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The A78V Mutation in the Mad3-like Domain of *Schizosaccharomyces pombe* Bub1p Perturbs Nuclear Accumulation and Kinetochore Targeting of Bub1p, Bub3p, and Mad3p and Spindle Assembly Checkpoint Function

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During mitosis, the spindle assembly checkpoint (SAC) responds to faulty attachments between kinetochores and the mitotic spindle by imposing a metaphase arrest until the defect is corrected, thereby preventing chromosome missegregation. A genetic screen to isolate SAC mutants in fission yeast yielded point mutations in three fission yeast SAC genes: *mad1*, *bub3*, and *bub1*. The bub1-A78V mutant is of particular interest because it produces a wild-type amount of protein that is mutated in the conserved but uncharacterized Mad3-like region of Bub1p. Characterization of mutant cells demonstrates that the alanine at position 78 in the Mad3-like domain of Bub1p is required for: 1) cell cycle arrest induced by SAC activation; 2) kinetochore accumulation of Bub1p in checkpoint-activated cells; 3) recruitment of Bub3p and Mad3p, but not Mad1p, to kinetochores in checkpoint-activated cells; and 4) nuclear accumulation of Bub1p, Bub3p, and Mad3p, but not Mad1p, in cycling cells. Increased targeting of Bub1p-A78V to the nucleus by an exogenous nuclear localization signal does not significantly increase kinetochore localization or SAC function, but GFP fused to the isolated Bub1p Mad 3-like accumulates in the nucleus. These data indicate that Bub1p-A78V is defective in both nuclear accumulation and kinetochore targeting and that a threshold level of nuclear Bub1p is necessary for the nuclear accumulation of Bub3p and Mad3p.

INTRODUCTION

During anaphase of mitosis, sister chromatids are separated by the mitotic spindle. The spindle assembly checkpoint (SAC) protects against genetic loss by preventing anaphase initiation until all chromosomes are properly attached to the spindle microtubules (for review see Wassmann and Ben-ezra, 2001). Screens in *Saccharomyces cerevisiae* identified the first SAC genes based on the sensitivity of bub and mad mutants to microtubule disruption (Hoyt et al., 1991; Li and Murray, 1991). MPS1 was identified as required for both the SAC and spindle pole body duplication (Weiss and Winey, 1996).

The first two SAC genes in *Schizosaccharomyces pombe*, *mad2* and *mph1*, were identified based on their ability to promote a metaphase arrest and inhibit proliferation when overexpressed in wild-type cells and to render cells checkpoint-defective when mutated (He et al., 1997, 1998). Fission yeast Mad2p was independently identified based on its interaction with the APC activator, Slp1p (Kim et al., 1998).

Homologues of the yeast SAC proteins have been identified in higher eukaryotes (for review see Millband et al., 2002). The SAC is not a linear genetic pathway, and it does not operate identically in all eukaryotes (for review see Bharadwaj and Yu, 2004), but a consensus model for SAC function is emerging (for review see Millband et al., 2002). The checkpoint proteins exist in small subcomplexes, the composition of which is altered during SAC-induced mitotic arrest (for review see Millband et al., 2002). Stabilization of a Bub1p-Mad1p-Bub3p complex in checkpoint-activated cells is required for checkpoint function (Brady and Hardwick, 2000). During checkpoint activation, the Mps1, Mad1, Mad2, Mad3, Bub1, and Bub3 checkpoint proteins localize to unattached kinetochores (for review see Cleveland et al., 2003). The Mps1, Mad2, BubR1, and Bub3 proteins associate dynamically with kinetochores, whereas Mad1p and Bub1p bind more stably (Howell et al., 2000, 2004; Shah et al., 2004). Mad2p, Mad3p, and Bub3p impose a metaphase arrest by binding to the anaphase promoting complex (APC) and inhibiting its ubiquitin ligase activity (for review see Yu, 2002), thereby preventing the ubiquitination and subsequent degradation of critical cell cycle regulatory proteins by the 26S proteasome.

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Bub1p is required for SAC function in mitosis (for review see Lew and Burke, 2003) and is essential for meiosis in fission yeast (Bernard et al., 2001; Yamaguchi et al., 2003) and flies (Basu et al., 1999). The evolutionarily conserved Bub1p domain structure includes a kinase domain, a Bub3p-binding domain, and a Mad3-like region, the function of which is unknown (see Figure 1A; Taylor et al., 1998; Hardwick et al., 2000; Warren et al., 2002). Bub1p kinase activity is required for efficient checkpoint function in fission yeast (Yamaguchi et al., 2003), but its in vivo targets are unknown. Bub1p localizes to kinetochores in every mitosis (Taylor and McKeon, 1997; Bernard et al., 1998; Jablonski et al., 1998; Ouyang et al., 1998; Basu et al., 1999; Sharp-Baker and Chen, 2001; Gillett et al., 2004) and regulates the kinetochore association of other SAC proteins (Basu et al., 1998; Sharp-Baker and Chen, 2001; Millband and Hardwick, 2002; Gillett et al., 2004; Johnson et al., 2004).

