Ras/cAMP-dependent Protein Kinase (PKA) Regulates Multiple Aspects of Cellular Events by Phosphorylating the Whi3 Cell Cycle Regulator in Budding Yeast*

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Eukaryotic cells monitor internal and external signals to commit themselves to cell division or to a switch to alternative developmental fates during the G1 phase. In the budding yeast *Saccharomyces cerevisiae*, this control network is called Start (1). Start is initiated by the G1 cyclin Cln3, which is associated with the cyclin-dependent kinase Cdc28 (2).

Whi3 in budding yeast was originally identified as a positive regulator of cell size control and is involved in the regulation of Start (3). The best characterized function of Whi3 is its ability to inhibit Cln3 function in the G1 phase by binding to CLN3 mRNA, thereby inhibiting the Cln3-Cdc28-mediated activation of two transcription factors, SBF (Swi4-Swi6) and MBF (Mbp1 and Swi4), which drive the expression of the G1 cyclin CLN1 and CLN2 mRNAs and lead to a delay in activation of Start of the cell cycle (4–7). In addition, Whi3 function is required for developmental options such as invasive growth and meiosis (4). However, little is known about the upstream regulator(s) of Whi3.

The Ras/cAMP-dependent protein kinase (PKA) pathway in yeast has been implicated in numerous cellular processes, including carbon storage, stress response, growth, differentiation, and life span (for reviews, see Refs. 8–10). The molecular mechanism by which yeast cells sense and respond to the specific stimuli generated by PKA has been studied extensively. One of the most prominent roles of PKA signaling is known to be the regulation of the critical cell size required for Start in response to nutrient conditions (for reviews, see Refs. 8–10). PKA has been implicated in cell size control by nutritional conditions such as nutrient levels, and decreased PKA signaling results in decreased cell size, whereas hyperactive PKA signaling leads to increased cell size (11–13), indicating that PKA is a positive regulator of cell size control.

In addition, the PKA pathway appears to play a crucial role in the connection between the availability of nutrient signals and G1/S transition. It has long been thought that the G1 length of cells growing rapidly in rich medium is shorter than that of cells growing slowly in poor medium and that cells in rich medium also have higher PKA activity than those in poor medium. Genetic evidence supports this idea that PKA increases the expression of the Cln3-Cdc28 kinase complex to promote passage through Start in rich medium (14). On the other hand, other data indicate that PKA delays Start during the shift from poor to rich medium (12, 13). These results indicate that PKA plays an important role in Start as a positive or negative regul-
lutor depending on the nutrient conditions/shifts. Thus, the involvement of PKA in the regulation of Start remains enigmatic. Despite the obvious importance of the PKA signaling pathway, only a few substrates/targets of PKA in the control of cell growth have been identified (9, 10).

In this study, we identify PKA as the Whi3 kinase. We show that the phosphorylation of Ser-568 in Whi3 by PKA plays an inhibitory role in Whi3 function. This mechanism is essential for the acceleration of G1/S progression. Furthermore, we demonstrate that the phosphomimetic S568D mutation of Whi3 prevents sporulation and invasive growth. On the basis of these findings, we propose that the phosphorylation of Whi3 by PKA is involved in multiple cellular events, including cell cycle control and developmental fate in response to environmental stimuli.

