence of LAT-A. Staining was scored as polarized when observed as an intense patch at the cell cortex. Examples of immunofluoresence analysis are shown for **(b,c)** actin, **(d,e)** Cdc42p and **(f,g)** Spa2p in the absence (b,d,f) or presence (c,e,g) of LAT-A. The bar is 5 μm.

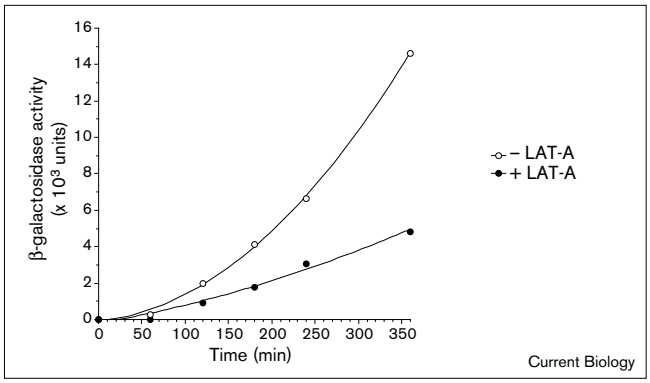
cortex. As the projections grew, the patches remained clustered at their tips (Figure 1a,b). In the presence of LAT-A, no actin cables or cortical actin patches were observed (Figure 1a,c). Cells treated with LAT-A were not able to form mating projections, although during the time course viability remained over 95%.

We repeated the procedure looking for polarized localization of 10 other proteins that normally localize to the mating projection (Figure 1a). In the absence of LAT-A, these proteins localized with kinetics very similar to those observed for actin (data not shown). In the presence of LAT-A, however, all of these proteins showed a dramatic reduction or a complete abrogation in their ability to become polarized at the cell cortex. Most significantly, the GTPase Cdc42p and Bem1p — an SH3-domain-containing protein involved in bud emergence and mating [6,7] — although able to develop a normal level of polarized localization in mitotically growing LAT-A-treated cells, were clearly unable to do so in LAT-A-treated cells exposed to α factor (Figure 1a,d,e). A marked reduction in polarity was also observed for the septin protein Cdc10p [8] and Spa2p, a protein that is highly polarized during growth and mating-projection formation [9], both of which were also able to develop a normal level of polarized localization in mitotically growing LAT-A-treated cells [5], but were not able to do so in LAT-A-treated cells exposed to α factor. In addition, proteins associated with vesicle trafficking (calmodulin, Myo2p and Sec4p [10–12]), Rho1p, which is associated with actin rearrangements and cell wall deposition [13,14], the actin-related protein Arp2p [15,16] and the actin-bundling protein Sac6p [17] showed no polarized localization in the absence of filamentous (F) actin.

As disruption of the actin cytoskeleton had such a dramatic effect on the localization of proteins with crucial roles in polarity establishment, we investigated whether signalling through the pheromone-responsive mitogen activated protein (MAP) kinase pathway was also affected. We generated a yeast strain carrying the *FUS1* promoter appended to the *LACZ* reporter gene. *FUS1* transcription is switched on rapidly following addition of α factor to these cells, and its expression requires a functional MAP kinase pathway. As shown in Figure 2, the absence of an intact actin cytoskeleton in cells treated with α factor for 4 hours caused a 60–70% reduction, with respect to control cells, in the level of β-galactosidase generated.

We postulated that the observed polarity and signalling defects might reflect an inability to polarize the α-factor receptor (Ste2p) in the absence of a functional actin cytoskeleton. Previous studies have shown that Ste2p becomes polarized within about 90–120 minutes of α-factor addition [18,19] and that during this time the receptors are first internalized, reappearing on the cell surface after about 60 minutes [20]. We constructed a yeast strain

Figure 2



The effect of LAT-A on signalling through the pheromone-responsive MAP kinase pathway. Cells carrying a *FUS1:LACZ* reporter construct were released from G0 in the presence of α factor. At the indicated time points, cells were processed for the β-galactosidase assay.

expressing Ste2p carrying a Myc epitope tag at its carboxyl terminus. Ste2p–Myc was functional as it was able to mediate both pheromone-induced mating projection formation and normal levels of signalling through the MAP kinase pathway, as detected by β-galactosidase production. As shown by immunofluorescence microscopy (Figure 3a), in the absence of α factor Ste2p localized over the entire surface of cells. Following addition of α factor, staining was lost from the cortex such that, between 10 and 30 minutes after the addition of α factor, only a small proportion of cells showed any Ste2p staining at the cell surface (Figure 3b). During this time, staining was observed in what appeared to be distinct cytoplasmic organelles. After 60 minutes, polarized Ste2p staining at the cell surface was observed, frequently in the vicinity of the Ste2p-containing organelles (Figure 3c). Once polarized, Ste2p remained at the tip of the mating projection throughout the time course (3 hours; Figure 3d).