The SAC can be activated by defects in microtubule/kinetochore interactions or by overexpression of some SAC components (for review see Bharadwaj and Yu, 2004). The cell cycle arrest due to mph1 overexpression requires mad2, indicating that mad2 acts downstream of mph1 (He et al., 1998). The observation that mad2 cells survive mph1 overexpression also indicates that kinetochore/microtubule interactions are unperturbed. A genetic screen to isolate fission yeast strains, like mad2/H9004, is insensitive to mph1 overexpression identified five novel mutant alleles of three checkpoint genes: bub1, bub3, and mad1. This study focuses on the bub1-A78V mutant, which is mutated in the Mad3-like region (Taylor et al., 1998; Hardwick et al., 2000; Warren et al., 2002), a domain that is evolutionarily conserved but for which the function is unknown.

MATERIALS AND METHODS

Yeast Methods

The strains used in this study are listed in Table 1. Standard yeast methods and media were used (Moreno et al., 1991). Spotting experiments were performed by growing cells to midlog phase in yeast extract medium (YE) or supplemented Edinburgh minimal media (EMM) and spotting an equal number of cells in fivefold dilutions onto YE or EMM plates in the presence or absence of 15 μg/ml thiabendazole (TBZ, Sigma-Aldrich, 3 mg/ml stock in isopropanol) as indicated. Asynchronous cultures were grown in YE or supplemented EMM to midlog phase at 25°C, whereas SAC-activated cells were grown to midlog phase in YE at 25°C, synchronized in hydroxyurea (HU, 10 mM) for 4 h at 30°C, washed, and released into YE/100 g/ml methyl benzimidazol-2-yl carbamate (carbendazim, MBC, Sigma-Aldrich, 5 mg/ml stock in dimethyl sulfoxide) for 2 h at 25°C as previously described (Millband and Hardwick, 2002).

Protein Methods

Yeast were grown in YE to midlog phase, and extracts were prepared in HB buffer (Moreno et al., 1991) or trichloroacetic acid (TCA) extraction buffer. For TCA extractions, 8 × 10^7 cells were washed in 20% TCA, pelleted, resuspended in 100 μL 20% TCA, lysed by bead beating, pelleted, washed with ice-cold acetone, resuspended in 50 μL 2× SDS loading buffer plus 25 μL 1 M Tris (pH 7.5), boiled 5 min, and centrifuged 5 min at 13,000 rpm to collect the supernatant. Proteins were separated by 4–20% or 10% Tris-HCl SDS-PAGE (Bio-Rad, Richmond, CA) and transferred to an Immobilon-P nylon membrane (Millipore, Bedford, MA). A polyclonal rabbit anti-S. pombe Bub1p
Table 1. Schizosaccharomyces pombe strains used in this study

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antibody (K. Hardwick, unpublished results) at 1:1000 dilution in BLOTTO, a 1:5000 dilution of anti-rabbit HRP-conjugated secondary antibody (Promega, Madison, WI), and ECL detection reagents (Amersham, Piscataway, NJ) were used to perform Western blots.

Communoprecipitations were performed essentially as described (Millband and Hardwick, 2002). A polyclonal Bub1p antibody was used to immunoprecipitate wild-type Bub1p and Bub1p-A78V from cycling cells. Coupled 9E10-Sepharose (Santa Cruz Biotechnology, Santa Cruz, CA) was used to communoprecipitate Myc-Bub3p (K. Hardwick, unpublished results) from wild-type and bub1-A78V cells. Western blots were performed using sheep anti-Myc antibody (1:1000 in BLOTTO) and rabbit polyclonal anti-Myc A14 (Santa Cruz Biotechnology; 1:2000) on nitrocellulose.

The mep1 Overexpression Suppressor Screen

SS446 and SS447 were transformed with the plasmid coding for the thiamine-repressible mep1 gene promoter (He et al., 1998), in which mep1 is transcribed from the thymine-repressible nmt1 gene promoter (Basi et al., 1993; Maundrell, 1993). Transformants were grown in EM with thiamine to repress mep1 expression and mutagenized with nitrosoguanidine in two batches to 61.5 or 73.7% killing efficiency. From 1.6 × 10^6 cells plated onto EM with appropriate amino acid supplements in the absence of thiamine to induce mep1 overexpression, four thousand mep1 overexpression suppressor strains were selected by their growth after replica plating to fresh EM plates lacking thiamine. Two hundred fifty suppressor strains were streaked to EM with thiamine + 15 μg/ml TBZ at 29°C to test whether they were likely to be defective in the SAC.

