Interactions Between the Kinetochore Complex and the Protein Kinase A Pathway in Saccharomyces cerevisiae

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ABSTRACT The kinetochore is a large structure composed of multiple protein subcomplexes that connect chromosomes to spindle microtubules to enable accurate chromosome segregation. Significant advances have been made in the identification of kinetochore proteins and elucidation of kinetochore structure; however, comparatively little is known about how cellular signals integrate with kinetochore function. In the budding yeast Saccharomyces cerevisiae, the cyclic AMP protein kinase A signaling pathway promotes cellular growth in response to glucose. In this study, we find that decreasing protein kinase A activity, either by overexpressing negative regulators of the pathway or deleting the upstream effector Ras2, improves the viability of ipl1 and spc24 kinetochore mutants. Ipl1/Aurora B is a highly conserved kinase that corrects attachment of sister kinetochores that have attached to the same spindle pole, whereas Spc24 is a component of the conserved Ndc80 kinetochore complex that attaches directly to microtubules. Unexpectedly, we find that kinetochore mutants have increased phosphorylation levels of protein kinase A substrates, suggesting that the cyclic AMP protein kinase A signaling pathway is stimulated. The increase in protein kinase A activity in kinetochore mutants is not induced by activation of the spindle checkpoint or a metaphase delay because protein kinase A activity remains constant during an unperturbed cell cycle. Finally, we show that lowering protein kinase A activity can rescue the chromosome loss defect of the inner kinetochore ndc10 mutant. Overall, our data suggest that the increased protein kinase A activity in kinetochore mutants is detrimental to cellular growth and chromosome transmission fidelity.

KEYWORDS budding yeast protein kinase A kinetochore chromosome segregation spindle checkpoint

Although the budding yeast Saccharomyces cerevisiae (S. cerevisiae) is able to utilize various carbon sources, glucose is the preferred carbon source. Upon addition of glucose to the cell, the cyclic AMP protein kinase A (cAMP-PKA) pathway is activated, and adenylyl cyclase, called Cyr1 in yeast, catalyzes the synthesis of cAMP from ATP. In S. cerevisiae, the activity of adenylyl cyclase is stimulated by the small G proteins Ras1 and Ras2 and a G protein coupled receptor system [supporting information, Figure S1, (Thevelein and de Winde 1999)]. Cdc25 and Sdc25 are two Ras guanine nucleotide exchange factor proteins that switch GDP-bound Ras to GTP-bound Ras, whereas Ira1 and Ira2 are redundant GTPase activating proteins that inactivate Ras by hydrolysis of the bound GTP to GDP (Dechant and Peter 2008; Tamaki 2007). The cAMP produced by adenylyl cyclase binds Bcy1, which is an inhibitory subunit of PKA. In budding yeast, PKA is a heterotetramer of two catalytic subunits (any two of Tpk1, 2 or 3) and two Bcy1 subunits. Once cAMP binds Bcy1, the two catalytic subunits are released from Bcy1, which allows these subunits to phosphorylate multiple target proteins, including phosphorylation and inhibition of the stress response transcription factors Msn2 and Msn4 (Gelade et al. 2003). cAMP accumulation in yeast is downregulated by both a low (Pde1) and high (Pde2) affinity phosphodiesterase, which hydrolyze cAMP to AMP (Nikawa et al. 1987; Sass et al. 1986).

In addition to mediating the cellular response to glucose, the PKA pathway also has cell cycle regulatory roles. For example, PKA activity mediates mitotic arrest in response to DNA damage by regulating...
phosphorylation of Cdc20 (Searle et al. 2004). Cdc20 is a specificity factor of the anaphase promoting complex (APC), and multiple studies suggest that PKA is an inhibitor of the APC (Anghileri et al. 1999; Bolte et al. 2003; Heo et al. 1999; Irminger et al. 2000). The PKA pathway has also been implicated in chromosome segregation. Three studies have demonstrated a potential interaction between the kinetochore, a large structure composed of multiple protein complexes that connects spindle microtubules to chromosomes, and the cAMP-PKA pathway. The inner kinetochore is comprised of the CBF3 centromere-binding complex and a modified nucleosome (Choy et al. 2012; Westermann et al. 2007). Spt1, which is required for assembly of the CBF3 inner kinetochore complex, physically interacts with Cdc20 and upregulates the activity of the cAMP-PKA pathway (Dubacq et al. 2002). Overexpression of negative regulators of the cAMP-PKA pathway rescues the lethality of kinetochore mutants (Li et al. 2005; Magtanong et al. 2011). However, no systematic analysis has been performed to analyze the effect of increasing or decreasing PKA activity on multiple kinetochore mutants, including spindle checkpoint active and inactive alleles, or what impact a kinetochore defect might have on PKA activity.

In this study, we find that decreasing PKA activity rescues the viability of strains carrying mutations in the highly conserved Ipl1/Aurora B kinase and the Ndc80 kinetochore complex. We also show that reduction of PKA activity rescues the chromosome loss defect of the inner kinetochore ndc10-1 mutant. Unexpectedly, we find that ipl1, spc24, and ndc10-1 kinetochore mutants have a high level of PKA activity that is not due to spindle checkpoint activation or metaphase arrest. We propose that the high level of PKA activity in kinetochore mutants is partially responsible for the chromosome loss and growth defects in these strains.

