Inhibitory GEF Phosphorylation Provides Negative Feedback in the Yeast Polarity Circuit

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Summary

Cell polarity is critical for the form and function of many cell types. During polarity establishment, cells define a cortical “front” that behaves differently from the rest of the cortex. The front accumulates high levels of the active form of a polarity-determining Rho-family GTPase (Cdc42, Rac, or Rop) that then orients cytoskeletal elements through various effectors to generate the polarized morphology appropriate to the particular cell type [1, 2]. GTPase accumulation is thought to involve positive feedback, such that active GTPase promotes further delivery and/or activation of more GTPase in its vicinity [3]. Recent studies suggest that once a front forms, the concentration of polarity factors at the front can increase and decrease periodically, first clustering the factors at the cortex and then dispersing them back to the cytoplasm [4–7]. Such oscillatory behavior implies the presence of negative feedback in the polarity circuit [8], but the mechanism of negative feedback was not known. Here we show that, in the budding yeast Saccharomyces cerevisiae, the catalytic activity of the Cdc42-directed GEF is inhibited by Cdc42-stimulated effector kinases, thus providing negative feedback. We further show that replacing the GEF with a phosphosite mutant GEF abolishes oscillations and leads to the accumulation of excess GTP-Cdc42 and other polarity factors at the front. These findings reveal a mechanism for negative feedback and suggest that the function of negative feedback via GEF inhibition is to buffer the level of Cdc42 at the polarity site.

Results and Discussion

Cdc24 Phosphorylation Reduces GEF Activity In Vitro

Guanosine triphosphate (GTP)-Cdc42 is known to activate p21-activated kinases (PAKs) that then phosphorylate the Cdc42-directed guanine nucleotide exchange factor (GEF) Cdc24 [9, 10]. To assess the effect of phosphorylation on Cdc24 GEF activity, we isolated hemagglutinin (HA) epitope-tagged Cdc24 from yeast cells that overexpressed the protein, and then we measured its ability to stimulate guanine nucleotide exchange on recombinant Cdc42 (Figure 1A). HA-Cdc24 displayed robust GEF activity that was abolished by mutations in the catalytic domain, indicating that the activity was intrinsic to Cdc24 and not due to a coprecipitating factor (Figure 1B). As judged by gel mobility, a majority of the HA-Cdc24 was not phosphorylated. To generate a preparation of highly phosphorylated Cdc24, we coexpressed the activated Cdc42 mutant Cdc42Q61L and a Bem1-Cla4 fusion protein [11] to promote Cla4 (PAK)-mediated hyperphosphorylation of HA-Cdc24 (Figure 1C). Phosphorylated Cdc24 was less active than unphosphorylated Cdc24 (Figure 1D). This was due to phosphorylation, because treatment with λ-phosphatase caused an increase in GEF activity (Figure 1E). We conclude that Cdc24 phosphorylation reduces GEF activity.

Assay for Cdc24 Activity In Vivo

To assess Cdc24 activity in vivo, we followed a strategy based on the expectation that if Cdc24 were targeted uniformly to the plasma membrane (rather than just to the polarity site), then it would generate GTP-Cdc42 all over the membrane (Figure 2A). In turn, that should dominantly interfere with polarization mediated by the endogenous Cdc24, blocking proliferation [12]. The level of expression needed to block polarization would depend on the activity of the membrane-targeted Cdc24 (MT-Cdc24), providing a measure for Cdc24 activity in vivo. We used a 28-residue N-terminal peptide from Psr1 [13] to efficiently target Cdc24 to the plasma membrane. Levels of expression were controlled by an estrogen-inducible promoter system (Figure 2A) [14]. High-level expression of MT-Cdc24 induced accumulation of GTP-Cdc42 all over the plasma membrane (Figure 2B) and blocked both polarization (Figure 2B) and proliferation (Figure 2D).