Genetic linkage between mep1 overexpression suppressor mutations and mutations in the SAC genes, mad1, mad2, mad3, bub1, and bub3, was determined by random spore analysis. For strains that carry mutations that were tightly linked to one of these checkpoint genes, PCR amplification (Qiagen TaqPCR Master Mix Kit, Chatsworth, CA) and automated nucleotide sequencing of the candidate mutated ORF were performed.

Sequence Analysis

S. pombe Bub1p conserved regions were identified based on homology to defined S. cerevisiae Bub1p domains (Taylor et al., 1998; Hardwick et al., 2000; Warren et al., 2002) using the European Molecular Biology Open Software Suite water program (http://www.hgmp.mrc.ac.uk/software/EMBOS5/Apps/water.html). A list of S. pombe proteins that contain a putative nuclear localization signal (http://cubic.bioc.edu/cgi/var/nair/predictNLS/Genome.pl) constructed by the Rost group at the Columbia University Bioinformatics Center (Nair et al., 2003) was used to determine if fission yeast spindle checkpoint proteins contain an identifiable NLS.

Strain Construction

Using a previously described method (Millband and Hardwick, 2002), a plasmid encoding the carboxy terminus of Bub1 fused to GFP (Hardwick, unpublished results) was used to construct Bub1-A78V-GFP by PaAl digestion of the tagging vector and subsequent integration into bub1-A78V cells. To construct bub1Δ strains carrying Mad1-GFP, Bub3-GFP, Mad3-GFP (Millband and Hardwick, 2002), or Ath2-GFP (Ding et al., 1998), TBZ-sensitive progeny were selected by replica plating random spores to plates containing 15 μg/ml TBZ and phloxine B and subsequently screened visually for the presence of GFP. To construct bub1Δ strains carrying Mad1-GFP, Bub3-GFP,
or Mad3-GFP, ura+ colonies were selected from a cross and screened visually for the presence of GFP. To construct Bub1-NLS-GFP and Bub1-A78V-NLS-GFP strains, complementary oligonucleotides (Integrated DNA Technologies, Coralville, IA) encoding the SV40 or nucleoplasmin NLS (for review see Yoshida and Sazer, 2004) were hybridized by boiling for 5 min and slowly cooling to room temperature and cloned into the *Sfi*I site of the Bub1-GFP tagging vector, located between the *bub1C* terminus and GFP. The cloned products were integrated into wild-type or bub1-A78V cells as described above. To construct a GFP-tagged Bub1p Mad3-like domain (amino acids 6–193), this region was PCR amplified and cloned into the *Bgl*II site of pEGP573 (Pasion and Forsburg, 1999), and the resulting plasmid was transformed into wild-type cells. To construct *pREP*4-SV40NLS-GFP, a fragment from *pREP* SV40 NLS-GFP-*)LaCZ* (Yoshida and Sazer, 2004) containing only SV40 NLS-GFP was subcloned into *pREP*4 (Maundrell et al., 1993).

**Site-directed Mutagenesis**

To recreate the bub1-A78V mutation, a wild-type *bub1* genomic plasmid (Bernard et al., 1998) was mutagenized using the Transformer Site-directed Mutagenesis Kit (Clontech, Palo Alto, CA). The *bub1*-A78V mutagenic oligonucleotide was 5'-gtgctcaaatgcttgatgatgttattcag tacttagaaagatg-3'.