MATERIALS AND METHODS

Yeast strains, plasmids, and media

The yeast strains used in this study are described in Table S1. The pRS326-PDE2 plasmid was obtained from a Ω yeast genomic DNA library (Connelly and Hieter 1996) as a high copy suppressor of spc24-9 lethality on 0.1M HU plates at 33°C (Ma et al. 2007). The genomic DNA coordinates for the PDE2 insert are Chr XV 1011626–1019437. A subclone was constructed that contained only the PDE2 ORF (Chr XV 1013176–1015806), which also rescued spc24-9 lethality (data not shown). The BCY1 plasmid (p416-GPD-BCY1) and vector control (p416-GPD) were kind gifts from Kevin Morano (Trott et al. 2005). The RAS2Δ19 plasmid was a kind gift from Paul Herman (Ramachandran and Herman 2011). The liquid media were rich medium (YPD) or supplemental minimal medium (SC) (Kaiser et al. 1994). The plates for the carbon source spot assays were glucose (2%), glycerol (2%), acetate (1%), ethanol (3%), and YEP (no added carbon source). Yeast genomic DNA was obtained from a 2 μm plasmid (Connelly and Hieter 1996) as a high copy suppressor of spc24-9 lethality.

Western blots

For Figure 3 and Figure 4, wild-type, spc24-10, spc24-9, spc24-8, spc24-8 mad2Δ, mad2Δ, ndc10-1, and ipl1-321 cells were grown to mid-logarithmic phase at 25°C (Figure 3A) or the semipermissive temperature of 31.5°C (Figure 3B) in YPD medium. Wild-type cells harboring the RAS2Δ19 plasmid were grown in SC-Ura medium. 15 μL of culture was harvested, and the pellet was washed once with cold dH2O, frozen at −80°C, and lysed the next day. For Figure 5B, wild-type cells were grown to mid-logarithmic phase in YPD medium, then treated with 20 μg/mL nocardazole or without nocardazole (Log) for 2 hr at 23°C before harvesting. Msna phosphorylation was detected with an α-P-CREB (S133) antibody (1:1000, Cell Signaling), Msna protein was detected with an α-Actin antibody (1:10,000, kind gift from Francisco Estruch), Pgk1 protein levels were detected with an α-Pgk1 antibody (1:10,000, Invitrogen), phosphorylation of PKA substrates was detected with a phospho-PKA substrate antibody (α-sub, 1:5,000, Cell Signaling #9624), and Clb2 was detected with an α-CIB2 antibody (1:5,000, Santa Cruz). For Figure 3, the blot was cut into two parts. The bottom part was probed with α-Pgk1; the top part was first probed with α-P-CREB, stripped for 30 min at 60°C with Tris-SDS buffer [62.5 mM Tris HCl (pH 6.8), 2% SDS, 100 mM β-Mercaptoethanol], and then probed with α-Msn2. For Figure 4, the blot was probed with α-sub and then probed with α-Pgk1 after stripping. Images were captured with a Chemidoc MP imaging system (Bio-Rad), and quantitation was performed using Image Lab software (Bio-Rad). At least three independent experiments were performed for each Western blot. For Figures 3 and 4, a one-way ANOVA test was used to determine which mutants had significantly different phosphorylation levels from wild-type. For Figure 5, an ANOVA with Tukey’s post-hoc analysis was done to compare values from all time points, except for time point zero (which has lower PKA activity due to pheromone treatment), with each other.

DAPI staining and budding index

For each time point in Figure 5A, 1 mL of cells was spun down at 2000 rpm, fixed with 70% ethanol for 1 hr at room temperature, resuspended in PBS, and stored at 4°C. For microscopy imaging, cells were lightly sonicated and mixed well with DAPI (data not shown). The top part was probed with α-P-CREB, stripped for 30 min at 60°C with Tris-SDS buffer [62.5 mM Tris HCl (pH 6.8), 2% SDS, 100 mM β-Mercaptoethanol], and then probed with α-Msn2. For Figure 3, the blot was probed with α-sub and then probed with α-Pgk1 after stripping. Images were captured with a Chemidoc MP imaging system (Bio-Rad), and quantitation was performed using Image Lab software (Bio-Rad). At least three independent experiments were performed for each Western blot. For Figures 3 and 4, a one-way ANOVA test was used to determine which mutants had significantly different phosphorylation levels from wild-type. For Figure 5, an ANOVA with Tukey’s post-hoc analysis was done to compare values from all time points, except for time point zero (which has lower PKA activity due to pheromone treatment), with each other.

Chromosome fragment loss assay

ndc10-1 and ndc10-1 ras2Δ mutants carrying a chromosome fragment (CF) (CIII TRP) were grown over night in SC-TRP media to select for the CF, and then ~12,000 colonies were spread onto SC plates with limiting adenine as described (Kosholsh and Hieter 1987). ndc10-1 pRS416 CIII TRP and ndc10-1 pRS416-CYB1 CIII TRP strains were grown overnight in SC-TRP-URA media to select for the plasmid and CF, and then ~6,000 colonies were spread onto SC-Ura plates (to select for the plasmid) with limiting adenine. Plates were incubated at 25°C for 3 days and then incubated for 2 days at 4°C to develop the red pigment. Colonies that were all red (CF lost while plating), half red/half white (CF lost during first cell division), and total colonies were counted.