EXPERIMENTAL PROCEDURES

Yeast Strains and Media—The yeast strains used in this study were as follows: W303-1A (MAT\(a\) trp1-1 leu2-3 ade2-1 ura3-1 his3-11 can1-100), SHI301 (MAT\(a\) Δwhi3::kanMX6), SHI294 (MAT\(a\) WHI3-3HA::kanMX6), YMM501 (MAT\(a\) Δbcy1::URA3 WHI3-3HA::kanMX6), YMM502 (MAT\(a\) WHI3-S568A-3HA::kanMX6), YMM504 (MAT\(a\) Δbcy1::URA3 WHI3-S568A-3HA::kanMX6), YMM505 (MAT\(a\) WHI3-S568D-3HA::kanMX6), YMM507 (MAT\(a\) WHI3-3HA::Δcln3::HI3 SMY1-3HA::kanMX6), YMM508 (MAT\(a\) WHI3-S568A-3HA::Δcln3::kanMX6), YMM509 (MAT\(a\) WHI3-S568D-3HA::Δcln3::kanMX6), YMM510 (MAT\(a\) Δwhi3::kanMX6 Δwhi3::kanMX6), YRT73 (MAT\(a\) Δcln3::HI3 WHI3-3HA::kanMX6), YRT76 (MAT\(a\) Δcln3::HI3 WHI3-S568A-3HA::kanMX6), YRT75 (MAT\(a\) Δcln3::HI3 WHI3-S568D-3HA::kanMX6), YRT74 (MAT\(a\) Δcln3::HI3 Δwhi3::kanMX6), YTO1 (MAT\(a\) HIS3 GAL1-WHI3-3HA::kanMX6), YTO2 (MAT\(a\) HIS3 GAL1-WHI3-S568D-3HA::kanMX6), YMM507 (MAT\(a\)/Δ WHI3-3HA::kanMX6/WHI3-3HA::kanMX6), YMM508 (MAT\(a\)/Δ WHI3-S568A-3HA::kanMX6/WHI3-S568A-3HA::kanMX6), YMM509 (MAT\(a\)/Δ WHI3-S568D-3HA::kanMX6/WHI3-S568D-3HA::kanMX6), YMM510 (MAT\(a\)/Δ whi3::kanMX6/whi3::kanMX6), YRT91 (MAT\(a\)/Δ cln3::HI3 WHI3-3HA::kanMX6/cln3::HI3 WHI3-3HA::kanMX6), YRT94 (MAT\(a\)/Δ cln3::HI3 WHI3-S568A-3HA::kanMX6/cln3::HI3 WHI3-S568A-3HA::kanMX6), YRT93 (MAT\(a\)/Δ cln3::HI3 WHI3-S568D-3HA::kanMX6/cln3::HI3 WHI3-S568D-3HA::kanMX6), and YRT92 (MAT\(a\)/Δ cln3::HI3 whi3::kanMX6/Δ cln3::HI3 whi3::kanMX6). The strains with chromosomally integrated genes for WHI3-S568A and WHI3-S568D with a 3-HA epitope tag were constructed by gene replacement. Genomic DNA was isolated from the whi3::kanMX4 strain on a BY4741 background (Invitrogen). The PCR-amplified fragments of whi3::kanMX4 were used to transform the W303-1A and SMLY41a strains (16). Deletion of the genomic CLN3 gene (CLN3 plasmids provided by I. Yamashita) and the BCY1 gene was performed using a disruption plasmid.

The strains with chromosomally integrated genes for WHI3-S568A and WHI3-S568D with a 3-HA epitope tag at their C termini were constructed as follows. The pWHI3-S568A-3HA and pWHI3-S568D-3HA plasmids were digested with NspV (within the WHI3 locus) and used to transform the appropriate strains. Insertion of these fragments into the original WHI3 locus was confirmed by DNA sequencing and Western blot analysis.

In Vitro Phosphatase Assay—Cell extracts (200 μg of total protein) were incubated with 400 units of λ-phosphatase (New England Biolabs) in 50 μl of λ-phosphatase buffer and 1 mM MnCl\(_2\) with or without phosphate inhibitors (50 mM NaF and 5 mM sodium orthovanadate) for 60 min at 30 °C. After the phosphatase treatment, 4 mM MgCl\(_2\) and 50 μM ATP were added, and the mixture was boiled for 10 min. Whi3-HA was detected by immunoblotting.