**Fluorescence Microscopy**

To observe microtubules in living cells, Atb2-GFP cells (Ding et al., 1998) expressing GFP-a-tubulin were grown in supplemented EMM in the presence of thiamine at 25°C, washed, and resuspended in supplemented EMM lacking thiamine for 24 hr at 32°C. Strains expressing untagged GFP, SV40-NLS-GFP, or Bub1p Mad3-like-domain-GFP were grown in the presence of thiamine at 25°C, washed, and resuspended in EMM lacking thiamine for 18 or more hours at 25°C. These four strains were visualized using a Zeiss Axioskop fluorescence microscope (Thornwood, NY) and photographed with a DVC 1300 Black and White CCD camera using QED software (Media Cybernetics, Silver Spring, MD). Bub1-GFP, Bub1-A78V-GFP, Bub1-A78V-SV40-NLS-GFP, Bub1-A78V-nucNLS-GFP, Bub3-GFP, Mad1-GFP, or Mad3-GFP were visualized using an Applied Precision DeltaVision Restoration Microscope System (Isisquah, WA), and images were acquired using a Photometrics CoolSnap HQ camera (Tucson, AZ). Equivalent exposure times and equal numbers of m stacks were used to accumulate a series of optical images that were summarized as two-dimensional projections. To calculate the ratio of nuclear GFP signal to cytoplasmic GFP signal, fluorescence values were determined using the sum protocol. Fluorescence values were determined using the sum algorithm of the DeltaVision software (SoftWoRx 5.2.3) to analyze stacks of Z sections, which were projected two-dimensionally using the sum protocol. Fluorescence values were determined for the nucleus and cytoplasm of at least five cells per sample, and the statistical SE was calculated for each data set. Hoechst 33342 (Sigma-Aldrich, St. Louis, MO) or 4',6-diamidino-2-phenylindole dihydrochloride (DAPI, Sigma-Aldrich) were used to visualize DNA in living or fixed cells respectively, as previously described (Moreno et al., 1991; Demeter et al., 1995). To determine the percentage of cells with condensed DNA, cells were fixed with formaldehyde, stained with DAPI, and visualized using a Zeiss Axioskop fluorescence microscope.

**RESULTS**

The *mph1* Overexpression Screen Identified Five New Alleles of Known SAC Genes

The observation that *S. pombe* cells mutated in *mad2* are insensitive to the checkpoint activation and cell cycle arrest caused by *mph1* overexpression (He et al., 1998) suggested that this characteristic could be used to identify strains mutated in other checkpoint genes that act downstream of *mph1*.

Approximately 1.6 × 10⁷ cells, transformed with a plasmid from which *mph1* was expressed from the thiamine-repressible *nmt1* gene promoter, were grown in thiamine to keep the level of expression low and then chemically mutagenized (see Materials and Methods for details). Four thousand candidate suppressor strains were identified by their ability to form colonies when expression of *mph1* was derepressed. To ask whether this screening strategy identified strains with defects in microtubules and/or components of the spindle assembly checkpoint, 250 of the suppressor strains were tested for growth in the presence or absence of the microtubule-stabilizing drug, TBZ. From among the 55 most TBZ-sensitive strains, 16 were initially chosen for further study because of their high sensitivity to TBZ and normal growth in the absence of TBZ.

To determine if they were mutated in a previously identified SAC gene, each of the 16 strains was crossed to mad1Δ, mad2Δ, mad3Δ, bub1Δ, and bub3Δ strains (He et al., 1997, 1998; Bernard et al., 1998; Millband and Hardwick, 2002). Five of the strains carried a mutation that is tightly linked to one of these checkpoint genes. PCR amplification of these putative mutant alleles and subsequent nucleotide sequencing of their open reading frames confirmed that three *bub1* alleles, one *bub3* allele, and one *mad1* allele were identified, indicating that the strategy of the screen was valid.

The three *bub1* mutations occurred in evolutionarily conserved regions of the protein (Figure 1A). The *bub1*-R888Stop mutation introduced a premature stop codon in the protein kinase domain and removed 82 amino acids from the carboxy-terminus of the protein. The *bub1*-A78V and *bub1*-G139D mutations both introduced a single amino acid change in the conserved Mad3-like region. Strains carrying each of these three mutant alleles exhibited a 25- to 625-fold increase in sensitivity to TBZ when compared with wild-type cells.

The *bub1*-A78V Mutant Is Defective in the SAC but Produces a Wild-type Level of Protein

A Western blot was performed to determine whether the three *bub1* mutants are checkpoint-defective because, like *bub1*a, they have a significant decrease in Bub1 protein level. The amounts of Bub1 protein in the *bub1*-R888Stop and *bub1*-G139D strains were significantly lower than in the wild-type strain, indicating that they are likely to be checkpoint-defective because of the near absence of Bub1p. In contrast, *bub1*-A78V produces a wild-type level of protein (Figure 2A). To confirm this finding, a Western blot was performed using serial dilutions of the same protein preparations shown in Figure 2A, and the band intensities were quantified (Figure 2B). For the wild-type sample, the ratio of Bub1p band intensity to the intensity of the control band was 3.1. This ratio was 3.7 for the *bub1*-A78V sample, demonstrating that the ability of *bub1*-A78V cells to survive *mph1* overexpression was not due to a decreased abundance of Bub1 protein.