In order to determine whether actin had a direct role in the polarization of Ste2p, in addition to its previously recognized role in endocytosis [21], cells were incubated in the presence of α factor for 30 minutes, after which time 86% of cells had no cortical staining for Ste2p. LAT-A was then added and further samples were taken to follow Ste2p localization. Disruption of the actin cytoskeleton resulted in an inability of Ste2p to localize in a polarized manner. Instead, we observed that Ste2p reappeared at the cell surface, but it was not polarized (Figure 3e,f). Quantitation of Ste2p localization in LAT-A-treated cells revealed that there did appear to be some level of polarity when Ste2p first reached the cell surface. After 60–90 minutes, some 40% of cells showed Ste2p staining over less than half of the cell surface, though this staining was rarely what we scored as 'polarized' in Figure 3f, in which only cells that stained in an intense patch on the cell surface were counted. This partial polarity

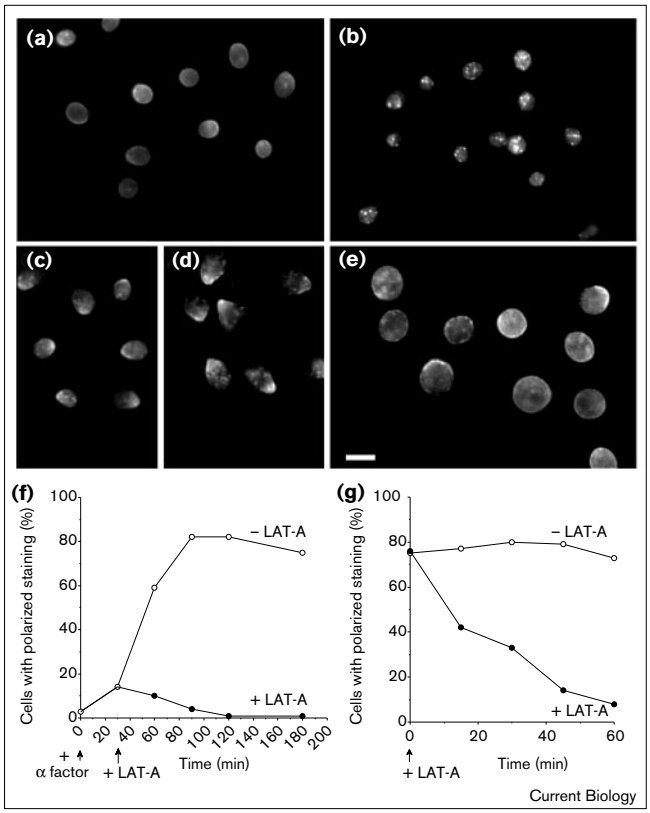
was lost fairly rapidly, however, such that after 120 minutes only 5% of cells showed any restriction in surface staining. This result suggests that Ste2p can achieve partial polarity in the absence of actin function, but requires the actin cytoskeleton to maintain this partial polarity and to achieve a tightly clustered distribution.

We also addressed whether an intact actin cytoskeleton is required to maintain, as well as establish, tight clustering of Ste2p. Cells were incubated in the presence of α factor for 2 hours to allow relevant cell components to localize to the mating projection tip. LAT-A was then added and samples were processed for immunofluorescence microscopy. As shown in Figure 3g, the disruption of actin caused the delocalization of Ste2p with a half-life for loss of polarity of about 20 minutes. When Cdc42p was localized in the same cells it was also observed to lose its polarized localization with similar kinetics to that of Ste2p (data not shown). Loss of F actin in these cells occurred faster than the loss of polarization of Ste2p or Cdc42p, with a half-life of 5 minutes for cortical actin structures, similar to the times for disassembly observed previously [5,22].