In Vitro Protein Kinase Assay—The procedure used for the in vitro kinase assay was carried out as described previously (17). Expression of the MBP-Whi3 fusion protein in Escherichia coli BL21 was induced by the addition of 0.1 mM isopropyl-β-D-thiogalactopyranoside to the culture medium. MBP-Whi3 protein was affinity-purified using amylose resin beads (New Eng-
Role of Whi3 via PKA in Multiple Cellular Events

land Biolals) according to the manufacturer’s instructions. Protein kinases from a collection of 119 yeast protein kinases fused to glutathione S-transferase (kindly provided by M. Snyder) (17) were overexpressed in yeast and affinity-purified using glutathione–Sepharose 4B beads (Amersham Biosciences). The MBP-Whi3 and GST-fused protein kinases on the beads were mixed in 50 μl of kinase buffer containing 0.5 μCi of [γ-32P]ATP, 100 μM ATP, and 10 mM MgCl₂ and incubated at 30 °C for 30 min. After the beads had been washed with the kinase buffer, the proteins were eluted by boiling the beads in SDS sample buffer for 5 min. The eluted proteins were resolved by SDS-PAGE and detected by autoradiography.

Invasive Growth Assay—Yeast cells (ΣMLY41a, provided by J. Heitman) were streaked onto a yeast extract/peptone/dextrose (YPD) plate and allowed to grow at 28 °C for 2 days. The plate was photographed before and after rinsing with a gentle stream of water to remove all of the cells from the agar surface.

Sporulation Conditions—For induction of sporulation, cells grown in YPD medium were shifted to 1% yeast extract, 2% peptone, and 2% potassium acetate; grown for at least three generations at 28 °C; and harvested at a density of 3–5 × 10⁷ cells/ml. The cells were washed twice with sporulation medium (1% potassium acetate), resuspended at 1.5 × 10⁷ cells/ml in the same medium, and further incubated at 28 °C. The formation of ascis was assessed by phase-contrast microscopy.

RNA Binding, Immunoprecipitation, and mRNA Detection—Exponentially growing cells (4 × 10⁸) were disrupted with glass beads in 200 μl of extraction buffer (50 mM Tris-HCl (pH 7.5), 150 mM KCl, and 2 mM MgCl₂) containing 40 units/μl RNasin (Qiagen), phosphatase inhibitors (10 mM NaF and 1 mM sodium orthovanadate), 0.1% Nonidet P-40, 1 mM DTT, 1 mM PMSF, 10 μg/ml aprotinin, and protease inhibitor mixture (α-Complete, Roche Applied Science). Extracts were cleared by centrifugation for 15 min at 14,000 × g. Anti-HA monoclonal antibody (HA.11, Berkeley Antibody Co.) and protein A-agarose beads were added to the cleared extracts, followed by incubation for 8 h at 4 °C. The beads were then washed four times with wash buffer (50 mM Tris-HCl (pH 7.5) containing 150 mM KCl and 2 mM MgCl₂) and subsequently eluted for 10 min at 65 °C in 50 mM Tris-HCl (pH 8.0) containing 100 mM NaCl, 10 mM EDTA, and 1% SDS. Eluted samples were extracted according to standard protocols (Qiagen). RT-PCR was performed with 2 μl of RNA as template using the One-Step SYBR PrimeScript RT-PCR Kit II (TaKaRa) and the conditions suggested by the manufacturer.

RESULTS

Identification of PKA as the Whi3 Kinase—Whi3 was identified previously as a negative regulator of the G₁ cyclin for cell size control (3) and shown to play an important role in regulating Cln3 activity in G₁ (4). Despite its linkage to multiple cellular events, including cell cycle progression and developmental fate, the upstream regulator(s) of Whi3 has still not been fully identified. Protein phosphorylation plays a pivotal role in the regulation of many cellular functions. As the phosphorylation of Whi3 had not been analyzed yet, we examined whether Whi3 could be phosphorylated in vivo. We detected slowly migrating bands of Whi3-HA on an SDS-polyacrylamide gel (Fig. 1A, lane 2). The phosphatase-treated Whi3-HA protein migrated faster

![Image](313x364 to 563x717)