The only mutation identified by sequencing the *bub1*-A78V allele (C237T) resulted in an alanine to valine mutation and this amino acid is not conserved among *Bub1* proteins but is located within a highly conserved region of the protein. To confirm that the single amino acid change in *Bub1*-A78V is sufficient to disrupt SAC function, the mutation was recreated by site-directed mutagenesis of a plasmid that was then transformed into *bub1*a cells to determine whether it encodes a defective protein. Although wild-type *bub1* expressed from its native promoter in a multicopy plasmid rescued the TBZ-sensitivity of the *bub1*a strain, the *bub1*-A78V plasmid only marginally improved growth (Figure 2C). Similar results were observed when the plasmids were integrated into *bub1*a cells (Figure 2D) and are consistent with the previous comparison between *bub1*-A78V and *bub1*a strains on TBZ plates (Figure 1B), suggesting that the mutant protein may retain partial function. These results indicate that the *bub1*-A78V mutation is solely responsible for the defects in the *bub1*-A78V strain.

Therefore, the *bub1*-A78V mutant strain can be used to examine the functional consequences of specifically disrupting the *Bub1p Mad3*-like region. Compared with wild-type cells, the growth of *bub1*-A78V cells was impaired when the SAC was activated by TBZ (Figure 1B), indicating that *bub1*-A78V cells fail to activate the SAC. To confirm that the SAC is not functional in *bub1*-A78V cells, it was activated in the

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absence of microtubule disruption by mph1 overexpression. Under these conditions, 18.5% of wild-type cells arrested at the metaphase-to-anaphase transition (Figure 2E) with a short mitotic spindle. In contrast, only 1.5% of bub1-A78V cells exhibited a short spindle after this treatment. This result confirmed that bub1-A78V cells are SAC-defective because they fail to arrest the cell cycle in response to SAC activation, which explains their ability to survive mph1 overexpression, during which the microtubules are unperturbed, and their impaired growth in the presence of TBZ. Taken together, these data demonstrate that alanine 78 in the Bub1p Mad3-like region is required for SAC activation in response to mph1 overexpression or microtubule damage.

The Bub1p, Mad3p, and Bub3p SAC Proteins, but not Mad1p, are Mis-localized in Interphase bub1-A78V Cells

Wild-type S. pombe Bub1-GFP localized predominantly but not exclusively to the nucleus in interphase cells (Figure 3B), with a short mitotic spindle. In contrast, only 1.5% of bub1-A78V cells exhibited a short spindle after this treatment. This result confirmed that bub1-A78V cells are SAC-defective because they fail to arrest the cell cycle in response to SAC activation, which explains their ability to survive mph1 overexpression, during which the microtubules are unperturbed, and their impaired growth in the presence of TBZ. Taken together, these data demonstrate that alanine 78 in the Bub1p Mad3-like region is required for SAC activation in response to mph1 overexpression or microtubule damage.

Functions of the Bub1p Mad3-like Domain

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These results indicate that in bub1-A78V cells, Bub1p, Bub3p, and Mad3p, but not Mad1p, fail to correctly accumulate in the nucleus of cycling cells.

**Bub1-A78V-GFP Does Not Localize to Kinetochores during a Normal Mitosis or When the SAC Is Activated**

During a normal fission yeast cell cycle, Bub1p localizes to two spots, corresponding to clustered kinetochores, during prometaphase and remains there until telophase (Bernard et al., 1998). When the SAC is activated, Bub1p is recruited to kinetochores, where it remains until the cell cycle resumes (Bernard et al., 1998; Toyoda et al., 2002). To test whether the bub1-A78V mutant is defective in the SAC because Bub1p cannot be properly recruited to kinetochores, Bub1p-GFP and Bub1-A78V-GFP localization was compared in cycling interphase cells and in cells synchronized in mitosis upon release from an S phase block imposed by HU into either fresh medium or medium containing the microtubule-destabilizing drug, MBC, which rapidly promotes depolymerization of microtubules in fission yeast (Tran et al., 2001; Sawin and Snaith, 2004) and activates the SAC (Millband and Hardwick, 2002).

In untreated cycling or mitotic cells, Bub1-GFP was predominantly nuclear, whereas Bub1-A78V-GFP was more uniformly distributed between the nucleus and the cytoplasm (Figure 4A). Bub1-GFP localized to kinetochore dots in early and late anaphase mitotic cells (Figure 4A). This distinct localization pattern was not seen with Bub1-A78V-GFP (Figure 4A). When the SAC was activated by MBC, 20.6 ± 1.9% of wild-type cells exhibited bright Bub1-GFP kinetochore dots, a number consistent with previously published reports (Tournier et al., 2004). This localization pattern was only observed in 3.0 ± 0.6% bub1-A78V-GFP cells. These results demonstrate that Bub1p-A78V is defective in kinetochore localization. To ensure that this observed mislocalization did not result because fewer mitotic bub1-A78V cells were present in the sample population due to the SAC defect, cells were examined 2 h after SAC activation. At this time point, equal numbers of mitotic cells were observed in the wild-type and mutant strains (see Figure 4B).

