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Ipl1p-dependent phosphorylation of Mad3p is required for the spindle checkpoint response to lack of tension at kinetochores

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The spindle checkpoint delays anaphase onset until all chromosomes are correctly attached to microtubules. Ipl1 protein kinase (Aurora B) is required to correct inappropriate kinetochore–microtubule attachments and for the response to lack of tension between sister kinetochores. Here we identify residues in the checkpoint protein Mad3p that are phosphorylated by Ipl1p. When phosphorylation of Mad3p at two sites is prevented, the cell’s response to reduced kinetochore tension is dramatically curtailed. Our data provide strong evidence for a distinct checkpoint pathway responding to lack of sister kinetochore tension, in which Ipl1p-dependent phosphorylation of Mad3p is a key step.

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Since errors in chromosome segregation lead to aneuploidy, cell death, and disease, cells have evolved mechanisms to ensure that their replicated chromosomes are accurately segregated. The spindle checkpoint, involving a conserved network of Mad and Bub proteins, acts as a surveillance system to monitor kinetochore–microtubule attachment or result from attachment of both sister kinetochores to the same spindle pole (syntelic or monopolar attachment). Syntelic attachments are thought to result in a lack of tension between sister kinetochores, and error correction mechanisms are required to promote biorientation. It is clear from both vertebrate and yeast studies that Aurora B kinase (Ipl1p in yeast) has a crucial role to play in promoting such biorientation, and that this involves breaking incorrect (syntelic) microtubule attachments (Tanaka et al. 2002; Hauf et al. 2003; Dewar et al. 2004; Lampson et al. 2004). Aurora kinase may also link the correction of inappropriate attachments with a spindle checkpoint-dependent mitotic delay, as it is required to delay anaphase in response to the lack of cohesion at sister centromeres, reduced microtubule dynamics (taxol), and certain kinetochore defects (Biggins and Murray 2001; Ditchfield et al. 2003; Hauf et al. 2003; Pinsky et al. 2003). However, the existence of a distinct “tension checkpoint” mechanism remains controversial (for a recent review, see Pinsky and Biggins 2005). In budding yeast, although in some circumstances Ipl1p can activate the spindle checkpoint through generation of unattached kinetochores when it promotes breakage of defective microtubule attachments (Pinsky et al. 2006), it may also play a direct role in spindle checkpoint signaling in response to a lack of tension.

Studies using Aurora B kinase inhibitors in vertebrate cells have indicated a possible link between Aurora B and BubR1: BubR1 phosphorylation and localization are perturbed when Aurora B is inhibited (Ditchfield et al. 2003). Here we use budding yeast to demonstrate that the yeast BubR1-related protein Mad3p is a substrate of Ipl1p, and we have mapped multiple Ipl1p phosphorylation sites in Mad3p. Blocking phosphorylation of these sites by mutation to alanine prevents cells from delaying anaphase specifically in response to lack of sister kinetochore tension. We therefore propose that a distinct, Ipl1p-dependent branch of the spindle checkpoint pathway monitors tension at sister kinetochores, and that phosphorylation of Mad3p by Ipl1p is a crucial component in this checkpoint response.

Results and Discussion

BubR1 kinetochore localization is sensitive to lack of tension (Skoufias et al. 2001; Taylor et al. 2001), and BubR1 phosphorylation is Aurora B-dependent (Ditchfield et al. 2003). In yeast, we and others have found that Mad3p shows both Polo (Cdc5p) and Aurora (Ipl1p) kinase-dependent phosphorylation in mitosis (Rancati et al. 2005; data not shown). Since Ipl1p is required for mitotic checkpoint activation in response to loss of tension (Biggins and Murray 2001), Ipl1p-dependent phosphorylation of Mad3p may be a mechanism by which Ipl1p
participates in the checkpoint-dependent anaphase delay. To test this, we therefore needed to analyze yeast cells in which kinetochores remain attached to microtubules but do not come under tension, a condition proposed to occur when cells dependent on SCC1 expression from the GAL promoter undergo DNA replication following transfer to glucose to block SCC1 expression (Indjeian et al. 2005). Scclp is a component of the cohesin complex responsible for sister chromatid cohesion, and depletion of Scclp prevents cells from establishing cohesion as they pass through S phase.

