Possible additional roles in mating for *Ustilago maydis* Rho1 and 14-3-3 homologues

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Both the Rho GTPases and 14-3-3 proteins each belong to ubiquitous families of proteins involved in a variety of cellular processes, including cytokinesis, cell polarity, cellular differentiation and apoptosis. In fungi, these components of signaling pathways are involved in cell cycle regulation, cytokinesis and virulence. We study cellular differentiation and pathogenesis for *Ustilago maydis*, the dimorphic fungal pathogen of maize. We have reported on the interactions of Pdc1, a *U. maydis* homologue of human 14-3-3ε, with Rho1, a small GTP binding protein; these proteins participate in cell polarity and filamentation pathways that include another small G protein, Rac1, and its effector PAK kinase, Cla4. Here we describe additional experiments that explore possible relationships of Pdc1 and Rho1 with another PAK-like kinase pathway and with the a mating-type locus.

Two highly-conserved signaling pathways are present in a variety of fungi and many of their components are conserved from fungi to humans. These pathways, the MAPK and cAMP-dependent PKA pathways, play roles in cellular differentiation, responses to environmental signals such as nutrient levels and stress, response to pheromone for mating, and virulence. The 14-3-3 proteins and the Rho small GTP-binding proteins are ubiquitous in eukaryotic cells as regulators of these and other signaling pathways and each belongs to a large family of related proteins.

The 14-3-3 proteins are members of a large family of highly conserved and ubiquitously-expressed proteins. Seven isotypes are present in mammals and up to fifteen different isotypes have been discovered in plants. Like *Drosophila melanogaster* and *Caenorhabditis elegans*, fungi tend to have two isotypes, though both *Candida albicans* and *U. maydis* each have only a single gene encoding their respective 14-3-3 homologues. Members of this protein family of small, ~30 kDa proteins, typically form dimers with one another to generate an active ligand-binding protein.

Their exact roles in the various cellular processes with which 14-3-3 proteins have been associated has been difficult to ascertain due to multiple isotypes per cell and the fact that over 200 interacting partners have been identified for 14-3-3 proteins. Thus, in *U. maydis* we were provided with a unique opportunity to investigate the role(s) of 14-3-3 proteins, since only one homologue was predicted from inspection of the genome sequence. We identified a 14-3-3ε homologue in *U. maydis*, which we called Pdc1 (phosphorylation-domain coupling protein) since 14-3-3 dimers couple phosphoserine/threonine target proteins. *U. maydis* Pdc1 is predicted to have 77% and 75% identity to *Saccharomyces cerevisiae* Bmh1 and Bmh2, respectively. In fact, when overexpressed in a *S. cerevisiae* Δbmh1bmh2 mutant, Pdc1 partially rescues the growth defect of the yeast mutant, suggesting at least some functional similarity as well.

It was subsequently determined that Pdc1 plays a role in cell cycle control. However, in contrast to the role of its *S. cerevisiae* homologue, Bmh1, *U. maydis* Pdc1/Bmh1 was found to be involved in G2 transition, a function dependent on the phosphorylation state of the dual-specificity phosphatase, Cdc25.
from overexpression of a synthetic peptide inhibitor of 14-3-3 proteins, difopein, seems to lend further support for the role of Pdc1 in cell cycle control. Cells expressing difopein, a competitive inhibitor that binds to the ligand-binding groove of 14-3-3 proteins, undergo polar growth extension (Fig. 1) in a fashion similar to b-mating locus-induced filament formation. However, unlike b-mating locus-induced filament, the apical-constricted growth extensions induced by treatment of cells with difopein appear tapered at the active growing end. Such extensions are consistent with failure of cells to undergo cell division. In addition to its role in cell cycle control, Pdc1 also is required for viability in haploid cells and plays critical roles in cytokinesis, cell polarity and filamentation.