In conclusion, this study demonstrates that in response to an external pheromone cue, yeast cells require an intact actin cytoskeleton for the polarized localization of several proteins including Cdc42p and the α-factor receptor itself. Studies focused on the clustering of acetylcholine receptors at the mammalian neuromuscular junction have revealed interactions between receptors and the actin cytoskeleton. Acetylcholine receptors can associate with β-dystroglycan via the rapsyn protein [23] and β-dystroglycan can indirectly associate with actin filaments by binding to utrophin [24,25]. The consequences of disrupting this interaction *in vivo*, however, have not been reported.

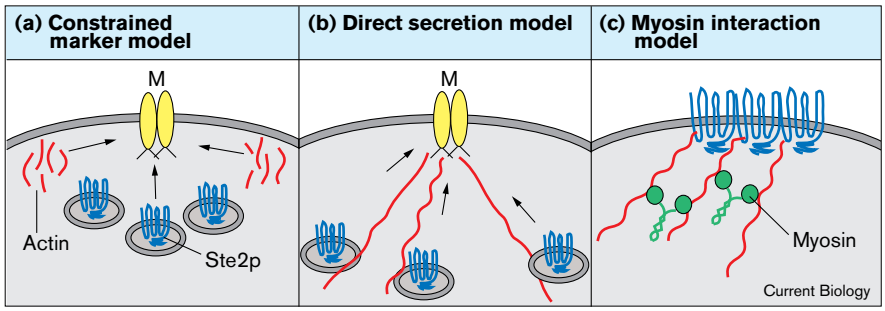
A number of models, described in Figure 4, can be suggested for how the actin cytoskeleton might bring about

Figure 3



Localization of Ste2p following addition of α factor and the effect of actin disruption. Cells were grown to log phase and α factor was added. Cells were fixed and processed for immunofluorescence at various time points (t); **(a)** t = 0 min, **(b)** t = 20 min, **(c)** t = 90 min, **(d)** t = 150 min. **(e)** The effect of LAT-A on Ste2p localization was also tested. LAT-A was added to cells at t = 30 min and the cells were fixed for immunofluorescence at t = 150 min. The bar is 5 μm. **(f)** LAT-A-treated cells were analyzed quantitatively by immunofluorescence at time points over a 3 h time period. **(g)** To analyze the effect of actin disruption on localized Ste2p receptors, LAT-A was added 120 min after the addition of α factor and the cells were processed for immunofluorescence over a 1 h time period.

Figure 4



Models proposed to explain the role of the actin cytoskeleton in receptor clustering during mating projection formation. Pheromone binding localizes a molecular marker (M), around which pheromone receptors cluster. The physical nature of such a positional marker is unknown, although a precedent for actin involvement in localization of such a marker has been established in studies of the ability of diploid yeast cells to identify their correct site for budding [30]. **(a)** An intact actin cytoskeleton is required to maintain localization of the marker. **(b)** The actin cytoskeleton facilitates directed secretion of receptor to the site of the marker. **(c)** Tight polarity of receptors is generated by the interaction of receptors with actomyosin.

This model is broadly similar to that proposed to account for integrin clustering in response to ligand binding in mammalian cells [31]. For all three models, consolidation of receptor polarity provides a cortical focus for localization of other proteins involved in polarity establishment and signalling.

receptor clustering and the resulting localization of the signalling machinery. We propose that pheromone binding to the receptor results in the positional marking of the cell surface. Actin might then be required to directly constrain this marker or to direct secretion to this site; alternatively, an interaction of actomyosin with the receptors could generate a contractile force to consolidate receptor localization. Although the cells in our experiment are exposed to pheromone isotropically, it may be that under these conditions cell polarity is determined by small differences in receptor occupancy across the cell surface. Whereas cells exposed to isotropic pheromone preferentially polarize towards an intrinsically determined site adjacent to the bud scar, in our experiments — in which cells have been released from G0 — this axial bud site marker is not maintained [26] and we have observed that the site of mating-projection formation is most often not adjacent to the previous bud site (data not shown). The evolutionary conservation of many proteins involved in polarity establishment suggests that there might be a general mechanism whereby actin-dependent receptor clustering mediates the concentration of signalling proteins at a single region of the cell cortex, allowing small differences in receptor occupancy to be amplified to generate pronounced cell polarity and spatially controlled morphogenesis.