To validate such GAL-SCC1 strains as a “lack of tension assay,” we visualized cells containing fluorescently labeled spindles (Tub1-CFP) and kinetochores (Mtw1-3GFP). Such cells were presynchronized in G1 with α-factor and released in the presence of glucose to allow DNA replication in the absence of SCC1 expression, and then kinetochores–microtubule attachment was monitored by microscopy as described by Pinsky et al. (2006). We found that in all mitotic Scclp-depleted cells analyzed (n = 650), Mtw1-3GFP localized exclusively along the spindle axis defined by Tub1-CFP fluorescence, indicative of highly efficient kinetochore attachment (Fig. 1A). We conclude that depletion of Scclp does not generate unattached kinetochores and can therefore be used to generate sister chromatids that are attached to microtubules but that lack tension. This finding is further supported by similar analysis of cells expressing Mtw1-3GFP and Tub1-CFP but whose cohesion defect was generated using the temperature-sensitive mcd1-1 allele of SCC1 as an alternative to Scclp depletion (data not shown).  

Ipl1p phosphorylates Mad3p in vitro

To determine whether Mad3p might be a direct substrate for Ipl1p, recombinant Mad3p was incubated with recombinant Ipl1p and its activator Sli15p (the yeast INCENP ortholog) [Kang et al. 2001], together with radion-labeled Mg²⁺-ATP to perform a kinase assay. Figure 2A shows that Mad3p is, indeed, phosphorylated by Ipl1p and that this is dependent on Sli15p as expected. When this ß-P-labeled Mad3p was subjected to tryptic digestion and the resulting peptides separated by HPLC, five phosphopeptides were evident (Fig. 2B). Detailed phosphorylation site analysis of Mad3p identified the major Ipl1p phosphorylation site (corresponding to peak 2) on Mad3p in vitro as Ser 373 (Fig. 2C). We then replaced Ser 373 with alanine, to prevent phosphorylation at this site, and examined the ability of Mad3p-S373A to be phosphorylated by Ipl1p–Sli15p (Fig. 2D). Wild-type Mad3p was phosphorylated efficiently in comparison with the Mad3p-S373A protein, confirming that Ser 373 is indeed the main site of Mad3p phosphorylation by Ipl1p in vitro. However, the low residual level of phosphorylation still evident on the mutant protein prompted us to identify the remaining phosphorylation sites using a more sensitive method employing LC-MS with precursor ion scanning. Three additional sites (Ser 10, Ser 303, and Ser 486) were definitively identified, and a fourth site was localized within a 16-amino-acid phosphopeptide (residues 460–475), although we were unable to distinguish which one of its five serines or threonines was phosphorylated.

We therefore established that Mad3p is phosphorylated directly in vitro on up to five different sites by the Ipl1p–Sli15p complex. Two out of the four iden-
Figure 2. Identification of Ipl1p-dependent phosphorylation sites in Mad3p. (A) Recombinant Mad3p was incubated with either Ipl1p or Ipl1p-D227A (kinase dead) in the presence or absence of Sl15p, together with Mg\(^{2+}\)-[\(\gamma\)-\(32\)P]ATP. Phosphorylation of Mad3p was visualized following polyacrylamide gel electrophoresis and autoradiography, locating the position of the Mad3p band by Coomassie staining. (B) Following phosphorylation with Ipl1p-S15p complex, \(32\)P-labeled Mad3p was digested with trypsin and separated on a C18 column, monitoring the \(32\)P elution profile. Peaks containing \(32\)P-labeled peptides are labeled 1-5. Peak 2 contained 57% of the applied \(32\)P radioactivity. (C) Peak 2 was analyzed by MALDI-TOF mass spectrometry and Edman degradation: The deduced amino acid sequence from the mass spectrometric analysis is plotted against the radioactivity released in each cycle of Edman degradation. Maximal release of \(32\)P occurred in the cycle corresponding to Ser 337. (D) Escherichia coli-expressed Mad3p and Mad3p-S337A were incubated with Ipl1p-S15p complex in the presence of Mg\(^{2+}\)-[\(\gamma\)-\(32\)P]ATP. Phosphorylation of Mad3p and Mad3p-S337A was determined following electrophoresis on a polyacrylamide gel and autoradiography, locating the position of the Mad3p band by Coomassie staining. The relevant section of the stained gel is shown to confirm equal loadings. (E) The Ipl1p consensus phosphorylation site [Cheeseman et al. 2002] is shown together with the four Ipl1p phosphorylation sites identified by LC-MS with precursor ion scanning (one of which had been previously identified in C), highlighting the phosphorylated serine, the basic residue at -2, and the hydrophobic residue at +1 present at two of the sites. A phosphopeptide containing a fifth site that could not be unambiguously placed within the sequence is also shown (possible sites of phosphorylation are indicated in gray). The location of each defined phosphoserine within the Mad3p sequence is also indicated in relation to two conserved domains [Hardwick et al. 2000] involved in binding Cdc20p/Mad2p (I) and Bub3p (II).