**FIGURE 1. Whi3 is phosphorylated by PKA in vitro and in vivo.** A, protein phosphatase treatment of Whi3-HA. Whole cell extracts of WT cells expressing Whi3-HA (lanes 2–4) were incubated with (+) or without (−) phosphatase (PPase) or a phosphatase inhibitor. Lane 1 is a negative control lacking the HA tag on Whi3. After separation by SDS-PAGE, Whi3-HA was detected by immunoblotting. Arrows indicate the phosphorylated (*) and dephosphorylated forms of Whi3-HA protein. A nonspecific band is indicated (***). B, recombinant MBP-Whi3 or MBP (control) was incubated with GST-Tpk1, GST-Tpk2, or GST-Tpk3 in the presence of [γ-32P]ATP (γ-32P). Phosphorylated Whi3-HA was separated by SDS-PAGE and detected by autoradiography (upper panel). CBB, Coomassie Brilliant Blue. D, MBP-RRM, MBP-RRM-S568A, or MBP (control) was incubated with GST-Tpk1 in the presence of [γ-32P]ATP. Phosphorylated RRM was separated by SDS-PAGE and detected by autoradiography (upper panel). Phosphorylation levels were quantified by phosphorimaging, and relative values normalized to MBP-RRM are indicated below each band. Means ± S.E. of three independent experiments are shown. The difference was statistically significant (middle panel). **, p < 0.005 by t test. MBP, MBP-RRM, and MBP-RRM-S568A proteins were purified from E. coli BL40 and stained with Coomassie Brilliant Blue (CBB, lower panel). C, schematic representation of full-length Whi3. The location of the RRM and the position of Ser-568 are indicated. D, MBP-RRM, MBP-RRM-S568A, or MBP (control) was incubated with GST-Tpk1 in the presence of [γ-32P]ATP. Phosphorylated RRM was separated by SDS-PAGE and detected by autoradiography (upper panel). Phosphorylation levels were quantified by phosphorimaging, and relative values normalized to MBP-RRM are indicated below each band. Means ± S.E. of three independent experiments are shown. The difference was statistically significant (middle panel). **, p < 0.005 by t test. MBP, MBP-RRM, and MBP-RRM-S568A proteins were purified from E. coli BL40 and stained with Coomassie Brilliant Blue (CBB, lower panel). E, mobility shift of Whi3-HA or Whi3-S568A-HA from WT and Δcy1 cells. WT and Δcy1 cells expressing Whi3-HA or Whi3-S568A-HA were grown in YPD medium to mid-log phase. Proteins of whole cell extracts were separated by SDS-PAGE and detected by immunoblotting with anti-HA (for Whi3-HA and Whi3-S568A-HA) and anti-PSTAIRE (for Cdc28 as a loading control) antibodies. Arrows indicate phosphorylated Whi3-HA proteins with different mobilities. F, protein phosphatase treatment of Whi3-HA. Whole cell extracts of WT and Δbcy1 cells expressing Whi3-HA (lanes 2–7) were incubated with (+) or without (−) a phosphatase or a phosphatase inhibitor. Lane 1 is a negative control lacking the HA tag on Whi3. After separation by SDS-PAGE, Whi3-HA was detected by immunoblotting. Arrows indicate the hyperphosphorylated (**), phosphorylated (*), and dephosphorylated forms of Whi3-HA protein. A nonspecific band is indicated (***).
than the untreated protein (Fig. 1A, compare lanes 2 and 3), indicating that Whi3 had been phosphorylated under normal growth conditions.

To identify the protein kinase(s) responsible for the phosphorylation of Whi3, we screened for the kinase that is capable of phosphorylating the recombinant Whi3 protein fused to MBP in vitro, using [γ-32P]ATP as the phosphate donor, from a collection of 119 potential protein kinases consisting of those previously characterized and predicted in the yeast genome database. From this screening, we found that Tpk1, one of the three isoforms of the cAMP-dependent protein kinase (PKA), exhibits the highest kinase activity for MBP-Whi3 but shows no activity toward MBP (Fig. 1B, lanes 1 and 2). The two other isoforms of PKA, i.e. Tpk2 and Tpk3, exhibit moderate kinase activity in this assay (Fig. 1B, lanes 3 and 4).