Rho (Ras homologue) GTPases are small molecular weight G-proteins belonging to the Ras superfamily, whose activity is dependent on the reversible binding of guanosine triphosphate and guanosine diphosphate. They are involved in actin organization, polarized cell growth, cytokinesis and regulation of cell cycle. In these processes, they play instrumental role(s) in establishing and maintaining cell polarity. Members of the Rho-family GTPases include Rho, Rac and Cdc42. In *U. maydis*, Rho1 is the only member of this family identified to date that is required for cell viability in haploid cells, as its deletion or depletion leads to cell death. The phenotypes following its depletion or overexpression mirror those of Pdc1, with the added observation of reduced mating of *U. maydis* cells when Rho1 is overexpressed in the mating partners. In addition, epistasis analyses suggest that both Pdc1 and Rho1 are upstream of cytokinesis and cell polarity pathways, ultimately regulated by another small G protein, Rac1, and its effector kinase, Cla4 [a p21-activated kinase (PAK)].

In additional experiments with both Pdc1 and Rho1, we sought to explore further the interactions between these proteins and other signaling components affecting cell morphology and differentiation. *U. maydis*, like other fungi, has several homologues of PAK-like kinases. In particular, it contains a Cla4 homologue and a homologue of the yeast Ste20p, Smu1. While neither of these kinases is required for viability, presumably due to some redundancy, at least in yeasts, deletion of both is lethal, suggesting overlap in function.

In *S. cerevisiae*, the 14-3-3 homologues, Bmh1 and Bmh2 regulate pseudohyphal development by binding to the p21-activated kinase Ste20 of the MAPK signaling pathway. Thus, we were initially curious to examine similar potential roles for Pdc1 and/or Rho1 with regard Smu1 in *U. maydis*. As seen in Figure 2, when either Pdc1 or Rho1 was overexpressed in a haploid strain deleted for the smu1 gene, we observed a multiple-budding phenotype, reminiscent of the ubc1 mutants that lack the regulatory subunit of PKA and thus have overactive catalytic subunit of the kinase. No such phenotype was observed in wild type cells when these genes were overexpressed, although overexpression of pdc1 in wild type led to slight elongation of the cells.

Our previous work with Pdc1 and Rho1 indicated that their role is likely upstream of the a and b mating-type loci. In *U. maydis* the a locus encodes pheromone and receptors that control the cell-cell recognition that serves as a prelude to cell fusion; normally such fusion is a prerequisite for subsequent filamentation and infectivity, in turn controlled by the b locus. In genetically engineered strains where the cell fusion step has been bypassed, once filamentation has been initiated, neither depletion nor overexpression of Pdc1 or Rho1 has any observable effects on filamentation, although their depletion is eventually lethal. The otherwise haploid SG200 strain has been engineered so that it is heterozygous at the b locus and so that it produces autocrine signaling of pheromone to the corresponding receptor. This leads to upregulation of pheromone gene expression and then to filamentous growth under control of the b locus. When Pdc1 were overexpressed in SG200, the result was both multiple-budding and clusters of cells resembling “balloon dachshunds” (Fig. 3).
Here, we have added to our previously published analyses by providing (1) additional evidence of the role of Pdc1 in cell cycle (Fig. 1); (2) evidence that Pdc1 and Rho1 participate in the same or a parallel pathway with Smu1 (Fig. 2); and (3) data showing a possible role of Pdc1 in phenotypes associated with expression of the a locus (Fig. 3). We showed previously that Smu1 is required for upregulation of the mfa gene in a2 cells upon exposure to pheromone from a1 cells. When a2 cells are deleted for smu1, they show reduced mating in both charcoal and confrontation mating assays, and they fail to increase mfa2 expression in such assays. Interestingly, based on yeast two-hybrid and co-immunoprecipitation analyses, Smu1 is predicted to interact with Rho1. If so, this would suggest a role for Rho1 (and its upstream partner, Pdc1) in cell morphology associated with expression levels of the a locus.

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