Mutation of Mad3 to prevent phosphorylation of Ser 303 and Ser 337 abrogates the tension checkpoint

Since MAD3 is essential for the checkpoint response to lack of tension, we next investigated whether phosphorylation of Mad3p at the Ipl1p phosphorylation sites, identified in vitro, was important in vivo. We initially mutated the major in vitro site [Ser 337] to alanine and introduced the mad3-S337A allele into strains in which the tension checkpoint could be activated by SCC1 deletion. Although cells without Mad3p (mad3Δ) quickly degraded Pds1p, we found that cells carrying either MAD3 or mad3-S337A maintained elevated levels of Pds1p (data not shown), indicative of a functional checkpoint. Thus the S337A mutation alone does not impair the tension checkpoint. Although Ser 337 is the major Mad3p site phosphorylated by Ipl1p in vitro, phosphorylation of one or more of the other candidate sites may function redundantly in vivo. We therefore made a double mutant in which both Ser 303 and Ser 337 were changed to alanine (mad3-2SA), and a quadruple mutant in which serines 10, 303, 337, and 486 were similarly mutated (mad3-4SA) to prevent phosphorylation. Figure 3 shows that both the mad3-2SA and mad3-4SA mutants were largely defective in the tension checkpoint delay. Since no reproducible difference was found between the double and the quadruple mutants, we conclude that S303 and S337 are likely to be the major phosphorylation sites required for a complete tension checkpoint response and that they function redundantly in vivo. However, cells carrying mad3Δ degraded...
Pds1p slightly earlier than the mad3-2SA and mad3-4SA mutants. This slight residual delay may represent the contribution of Ipl1p-generated unattached kinetochores to the tension checkpoint response. Alternatively, Mad3p phosphorylation may be only part of a more complex response, and complete absence of Mad3p may additionally destabilize checkpoint protein complexes. The requirement for Ipl1p-dependent Mad3p phosphorylation for a fully functional checkpoint is consistent with a recent proposal that Ipl1p activity cooperates with vertebrate spindle checkpoint proteins to inhibit the APC/C (Morrow et al. 2005).

**Figure 4.** Blocking phosphorylation of Mad3p does not abrogate the checkpoint response to unattached kinetochores

To assess the ability of strains to mount a checkpoint response to unattached kinetochores, we tested the benomyl sensitivity (Li and Murray 1991) of our alanine substitution mutants alone or in combination. Figure 4A shows that all of the mad3 mutant strains tested behaved identically to a MAD3 wild-type control and were able to grow well on rich medium containing 12.5 and 15 µg/mL benomyl, respectively. Furthermore, when synchronous mad3-2SA cells were treated with nocodazole and the stability of Pds1p was examined, they were found to display a similar mitotic arrest to wild-type cells (Fig. 4B). Thus all of these mad3 mutant alleles support a normal checkpoint response to unattached kinetochores, and the effect of the mad3-2SA allele is therefore highly specific for checkpoint activation in response to lack of tension. In support of distinct pathways responding to tension or attachment, vertebrate localization studies have shown that checkpoint components behave in a manner consistent with differential monitoring of these two properties. For example, Mad1 and Mad2 tend to dissociate from kinetochores upon stable microtubule attachment, whereas BubR1, the functional equivalent of budding yeast Mad3p in vertebrates, remains on attached chromosomes if they lack tension, such as in the presence of the microtubule-stabilizing agent taxol (Skoufias et al. 2001; Shannon et al. 2002; Logarinho et al. 2004).