**Mad1p Localizes Correctly to Kinetochores when the SAC Is Activated in bub1-A78V and bub1Δ Cells**

Mad1p is recruited to unattached kinetochores when the SAC is activated in fission yeast (Hardwick, unpublished results), budding yeast (Gillett et al., 2004), and higher eukaryotes (for review see Bharadwaj and Yu, 2004), and experiments in budding yeast and *Xenopus laevis* show that this recruitment requires Bub1p (Sharp-Baker and Chen, 2001; Gillett et al., 2004). In *S. pombe*, Mad1p localization to the nucleus and enrichment at the nuclear periphery in interphase was the same in wild-type, bub1-A78V, and bub1Δ cells (Figures 3C and 5A). When the checkpoint was activated by MBC treatment for 2 h, Mad1-GFP localized to kinetochores in wild-type, bub1-A78V, and bub1Δ cells (Figure 5A). These results indicate that Bub1p is
yet neither bub1-A78V nor bub1Δ cells arrested at metaphase in response to checkpoint activation, demonstrating that the kinetochore recruitment of Mad1p alone is unable to promote a SAC response.

Bub3p and Mad3p Do Not Localize to Kinetochores when the SAC Is Activated in bub1-A78V or bub1Δ Cells

In Xenopus extracts and Drosophila cells, Bub3p localization to unattached kinetochores requires Bub1p (Basu et al., 1998; Sharp-Baker and Chen, 2001), and experiments in S. pombe have shown that Mad3p localization to unattached kinetochores is dependent on Bub1p (Millband and Hardwick, 2002). The observation that the interphase nuclear accumulation of Bub3p and Mad3p was reduced in bub1-A78V and bub1Δ cells (Figure 3, D and E) raised the question of whether Bub3p-GFP and Mad3p-GFP can localize to kinetochores when the SAC is activated in bub1-A78V cells.

In wild-type cycling cells or synchronized mitotic cells, Bub3p-GFP and Mad3p-GFP localized predominantly inside the nucleus, but this nuclear accumulation was reduced in bub1-A78V and bub1Δ cells (Figure 5, B and C). When the checkpoint was activated by MBC treatment for 2 h, Bub3p-GFP and Mad3p-GFP localized to kinetochores in wild-type cells but not in bub1-A78V or bub1Δ cells (Figure 5, B and C). This mis-localization of Bub3p-GFP and Mad3p-GFP was not a result of decreased production of Bub1p-A78V in these cells (Figure 5D).

**Bub1p-A78V Retains Its Ability To Bind to Bub3p**

Because Bub1p binds to Bub3p constitutively in budding yeast and higher eukaryotes, and Bub3p is required for kinetochore localization of Bub1p (for review see Millband et al., 2002), it is possible that Bub1p-A78V does not localize correctly because mutation of the Mad3-like region prevents its binding to Bub3p. This question was addressed by asking if these two proteins coimmunoprecipitate. When Bub1p or Bub1p-A78V was immunoprecipitated from cycling cells, Bub3p was copurified (Figure 6). Likewise, when Bub3p was immunoprecipitated from cycling cells, Bub1p or Bub1p-A78V was coprecipitated. Therefore, the SAC defect in bub1-A78V cells is not a result of disruption of the Bub1p-Bub3p complex.

Increased Nuclear Accumulation of Bub1p-A78V Does Not Significantly Improve Kinetochore Targeting of Bub1p-A78V or SAC Function

Bub1p-A78V did not localize to kinetochores during SAC activation and failed to accumulate to the wild-type level in the nucleus. The SAC defect caused by the A78V mutation may result from either or both of these defects, because adequate nuclear accumulation in interphase could be required for sufficient kinetochore localization upon SAC activation. To distinguish between these possibilities, an exogenous SV40 or nucleoplasmic nuclear localization signal (NLS), each of which functions efficiently in S. pombe (for review see Yoshida and Sazer, 2004), was fused to Bub1p-A78V in an attempt to drive more protein into the nucleus. bub1p-A78V-SV40NLS-GFP or bub1p-A78V-nucNLS-GFP was expressed from the genomic bub1 promoter at the endogenous locus. Although the ratio of nuclear to cytoplasmic GFP fluorescence in cells expressing Bub1p-A78V-GFP was 1.1 ± 0.1, this ratio was increased to 2.9 ± 0.2 in bub1p-A78V-SV40NLS-GFP or bub1p-A78V-nucNLS-GFP.
bub1-A78V-SV40NLS-GFP cells and 2.0 ± 0.1 in bub1-A78V-nucNLS-GFP cells (Figure 7A). However, the increased nuclear accumulation of Bub1p-A78V did not significantly improve growth on TBZ (Figure 7B). Although a slight improvement in kinetochore targeting was observed when either exogenous NLS was fused to Bub1p-A78V (Figure 7C), these observed differences were not statistically significant as determined by an unpaired $t$ test. These data indicate that the bub1-A78V mutation impairs SAC activity by disrupting two aspects of Bub1p function, namely nuclear accumulation and kinetochore localization. Because the kinetochore localization defect could not be overcome by increased nuclear accumulation of the mutant protein, these results indicate that the kinetochore localization defect is not a direct consequence of the nuclear accumulation defect.