To identify the site(s) of Whi3 phosphorylated by PKA, we searched for the consensus sequence for PKA-mediated phosphorylation (R(R/K)X(S/T)) (18) in Whi3 and found only one consensus sequence (Ser-568) situated within the RRM of Whi3 (Fig. 1C). To examine whether Ser-568 is phosphorylated in vitro, we generated a RRM of Whi3 that contained amino acids 539–621 and then constructed non-phosphorylatable mutant RRM-S568A recombinant protein by site-directed mutagenesis, after which we performed an in vitro kinase assay. The phosphorylation level of MBP-RRM-S568A was statistically significantly reduced compared with that of MBP-RRM but to a modest extent (Fig. 1D), suggesting that Ser-568 of Whi3 is one of the phosphorylation sites mediated by PKA in vitro.

To confirm that PKA phosphorylates Whi3 in vivo, we examined whether the phosphorylation level of Whi3 would be elevated by deletion of the PKA negative regulator BCY1 gene, in which case, PKA becomes constitutively active. As expected, in the bcy1 cells, the phosphorylation level of Whi3-HA increased (Fig. 1E, lanes 1 and 2). The phosphatase-treated Whi3-HA protein in bcy1-deleted cells migrated faster than the untreated protein (Fig. 1F, compare lanes 3 and 5), confirming that Whi3 had been phosphorylated by PKA in vivo. To examine whether Ser-568 is a phosphorylation site of Whi3 in vivo, we constructed the non-phosphorylatable mutation Whi3-S568A by site-directed mutagenesis and compared the phosphorylation levels of Whi3 HA and the Whi3-S568A HA mutant protein in both WT and bcy1-deleted cells (Fig. 1E). In the WT cells, the phosphorylation level of the Whi3-S568A HA mutant protein was indistinguishable from that of the Whi3 HA protein (Fig. 1E, lanes 1 and 3), suggesting that an additional phosphorylation site(s) of Whi3 recognized by PKA might exist in Whi3. In contrast, in the bcy1 cells, the major mobility shift in the Whi3-HA protein was not observed in the Whi3-S568A HA mutant protein (Fig. 1E, lanes 2 and 4). These results indicate that Ser-568 of Whi3 is the site phosphorylated by PKA in vivo.

Phosphomimetic Whi3-S568D Mutation Decreases Cell Size—The above results indicate that Ser-568 of Whi3 is one of the major phosphorylation sites recognized by PKA in vivo. Thus, we focused on the role of this phosphorylation of Whi3 in multiple cellular events. As the Whi3 gene was originally identified as a positive regulator of cell size control (3), we first investigated whether the phosphorylation state of Ser-568 in Whi3 participates in this control. Wild-type (Whi3-HA) and Whi3-S568A-HA cells growing exponentially in normal YPD medium were similar in size. In contrast, the size of phosphomimetic Whi3-S568D-HA mutant cells, like that of the deletion mutant (Δwhi3), was smaller compared with the WT cells (Fig. 2A). These results indicate that the phosphorylation of Whi3 by

Role of Whi3 via PKA in Multiple Cellular Events

![FIGURE 2. PKA controls cell size by phosphorylating Whi3. A, Whi3-HA, Δwhi3, Whi3-S568A-HA, and Whi3-S568D-HA cells were grown in YPD medium. Relative cell size was determined by measuring forward angle light scattering with a FACS Calibur (BD Biosciences). Each histogram was obtained from 2 × 10^5 cells. B, representative size distributions of log-phase cultures of the indicated strains in YP + 2% glucose (solid lines) or YP + 2% ethanol (dotted lines). Cells were grown in YPD medium to mid-log phase, and cells were re-inoculated into YP + 2% glucose or YP + 2% ethanol and grown to mid-log phase. The cell size of samples was determined using a FACS Calibur.](image-url)
PKA causes a decrease in cell size by down-regulating Whi3 function.