Based on our observations and previously published work, we propose a dual role for Ipl1p: resolution of maloriented sister kinetochores and phosphorylation of Mad3p so as to activate or enhance the checkpoint delay in response to lack of sister kinetochore tension (see Fig. 5). We have demonstrated that the checkpoint protein Mad3p is a substrate for Ipl1 protein kinase and shown that blocking phosphorylation at two critical sites abrogates the spindle checkpoint response to lack of sister kinetochore tension. In contrast, the same mutations have no effect on the checkpoint response to unattached kinetochores resulting from microtubule depolymerization. Our model is therefore analogous to that of Morrow et al. (2005), who have proposed a distinct, Aurora B-dependent link between kinetochores not under tension and the core checkpoint machinery. Pinsky et al. (2006) have argued that the principal role of Ipl1p kinase in the spindle checkpoint is to generate unattached kinetochores upon lack of tension, and it clearly can function in this manner when the checkpoint is activated in response to kinetochore defects produced when cells expressing thermosensitive alleles of genes encoding a range of kinetochore components are shifted to the restrictive temperature. However, using the same assay to monitor kinetochore attachment, we have failed to find any evidence for unattached kinetochores in Scc1p-depleted cells, and yet such cells show a Mad3p-dependent anaphase delay that requires the presence of two Ipl1p phosphorylation sites.
in Mad3p. Thus, in contrast to cells with defective kinetochore components where reattachment of microtubules may be impaired, kinetochore–microtubule interactions destabilized by Ipl1p in Sec1p-depleted cells may be rapidly reinitiated and therefore remain undetected through the “attachment” pathway. Mad3p may acquire different patterns of phosphorylation when the checkpoint machinery is activated in different ways, although how this leads it to become a more potent inhibitor of the APC/C remains to be determined. Ipl1-dependent Mad3p phosphorylation may be particularly important in response to lack of tension to ensure that APC/C Cdc20 is rapidly and efficiently inhibited. Such Mad3p phosphorylation may also occur more rapidly than a response to lack of tension via the “attachment” pathway, which would first require breaking the incorrect microtubule attachments, then recruitment of kinetochore-bound signaling scaffolds [Mad1p and/or Bub1p], and finally interaction with Mad2p and Mad3p to produce new anaphase inhibitors. Phosphorylated Mad3p may also be more potent as an inhibitor, acting as an amplifier of the intermittent checkpoint signal generated by rapidly detached and reattached kinetochore microtubules.

In conclusion, our findings provide the first clear mechanistic basis for a direct role of Ipl1p kinase activity in spindle checkpoint signaling in response to a lack of sister kinetochore tension and argue strongly in favor of two distinct responses within the spindle checkpoint: an attachment response independent of Ipl1p activity and a tension response requiring Ipl1p activity and Mad3p phosphorylation (Fig. 5).

Materials and methods

Yeast strains and general methods

All yeast strains [listed in Supplementary Table 2] were derivatives of W303 {ade2-1 his3-11,15 leu2-3,112 trpl-1 ura3-1 can1-100 ssd1-d2 Gal-1}. Strain and plasmid construction is described in the Supplemental Material. Basic yeast methods and growth media and routine recombinant DNA methodology were performed as previously described (Gietz et al. 1992; Kaiser et al. 1994; Sambrook and Russell 2001). Benomyl sensitivity was assessed by spotting strains, in 10-fold dilutions, onto plates containing 12.5 µg/mL or 15 µg/mL benomyl, respectively, followed by growth for 3 d at 23°C.