**The Bub1p Mad3-like Region has NLS Activity**

*S. pombe* Bub1p-GFP is localized predominantly to the nucleus (Figure 3B) although it does not contain a consensus nuclear localization signal (see Materials and Methods). However, the A78V mutation in the Mad3-like domain disrupts the nuclear accumulation of Bub1p. To determine if this domain is sufficient to promote nuclear accumulation, the Mad3-like region (amino acids 6–193) was fused to GFP, and the localization of this fusion protein was monitored in wild-type cells. When untagged GFP was expressed in wild-type cells, the GFP fluorescence was distributed throughout the cell (Figure 8A), whereas robust nuclear fluorescence was observed when the classical SV40 NLS was fused to GFP (Figure 8B). Fusing the Bub1p Mad3-like region to GFP also promoted nuclear accumulation, although to a lower level than the SV40 NLS. These results indicate that the Bub1p Mad3-like region is sufficient to direct an exogenous protein into the nucleus.

**DISCUSSION**

The mph1 Overexpression Survival Screen Identified Point Mutations in bub1, bub3, and mad3

A screen to identify fission yeast mutants that survive an mph1 overexpression-induced SAC arrest identified novel mutant alleles of bub1, bub3, and mad3. The bub1-A78V mutation is in the conserved Mad3-like domain (Taylor et al.,...
1998; Hardwick et al., 2000; Warren et al., 2002), the functional significance of which was previously unknown. Because it produces a stable protein, bub1-A78V provides a unique tool for investigating the importance of the Mad3-like region for checkpoint function. Cells in which the Mad3-like domain was deleted from Bub1p (Hardwick, unpublished results) have defects in SAC function and kinetochore targeting similar to those described here for the bub1-A78V point mutant, strengthening our hypothesis that this mutation disrupts the function of the Mad3-like domain.

Disruption of the Mad3-like Domain Interferes with the Nuclear Accumulation and Kinetochore Targeting of Bub1p

Because the nuclear envelope remains intact throughout the cell cycle in yeast, all proteins necessary for nuclear functions, including SAC proteins, must be transported into the nucleus. Bub1p, but not Bub1p-A78V, accumulates in the nucleus of interphase fission yeast cells and is enriched at kinetochores during normal mitoses and upon checkpoint activation (Figure 4A). Even when the GFP fluorescence was enhanced to demonstrate that Bub1p-A78V is not excluded from the nucleus, kinetochore localization was not observed in mitotic cells. Increasing the intranuclear concentration of Bub1p-A78V by fusing it to two different exogenous NLSs did not restore kinetochore localization or SAC function (Figure 7). The Bub1p-A78V mutant protein is therefore defective in kinetochore targeting.

These data raise the question of whether the Mad3-like domain is both necessary and sufficient for the nuclear and kinetochore accumulation of Bub1p. The observations that...
Bub1p-A78V does not accumulate in the nucleus and that the isolated Mad3-like domain has NLS activity when fused to GFP (Figure 8) show that this domain is necessary and sufficient to promote nuclear targeting. In contrast, the Mad3-like domain alone is not sufficient for kinetochore targeting (unpublished data).

**Bub1p-A78V mis-localization results in the mis-localization of Bub3p and Mad3p, but not Mad1p**

Fission yeast Bub1p is required for the kinetochore localization and nuclear accumulation of Bub3p and Mad3p and for checkpoint activation. Conversely, the kinetochore localization of Bub1p may require Bub3p and Mad3p, as has been shown in higher eukaryotes (Taylor et al., 1998; Chen, 2002). If so, the decreased nuclear accumulation of Bub3p and Mad3p in bub1-A78V cells could further reduce the ability of mutant Bub1p to localize to kinetochores.