PKA activation leads to larger cell size in response to nutrient conditions (13). We examined whether Whi3 is involved in this cell size control, using glucose and ethanol as carbon sources. As reported previously (13), the cell size of wild-type cells (Whi3-HA) growing in glucose medium was larger than that in ethanol medium (Fig. 2B). The cell size of all whi3 mutant strains (Δwhi3, Whi3-S568D-HA, and Whi3-S568A-HA) was also similarly responsive to these carbon sources as the wild-type cells. These results suggest that Whi3 is not a major target of PKA in terms of cell size control in response to a nutrient signal.

We noted that the effect of a whi3 mutation (Whi3-S568D and Whi3-S568A) on the expression levels of these proteins was comparable (Fig. 3A). Because phosphomimetic Whi3-S568D-HA mutant cells seemed to behave like the Δwhi3 cells, we asked whether overexpression of Whi3-S568D would produce a phenotype or not. WHI3 expressed from the GAL1 promoter is lethal and causes an increase in cell size (3).
These results suggest that the PKA-mediated Whi3 phosphorylation is important for restraining Cln3 function. Determination of the timing of the cell cycle progression and CLN2 transcription of Whi3 mutant cells synchronized in the G1 phase. We examined the cell cycle progression and CLN2 transcription of Whi3 mutant cells synchronized in the G1 phase. Like the deletion mutation (∆whi3), the Whi3-S568D mutation led to a shortened G1 phase compared with the length of the G1 phase in WT cells (Fig. 4A). In contrast, the Whi3-S568A mutation led to a prolonged G1 phase (Fig. 4A). Consistent with the effect of these mutations on cell cycle progression, transcription of the CLN2 mRNA was accelerated by the Whi3-S568D mutation and decelerated by the Whi3-S568A mutation (Fig. 4B and C). These results suggest that the PKA-mediated Whi3 phosphorylation at Ser-568 inhibits Whi3 function, thus promoting the start of the cell cycle.

Phosphorylation of Ser-568 in Whi3 is important for cell fate determination—Whi3 is important for restraining Cln3 function in the G1 phase, leading to sporulation or invasive growth (4). Coincidentally, PKA has been shown to be required for the growth regulation of the switch to sporulation and filamentous growth (9). Thus, we examined the effects of the Whi3 mutations on sporulation and invasive growth. The haploid Whi3-S568D-HA mutant, but not the Whi3-S568A-HA mutant, was defective in invasive growth (Fig. 5A). Furthermore, the homozygous diploid Whi3-S568D-HA mutant, but not the Whi3-S568A-HA mutant, failed to sporulate (Fig. 5B). As it has been reported that defects in both sporulation and invasive growth of ∆whi3 cells are alleviated by the loss of CLN3 (4), we examined whether these deficiencies in the Whi3-S568D-HA mutant are caused by promotion of the G1/S transition through the up-regulation of Cln3. As expected, both defects in the Whi3-S568D-HA mutant were reversed by the CLN3 deletion (Fig. 5A and B). These results indicate that the PKA-mediated phosphorylation of Ser-568 in Whi3 is essential for cell fate determination in the G1 phase.