RESULTS

Reduction of PKA activity alters the viability of kinetochore mutants:

The Ndc80 complex is a highly conserved essential kinetochore complex composed of Ndc80, Nuf2, Spc24, and Spc25 in which the Ndc80/Nuf2 subcomplex binds directly to microtubules and the Spc24/Spc25 subcomplex interacts with the kinetochore (Toole and Stukenberg 2011). We previously performed a genome-wide synthetic
lethal (SL) screen with three phenotypically distinct alleles of the \textit{SPC24} gene and identified SL or synthetic sick interactions between multiple \textit{spc24} alleles and two negative regulators of the \textit{cAMP-PKA} pathway, \textit{ira2Δ} and \textit{pde2Δ} (Montpetit et al. 2005). Consistent with our previous SL data, we found that overexpression of \textit{PDE2}, which encodes the high affinity \textit{cAMP} phosphodiesterase that converts \textit{cAMP} to \textit{AMP} (Sass et al. 1986), rescued the temperature-sensitive (Ts) lethality of \textit{spc24-8}, \textit{spc24-9}, and \textit{spc24-10} mutants (Figure 1, A and B). Likewise, overexpression of \textit{BCY1}, which negatively regulates PKA, also rescued the Ts lethality of all three \textit{spc24} mutants (Figure 1, A and B) (Toda et al. 1987). The genetic interactions between negative regulators of the PKA pathway and \textit{spc24} mutants suggest that high PKA activity is particularly detrimental to strains with defective kinetochore-microtubule attachments.

To determine whether other kinetochore mutants were affected by PKA activity, we overexpressed \textit{BCY1} and \textit{PDE2} in the \textit{ipl1-321} mutant, which suffers from inappropriate attachment of sister kinetochores to the same spindle pole (Biggins and Murray 2001; Cheeseman et al. 2002; Tanaka et al. 2002). We found that the growth of the \textit{ipl1-321} mutant was modestly rescued by \textit{BCY1} or \textit{PDE2} overexpression (Figure 1C). However, overexpression of \textit{BCY1} or \textit{PDE2} was detrimental to a \textit{ndc10-1} mutant that lacks an assembled kinetochore at a restrictive temperature [Figure 1G, (Goh and Kilmartin 1993)].

The small GTP-binding protein Ras2 interacts with adenylate cyclase and stimulates production of \textit{cAMP} (Field et al. 1990; Suzuki et al. 1990). Consistent with the \textit{BCY1} and \textit{PDE2} overexpression data, we found that the double \textit{spc24 ras2Δ} and \textit{ipl1-321 ras2Δ} mutants grew better than \textit{spc24} or \textit{ipl1-321} single mutants, whereas the \textit{ndc10-1 ras2Δ} double mutant grew more poorly (Figure 2, A and C). Therefore, lowering PKA activity is beneficial to mutants that have kinetochore-microtubule attachment defects but is detrimental to strains that have no kinetochore assembled. To determine how kinetochore mutants respond to an increase in PKA activity, we introduced a dominant allele of Ras2, \textit{RAS2val19}, which activates the PKA pathway, into \textit{ipl1-321, ndc10-1}, and \textit{spc24} mutant strains. As expected, the temperature sensitivity of \textit{ipl1-321} and \textit{spc24} strains was exacerbated by the dominant \textit{RAS2val19} allele and, surprisingly, so was the Ts of the \textit{ndc10-1} strain (Figure 2D). Therefore increasing PKA activity is detrimental to mutants with either no kinetochore or kinetochore-microtubule attachment defects.

The kinetochore monitors its state of microtubule attachment and signals to the spindle checkpoint to prevent anaphase onset in the presence of incorrectly attached or unattached kinetochores. Depending on the nature of the mutation, a kinetochore mutant may or may not activate the spindle checkpoint and arrest the cell cycle in metaphase. For example, of our three \textit{spc24} alleles, only \textit{spc24-8} cells are able to activate the spindle checkpoint at a restrictive temperature (Montpetit et al. 2005). To determine whether decreasing PKA activity can bypass the requirement for the spindle checkpoint, we impaired the spindle checkpoint in \textit{spc24-8} by deletion of the Mad2 checkpoint protein. The \textit{spc24-8 mad2Δ} strain was SL at 34°C, but the SL was rescued by deletion of \textit{RAS2}, suggesting that lowering PKA levels may allow for partial bypass of the spindle checkpoint (Figure 2B).