Biochemical and immunological techniques

Cells were synchronized in G1 using α-factor at 1 µg/mL and were released by washing three times before resuspension in the appropriate media. To prevent cells from entering the next cell cycle, α-factor was added back when small buds appeared. Where cells were released into YPD containing nocodazole, the drug was used at a concentration of 15 µM at 23°C. To study tension checkpoint activation, strains expressing SCC1 from a GAL promoter, were arrested in G1 in YP medium containing raffinose and galactose for 150 min at 30°C and then transferred to YPD containing 1 µg/mL α-factor and incubated for a further 2 h at 30°C. Cells were finally released into YPD at 30°C.

Cell cycle progression was followed by monitoring Pds1p levels in strains containing PDS1-mycC. Samples taken over a 180-min time course for immunoblotting were resuspended in an equal volume of sample buffer containing 4% (w/v) SDS, 80 mM Tris-HCl (pH 6.8), 10% (v/v) glycerol, 20 mM EDTA, bromophenol blue, 0.1 M DTT, 100 µM Pefabloc, 10 µg/mL leupeptin, 10 µg/mL pepstatin, 10 µg/mL chymostatin, 1 mM sodium pyrophosphate, 50 mM NaF, and 100 µM microcystin. An equal volume of glass beads (0.5 mm diameter) was added, and cells were lysed using a Ribolysar [Hybaid] before separation of proteins by SDS-PAGE and Western blotting. Pds1p-mycC was detected with an anti-myc antibody [c-myc A14; Santa Cruz Biotechnology] used at 1:1000 dilution followed by goat anti-rabbit-HRP secondary antibody ( Pierce) at 1:1000 dilution, using ECL detection. Membranes were blotted with a rabbit anti-Mad1p antibody [Hardwick and Murray 1993] as a loading control.

Protein kinase assay and phosphorylation site mapping

Purified GST-Ipl1p (0.2 µg) and GST-Mad1p (0.04 µg) or Mad3p (0.6 µg) were incubated in 20 µL of 50 mM Tris-HCl (pH 7.5), 0.1% β-mercaptoethanol, 0.1 mM EGTA, 10 mM MgCl₂, and 100 µM γ-32P-ATP (826 cpm/pmol) for 15 min with 10 µM Microcystin (gift from Professor C. MacKintosh, University of Dundee, Dundee, Scotland, UK) plus recombinant substrate, as appropriate. Reactions were stopped by adding 2x sample buffer and separated by SDS-PAGE, the proteins were transferred to Immobilon-P transfer membrane (Millipore), and the membrane was exposed to film. Preparation of recombinant GST-Ipl1p, GST-Mad1p, and Mad3p is described in the Supplemental Material. To map phosphorylation sites, 32P-labelled Mad3p (6 µg) was generated in a similar manner but using high specific activity γ-32P-ATP (5000 cpm/pmol), excised as a gel slice following SDS-PAGE, and digested with trypsin. Peptides were separated by HPLC and analyzed by MALDI-TOF-TOF mass spectrometry and Edman degradation as described previously (Lizzcano et al. 2004). Alternatively, Mad3p tryptic digests prepared from in vitro kinase assays (carried out as above but with nonradioabeled ATP) were processed and analyzed essentially as described by Williamson et al. (2006), detecting phosphopeptides by precursor ion scanning in the negative ion mode and switching to the positive ion mode to obtain the peptide sequence by an ms/ms product ion scan.

Microscopy

GAL-SCC1 cells containing Tub1-CPF and Mtw-3GFP (Pinsky et al. 2006) were synchronized by addition of α-factor and then depleted for Sec1p by growth in glucose media for 120 min followed by release from G1 into glucose medium. At 15-min intervals over a 2-h time course, samples of cells were fixed briefly (5 min) in 3.7% (w/v) formaldehyde, washed, and then imaged using an Intelligent Imaging Innovations [3i] Marianas system, which incorporates a Zeiss Axiovert microscope, CoolSnap CCD, and Slidebook software.

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