In principle, the effect of MT-Cdc24 could stem from its catalytic activity (promoting uniform cortical GTP-Cdc42 accumulation), or from titrating Cdc42 interactors (e.g., Bem1 [15] and Rsr1-GTP [16]) away from the endogenous Cdc24, or both. We found that a membrane-targeted but catalytically dead Cdc24 (MT-Cdc24-AA) was no longer effective in promoting accumulation of GTP-Cdc42 all over the membrane (Figures 2B and 2C) but was still able to block proliferation at high expression levels, presumably via titration (Figure 2D). Membrane targeting of a Bem1-binding-deficient mutant (MT-Cdc24-KR: D824K, D831R [17]) was also able to block proliferation at high expression levels (Figure 2D). At lower expression levels, only MT-Cdc24 (and not MT-Cdc24-AA or MT-Cdc24-KR) blocked proliferation (Figure 2D). A double mutant lacking both catalytic activity and Bem1-binding ability did not block proliferation (Figure 2D). Thus, the polarity-blocking effect of MT-Cdc24 reflects additive contributions from its catalytic activity and from Bem1 titration.
Nonphosphorylatable Cdc24 Is Hyperactive In Vivo

MT-Cdc24 exhibited a gel mobility shift compared with wild-type Cdc24, and phosphatase treatment reversed the shift (Figure 2E), suggesting that MT-Cdc24 is highly phosphorylated. A previous study mapped 35 phosphorylation sites on Cdc24 isolated from yeast is incubated with recombinant GDP-Cdc42 and γ[35S]GTP. GTP loading is assessed by a filtration assay. (B) Cdc42 GTP loading by wild-type Cdc24 or a catalytic domain mutant N452A/E453A (designated Cdc24-AA). Arbitrary units (AU) represent the amount of radioactive GTP loaded divided by the amount of HA-Cdc24 in the immunoprecipitate (inset). Activity is normalized to the wild-type Cdc24 sample. Mean ± SEM is shown (n = 3). (C) Western blot of Cdc24 isolated from wild-type cells (DLY15284) or cells overexpressing Cdc42 Q61L and Bem1-Cla4 (DLY15332). The latter (designated Cdc24 33A) has reduced GEF activity. Assay was as in (B). Mean ± SEM is shown (n = 3). (D) Cdc24 33A has reduced GEF activity. Assay was as in (B). Mean ± SEM is shown (n = 3). (E) Phosphatase treatment of Cdc24 33A increases GEF activity. Cdc24 33A was treated with buffer, λ phosphatase, or λ phosphatase plus phosphatase inhibitors, as indicated. Assay was as in (B). Mean ± SEM is shown (n = 3). ***p < 0.001, *p < 0.02 (two-tailed t test).

Computational Modeling of Negative Feedback through Cdc24 Phosphorylation

Because polarization can be initiated by stochastic fluctuations, which can occur at multiple locations, cells often begin...
Figure 2. Phosphosite Mutant GEF Is More Active In Vivo

(A) Assay for Cdc24 activity in vivo. An estradiol-regulated derivative of Gal4 (Gal4-ER) allows graded induction of the GAL1 promoter, driving overexpression of wild-type (WT) or MT GFP-Cdc24. WT Cdc24 is mostly cytoplasmic, and overexpression is well tolerated: cells polarize GTP-Cdc42 and

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Mean Cdc24, which is largely unphosphorylated when overexpressed, has comparable GEF activity to Cdc24 33A. Mean MT-Cdc2433A displays higher GEF activity than MT-Cdc24. Assays were as in Figure 1B, except that the GEF was immunoprecipitated using anti-GFP.

24 versions of Cdc24 were treated with 50 nM (C) Quantification of the cortical-to-cytoplasmic fluorescence ratio for the GTP-Cdc42 probe in cells expressing MT-Cdc24 derivatives. Mean versions blocked polarization at this high level of expression, leading to accumulation of large unbudded cells.

Given our findings, we asked whether adding inhibitory multistate phosphorylation of Cdc24 to the previous positive-feedback-only model (Figure 3C) could produce oscillatory clustering. Because multistate phosphorylation can yield ultrasensitive behavior [22–24], we modeled phosphorylation as a Hill function of the concentration of Cdc42-bound Bem1 complexes (see “Computational Modeling” in the Supplemental Experimental Procedures). However, ultrasensitivity was not essential to produce the predictions discussed below.