Chromosome loss defects in \textit{ndc10-1} mutants are suppressed by deletion of \textit{RAS2}

\textit{spc24, ndc10-1}, and \textit{ipl1-321} mutants have defects in chromosome stability (Biggins et al. 2001; Goh and Kilmartin 1993; Montpetit et al. 2005). The chromosome fragment (CF) loss assay is a colony color-based sector assay to determine whether a strain is able to maintain a nonessential CF in the cell (Koshland and Hieter 1987). Colonies that contain the CF are white, whereas colonies that lose the CF are red. If a haploid cell loses the CF in the first cell division, the colony is half-red and half-white (red/white half-sector). We asked whether reduction of PKA activity was able to rescue the chromosome segregation defects of kinetochore mutants using the CF loss assay. We overexpressed \textit{BCY1} or deleted \textit{RAS2} in \textit{spc24-9, ndc10-1,} and \textit{ipl1-321} strains carrying a CF and screened qualitatively for rescue
of CF loss at a variety of temperatures compared with kinetochore mutant alone. We determined that \textit{ndc10-1} CF loss, at a permissive temperature, was rescued by both overexpression of \textit{BCY1} or deletion of \textit{RAS2}, whereas \textit{ipl1-321} and \textit{spc24-9} CF loss was rescued by neither at a permissive or semirestrictive temperature. We next performed quantitative analysis of CF loss in \textit{ndc10-1} strains by growing cells
in media to select for the CF, then plating on nonselective minimal media that contains limiting adenine, which enriches for the red color that arises when cells lose the CF. We scored for colonies that lost the CF in the first cell division (half-white, half-red) and for complete CF loss (red colonies, Table 1). The CF loss events in ndc10-1 were dramatically suppressed by deletion of RAS2 (~20-fold) with a 1.3 × 10^{-2} CF loss rate in ndc10-1 compared with a 6.4 × 10^{-3} CF loss rate in ndc10-1 ras2Δ mutants (Table 1). Overexpression of BCY1 also suppressed CF loss rates in ndc10-1 cells by ~4-fold (Table 1, 8.3 × 10^{-3} CF loss events in ndc10-1 cells compared with 1.9 × 10^{-3} CF loss events in ndc10-1 cells carrying BCY1). In addition to reduction of half sector colonies, inhibition of PKA activity reduced the frequency of red colonies (total CF loss) in ndc10-1 cells by ~17-fold in the absence of RAS2 and ~2-fold when BCY1 was overexpressed (Table 1). In summary, we have found that decreasing PKA activity rescues chromosome loss in ndc10-1 mutants at a permissive temperature.

**Kinetochore mutants have increased levels of PKA activity**

One rational for the rescue of kinetochore defects when PKA activity is reduced is that PKA activity is elevated in kinetochore mutants. We employed two markers to determine if PKA levels are perturbed in spc24 mutants - the PKA dependent phosphorylation of the Msn2 transcription factor, and the phosphorylation profile of PKA substrates. We analyzed Msn2 phosphorylation in wild-type vs. kinetochore mutants using a phospho-CREB antibody that specifically recognizes PKA-dependent phosphorylation (Gorner et al. 2002). As a control, we analyzed Msn2 phosphorylation in wild-type strain expressing the dominant RAS2^{val19} allele. We quantified Msn2 PKA-specific phosphorylation compared with total Msn2 protein levels for all the samples. At a permissive temperature (25°C) all spc24 mutants had similar Msn2 PKA-phosphorylation levels to wild-type cells (Figure 3A), whereas at a semipermissive temperature (31.5°C), spc24-9 and spc24-8 mad2Δ cells had significantly higher (2.5- to 3-fold) Msn2 PKA-dependent phosphorylation levels compared with wild-type cells (Figure 3B). As expected, the phosphorylation of Msn2 in cells carrying RAS2^{val19} was dramatically increased by 5.7-fold at 25°C and 4.1-fold at 31.5°C compared with wild-type cells due to hyperactivation of the PKA pathway. Notably, ipl1-321 mutants had a 1.6-fold increase in Msn2 PKA-phosphorylation compared with the wild-type cells at 25°C and ndc10-1 had a 3.7-fold increase at 31.5°C. Therefore, cells with defective kinetochore assembly or kinetochore-microtubule attachments have higher PKA activity. There was a reduction in total Msn2 protein levels in spc24-10, ndc10-1, and ipl1-321 cells, possibly due to a reduction in overall protein synthesis in these mutants that are at a semipermissive temperature or because defects at the kinetochore affect Msn2 stability. Because Pkg1 levels are similar in all kinetochore mutants tested and spc24-10 mutants are not sensitive to cycloheximide (data not shown), we do not think that protein synthesis is reduced in spc24-10, ndc10-1, and ipl1-321 mutants.