Phosphorylation of Whi3 by PKA leads to its decreased interaction with CLN3 mRNA—All of the above results are consistent with the idea that PKA-mediated phosphorylation of Whi3 at Ser-568 inhibits Whi3 function. How did PKA down-regulate it? Whi3 negatively regulates CLN1, CLN2, and CLN3 mRNA levels by binding to these mRNAs, most efficiently to CLN3 mRNA (4). Thus, we speculated that the phosphomimetic Whi3-S568D mutant and the hyperphosphorylated form of Whi3 in the ∆bcy1 strain would show decreased interaction with CLN3 mRNA. To examine this possibility, we carried out Whi3 immunoprecipitation followed by RT-PCR analysis to measure Whi3 association with CLN3 mRNA. As expected, both the phosphomimetic (Whi3-S568D-HA) and hyperphosphorylated (∆bcy1 Whi3-HA) forms of Whi3 showed reduced interaction with CLN3 mRNA in ∆bcy1 strain (Fig. 6A). Conversely, the Whi3-S568A mutant displayed increased interaction with CLN3 mRNA (Fig. 6A). Moreover, the increase in the ability of Whi3-S568A to bind to CLN3 mRNA was still observed in the ∆bcy1 mutation background (see FIGURE 4. Phosphorylation state of Whi3 at Ser-568 by PKA affects the timing of CLN2 transcription and cell cycle progression. A, effect of the Whi3-S568A and Whi3-S568D mutations on cell cycle progression. The Whi3-HA, Whi3-S568A-HA, Whi3-S568D-HA, and ∆whi3 strains were synchronized with α-factor and released into YPD medium. B, effect of the Whi3-S568A and Whi3-S568D mutations on CLN2 transcription. Northern blot analysis was performed for CLN2 and ACT1 (control) mRNA levels in the cells taken periodically after synchronization with α-factor in YPD medium. C, changes in the relative density of the CLN2 and ACT1 mRNA bands. The amount of the CLN2 mRNA in B was normalized to that of the ACT1 mRNA. For each strain, the maximum value for the first cell cycle is referred to as 1.
Role of Whi3 via PKA in Multiple Cellular Events

Δbcy1 Whi3-S568A mutant), although the binding ability was slightly reduced by the Δbcy1 mutation. This result indicates that the Whi3-S568A mutation is epistatic to the Δbcy1 mutation and further supports our model that Ser-568 of Whi3 is a major regulatory site phosphorylated by PKA for down-regulating the ability of Whi3 to bind to CLN3 mRNA. Altogether, these observations suggest that PKA promotes G₁/S progression by phosphorylating Whi3, which in turn causes reduced interaction with CLN3 mRNA.

Whi3-associated mRNAs are enriched in clusters of the tetranucleotide GCAU, and mutation of these clusters in the CLN3 mRNA causes a reduction in its association with Whi3 (7). If Whi3-S568A binds through GCAU sites in the CLN3 mRNA, Whi3-S568A would not be able to bind to the CLN3mGCAU mRNA, and so Whi3-S568A cells harboring CLN3mGCAU should show a decrease in cell size. We therefore tested whether Whi3 Ser-568 contributes to the interaction with GCAU sites in the CLN3 mRNA by monitoring cell size. As reported previously (7), the size of the Δcln3 Whi3-HA cells harboring the CLN3mGCAU mRNA allele was smaller than that of those harboring CLN3 (Fig. 6B). As expected, the size of Δcln3 Whi3-S568A-HA cells harboring the CLN3mGCAU mRNA allele was also similarly smaller than that of the control CLN3, as in the case of the Δcln3 Whi3-HA cells (Fig. 6B). In contrast, the CLN3mGCAU mRNA had no significant effect on the size of the Δcln3 Whi3-S568D-HA or Δcln3 Δwhi3 cells (Fig. 6B). These results support the idea that phosphorylation of Ser-568 in Whi3 by PKA inhibits the interaction of Whi3 with GCAU clusters in the CLN3 mRNA.

DISCUSSION

Whi3 function is important not only for control of the G₁/S transition but also for the switch to developmental options such as invasive growth and meiosis (3, 4, 6, 7). None of the above studies addressed the role of the phosphorylation of Whi3 in such cellular functions. To this end, we identified PKA as the Whi3 kinase and examined the consequences of phosphorylation of Whi3 by PKA in multiple cellular events using the Whi3-S568D mutation and Whi3-S568A mutations. In this study, we showed that PKA acts as a negative regulator of Whi3. Furthermore, we demonstrated that the phosphorylation of Ser-568 in Whi3 by PKA contributes to cell cycle control and cell fate determination (Fig. 7).