**Bub3p Localization Depends on Bub1p**

Consistent with previous studies in higher eukaryotes (Basu et al., 1998; Sharp-Baker and Chen, 2001), Bub3p localization to kinetochores depends on functional Bub1p. In cells carrying the bub1-A78V mutation, Bub3p also fails to accumulate in the nucleus. In both yeast and higher eukaryotes, Bub1p constitutively binds to Bub3p (for review see Millband et al., 2002), and this interaction is maintained in the bub1-A78V mutant. Therefore, the mis-localization of Bub1p-A78V may directly prevent the Bub3p to which it is bound from localizing properly.

**Mad3p Localization Depends on Bub1p**

Mad3p-GFP does not localize to kinetochores when the checkpoint is activated in either bub1Δ cells in fission yeast (Millband and Hardwick, 2002) or in the bub1-A78V mutant (Figure 5C). Although binding of Bub1p to Mad3p has not been reported, the human homologue of Mad3p, BubR1, does physically interact with Bub1p (Taylor et al., 2001). A similar interaction between *S. pombe* Bub1p and Mad3p would provide a possible explanation for the mis-localization of Mad3p in bub1-A78V cells. Alternatively, because Bub3p constitutively binds to Mad3p (for review see Millband et al., 2002), failure of Bub3p to accumulate at kinetochores could mis-localize Mad3p.

**Mad1p Localization Does Not Depend on Bub1p**

In budding yeast and frogs, Bub1p recruits Mad1p to unattached kinetochores (Sharp-Baker and Chen, 2001; Gillett et al., 2004). In contrast, the localization of Mad1p does not depend on Bub1p in fission yeast (Figure 5A), which may be explained by the observation that fission yeast Mad1p, but not its budding yeast homologue, has a consensus NLS. Therefore, a Mad1p-Bub1p-Bub3p complex, which forms during mitosis in *S. cerevisiae* (Brady and Hardwick, 2000), is not required for Mad1p targeting to kinetochores in *S. pombe*. Fission yeast Mad1p may have its own kinetochore-targeting domain or may rely on a protein other than Bub1p, Bub3p, or Mad3p for its localization.

**Model for Regulation of the intracellular localization of Bub1p**

**Nuclear Accumulation May Be Regulated by Nuclear Import**

The A78V mutation in the Mad3-like domain of Bub1p interferes with its nuclear accumulation, although Bub1p does not contain an identifiable NLS (see Materials and Methods; Nair et al., 2003). Bub1p may carry an atypical NLS or it may bind to another protein that mediates its nuclear import. Of the proteins known to bind to Bub1p, neither Bub3p nor Mad3p have an identifiable NLS sequence.

Both the SV40 and the nucleoplasmin NLS can target *S. pombe* proteins exclusively to the nucleus (see Yoshida and Sazer, 2004), yet they are unable to restore the wild-type level of Bub1p-A78V to the nucleus. This observation and the lack of exclusive nuclear localization of wild-type Bub1p are consistent with the hypothesis that Bub1p has both an NLS and a nuclear export signal (NES) and that it shuttles between the nucleus and the cytoplasm. If so, Bub1p’s intracellular localization could be regulated by the balance between nuclear import and export and/or by the retention of Bub1p in the nucleus.

**Nuclear Accumulation of Bub1p May Be Regulated by Nuclear Retention**

Bub1p may bind to a nuclear anchoring protein that retains the protein in the nucleus, but such an anchor is unlikely to be Bub3p, because the bub1-A78V mutation does not disrupt the Bub1p-Bub3p complex.

Another candidate for the Bub1p nuclear anchor in interphase cells is the kinetochore, although such an interaction may be too transient to have been observed previously. Consistent with this possibility is a report that Bub1p colocalizes with kinetochore proteins recognized by the CREST antiserum in interphase human cells (Ouyang et al., 1998). If this were the case in *S. pombe*, the inability of Bub1p-A78V to bind to kinetochores in interphase cells might contribute to the defects in both nuclear and kinetochore accumulation in mitotic or checkpoint-activated cells.

**SUMMARY**

The kinetochore localization of Bub1p and its checkpoint function are abrogated by the A78V point mutation in the previously uncharacterized Mad3-like domain of *S. pombe* Bub1p. This domain is necessary and sufficient for nuclear accumulation. In bub1-A78V mutant cells, Bub3p and Mad3p also fail to accumulate at kinetochores when the SAC is activated. The essential role of Bub1p in the nuclear accumulation of Bub3p and Mad3p in unperturbed interphase and mitotic cells is a function not previously reported for any other checkpoint protein. In contrast, Mad1p is not dependent on Bub1p for its proper localization, an observation unique to fission yeast.

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**Note added in proof.** Reagents and observations listed as “Hardwick, unpublished results” have now been published as Vanooosthuyse et al. (2004), Mol. Cell. Biol., 22: 9786–9801.

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