Next, we examined the phosphorylation profile of PKA substrates in the kinetochore mutants using a phospho-PKA substrate antibody (α-sub). Strains were grown to log phase at permissive (25°C) and semipermissive (31.5°C) temperatures, lysates were generated, and Western blot analysis was performed. A wild-type strain expressing RAS2^{val19} was used as control to detect PKA substrates. We used a chemiluminescence detection system and exposed the blot for a very short time (15 sec) before any protein band was saturated. Our image software detected six prominent PKA-phosphorylated substrates at 25°C and five substrates at 31.5°C that migrated between 70 kD and 200 kD (Figure 4, A and B). All six bands have a stronger signal in Ras2^{val19} compared with the wild-type cells at 25°C, suggesting that these substrates are PKA phosphorylated. We quantified two proteins, Pa and Pb, as representative PKA substrates for all the strains. At 25°C, all mutants have Pa phosphorylation levels similar to wild-type cells. However, when compared with wild-type cells, the phosphorylation level of Pb is increased by 2.6-fold in spc24-8 mad2Δ, 3.8-fold in ndc10-1 cells, and 7.2-fold in Ras2^{val19} cells. At 31.5°C, all the mutants, except for spc24-8 and mad2Δ, have significantly increased phosphorylation of Pa by ~2-fold compared with wild-type cells, whereas the phosphorylation of Pb does not change. We noticed that the 100 kD PKA substrate was absent from the ndc10-1 strain and the ~160 kD band had reduced mobility (Figure 4B, ndc10-1 lane). Because Ndc10 is a 112 kD protein, we wondered whether Ndc10 was the 100 kD PKA substrate that was absent in ndc10-1 strains. However, we analyzed the PKA profile of strains carrying tagged versions of Ndc10 (Ndc10-13Myc and Ndc10-3HA), which should decrease mobility of the 100 kD band, and we did not detect any change in migration, suggesting that Ndc10 is not the 100 kD PKA substrate (data not shown). Therefore the 100 kD substrate could be an Ndc10-interacting protein that is destabilized at a semipermissive temperature in the ndc10-1 strain. Overall, these data demonstrate that PKA substrates have increased levels of phosphorylation in multiple kinetochore mutant strains, suggesting that PKA activity is elevated in these strains.

**PKA activity does not fluctuate during an unperturbed cell cycle**

Strains defective in kinetochore function tend to accumulate in either metaphase or anaphase with a 2N population of DNA. Therefore, it is possible that we detect an increase in PKA activity in kinetochore mutants due to an overall increase in PKA activity in metaphase or anaphase. To address this possibility, we investigated Msn2 phosphorylation levels during the cell cycle. Wild-type cells were arrested in G1 phase at 25°C with the mating pheromone α-factor, released into the cell cycle for 30 min and time points taken every 15 min until the cells had divided. We monitored Msn2 PKA-dependent phosphorylation, Msn2 protein levels, protein levels of the Clb2 mitotic cyclin, and nuclear division. At 0 min, Msn2 phosphorylation levels were low because mating pheromone inhibits adenylate cyclase (Liao and

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**Table 1 Reduction of PKA activity rescues CF loss in the ndc10-1 kinetochore mutant**

<table>
<thead>
<tr>
<th>Genotype (MATα)</th>
<th>Total Colonies</th>
<th>Chromosome Loss (Red)</th>
<th>Chromosome Loss in First Division (Red/White Half Sectors)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ndc103-1::HIS3 ndc10-1::kanMX6 CFI11</td>
<td>11,632</td>
<td>6.9 × 10^{-2} (801)²</td>
<td>1.3 × 10^{-2} (143)</td>
</tr>
<tr>
<td>ndc103-1::HIS3 ndc10-1::kanMX6 ras2ΔLEU2 CFI11</td>
<td>11,118</td>
<td>4.1 × 10^{-3} (46)</td>
<td>6.3 × 10^{-2} (7)</td>
</tr>
<tr>
<td>ndc103-1::HIS3 ndc10-1::kanMX6 CFI11 pRS416</td>
<td>5,926</td>
<td>1.8 × 10^{-2} (104)</td>
<td>8.3 × 10^{-2} (49)</td>
</tr>
<tr>
<td>ndc103-1::HIS3 ndc10-1::kanMX6 CFI11 pRS416-BCY1</td>
<td>6,409</td>
<td>9.7 × 10^{-3} (62)</td>
<td>1.9 × 10^{-3} (12)</td>
</tr>
</tbody>
</table>

² Numbers in brackets represent the total number of red or red/white colonies.
Thorner 1980). The metaphase-to-anaphase transition occurred between 75 and 90 min as Clb2 levels peaked and the nuclei divided (Figure 5A). Although Msn2 PKA-dependent phosphorylation appeared to increase at 75 to 90 min, so did Msn2 protein levels; therefore, when quantitated, no increase in Msn2 phosphorylation was detected at the metaphase-to-anaphase transition (Figure 5A). In fact, no significant difference was detected in Msn2 PKA-dependent phosphorylation between 30 and 120 min, suggesting that PKA activity remains constant during the cell cycle.

To specifically analyze Msn2 PKA-dependent phosphorylation in a population of metaphase-arrested cells, we treated wild-type cells with the microtubule depolymerizing agent nocodazole. Interestingly, the Msn2 PKA-dependent phosphorylation was significantly reduced upon nocodazole treatment (Figure 5B). In summary, our cell-cycle analysis suggests that Msn2 PKA-dependent phosphorylation does not specifically increase at the metaphase-to-anaphase transition. On the contrary, under conditions of cell-cycle arrest in G1 phase (mating pheromone treatment) or metaphase arrest (nocodazole treatment), Msn2 PKA-dependent phosphorylation is decreased. Therefore, our data suggest that the increase in PKA activity in kinetochore mutants is not simply due to an increase in PKA activity at the metaphase-to-anaphase transition.