Materials and methods

Growth of cells from G0, fluorescence procedures and analysis

For analyzing protein localization in cells emerging from G0, 1 ml samples of cells were removed at intervals following release from G0 as described previously [5] in the presence of α factor (2.5 μg/ml). LAT-A (200 μM) was added to cultures from a 50 mM stock in DMSO. Cells were processed for immunofluorescence essentially as described [27,28]. The staining patterns for each antibody, in the presence or absence of LAT-A, were examined at each time point after release from G0. Time courses were repeated three times and the number of cells counted was at least 200 for each time point. The β-galactosidase assay performed was as described by Kaiser *et al.* [29].

Supplementary material

Further details of methodology and information about the yeast strains and antibodies used are published with this paper on the internet.

Acknowledgements

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Supplementary material

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Materials and methods

Materials

Unless otherwise stated, chemicals used were obtained from Sigma Chemical Company or Merck/BDH. LAT-A was a gift from P. Crews and is also available from Molecular Probes; α factor was synthesized at UC Berkeley by David King.

Yeast strains, construction, growth conditions and measuring cell growth

Unless otherwise stated, cells were grown with rotary shaking at 26°C in liquid YPD medium (1% yeast extract, 2% bacto peptone, 2% glucose). Cell growth was measured using a haemocytometer and by measuring the turbidity at OD 600 nm. For studying mating projection formation, α factor was added to 2.5 μg/ml. For studies of F-actin disruption, LAT-A was added from a 50 mM DMSO stock to a final concentration of 200 μM. The strains used in this study were KAY111 (*MAT***a** *ura3-52, lys2-801, ade2-101, trp1-Δ63, his3-Δ200, leu2-Δ1, FUS1:LACZ::URA3*) and KAY316 (*MAT***a** *ura3-52, lys2-801, ade2-101, trp1-Δ63, his3-Δ200, leu2-Δ1, FUS1:LACZ::URA3, STE2-9xmyc::TRP1*). The integrated *FUS1:LACZ* was generated by transforming into strain DK499 (a gift from J. Thorner) the plasmid pSB286 (also from J. Thorner) cut with *BstE*II to integrate the reporter at the *FUS1* locus. The parent strain, DK499, was also from J. Thorner. The Myc-tagged version of Ste2 was made by generating a linear PCR fragment carrying the Myc tag and the *K. lactis TRP1* gene. The plasmid used as the template for the PCR was a gift from Kim Nasmyth. PCR reactions used Taq polymerase (BioLine), 1.5 mM $MgCl_2$ and the commercially supplied buffer. The PCR primers contained 60 bp overlap of the carboxyl terminus of *STE2* to allow homologous recombination at the *STE2* locus. The fragment was transformed into the strain KAY111 (genotype shown above). After transformation, strains that were Trp^+ were then tested by PCR to show that insertion was in the correct site in the genome. Western blotting with 9E10 antibody showed a protein of the correct size only in those transformed strains that showed an appropriate band by PCR.

Fluorescence procedures

The primary antibodies used in this study were as follows: anti-actin, anti-Cdc42p, anti-Rho1p and anti-Sac6p (Drubin lab); anti-Cdc10p and anti-Bem1p (from J. Pringle); anti-Myo2p (from S. Brown); anti-Arp2p (from B. Winsor); anti-Sec4p (from Peter Novick); anti-calmodulin (from Mike Stark); anti-Spa2p (from M. Snyder); and anti-Myc (Santa Cruz Biotechnology Inc.). Secondary antibodies used were fluorescein isothiocyanate (FITC)-conjugated goat anti-guinea-pig (Cappell/Organon Technika) at a dilution of 1:1000 and CY3-conjugated sheep anti-rabbit (Sigma Chemical Co.) at a dilution of 1:200. Cells were viewed with a Olympus BX-60 fluorescence microscope with a 100 W mercury lamp and an Olympus 100X Plan-NeoFluar oil immersion objective. Images were captured electronically using a Sys3000 cooled CCD camera (Digital PixelAdvanced Imaging Systems) and displayed on an Apple Macintosh computer using IP lab software (Scanalytics Inc.).