Simulations incorporating negative feedback displayed dampened oscillations, as well as reduced Cdc42 accumulation at the polarity site (Figure 3D). We also simulated cells in which two slightly unequal polarity clusters were formed at opposite ends of the cell and followed the relative amount of Cdc42 at a 45:55 ratio, and the upper lines show growth of the winning clusters, whereas the lower lines show disappearance of the losing clusters. Competition is resolved more rapidly with (blue) than without (red) negative feedback.

to concentrate Cdc42 at more than one site [6, 19]. However, Cdc42 clusters then appear to compete with each other, so that one cluster grows to become the front while all of the other clusters disappear [6, 20]. Theoretical studies have shown that the known biochemical interactions and activities of Cdc42, the PAK-Bem1-Cdc24 complex, and a guanine nucleotide dissociation inhibitor are sufficient in principle to explain both clustering of polarity factors and competition between clusters [20, 21]. To explain oscillatory clustering, an additional negative feedback loop was posited [6], although the mechanism of negative feedback was unknown.

form buds (top). A lipid-modified Psr1 peptide (blue) targets MT-Cdc24 uniformly around the plasma membrane, leading to uniform GTP-Cdc42 accumulation, loss of polarity, and failure to proliferate (bottom). (B) Localization of the GTP-Cdc42 probe Gic21–208-tdTomato in cells overexpressing WT or MT GFP-Cdc24 derivatives. Cells overexpressing the indicated versions of Cdc24 were treated with 50 nM [1-estradiol for 3–4 hr, and single confocal planes were imaged (GFP, left; RFP, middle; merge, right). All MT versions blocked polarization at this high level of expression, leading to accumulation of large unbudded cells. (C) Quantification of the cortical-to-cytoplasmic fluorescence ratio for the GTP-Cdc42 probe in cells expressing MT-Cdc24 derivatives. Mean ± SEM is shown (n = 5 fields of cells with ~ 40 cells/field). ***p < 0.001, **p < 0.01 (two-tailed t test). Cortical Cdc42 was reduced by catalytic mutations but increased by phosphoryte mutations.

(D) MT-Cdc24 overexpression blocks proliferation by a combination of catalytic activity and Bem1 titration. Left: cells were spaced (10-fold serial dilutions) onto plates containing the indicated concentrations of [1-estradiol to induce expression of Cdc24 or MT-Cdc24 derivatives and then incubated for 2 days at 24°C. MT-Cdc24-AA can titrate Bem1 but lacks catalytic activity, whereas MT-Cdc24-KR has catalytic activity but cannot titrate Bem1: both block proliferation, but less effectively than MT-Cdc24. Right: blot shows levels of induction (MT-Cdc24 has slower mobility due to phosphorylation). Cdc11 is the loading control.

(E) MT-Cdc24 is highly phosphorylated. Mobility shift is reduced upon treatment with λ phosphatase, but not λ phosphatase plus phosphatase inhibitors. (F) MT-Cdc24 33A is more potent at blocking proliferation than MT-Cdc24. Assay and blots were as in (D).

(G) Catalytically dead MT-Cdc24 33A is comparable at blocking proliferation to catalytically dead MT-Cdc24. Assay and blots were as in (D).

(H) MT-Cdc24 33A displays higher GEF activity than MT-Cdc24. Assays were as in Figure 1B, except that the GEF was immunoprecipitated using anti-GFP. Mean ± SEM is shown (n = 3). ***p < 0.001 (two-tailed t test).

(I) Cdc24, which is largely unphosphorylated when overexpressed, has comparable GEF activity to Cdc24 33A. Mean ± SEM is shown (n = 3).
Figure 4. Negative Feedback via GEF Phosphorylation Promotes Oscillatory Clustering and Lowers GTP-Cdc42 Levels at the Polarity Site

(A) Dynamics of Bem1-GFP clustering in CDC24 and CDC24^38A cells. Cells were imaged side by side to allow direct comparison of Bem1-GFP intensities. Maximum projections of confocal stacks are shown, and intensity of the probe in the cluster is quantitated. Blue arrowhead, signal from old neck or neighboring cell (not polarity site). Arrows show clustering peak(s): red, CDC24 cells (control); green, CDC24^38A cells.