Nonfermentable carbon sources permit growth at a higher restrictive temperature for mutants of the Ndc80 complex

Cells grown in glucose media are presumed to have high PKA activity, whereas cells growing in nonfermentable carbon sources are presumed to have low PKA activity. This hypothesis was tested by growing cells in media supplemented with ethanol or glycerol, which are nonfermentable carbon sources. The results showed that Msn2 PKA-dependent phosphorylation was higher in cells grown in nonfermentable carbon sources compared to glucose-grown cells. This suggests that the increase in PKA activity in kinetochore mutants is not simply due to an increase in PKA activity at the metaphase-to-anaphase transition.

Figure 3 Kinetochore mutants have higher levels of Msn2 PKA-dependent phosphorylation. Cells were grown to mid-logarithmic phase at 25°C (A) or the semipermissive temperature of 31.5°C (B) and lysed for immunoblot analysis. The blots were probed with α-P-CREB antibody to detect PKA-dependent Msn2 phosphorylation (P-Msn2) and α-Msn2 and α-Pgk1 as loading controls. One representative blot is shown out of three independent experiments. The bar graphs are the relative ratio of P-Msn2/total Msn2 analyzed from the three independent experiments. The value of 1 was assigned to the ratio of P-Msn2/total Msn2 in the wild-type cells. The asterisk represents values that are significantly different from the wild-type value (P < 0.05).

Figure 4 PKA substrates have higher levels of phosphorylation in kinetochore mutants. (A) The lysates described in Figure 3 were probed with the anti-PKA substrate antibody (α-sub) and α-Pgk1 as a loading control. Two protein bands from the α-sub blot, Pa and Pb, were selected for quantification. One representative blot from three independent experiments is shown. Bar graphs are the relative ratio of Pa/Pgk1 or Pb/Pgk1 averaged from three independent experiments. The value of 1 was assigned to the ratio of Pa/Pgk1 or Pb/Pgk1 in the wild-type cells. The asterisk represents values that are significantly different from the wild-type value (P < 0.05).
to have low PKA activity (Thevelein and de Winde 1999). Because reducing PKA activity increases the restrictive temperature of spc24 mutants, we tested the growth of spc24 mutants by plate assay on glucose vs. nonfermentable carbon sources (glycerol, acetate, and ethanol) or no added carbon source except for the yeast extract and peptone in the media (YEP) (Figure 6). spc24 mutants grew at a higher restrictive temperature on almost every media tested compared with glucose media (Figure 6, A and C). Notably, spc24-8 is rescued on nonfermentable carbon sources when the spindle checkpoint is active (spc24-8 or inactive (spc24-8 mad2Δ, Figure 6C). Therefore, exposing spc24 mutants to glucose, which triggers high PKA activity, is inhibitory to growth. We asked whether the glucose growth inhibition phenotype was specific to spc24 mutants or shared with other mutants in the Ndc80 complex. Ts mutants of other proteins of the Ndc80 complex, spc25-1, ndc80-1, and nuf2-61, were also rescued by growth on nonfermentable carbon sources (Figure 6B). To determine whether the glucose sensitivity was specific to the Nd80 complex, we spotted ndc10-1 and ptea321 cells on nonfermentable carbon sources (Figure 6D). Contrary to mutants in the Nd80 complex, the fitness of the ndc10-1 mutant was optimal on glucose media, which is consistent with decreased fitness of ndc10-1 cells when PKA activity is reduced (Figures 1C, 2C, and 6D). Surprisingly, glucose was also the preferred carbon source of the pte11-321 mutant, despite fact that lowering PKA activity improves the fitness of pte11-321 cells (Figures 1C, 2C, and 6D). A previous report also found that the pte11-2 mutant cannot be rescued by plating on media that slows growth, such as nonfermentable carbon sources (Tatchell et al. 2011). Because the growth rate of yeast is slower on nonfermentable carbon sources, it was possible that the rescue of spc24 mutants on nonglucose plates was due to a reduction in growth rate. Therefore, we tested growth of a subset of spc24 mutants on limiting nitrogen, cycloheximide, and rapamycin plates, all of which cause a reduction in growth rate. We did not detect any rescue of the spc24 Ts phenotype in any condition (data not shown). Therefore, spc24 mutants are rescued specifically by growth on non-glucose carbon sources and not generally by slowing cell growth rate.

**DISCUSSION**

This work stemmed from the initial observation that decreasing cAMP-PKA activity, either by overproducing BCY1 or PDE2 or by deleting RAS2, rescued the lethality of spc24 kinetochore mutants (Figures 1 and 2). In addition, we had previously identified genetic interactions between pde2 and ira2, which are mutants of negative regulators of the cAMP-PKA pathway, and spc24 mutants (Montpetit et al. 2005). A simple model of these observations is that the cAMP-PKA pathway inhibits kinetochore function. However, our data suggest that the interaction between the cAMP-PKA pathway and the kinetochore is more complex. Inhibition of PKA activity is beneficial to the growth of spc24 and pte11-321 mutants but detrimental to the ndc10-1 mutant, in which no kinetochore is assembled, and the spc24-8 mad2Δ double mutant, in which the spindle checkpoint has been abolished (Figure 1). In addition, all kinetochore mutants, except for the spindle checkpoint proficient spc24-8 mutant, have higher levels of PKA activity (Figures 3 and 4). The increased PKA activity is not due to accumulation of cells in metaphase or anaphase because during an unperturbed cell cycle, PKA-dependent Msn2 phosphorylation levels remain constant (Figure 5A). As well, metaphase-arrested cells have low levels of PKA activity (Figure 5B). Therefore, defects in the kinetochore, and not cell cycle arrest, cause an increase in PKA activity. Finally, we show that reduction of PKA activity rescues chromosome loss defects of the ndc10-1 mutant at a permissive temperature (Table 1). Therefore, the kinetochore is highly sensitive to fluctuations in PKA activity.