The PKA pathway has been implicated in numerous cellular processes such as cell cycle progression, stress response, and differentiation (for reviews, see Refs. 9 and 10). Although several PKA substrates have been described, the biological activities of these proteins are not sufficient to explain the global effect of PKA on multicellular events. In this study, we showed that like the Δwhi3 mutation, the S568D mutation accelerated
the G1/S transition (Fig. 4). In addition, we demonstrated that the S568D mutation was defective in invasive growth and sporulation as clearly as the \( \psi 6004 \) whi3 mutation (Fig. 5). These findings suggest that the phosphorylation of Whi3 at Ser-568 by PKA plays a critical role in the decision for commitment to the cell division cycle or to alternative developmental fates such as meiosis and invasive growth. The regulation of Whi3 function by PKA appears to play an essential role in the mechanism governing the cell fate decision. In rich medium, the inhibition of Whi3 function would suppress diverse developmental fates, thus allowing the cells to maintain growth appropriately. Conversely, in poor medium, the activation of Whi3 would promote diverse developmental fates for survival. We propose that the PKA signaling pathway is coordinated to control multicellular processes by regulating Whi3.

Consistent with our results, PKA was reported to be required for acceleration of the passage through G1 in response to glucose (14). Conversely, a sudden change from a poor carbon source to glucose leads to a G1 delay by activation of PKA to maintain a critical cell size (12, 13). This discrepancy may lie in the different effects on G1 cell cycle progression depending on the signaling pathways and/or the type of experiments. Thus, Whi3 may not be a major target of PKA with regard to G1 cell cycle progression when cells respond to a sudden change in nutrition. As shown here, elucidation of the role of PKA in cell cycle control can be facilitated by the isolation and characterization of a direct target of PKA.

PKA seems to act as a positive regulator of cell size (for reviews, see Refs. 8–10). In contrast, our results suggest that PKA appears to act as a negative regulator of cell size through the regulation of Whi3. As nutrients affected the cell size independently of Whi3 (Fig. 2B), the physiological roles of Whi3 regulated by PKA in the control of cell size remain unclear.

What is the mechanism for the down-regulation of Whi3 by PKA? The RRM of Whi3 would bind the CLN3 mRNA and restrict Cln3 synthesis in the endoplasmic reticulum (3, 4, 6, 7). Here, we showed that PKA phosphorylated Ser-568 of this RRM, thus inhibiting Whi3 function. Therefore, this phosphorylation might have disturbed the association between Whi3 and CLN3 mRNA, thus escaping the retention mechanism. Indeed, the phosphomimetic Whi3-S568D mutants and the hyperphosphorylated form of Whi3 in the \( \psi 6004 \) bcy1 strain showed reduced interaction with CLN3 mRNA (Fig. 6A).

We have shown that the \( WHI3-S566D \) allele behaved like the whi3 null mutation in all in vivo assays described here. However, prominent effects of the Whi3-S568A mutation were not observed, except for cell cycle progression. These results suggest that phosphorylation by PKA and/or other kinase(s) at some additional site(s) also contributes to down-regulation of Whi3 function.
Role of Whi3 via PKA in Multiple Cellular Events

Among the 119 kinase tested, we identified four kinases (Tpk1, Tpk2, Tpk3, and Ptk2) as Whi3 kinases. Of these, the Ptk2 kinase is involved in the regulation of ion transport and enhances spermine uptake (19, 20). These results indicate that Whi3 is phosphorylated not only by PKA but also by another kinase(s). Thus, Whi3 would be regulated in multiple ways. Further analysis will be needed for clarification of the physiological roles underlying these processes.

The identification of the phosphorylation site of Whi3 enabled us to directly analyze Whi3-dependent cyclin expression/cell cycle progression. Our data suggest that phosphorylation of Whi3 by PKA plays an important role as a direct modulator of a cell fate decision in response to external signals. Because Whi3 homologs are present in some organisms (3), it will be of great interest to study whether a similar mechanism operates in higher eukaryotes.

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