(B) Dynamics of GTP-Cdc42 clustering visualized with Gic2^1-208-tdTomato, as in (A).

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Cdc24 Phosphorylation Is Required for Oscillatory Polarization but Does Not Affect Competition between Polarity Clusters

Previous work showed that nonphosphorylatable Cdc24<sup>38A</sup> was functional, but the dynamics of polarization was not examined [18]. We generated yeast strains in which endogenous CDC24 was precisely replaced by CDC24<sup>38A</sup> at its endogenous locus and monitored polarization using Bem1-GFP [11], GFP-Cdc42, or the GTP-Cdc42 reporter Gic2<sup>1–208</sup>-tdTomato [25] as probes. We focused on diploid cells lacking the bud-site-selection protein Rsr1, where we had observed oscillatory cycles of clustering and dispersal of polarity factors [6]. Control experiments with HA-tagged constructs showed that Cdc24<sup>38A</sup> was expressed at similar levels to Cdc24 (Figure 4G), as reported previously [18].

During initial polarity establishment, CDC24 cells displayed oscillations in the concentration of polarity factors at the front (Figures 4A–4C; Figure S1 available online; Movie S1), as reported previously [6]. In contrast, CDC24<sup>38A</sup> cells kept polarity protein levels high at the front, without discernible oscillations (Figures 4A–4C; Figure S1; Movie S2). Unlike in the model with no negative feedback (Figure 3D), however, levels of polarity factors did gradually decline from the peak in mutant cells (Figures 4A–4C; Figure S1), perhaps reflecting additional sources of negative feedback. Two other negative feedback loops were recently proposed to act through the cytoskeletal targets of Cdc42: actin [26, 27] and septins [28]. These slower feedback loops cannot account for the rapid oscillations [6], but they may contribute to the slower decline in polarity protein levels at the front observed in the CDC24<sup>38A</sup> mutants.

As with CDC24 cells, several of the CDC24<sup>38A</sup> cells we imaged initially developed more than one polarity cluster (Figure 4D; Movie S2). To assess whether competition between clusters was altered in the mutant cells, we measured the coexistence time (the interval for which two or more clusters were present) for this subset of cells. Quantification indicated that competition timing was generally similar in CDC24 versus CDC24<sup>38A</sup> cells (Figure 4E). Thus, Cdc24 phosphorylation does not seem to be required to enforce rapid competition.

Cdc24 Phosphorylation Lowers Polarity Factor Concentrations at the Front

To compare the relative intensity of polarized signals, CDC24 and CDC24<sup>38A</sup> cells were imaged side by side on the same slide (Movie S3). Peak levels of Bem1, Cdc42, and GTP-Cdc42 were all higher in CDC24<sup>38A</sup> cells than in CDC24 cells (Figure 4F). This was not due to an increase in total cellular levels of Bem1 or Cdc42 (Figure 4G). The effect of CDC24<sup>38A</sup> was dominant to wild-type, as expected for a hyperactive protein (Figure S1D). Thus, negative feedback through Cdc42 phosphorylation acts to reduce polarity factor accumulation at the cortex.

CDC24<sup>38A</sup> cells did not display any major changes in polarization efficiency, proliferation rate, or cell morphology. Thus, it appears that cells can tolerate increased levels of polarized GTP-Cdc42 without severe ill effects, at least under lab growth conditions. Negative feedback may become more important when polarity-affecting stresses occur in more physiological environments. Alternatively, like cell-cycle checkpoints and DNA repair pathways, negative feedback may only be critical for a minority of cells that experience polarity problems (e.g., because they contain potentially dangerous levels of polarity factors). Consistent with this hypothesis, preliminary observations suggest that negative feedback becomes important for proliferation when Cdc42 is overexpressed. It will be of great interest to determine whether polarity establishment in other systems also employs inhibitory GEF phosphorylation to regulate polarization.

Supplemental Information

Supplemental Information includes Supplemental Experimental Procedures, one figure, and three movies and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2014.02.024.

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