We demonstrate that, in addition to suppression of lethality by lowering PKA activity, mutants of the Nd80 kinetochore complex are sensitive to glucose, and their growth defects are suppressed on nonfermentable carbon sources (Figure 6). It was shown previously...
that mutants of the APC are also suppressed by reducing Ras signaling and growth on nonglucose carbon sources (Irniger et al. 2000). Therefore, we expected that all kinetochore mutants that were rescued by reducing PKA signaling would preferentially grow on nonglucose carbon sources. However, we tested a variety of kinetochore mutants in addition to the mutants presented here and found no strict correlation between growth rescue by inhibition of PKA signaling and growth rescue on nonglucose carbon sources. For example, a strain carrying a mutation in CTF13, which codes for a protein in the CBF3 inner kinetochore complex, displayed no growth changes upon overexpression of BCY1 but was rescued by growth on glycerol or galactose media (data not shown). For example, a strain carrying a mutation in CTF13, which codes for a protein in the CBF3 inner kinetochore complex, displayed no growth changes upon overexpression of BCY1 but was rescued by growth on glycerol or galactose media (data not shown). The growth of the ipl1-321 mutant is improved upon deletion of RAS2 or overexpression of BCY1 but not when plated on nonfermentable carbon sources (Figures 1C and 2C). Although the viability of all mutants of the Ndc80 kinetochore complex was rescued by reducing PKA signaling or plating on a nonglucose carbon source. For example, a strain carrying a mutation in CTF13, which codes for a protein in the CBF3 inner kinetochore complex, displayed no growth changes upon overexpression of BCY1 but was rescued by growth on glycerol or galactose media (data not shown). Therefore, the sensitivity of kinetochore mutants to fluctuations in PKA activity is not strictly correlated to the presence of glucose in the media.

The chromosome loss defect of ndc10-1 cells is reduced by 20-fold upon deletion of RAS2 and 4-fold overexpression of BCY1 at 25°C (Table 1). However, when grown at 30°C, we find that overexpression of BCY1 causes lethality to ndc10-1 strains and that ndc10-1 ras2Δ strains grow more poorly than do ndc10-1 strains at 32°C (Figures 1C and 2C). At a permissive temperature, kinetochore complexes still associate with centromere DNA in the ndc10-1 mutant, whereas at a restrictive temperature, no kinetochore complexes are able to assemble on the centromere (He et al. 2001; Janke et al. 2001; Nekrasov et al. 2003; Ortiz et al. 1999). Therefore, lowering PKA activity only rescues CF loss of the ndc10-1 mutant when there is an assembled kinetochore that is able to attach to microtubules. Perhaps this is why we did not detect rescue of CF loss in ipl1-321 or spc24-9 mutants in which kinetochore-microtubule attachment is impaired.

We find that the ipl1-321 growth defect is suppressed by overexpression of PDE2 and BCY1 or by deletion of RAS2, all of which lower PKA activity (Figures 1C and 2C). In addition, expression of the dominant RASval19 allele is detrimental to ipl1-321 growth (Figure 2D). The rescue of ipl1-321 growth defect by lowering PKA activity is intriguing in light of recent data demonstrating that reduction of target of rapamycin (TOR) complex 1 (TORC1) activity also suppresses the growth defect of an ipl1-2 mutant (Tatchell et al. 2011). TOR and PKA are the two major signaling pathways that activate cell growth in response to nutrients by regulating processing, such as translation, ribosome biogenesis, and glucose metabolism (Smets et al. 2010; Soulard et al. 2009). TORC1 and PKA have been shown to regulate common target proteins, and recent data demonstrate that TOR can activate PKA toward a subset of substrates (Soulard et al. 2010). Reduction of TORC1 activity suppressed the chromosome loss
defect in *ipl1-2* mutants, whereas we did not detect rescue of CF loss in *ipl1-321* mutants upon reduction of PKA activity (Tatchell et al. 2011). Nonetheless, our data combined with Tatchell et al. (2011) strongly support a link between nutritional status and kinetochore function.

Activation of the PKA pathway is known to be inhibitory to the APC, possibly via phosphorylation of Cdc20 (Anghileri et al. 1999; Bolte et al. 2003; Irniger et al. 2000; Searle et al. 2004). For example, the growth defect of *apc10-22* mutants, which stabilizes Pds1, is suppressed by deletion of *RAS2* (Irniger et al. 2000). Stabilization of Pds1 (securin) prevents chromosome separation due to inhibition of Esp1 (separate). The APC is also inhibited, and Pds1 is stabilized upon activation of the spindle checkpoint by the interaction of the Mad2 checkpoint protein with Cdc20 (Clarke and Bachant 2008). However, no study has addressed the interaction between the PKA pathway and the spindle checkpoint. In this work, we monitored Msn2 PKA-dependent phosphorylation in cells arrested in metaphase due to induction of the spindle checkpoint upon nocodazole treatment (Figure 5B). We found that Msn2 PKA-dependent phosphorylation is reduced under these conditions, suggesting that PKA activity may be reduced during the spindle checkpoint. Interestingly, the only kinetochore mutant that did not display high levels of PKA activity was *spc24-8*, which arrests in metaphase due to activation of the spindle checkpoint (Montpetit et al. 2005) (Figures 3 and 4). It is possible that reducing PKA levels suppresses *spc24* and *ipl1* mutants due to spindle checkpoint activation; however, this does not explain why the viability of the *spc24-8* mutant, which is spindle checkpoint proficient, is improved by reducing PKA activity (Figures 1 and 2). Activation of the PKA pathway causes phosphorylation of the Msn2 nuclear localization sequence on serine residues, prevention of Msn2 nuclear import, and restoration of Msn2 to the cytoplasm (Gorner et al. 1998, 2002; Jacquet et al. 2003). Inhibition of PKA activity upon nocodazole treatment might evoke a general stress response that results in dephosphorylation of Msn2 and subsequent localization to the nucleus. In fact, microarray studies performed after treatment of yeast cells with benomyl, another microtubule poison, demonstrated that expression of Msn2 genes is induced (Lucan-Danila et al. 2005). Whether inhibition of PKA activity rescues *spc24* mutants due to activation of a Msn2-dependent stress response remains to be tested.

Because it is difficult to separate the spindle checkpoint response from a general cell stress response upon addition of microtubule poisons, deletion of the Mad2 spindle checkpoint protein is an alternative method to assess the spindle checkpoint. We find that overexpression of *BCY1* and *PDE2* only rescues viability of strains that have a partially assembled kinetochore and Mad2 present in the cell (Figure 7). For example, overexpression of *BCY1* and *PDE2* rescues the growth defect of the *spc24-8* (spindle checkpoint active) mutant but is detrimental to the growth of the *spc24-8 mad2Δ* (spindle checkpoint defective) mutant (Figures 1B and 7). The APC specificity factor Cdc20 is phosphorylated on PKA consensus sites after DNA damage, and inactivation of PKA accelerates Pds1 destruction (Searle et al. 2004). If phosphorylation of Cdc20 by PKA prevents an interaction between Cdc20 and Mad2, then decreasing PKA activity may enrich the Cdc20-Mad2 interaction and spindle checkpoint response. If Mad2 is not present, then decreasing PKA activity may accelerate Pds1 destruction, which would force *spc24-8 mad2Δ* cells into anaphase with defective chromosome attachments. One complication to this argument is that deletion of *RAS2* rescues the growth defect of both *spc24-8* and *spc24-8 mad2Δ* strains (Figures 2B and 7). Likely, there is more than one mechanism by which a reduction in PKA activity rescues viability of kinetochore mutants.

An unexpected finding from our study is that kinetochore mutants have increased levels of PKA activity when grown at a semipermissive temperature (Figures 3 and 4). These data suggest that defects in the kinetochore may trigger activation of the PKA pathway, possibly via components of the cAMP-PKA pathway, that are nuclear localized. Bcy1, the PKA regulatory subunit, is predominantly in the nucleus when cells are grown in glucose (Griffioen et al. 2000). It remains to be determined whether a nuclear pool of Bcy1 or a nuclear-localized PKA catalytic subunit, such as Tpk1, interacts with the kinetochore and whether disruption of kinetochore function releases active Tpk1. It has been demonstrated that loss of one copy of *BCY1* in a diploid strain increases the rate of chromosome loss, suggesting that Bcy1 may be important for kinetochore function (Magtanong et al. 2011). How the kinetochore impinges upon the cAMP-PKA pathway and whether this function is conserved in higher eukaryotes will be an interesting subject of future research.

Figure 7 Summary of kinetochore and PKA interactions. At a restrictive temperature, the *ndc10-1* mutant does not assemble a kinetochore; therefore, microtubules cannot attach and Mad2 cannot be recruited. The *ipl1-321* mutant has functional kinetochores that attach to the same pole, but Mad2 is not recruited (Gillett et al. 2004). The *spc24-8* mutant is checkpoint active; therefore, Mad2 is presumed to be properly localized to the kinetochore. The *spc24-8 mad2Δ* mutant lacks Mad2 and is spindle checkpoint defective; however, kinetochores are still present. The state of the kinetochore-microtubule interaction in *spc24-8 mad2Δ* cells has not been investigated. The kinetochores in *spc24-9* and *spc24-10* mutants have defects in microtubule attachment and are presumed to mislocalize Mad2 as the spindle elongates in both mutants, despite attachment defects. In addition, similar mutants in the Ndc80 complex mislocalize Mad2 (Gillett et al. 2004). Genetic interactions upon overexpression of *BCY1* or *PDE2*, deletion of *RAS2* (ras2Δ), or expression of *RAS2Δ19* in each kinetochore mutant is represented with a plus (+) sign if growth defects were improved and minus (−) sign if growth defects were exacerbated.
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